Establishing methods for cultivation of liver- and muscle cells from Atlantic cod
Special emphasis on morphological and metabolic characterisation of cells from liver during in vitro differentiation

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Thesis submitted for the degree of Master of Science

by

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Table of contents

Table of figures

List of tables

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You raise me up!!!~O (∩_∩) O~

Abbreviations

AA	Arachidonic acid
ACC	acyl-CoA-carboxylase
ACO	Acetyl CoA-oxidase
AFAP	Adipocyte fatty acid binding protein
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BMP	Bone morphogenetic protein
cDNA	Complementary DNA
CE	Cholesterol esters
CPT	Carnitine palmitoyl tranferase
COA	Coenzyme A
DAG	Diacylglycerol
DEF	Digestive-organ-expansion-factor
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DNL	*de novo* lipogenesis
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene diamine tetra-acetic acid
EFAs	Essential fatty acids
EF1a	Elogation factor 1 alpha
EPA	Eicosapentaenoic acid
FAS	Fatty acid synthase
FABP	Fatty acid binding protein
FA	Fatty acid
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
FFA	Free fatty acid
FGF	Fibroblast growth factors
G6PD	Glucose-6-phosphate dehydrogenase
GPDH	Glycerol-3-phosphate dehydrogenase

GLUT	Glucose transporter
GK	Glucokinase
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHEX	Hematopoietically-expressed homeobox
HUFA	Highly unsaturated fatty acids
HSI	Hepatosomatic index
IBMX	3-Isobutyl-1-methylxanthine
L-15	Leibowitz-15
LCFAs	Long chain fatty acids
LEG	Liver-enriched gene
LPL	Lipoprotein lipase
MAG	Mono-acylglycerols
ME	Malic enzyme
MLC	Myosin light chain
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
MyoD	Myogenic Differentiation
NADPH	Nicotinamide adenine dinucleotide phosphate
PA	Palmitic acid
PBS	Phosphate buffered saline
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acids
RT-qPCR	Real-time quantitative polymerase chain reaction
RA	Retinoic acid
REST	Relative expression software tool
RNA	Ribonucleic acid
RNase	Ribonuclease
rRNA	Ribosomal RNA
RT	Reverse transcription
T3	3,3′,5-Triiodo-L-thyronine sodium salt
TAG	Triglycercide

TCA	Tricarboxylic acid
TLC	Thin layer chromatography
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

Abstract

Atlantic cod is regarded as one of the most promising future aquaculture species in Norway. However, the cod aquaculture industry is facing major problems with occurrence of "fatty livers" leading to high degree of tissue offal at slaughter. Only a small extent can be utilized in fish oil production. One of the aims of this thesis was therefore to establish a cod liver cell culture model enabling detailed studies of how nutritional factors may influence liver growth and fat deposition. It is further a goal for the industry to optimize the cod's utilization of energy from the diet for muscle growth and to less extent for deposition of fat in the liver. We therefore also wanted to establish a muscle cell culture model enabling future studies of how nutrients affect cod muscle-growth and metabolism. *In vitro* culture of unspecialised precursor cells from liver show that these cells possess high proliferation capacity. Hormones or lipids were added in order to study the effects on cell development (differentiation). The expression of several genes were analysed at different stages during the cell differentiation process. Our results show that liver cells at confluence, given a lipid mixture or a differentiation mixture containing hormones, went through significant morphological changes with time. Cells cultivated in a media with lipid supplementation showed a significant increase in the gene expression of PPARβ. We also tested some genes known to be highly expressed at late differentiation stages. Although no significant effects, all the genes GPDH, FABP-6, GLUT4, Annexin2, and Annexin3 showed tendencies to increasing expression with time during the differentiation process. We also succeeded in isolating and differentiating cod muscle cells. Q-PCR results from two stages of differentiating muscle cells showed that the expression of the early muscle specific marker MyoD decreased and the late marker α-actinin gene increased significantly from day 2 to day 9, verifying, together with morphology data that these cells develop from un-mature muscle cells to more mature multinucleated myotubes with time. The lipid class distribution of liver cells at confluence showed that the cells at this stage had approximately equal distribution of TAGs (24.0%), phospholipids (22.5%) and CE (21.4%) and lower levels of DAG (16.6%), FFA (10.4%) and MAG (5.2%). The cells at confluence had high capacity for

uptake of palmitic acid (PA), in agreement with high degree of immunostaining of the fatty acid transporter FATP1. Approximately 88% of radioactivity from 1-^{14}C-PA taken up into the cells was found in phospholipids. We also found that the radioactive PA was to a major extent (90%) elongated and desaturated to 18:1n-9 (oleic acid) and only 10% remained as 16:0.

1 Introduction

1.1 Cod (Gadus morhua) aquaculture development

Approximately 51.7 million tons of seafood products excluding aquatic plants was produced by the aquaculture industry in 2006 (FAO 2008). Many kinds of fish species are cultured and an increasing number of fish farms establish in most continents, especially in Europe and Asia. At present, more and more fish products are obtained from aquaculture compared to traditional fishery industry (Fig1).

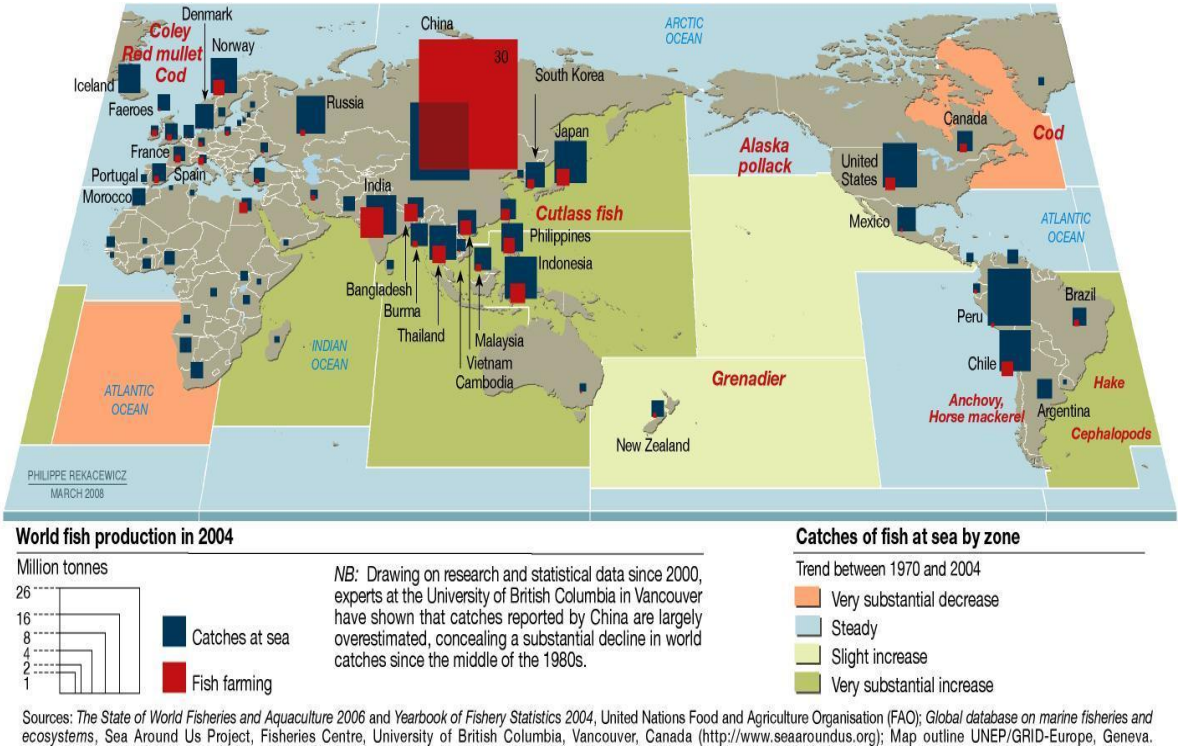


Fig 1. State of world fisheries and aquaculture in 2004. (2009 UNEP/GRID-Arendal). The red squares represent fish farming and the blue squares represent catch in sea.

Fish is one of the pillar industries in Norway. Fish aquaculture started in the early 1960's in Norway. In 2004, aquaculture accounted for 52% of the Norwegian seafood exports. All along, Atlantic salmon (*Salmo salar*) has been the leading cultured species,

and the estimated production in 2006 was 629,888 tons (Fig. 2) (Paisley et al. 2010). In addition, the production of farmed cod (*Gadus morhua*) has increased rapidly from 2000 (Fig. 3). Cod is extensively valued for fish fillet, liver oil, smoked and frozen roes. In Norway, the initial attempt of cod cultivation started at the Flødevigen Biological station in 1884 (Morais et al. 2001). In 2006, the production raised to 3168 tons (Paisley et al. 2010). Along with the world economic development, the requirement of fish food products gradually increases. Due to the relative high market value and growth rate, cod is regarded as an alternative and highly anticipated aquaculture species for Norway in the future (Jobling & Pedersen 1995).

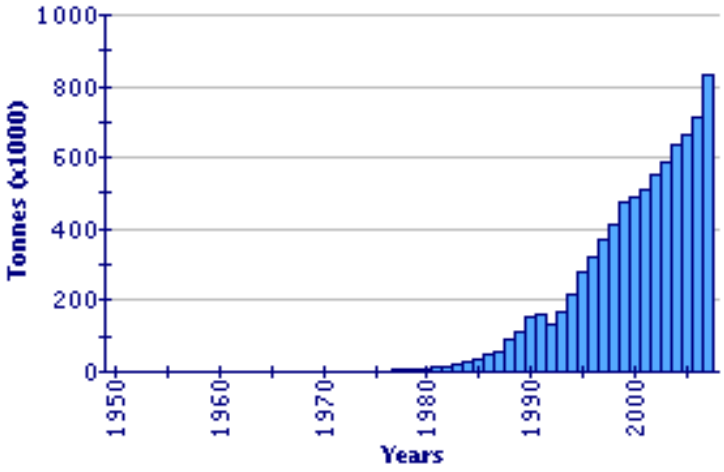


Fig 2. Aquaculture production of Atlantic salmon (Salmo salar) in Norway from 1950 to 2007 (FAO Fishery Statistic).

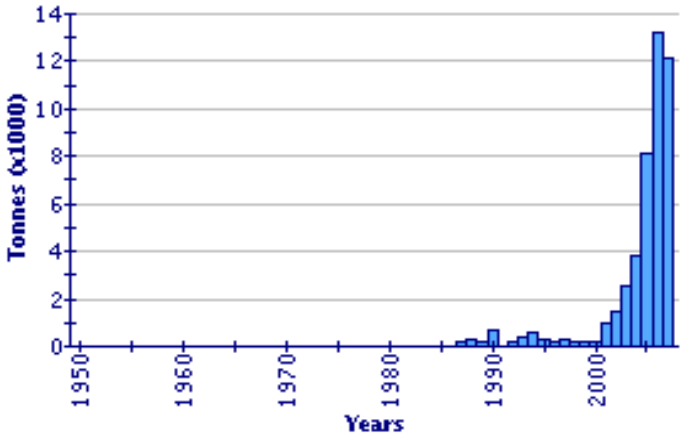


Fig 3. Global aquaculture production of cod (*Gadus morhua*) from 1950 to 2007 (FAO Fishery Statistic).

As such a promising species, both fish farmers and fishery companies want to increase the growth rate and relatively cut down the feeding costs. The high energy feeds turn to be the first choice for them, due to the fact that lipid and protein are both important energy sources in fish. In this article, we mainly focus on lipids. As a matter of fact, high energy feeds do make a comparable growth rate increase, and play an effective role on the culture of some commercial fish species, such as salmonids. Atlantic cod require higher protein levels than Atlantic salmon in their diets (Arnason et al. 2010; Hillestad & Johnsen 1994).

Dietary lipids function as a basic energy source and also supply essential fatty acids (EFAs), (Morais et al. 2001). In oily fish, such as farmed Atlantic salmon, dietary lipids are mainly deposited in the muscle and in the visceral adipose tissue (Polvi & Ackman 1992). Cod however, as lean fish, accumulates the majority of their dietary lipids in the liver, and the proportion is around 80-90% of the whole body lipid content (Lie et al. 1986). Cod fed with high energy diets have a distinctly larger liver compared to the one fed with low energy diets (Jobling et al. 1991; Jobling et al. 1994). It has been proved that the relationship between increased dietary lipid level and the relative amount of lipid found in the liver is linear and positive (Hansen et al. 2008). Therefore, high energy diets give rise to disproportionate fatty livers which can account for more than 18% of the total weight of the cod (Karlsen et al. 2006). In natural conditions, this fatty liver is used for storage of the energy which helps wild cod to get through the season of starvation. However for the farmed cod, feed is available continuously (Kjær et al. 2009). Normally, the hepatosomatic index (HSI) of wild cod is 2–6% and the HSI in cultured cod is usually more than 12% (Jobling 1988). Cod as a gadoid species can store less than 2% lipid in the muscle (Nanton et al. 2001), and mainly as phospholipids, whereas more than 90% of lipids deposited in liver are triacylglycerols (TAGs) (Dossantos et al. 1993). Thus, huge amounts of the energy from diets incorporate into the liver while the somatic growth is affected. Due to the higher value of cod fillet compared with cod liver, majority of limited marine lipids ideally should incorporate into fillet rather than liver. Researchers reported that higher protein and lower lipid proportion in diets help to decrease the fatty liver (Kim & Lall 2001; Rosenlund et al. 2004), but then the feeds will cost more and it

will also lower nitrogen utilization at a given intake as a result of high ration protein diets (Hatlen et al. 2007).

1.2 Lipid metabolism

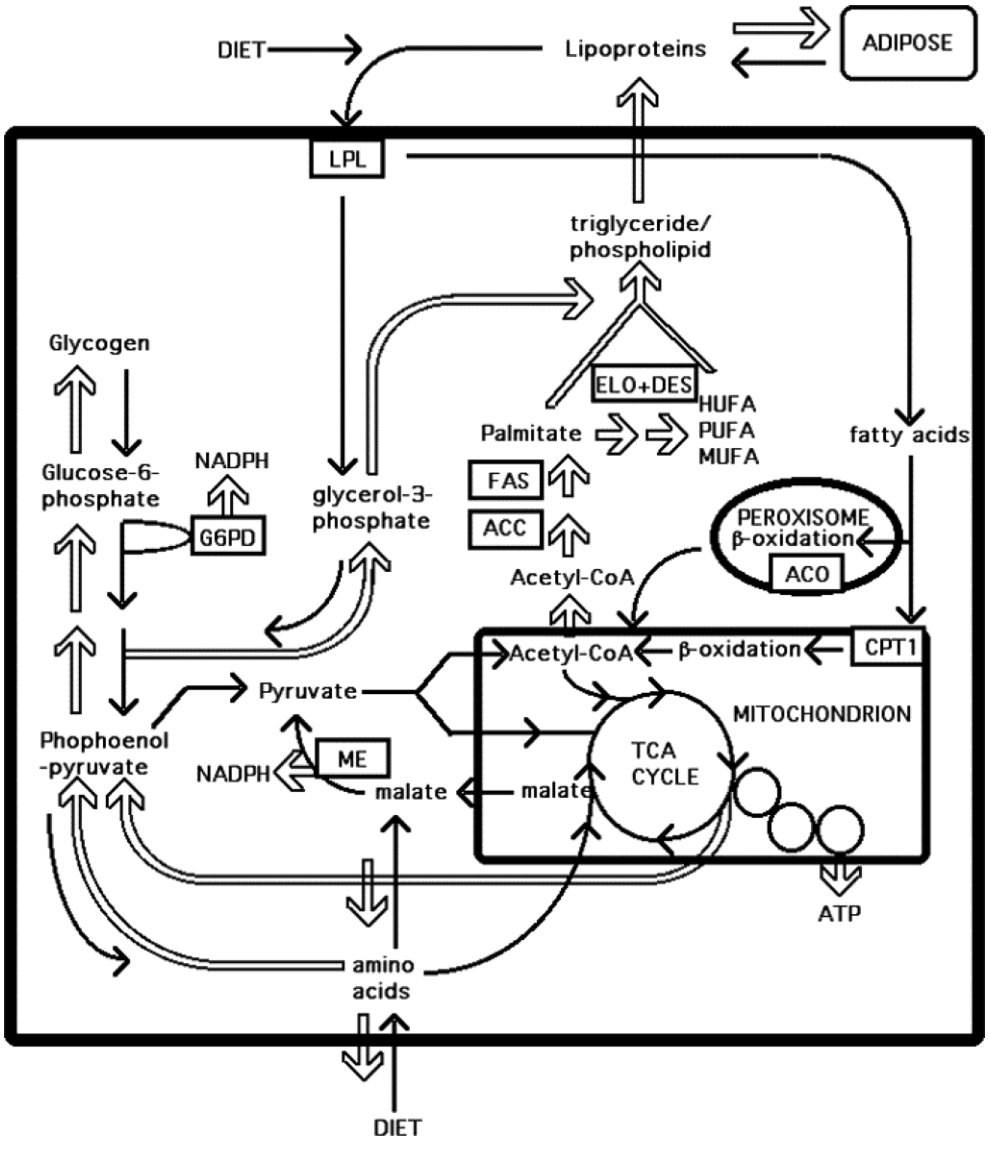


Fig.4 Schematic view of the main pathways of dietary fuel metabolism in fish liver (Leaver et al. 2008). Open arrows indicate anabolic pathways and normal arrows indicates catabolic routes.

The figure above (fig. 4) shows that fatty acids (FAs) supplied from diets can go to β-oxidation in peroxisomes or mitochondria in hepatocytes for producing energy, or be stored in adipose tissue. This step is catalyzed and modulated by lipoprotein lipase (LPL). Acetyl CoA-oxidase (ACO) and the carnitine palmitoyl tranferase (CPT) system are the key enzymes in peroxisomal and mitochondrial membranes, respectively. They control the FA oxidation rate in both organelles. Following β-oxidation, the product acetyl-CoA can enter into the TCA cycle and release ATP for energy generation or form phophoenol pyruvate and malate, to join the glycometabolism and amino acid metabolism. The β-oxidation product acetyl-CoA can also go to the lipid synthesis pathway. In lipid synthesis, acetyl-CoA is catalyzed by both FA synthase (FAS) and acyl-CoA-carboxylase (ACC) and NADPH. NADPH was provided from pentose-phosphate pathway. In this pathway, glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) are the catalyzers. Thereafter, monounsaturated FAs (MUFA) and polyunsaturated FAs (PUFA) are synthesized via FAs intermediates (e.g., palmitate) by means of elongation (FA elogase) and desaturation (FA desaturase). Finally TAGs or phospholipids can be formed. The participation of glycerol-3-phosphate is necessary for phospholipid synthesis. TAGs and phospholipids can be transported in the form of lipoproteins to adipose tissue. Then the lipid depositions are formed (Leaver et al. 2008).

The formation of fatty liver in cod can be due to a disorder of lipid metabolism. Based on recent research, the main reasons causing the fatty liver can be: an imbalanced ratio of protein and lipid in dietary diets or a deficiency of lipid degradation factors such as some EFA, vitamins and cholines (Nanton et al. 2001; Rodríguez et al. 1997; Russell et al. 2001; Yang et al. 2003). For instance, disorder in the ratio of protein and energy in the diet causes a deposition of TAGs in cod liver and increase the degree of vacuolation in hepatocytes (Morais et al. 2001). Therefore, we hypothesize that it might be possible to decrease the fatty liver by regulating the lipid metabolism.

Unlike the freshwater fish, long chain (C>20) n-3 highly unsaturated fatty acids (HUFA) are EFA for marine fish (Sargent et al. 1999). The ratio of eicosapentaenoic acid (EPA, 20: 5 n-3), docosahexaenoic acid (DHA, 22: 6n-3) and arachidonic acid (AA, 20:4n-6) in the diet is very important for the health and growth of marine fish. For cod, EPA and DHA are of vital importance (Takeuchi et al. 1994; Zheng et al. 1996).

Deficiency of EFA cause symptoms such as growth reduction, higher mortality, fin erosion and fatty liver (Tocher 2010).

Phospholipids are important components of lipoproteins which are the main mediators of transporting TAGs out of the liver (Coutteau et al. 1997). Therefore, phospholipid deficiency is also one of the critical factors for the formation of fatty liver.

In face of this fatty liver issue, nowadays there are several methods that are being used to try to control this superfluous fatty liver. First of all, different dietary ingredients are used to decrease the lipogenesis by regulating key enzymes which act on the TAG anabolism. Glucokinase (GK) plays a pivotal role by catalyzing glucose to glucose-6-phosphate. GK helps to transform surplus saccharides into TAG (Nordlie et al. 1999). Gilthead Sea bream *(Sparus aurata)* fed low protein/high carbohydrate diets increase the mRNA expression of GK whereas almost no expression was seen when fish fed low carbohydrate/high protein diets (Caseras et al. 2002; Meton et al. 2004). Another experiment in rainbow trout *(Oncorhynchus mykiss)* liver showed that GK activity and mRNA expression increase in proportion to the content of starch in the diet (Capilla et al. 2003). FAS catalyzes the last step in the FA biosynthesis. High protein diets can lessen the mRNA expression of the FAS gene, thereby decreasing the deposition of TAG (Clarke 1993). LPL is a glycoprotein enzyme which hydrolyzes TAGs in lipoproteins into free fatty acids(FFA) that can either be stored or used as direct energy (Auwerx et al. 1992). An experiment with red sea bream *(Pagrus major)* showed that several kinds of unsaturated FA in the diets could increase the mRNA expression of LPL, thus declining the deposition of TAGs in liver (Liang et al. 2002). In addition, the TAG catabolism can be modulated. As a carrier, carnitine transfer FAs into mitochondria for β-oxidation. An experiment of sea bream *(Paprus major)* showed that carnitine added to the diets raised the FA oxidation and lowered the content of TAG in the liver (Chatzifotis et al. 1996). Similar functions of carnitine are also found in sea bass *(Dicentrarchus labrax)* (Santulli et al. 1988).

Furthermore, increasing lipoprotein synthesis is a good way to decrease the content of TAG (Coutteau et al. 1997). As mentioned above, phospholipid is a key component of lipoproteins. In rainbow trout *(Oncorhynchus mykiss)*, phospholipids added in the feed

caused an increase of TAG transportation out of liver, thus increasing the fat mobilization and utilization (Poston 1991).

Other anti-fatty liver factors found are methionine and choline. They are found to decrease the liver lipid content in channel catfish *(Ictalurus punctatus)* (Wilson & Poe 1988). A similar effect of choline was found in juvenile red drum *(Sciaenops ocellatus)* (Craig & Gatlin 1997). Betaine, as a source of methyl groups, attends to many methylated reaction. It can also be an alternative source of choline in the diet of rainbow trout that helps to decrease TAG *(Oncorhynchus mykiss)* (Rumsey 1991). Also small amount of lysine added to the diet can lower the liver lipid level in channel catfish *(Ictalurus punctatus)* (Burtle & Liu 1994). On the other hand, excessive C-18:1 MUFAs promote the formation of lipid droplets in liver of marine teleosts (Spisni et al. 1998).

1.3 Liver development and cell differentiation

As I mentioned before, liver is one of the most important and largest internal organs related to fish metabolism. The development of the liver and liver cells critically affects the individual growth of each fish. In a zebra fish liver research, the formation of liver is described as follows (fig.5): during liver development of zebra fish, the first step is called specification. This step describes the development from endoderm cells to rod formation. The signaling molecules Wnt2bb, bone morphogenetic protein (Bmp) 2b, fibroblast growth factor (Fgf) and retinoic acid (RA) function to specify hepatoblasts. After that, the initiation step follows, and the liver starts to bud under the action of hematopoietically-expressed homeobox (Hhex) protein. Finally, the outgrowth and expansion step ends the liver development. In this last step, the differentiation of hepatocytes starts, and the liver forms and acquires functions. In the process of development, Prox1 help to promote hepatoblast migration, and digestive-organ expansion factor (Def), liver-enriched gene 1 (Leg1), and Δ113p53 play crucial roles in expansion of embryonic liver growth (Ting & Peng 2009).

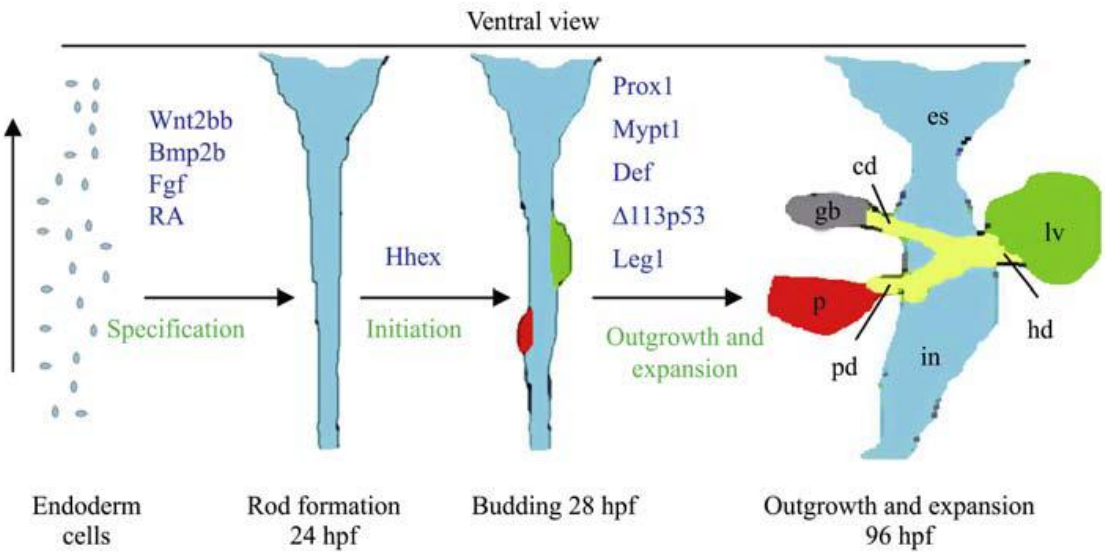


Fig.5. Ventral view of liver development in zebrafish (hpf = hours post-fertilization) (Ting & Peng 2009). lv: liver, green; p: pancreas, red; in: intestine, cyan; gb: gall bladder, gray; es: esophagus, cyan; hd: hepatic duct, yellow; pd: pancreatic duct, yellow; cd: cystic duct, yellow. A: anterior; P: posterior.

Hepatocytes are the main cell species, which accounts for approximately 80% of the volume in the whole liver. The non-hepatocyte cells include endothelial cells, Kupffer cells and fat-storing cells (Blouin et al. 1977). Hepatocytes are also responsible for the majority of the functions in liver, being the center of most metabolisms and storage of glycogen, amino acids and fat (Ting & Peng 2009). However, it is not really known what cells exist in cod liver, and which kind of cells that store the huge amount of lipid in the liver.

According to the definition from the dictionary of U.S National Cancer Institute, "cell differentiation is the process during which young, immature (unspecialized) cells take on individual characteristics and reach their mature (specialized) form and function." The hepatic differentiation can briefly be divided into 4 stages during the whole liver development process of mammals. In the beginning, the initial cell type is undifferentiated embryonic stem cells. Then they differentiate toward embryonic endoderm cells (Soto-Gutierrez et al. 2006). When the hepatic endoderm is specified and the above-mentioned liver bud start to grow, the cells in this stage are called hepatoblasts

(Lemaigre & Zaret 2004). In the last stage, hepatoblasts gradually differentiate into mature hepatocytes. Hepatocytes in different stages of hepatic differentiation vary morphologically and functionally (Cai et al. 2007; Lavon & Benvenisty 2005) .

Besides liver itself, adipose tissue also plays a pivotal role in the research of cod fatty liver since it is still not known whether the liver cells in cod maintain their liver functions when they store more and more lipids, or if they rather acquire the function of the specialized lipid storing cells of adipose tissue. Adipose tissue function primarily to safely store excessive energy in the form of TAG and reuse them during energy deprivation (Dani et al. 1997). Adipose tissue of human body consist of adipocytes, adipose precursor cells, blood cells, endothelial cells, fibroblasts, and monocytes/ macrophages (Harmelen et al. 2005). The main cell type in adipose tissue is adipocytes (Pierleoni et al. 1998). The approximate differentiation of adipocytes is separated into 4 stages. The cell type in the first stage is pluripotent stem cells. They give rise to the multipotent mesenchymal precursor cells which have potential to differentiate toward myoblasts, chondroblasts, osteoblasts and adipoblasts. Adipoblasts further differentiate to determinated preadipocytes, which through terminal differentiation finally change into mature adipocytes (Gregoire et al. 1998; Poliard et al. 1995). Above differentiation research is also based on mammalian research. However, the differentiation process of adipocytes in salmon is shown to be very similar to the one in mammals (Todorcevic et al. 2009). Hepatocyte and adipocyte differentiation processes have previously not been studied in cod, and few specific markers for the different cell types are available. However, for instance peroxisome proliferator-activated receptorβ (PPARβ) is available, and known to be an important transcription factor involved in adipocyte differentiation in salmon (Todorcevic, M. et al. 2008). Generally, the expression of genes involved in metabolism are increased the more differentiated the cells get. In this way, increased gene expression of markers for metabolism, such as glycerol-3-phosphate dehydrogenase (GPDH), glucose transporter (GLUT) family proteins and fatty acid binding proteins (FABP) can be used as a measure of degree of differentiation.

Muscle cell development has not been studied in cod either. According to research on cultivated Atlantic salmon skeletal muscle cells, the differentiation process of muscle

cells can roughly be divided into 3 stages. In the beginning of the culture, the main cell type is the un-specialized precursors called myosatellite cells which have a spindle shape. The early stages of muscle cell cultures are not homogenous but contain also fibroblast-like cells with triangular shape. In a later developmental stage, the myosatellite cells become elongated, and connect with each other and form a continuous net of multinucleated muscle cells (myotubes). When the muscle cells in culture reach confluence, the cultures are more homogenous, consisting mainly of post-mitotic muscle cells without fibroblast-like cells or myosatellite cells (Vegusdal et al. 2004). Although not known for cod, the muscle-specific factor MyoD is known to be expressed early in embryogenesis, at a time when muscle lineage decisions are established. MyoD are required for myogenic determination, whereas actin is a structural protein highly expressed at later stages of differentiation (Khaitlina 2001; Megeney & Rudnicki 1995; Rudnicki & Jaenisch 1995).

1.4 Cell culture

The definition of cell culture in Encyclopædia Britannica indicates "the maintenance and growth of the cells of multicellular organisms outside the body in specially designed containers and under precise conditions of temperature, humidity, nutrition, and freedom from contamination". Animal cell culture started from nerve fiber in frogs in 1907 (Harrison et al. 1907). Along with the first cell line "RTG-2", a gonadal cell line derived from rainbow trout reported in 1962 (Wolf & Quimby 1962), plenty of different fish cell lines had been reported (Fryer & Lannan 1994). The first teleost applied to isolated hepatocytes was goldfish in 1976 (Birnbaum et al. 1976). The *in vitro* culture system helps a lot in the hepatocyte research. According to the traditional definition, the first harvesting and subculturing of this cell population is called primary cells (Freshney 1987). Technically, isolated hepatocytes can be considered as primary cells with all the cell properties and *in vivo* functions, while avoiding other influences from whole fish. So it is relatively reasonable to set defined experimental conditions, then carry out the research

process and finally analyze and summarize the results. Besides, ethical issue also encourages us to use the isolated cells or tissue to replace the use of experimental fish (Segner 1998).

Cell culture is divided into primary culture and subculture. In our experiments, only primary cultures were applied. Enzymatic dissociation or disaggregation is a method that is used to remove the cells from the tissue and let them attach and grow in the culture container. The main steps are as shown in figure 6: tissue remove; tissue chop; digestion with proteolytic enzymes (such as trypsin or collagenase) and transfer to culture vessels with culture medium. Afterwards, cell suspension start to attach to the solid substrate and form a monolayer. Then the cells begin to proliferate and get more and more dense, finally, achieving confluence. (Freshney 2005)

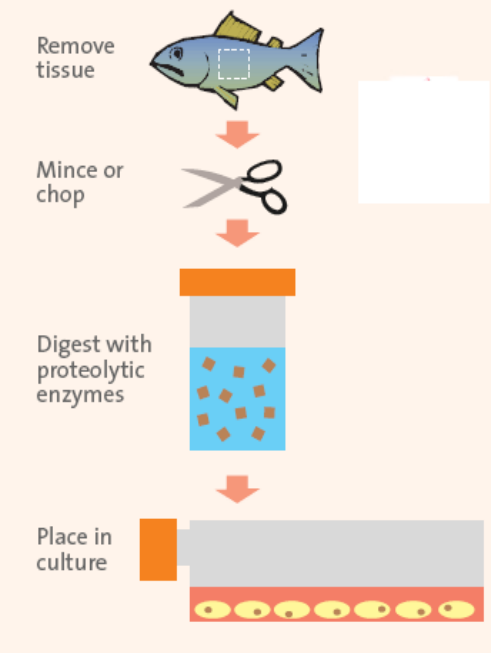


Fig 6. Enzymatic Dissociation in fish cell

(This figure is from the Technical Bulletin "Introduction to Animal Cell Culture" of Corning Incorporated Life Sciences, made by John A. Ryan, Ph.D.)

2 Aims of the studies

The major aim of this thesis was to establish the methodology for isolation and culturing of cod liver cells. With a cod cell culture model we will be able to perform detailed studies of the mechanisms which may determine liver growth and further to study how the growth is affected by dietary means.

- A subgoal was to test whether lipids and hormones (insulin) may trigger trans-differentiation of precursor cells from liver towards adipocyte-like cells.
- Another subgoal was to establish a cod muscle cell culture model, and test if unspecialised precursor cells isolated from cod muscle can proliferate and differentiate into mature multinucleated myotubes in culture.

3 Materials and Methods

3.1 Materials

Cod were obtained from Nofima (Sunndalsøra, Norway) and Norwegian Institute for Water Research (Drøbak, Norway). Metacain (MS-222) was from Norsk Medisinaldepot (Oslo, Norway). Cell flasks and cell scrapers were obtained from Nalge Nunc International (Rochester, NY, USA). Fish serum was purchased from BIODESIGN International (Saco, ME, USA). Antibiotics (mixture of penicillin, streptomycin and amphotericin B), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamin, lipid mixture, laminin, Thermanox cover slips, Hank's balanced salt solution (HBSS), phosphate buffered saline solution (PBS), Leibowitz-15 (L-15), albumin-solution, formalin, fetal bovine serum (FBS), ethylene diamine tetra-acetic acid (EDTA), sodium bicarbonate solution, Tween-20, 3,3′,5-Triiodo-L-thyronine sodium salt (T3), transferrin, dexamethasone, 3-Isobutyl-1-methylxanthine (IBMX) and Triton x-100 were all supplied from Sigma-Aldrich (St. Louis, MO, USA). Trypsin-EDTA solution was bought from SAFC Biosciences Ltd. (Hampshire, UK). Collagenase (type I, 220 U/mg) was obtained from Worthington (Lakewood, NJ, USA). Chloroform, methanol and acetic acid were obtained from VWR International (West Chester, PA, USA). Sodium chloride, sodium hydroxide, hexane, β-ME and thin layer chromatography (TLC) plates (silica gel 60) were all purchased from Merck (Darmstadt, Germany). Whatman filter paper was obtained from Schleicher & Schuell (Dassel, Germany). Nylon filters 250/100 were obtained from Sefar AG (Heiden, Switzerland). RNeasy Mini Kit, QIAshredder columns and RNase-free DNase I were purchased from Qiagen (Valencia, CA, USA). TaqMan® Gold RT-PCR Kit was bought from Applied Biosystems (Foster City, CA, USA). Insulin, ProLong Antifade Reagent, Alexa-647 Goat-anti-Mouse IGg-2b antibody and the primers for RT-PCR analysis were obtained from Invitrogen (Carlsbad, CA, USA). The radiolabeled FAs [1-^{14}C] 16:0 were obtained from American Radiolabeled Chemicals, Inc. (St.Louis, MO, USA). The lipid marker pen was obtained from Electron Microscopy

Science (Fort Washington, PA, USA). Anti-human FATP1 (MAB3304) was obtained from R&D Systems, MN, USA)

Liquid scintillation counter Model 1900 TR TRI-CARB Liquid Scintillat Analyzer was obtained from Packard Instrument (Downers Grove, IL, USA). Radioactive detector A-100 was obtained from Radiomatic Instrument & Chemicals (Tampa, FL, USA). NanoDrop 1000 Spectrophotometer was obtained from NanoDrop Technologies, Inc (Wilmington, USA). Zeiss AxioVision microscope was obtained from Carl Zeiss, Inc (Göttingen, Germany). LightCycler® 480 Real-Time PCR System by Roche Applied Science (Mannheim, Germany). CAMAG TLC system was bought from CAMAG Scientific Inc. (Wilmington, N.C, USA). Bioscan AR-2000 system was obtained from Bioscan, Inc (Washington, DC, USA).

3.2 Isolation and culture of cod liver cell

Cods (of approximately 800 gram fish) were obtained from Norwegian Institute for Water Research (NIVA). They were fed a commercial diet prior to the trial. No protocol exists for cod liver cell isolation, so we based our isolation on a protocol for isolation of salmon adipocytes (Todorcevic, M. et al. 2008). We hypothesized that this was the most similar tissue to cod liver because of their high fat level. The fish were anesthetized with MS-222, and then the fish were killed by a blow to the head. After that the abdomen was cut open to expose the liver tissue, which was carefully excised in order to avoid contamination with intestinal contents. Then we put the liver tissue into tubes (approximately 20 g tissue per tube) with HBSS. The tissue were kept on ice and cut into smaller pieces with an autoclaved scissor. Total amount of dissected liver tissue we used were approximately 300 g (approximately 55 gram liver tissue per fish). Firstly, the tissue were centrifuged at 1250 rpm for 5 min at 4°C to get rid of the blood; by transferring everything, except the blood which stayed in the bottom layer, with a spoon to a new 50 ml sterile Falcon tube. The tubes were filled up with HBSS and centrifuged again. Subsequently, that the top-layer (tissue) was transferred to Erlenmeyer bottles with 0.25%

trypsin-solution (150 gram tissue / 550 ml trypsin). Then they were left on a shaking board for 30 min at 11°C.

Thereafter, 50 ml stop-solution (HBSS with 25% FBS) were added to stop the reaction. Then the tissue solution was filtrated through a 100 µm filter. The filtrate was centrifuged at 2000 rpm for 10 min at 4°C. Then the supernatant was thrown away, and the pellets were solved in growth-media containing L-15, 5% FBS, 1% glutamine, 1% HEPES, 0.5% bi-carbonate and 1% antibiotics (mixture of 10000U penicillin, 10 mg/ml Streptomycin and 250 ug/ml Amphotericin B). Finally, the ultimate solution were plated out into laminin pre-coated 6-well cell plates (diameter of 34.8 mm) and glass-bottom cells dishes which had been pre-coated with 15 µl laminin and dried for 1 day. Approximately 1.5 gram of tissue was seeded out per ml of growth media.

After seeding, the cells were incubated at 13°C during the entire period. Most cells were attached to the laminin on the next day and they were carefully washed with L-15 medium every 3 days and cultured in growth media.

3.3 Isolation and culture of cod muscle cell

Cod (approximately 25 g per fish) for muscle cell isolation were obtained from Nofima (Sunndalsøra, Norway). The isolation procedure was based on the protocols used for isolation of muscle cells of other fish species (Koumans et al. 1990; Matschak & Stickland 1995; Vegusdal et al. 2004). A total of 30 g muscle tissue was taken from the upper part of the lateral area of 15 fish. Then, tissue was put into 50 ml tubes in L-15 medium and cut into pieces. Further, the tissue was centrifuged at 300 g for 5 min at 4° C, and the supernatant thrown away. The pellet was re-suspended gently in 30 ml of L-15 with 1% Antibiotic-Antimycotic. After that, they were centrifuged again at 300 g for 5 min at 4°C and the supernatant was discarded. This washing procedure was done twice.

Afterwards, 90% L-15 including Antibiotic-Antimycotic (1%) and collagenase (5 ml / 1 g tissue) was prepared. The tissue was added to the collagenase solution in a sterile E-

flask with magnet and placed in a polystyrene box in 11°C water on a magnet stirrer for gentle stirring for 90 min. Subsequently, the tissue was transferred to new 50 ml tubes and centrifuged at 300 g for 5 min at 4°C. The supernatant was poured off and tissue washed twice, as described above. Then the pellet was re-suspended in 0.1% trypsin (4 ml / 100 ml L-15) in 90% L-15 with Antibiotic-Antimycotic (1%) in a sterile E-flask with magnet and placed in a polystyrene box in 11°C water on a magnet stirrer for gentle stirring for 30 min.

Afterwards, the suspension was centrifuged for 1 min at 300 g at 4°C. 30 ml of the supernatant were poured into a new 50 ml tube and mixed with 15 ml ice-cold stop-solution (L-l5 with 25% FBS and Antibiotic-Antimycotie). All resulting cell suspensions were spun down at 4°C and 300 g for 15 min. The supernatant was carefully poured out and the pellet re-suspended in L15 / Antibiotic-Antimycotic. The re-suspended cells were filtered through a 100 μm filter, followed by a 50 μm filter and finally a 20 μm filter. The cell suspension was centrifuged for 15 min at 300 g at 4°C and supernatant was carefully removed. Pellets were re-suspended in L-15 growth medium containing 10% FBS and centrifuged for 15 min in 300 g at 4°C. The supernatant was discarded.

Finally, the cells were re-suspended in muscle cell growth media (Leibowitz-15, 10% FBS, 1% Hepes, 1% Antibiotic-Antimycotic) and seeded out into laminin pre-coated 6-well cell plates (diameter of 34.8 mm). Cells were washed with L-15 and given new muscle growth media every 3 days.

3.4 Differentiation of cod liver cells

To test if the liver cells isolated can proliferate and differentiate into a mature cell type similar to adipocytes, we tried to stimulate the cells with different differentiation media. We used differentiation conditions previously described for salmon adipocytes (Todorcevic, M. et al. 2008). On day 7 after seeding, when cod liver cells had reached confluence, they were treated with either differentiation mix (12.5 μM IBMX, 0.5 μM

dexamethasone, 10 µg/ml Insulin, 7.5 ug/ml Transferrin, 5 µM T3 , 2.5% FBS, 2.5% fish serum, final concentrations in cell media) or lipid mix (1 µl/ml; corresponding to 4.5 µg/ml cholesterol, 10 µg/ml cod liver oil FAs (methyl esters), 25 µg/ml polyoxyethylene sorbitan monooleate, 2 µg/ml D-α-tocopherol acetate and insulin (5 µg/ml)), or left untreated. After 2 days of treatment (day 9), cells were washed twice and given new treatment. Control cells and cells on insulin and lipid mix were given the same treatment at day 7. Cells on differentiation mix were given lipid mix and fish serum. The treatments continued for 10 more days (until day 19), with washing and changing of media every 3 days.

3.5 RNA isolation, purification and cDNA synthesis

RNA was isolated from cod liver cells and cod muscle cells. For liver cells, RNA was isolated at 4 stages during cell development, at day 2, 7, 9 and 19. For muscle cells, RNA was isolated at 2 stages during cell development, at day 2 and day 9.

The cells were first washed twice with PBS. RNA isolation was carried out by using an RNeasy Mini Kit. First of all, the cells were added 600 µl RLT buffer to each well. RLT buffer (lysis buffer for disrupting and denaturating cells) was mixed with 1% β-ME which helps to inhibit RNase. Further, the cells were scraped off and transferred to QIA-shredder columns. The QIA-shredder columns were centrifuged at 13000 rpm for 2 min, and then the lysate, which load onto the upper part of the columns, was thrown away. Thereafter, 1x volume (600 µl) with 70% ethanol was added to the suspension in the bottom of the tube. The solution was mixed and transferred to Rneasy columns. After 15 seconds of 13000 rpm centrifugation, the bottom part of the tube was thrown away. Further, Dnase mix was made by mixing 10 µl Dnase I + 70 µl RDD buffer per sample (without vortex). This Dnase mix was added to each tube and incubated for 15 min in room temperature. 350 µl RW1 buffer (wash buffer) was added and centrifuged at 13000 rpm in 15 seconds. The bottom part of the tube and its content was discarded. Then a new tube was added on the bottom part, 500 µl RPE buffer with ethanol (wash buffer) was

added. After 15 seconds of 13000 rpm centrifugation, the content in the bottom part was thrown away. Then 500 µl RPE buffer with ethanol was added again. After 2 min centrifugation at 13000 rpm, the bottom part of the tube and its content was discarded. A new tube was again added on the bottom part, and centrifuged at 13000 rpm for 1 min. The content in the bottom part was thrown away. Subsequently, the upper part of the column was moved to an eppendorf tube and eluted with 40 µl Rnase free water. Samples were centrifuged at 13000 rpm for 1 min. The same elution process was done again by transferring the content back over the column and centrifuged once more. DNA was removed by using an RNase-free DNase Set during the RNeasy procedure.

After RNA isolation, the concentration of each RNA sample was tested by spectrophotometry (NanoDrop 1000 Spectrophotometer). If the RNA was contaminated (260/230 < 1.75), RNA purification was necessary. Upon purification, 0.1 x volumes of 3 M Na-Acetate and 3x volume of 99% ethanol were added to the RNA sample (40 µl each) and incubated in room temperature for 30 min. Then, the RNA samples were centrifuged at 10000 g in 4°C for 10 min. Afterwards, the supernatants were removed, and the samples were dried in room temperature. Finally, 30 µl of RNase-free water was added to each sample. Then all samples were tested again by NanoDrop 1000 Spectrophotometer.

To synthesize cDNA from muscle cell RNA, 0.5 µg of RNA of each sample was reverse-transcribed by using a TaqManR Gold RT-PCR Kit. All processes were carried out in accordance with following procedure: 0.5µg of total RNA was used in a 50 µl reaction with a final concentration of 1×TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM each of dNTP, 2.5 µM oligo $d(T)_{16}$, 0.4 U/µl RNase inhibitor, and 1.25 U/µl reverse transcriptase. The cDNA synthesis was performed with a 10 min primer incubation step at 25 °C, a 60 min RT step at 48 °C, and 5 min of RT inactivation at 95 °C.

cDNA from liver cell RNA was made by using a 96-well High Capacity RNA-to-cDNA system (ABI) with mixed primers. cDNA was diluted 1:50 before continuing to the real-time quantitative polymerase chain reaction (RT-qPCR).

Vector NTI Advance 10 (Invitrogen) or Roche Diagnostics primer design software was used to design real time PCR primers (table 1) based on available cod sequences in the GenBank.

Table 1

Primers for quantitative real-time PCR analysis

Genes	Accesion no.	Primer forward	Primer reverse	Amplicon size
Ppar b	FG 324860	aaggaagcccttcagtgaga	tccaactccagagcattgaac	72
Glut4	DQ 109810	cgatgggatggaggagaac	gcatctcttccggcttga	60
Glut2	AY 795481	tcttggtggccctcttga	aaaagattgcgttgataccagag	60
Afap	EB 677062	cagtcaccaccggcactaa	ggtgacggtttcaaggtcag	74
Gpdh	AY 635584	gctccaaagaaagtgtgcatc	ccaatgatcttggcaatgg	65
Fabp6	EB 677060	gggcgtgactacaagatggt	gggtccaggtgaagtcctc	61
annexin A2	EB 677089	ctccacagaccccgacag	tgaaaagattgccaaaaaggat	64
annexin III	CO 542785	actgtcgggagatcatcagc	ccgtcaggacactttctgtg	60
MyoD	AF 329903	gacaggaggacacttacttccaggtg	gtacatgggggaccgttgaaatc	118
MLC	EB 677090	gaaaacgtgagggtgacaaacagg	cgcaaacggctgcatcaatt	115
α-actinin	AF 500275	cagttacgtgattcaccaggaagagg	caagtgggagttacaccatgctgtg	130
EF1a	DQ 402371	caggtcatcatcctgaacca	atccaggactggggcatag	60

F = forward, R = reverse, E=efficiency, ppar beta = peroxisome proliferator-activated receptor, glut4 = glucose transporter 4, glut2 = glucose transporter 2, afap = adipocyte fatty acid binding protein, gpdh = glycerol-3-phosphate dehydrogenase, fabp6 = fatty acid binding protein 6, MyoD = Myogenic Differentiation 1, MLC = myosin light chain, EF1a = Elogation factor 1 alpha.

3.6 Real-time PCR and data analysis

Real-time PCR was performed with the LightCycler® 480 Real-Time PCR System and gene specific primers. The PCR Master mix consisted of 1 μl 0.5 μM reverse and forward primer, 5 μl LightCycler 480 SYBR Green I Mastermix, 4 μl 1:10 diluted cDNA template samples. Standard curves for each primer pair were calculated by serial dilution of cDNA and primer efficiencies were calculated from these. The conditions of PCR reaction were 95°C for 5 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in duplicate. A melting curve analysis (95°C for 5 seconds and 65°C for 1 min, 97°C) was run to confirm the presence of a single PCR product.

The relative gene expression level was determined by using the Relative Expression Software Tool (REST) (Pfaffl et al. 2002). Mean values were used in REST. The REST software was based on the formula:

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

The gene expression levels were normalized towards the reference gene EF1α. Normalized gene expression of stage 1 was set to 1, and the rest of the expression of each target gene for the other stages was expressed relative to stage 1, which is made in terms of fold induction principle.

3.7 Statistics

All data, except for gene expression data, were subjected to one-way analysis of variance (ANOVA). Differences were ranked by Duncan's multiple range test. We used the software package UNISTAT (London, England). The significance level was set at 0.05.

3.8 Immunocytochemistry

Cod liver cell samples (controls, at confluent stage) were firstly washed twice in PBS and fixed in formalin (4%) for 10 min. Subsequently, they were washed in PBS and dehydrated in series of ethanol (25%, 50% and 75%, 5 min each). Finally, samples were stored at -20°C in 75% ethanol until further analysis. Then, the samples were rehydrated in 50% and 25% ethanol and dH_2O each for 5 min in a row, and then briefly washed 3 times in PBS.

The bottom of the cell flasks were cut out. We used a lipid marker pen to divide the flask bottom into 4 parts. The cells were further covered with PBS-Triton-X100 (0.1%) for 15 min, then briefly washed twice in PBS followed by a wash in PBS for 5 more minutes. After that, the cells were blocked with 5% skimmed milk for 1.5 hour. After discarding the skimmed milk, the cells were incubated with the primary antibody anti-human FA transport protein 1 (FATP1) Mouse IGg-2B (100 µg/ml; diluted 50X in PBS with 0.05% Tween-20 and 2% skimmed milk) overnight at 4°C in a humid chamber. The next morning the cells were washed 3 times in PBS, 15 min each time.

Then, the cells were incubated with secondary antibody, Alexa-647 Goat-anti-Mouse (2 mg/ml) (diluted 300X in PBS with 0.05% Tween-20 and 2% skimmed milk), and DAPI (2 µl/ml) for 1.5 hours. Subsequently, cells were washed twice in PBS with 0.1% Tween-20 for 15 min. From the secondary antibody, the work was done in dark circumstances and covered with hood while incubating. Finally, the cells were mounted in ProLong Antifade Reagent and stored in fridge.

3.9 Microscopy

The cod liver cells and the cod muscle cells were followed microscopically by a Zeiss AxioObserver microscope. Phase contrast micrography was used approximately every

second day with 10X and 40X magnification to see and picture the change of the cells before and after the treatments. Fluorescence microscopy was also used for detection of the FATP immuno-staining.

3.10 Preparation of radioactive substrates of 16:0 (palmitic acid)

2 mM palmitic acid (PA) substrate was made. 0.1 gram non-radioactive PA was dissolved in 25 ml of chloroform, giving a stock solution of 15.6 mM PA. Further, 256.4 µl of the stock solution was taken out and evaporated under N_2 gas to get rid of the chloroform. The radioactive PA (25 µCi) was added to the vial containing the dried non-radioactive PA and dried under N_2 gas. 400 µl 0.1 M NaOH (pre-heated to 60°C) was added to the PA. Then this NaOH/PA solution was transferred droplet by droplet to a tube with 1.6 ml albumin-PBS solution (pre-heated to 60°C, albumin: FA (1: 2.7)). The blended solution should not have any sediment. In the end, pH was adjusted to 7 by adding HCl. The specific radioactivity in the substrate solution was 5.56 Ci / mol PA. Finally, the substrate solution was kept in the freezer at -20°C.

3.11 Incubation of cod liver cells with ^{14}C-palmitic acid

After 7 days in culture, when confluence was reached, 6 wells of cod liver cells were incubated with radioactive 16:0 for 48 hours. 3 wells were given a final 16:0 concentration of 20 µM, and another 3 wells were given a final 16:0 concentration of 200 µM. In addition we gave 2 wells a non-radioactive 16:0 solution (final concentration of 200 µM) as a control.

3.12 Lipid extraction from cod liver cells

After 9 days in culture, the cod liver cells were washed twice in PBS (with 5% albumin) and then scraped off the plates with 0.5 ml PBS. Cells were pelleted by centrifugation (13000 rpm, 1 min). Folch extraction method (Folch et al. 1957) were used for extracting total lipids from the cells. First, the samples were added 9 ml of chloroform: methanol (2: 1) solution, mixed, and then added 2 ml of 0.9% saltwater (NaCl) solution in a test tube. Afterwards, the mixed solution started to separate in two layers. The top layer was the water soluble phase, which mainly consisted of water and methanol and should be discarded. The bottom layer was the lipid phase, which mainly included lipids and chloroform and was used for the further analysis. The test tube was placed in fridge overnight for further separation. The next day, the water phase was pipette off and 6.6 ml lipid phase was transferred and divided into 2 new tubes (each for 3.3 ml). One was used for the lipid class composition analysis and the other one was used for the saturated and monounsaturated radiation analysis. The chloroform in the lipid phase was evaporated off by heating under N_2 –gas. The residual lipid extract was dissolved in 50 µl chloroform per tube. Further, the samples were methylated overnight by incubation with 2 ml benzene, 2 ml methanolic-HCL and 200 µl 2, 2-di-methoxy-propane at room temperature as described by Mason and Waller and by Hoshi (Hoshi et al. 1973; Mason & Waller 1964). The next day 2 ml hexane was added and the samples were neutralized by $NaHCO_3$. Then the benzene/hexane phase which included methylated lipids was collected, dried at 60°C and re-dissolved in 50 µl chloroform.

3.13 Analysis of lipid classes of cod liver cells

The composition of total lipid classes was analyzed with thin layer chromatography (TLC). Glass, high pressure TLC plates (20×10 cm; pre-coated silica gel 60; 0.20 mm layer) used in this analysis were firstly pre-developed to full length with hexane: diethyl

ether; acetic acid (85:15:1) as mobile phase and dried in the air, then they were activated at 110°C for 1 hour and finally placed in a vacuum desiccators for further use.

The total lipid extract from the cod liver cells, (prepared as described above) was applied onto the TLC plate with a TLC sampler 4 (CAMAG LINOMAT 5). The working gas was N_2 gas. The dosing amount of each track was 50 μl and the distance between tracks was 10 mm. After sample application, the TLC plate was placed into a Twin through Chamber 20×20 and the lipids were separated by using 25 ml hexane: diethyl ether: acetic acid (85:15:1) as mobile phase. The gas saturation environment was provided by placing a piece of filter paper in the chamber. The TLC plate was taken out from the chamber when the developing solvent developed to 7 cm from the starting point, and the plate was dried in room temperature. When dried, the TLC plate was dipped into a glass tank with 3% copper acetate and 8% phosphoric acid, followed by heating the plate at 130°C for 4 min. During this process, the belts on the silica gel gradually visualized. Finally, the TLC plate was placed into the TLC Scanner 3. Total lipid classes were identified in comparison with commercial standards and quantified by scanning densitometry using TLC Scanner 3. The scan lines were analyzed using an integrator (WinCats-planar Chromatography, version 1.3.3).

3.14 Radiation measurement

The radiation measurements were divided into two parts: the radioactive ratio between lipid classes (Phospholipids, FFAs and TAGs) and the radioactive ratio between saturated and monounsaturated FAs.

For the measurement of radioactive ratio between lipid classes, the above TLC plate was measured by Bioscan AR-2000 radio-TLC Imaging Scanner and the results were shown from the radio-chromatogram. The different positions of peaks in each sample were compared with commercial standards. According to the peak net area in the radio-

chromatogram of different lipids, the radioactive ratio in phospholipids, FFAs and TAGs could be calculated.

For the measurement of radioactive ratio between saturated and monounsaturated FAs, another part of the cell lipid extract was used. These samples were hydrolyzed and methylated before application to TLC plates as described above. However, these silical gel plates used here were impregnated with silver nitrate (4% silver nitrate in methanol/water 9:1, v/v). Further, the same TLC system was used as already described except the mobile phase used to separate saturated from monounsaturated FAs were toluene/ ethyl acetate (90:10, v/v). Finally, the plates were analyzed by Bioscan AR-2000 radio-TLC Imaging Scanner. For further accurate results, these samples were in addition analyzed by liquid scintillation counter. The silica powders of different samples were scrapped manually from the TLC plate and collected in scintillation bottles. 5 ml of scintillation liquid was added to each bottle, and samples were measured.

4 Results

4.1 Morphology of cod liver cells and cod muscle cells

4.1.1 Cod liver cells

Cell cultures from liver tissue in cod were successful (fig. 7-18). The morphology of the cells varied between the different stages and different treatments. In the beginning (day 5), when the cultures reached confluence, most of the cells appeared oval in shape. We wanted to test whether these cells have the same potential as stem cells to differentiate. After some days in culture (day 7), more and more cells stretched to spindle shaped or fibroblastic-like structure and started to produce lipid droplets at the same time. Some of the cod liver cells clumped together and produced some large lipid cluster. After one week, the cells which were treated with differentiation mix (for 2 days), followed by lipid mix showed that some cells converted back to round or oval in shape, the others were still stretched as before. The cells treated with only lipid mix kept their stretched shape. At day 12, distinct differences between the cells which were treated with only lipid mix and cells treated with differentiation mix (followed by lipid mix) appeared. Most of the cells which were treated with differentiation mix (followed by lipid mix) changed into oval or round in shape, but the ones treated with only lipid mix kept their stretched shape. At day 19, the control cells tended to be particularly long and extended, and almost no oval or round ones existed. Cells which were treated with differentiation mix (followed by lipid mix) kept their oval or round shape and cells given only lipid mix gathered together and stuck to the lipid clusters.

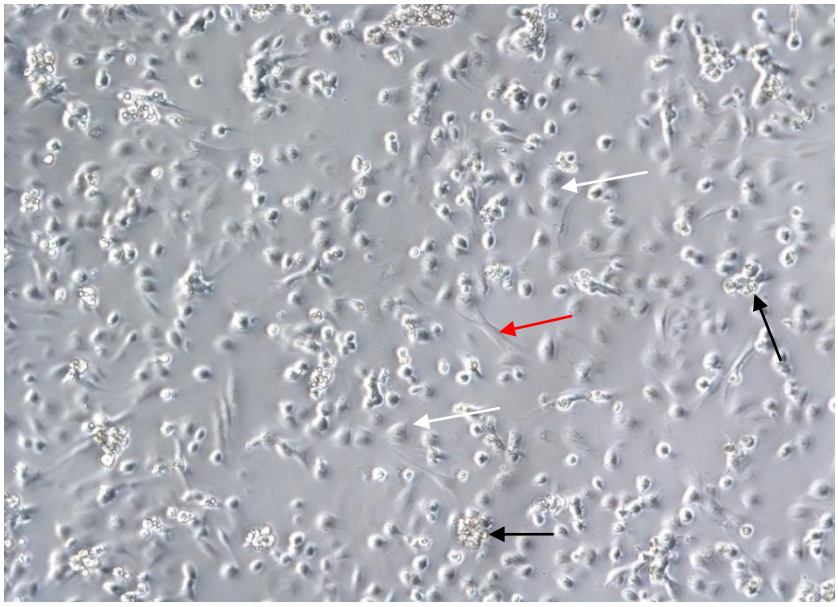


Fig.7. Morphology of cod liver cells. This light micrograph is taken at day 5 after cell seeding (10X magnification). We see cell we expect are mature hepatocytes, appearing as golden droplets (black arrow) and cells we expect are un-mature liver cells are round and oval in shape (white arrow), and there are some fibroblastic-like cells in between (red arrow).

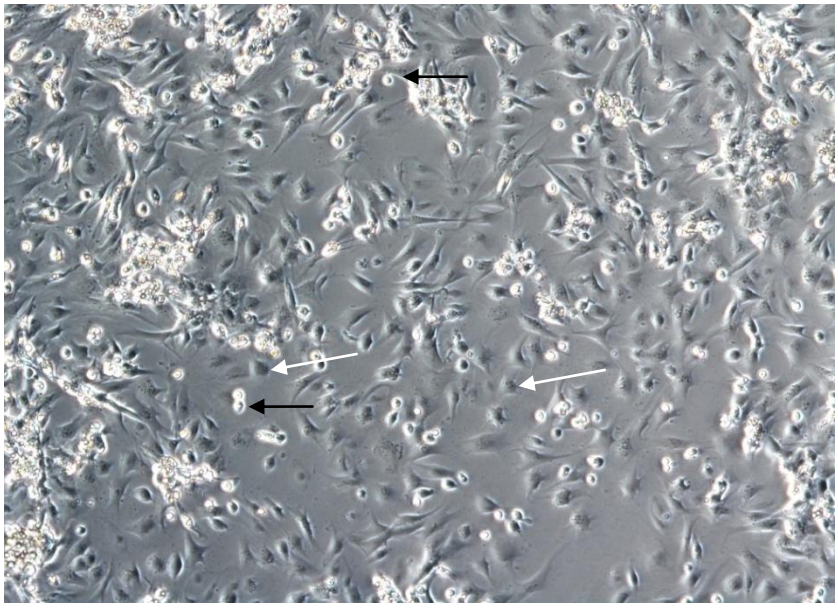


Fig.8. Morphology of cod liver cells. This light micrograph is taken at day 7 after cell seeding (10X magnification). We see cells that we expect are mature hepatocytes, appearing as golden droplets (black arrow). The cells we expect are un-mature liver cells (white arrow) start to stretch and connect with each other.

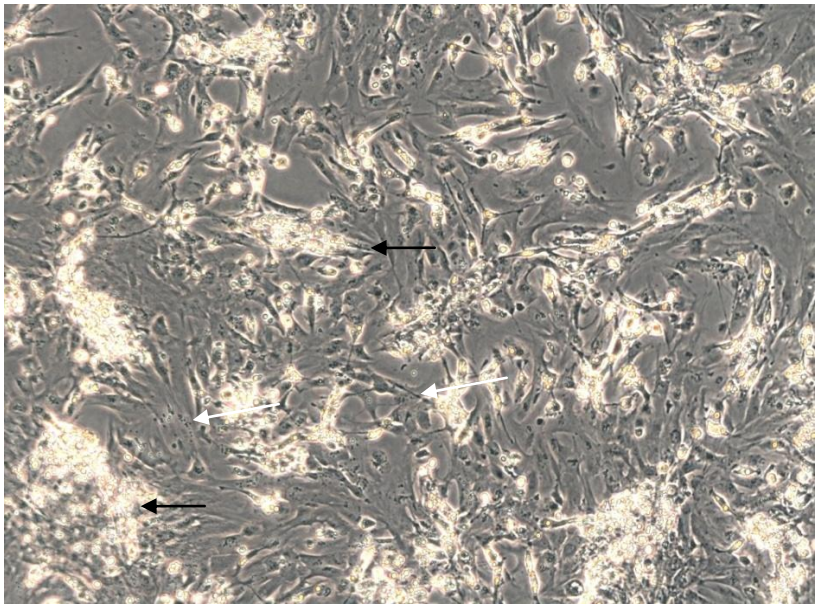


Fig.9. Morphology of cod liver cells. This light micrograph is taken at day 9 after cell seeding (10X magnification) Control cells. Large amount of lipids are present in the mature cells (black arrow). The cells we expect are un-mature liver cells get more and more stretched and spindle shaped in structure (white arrow).

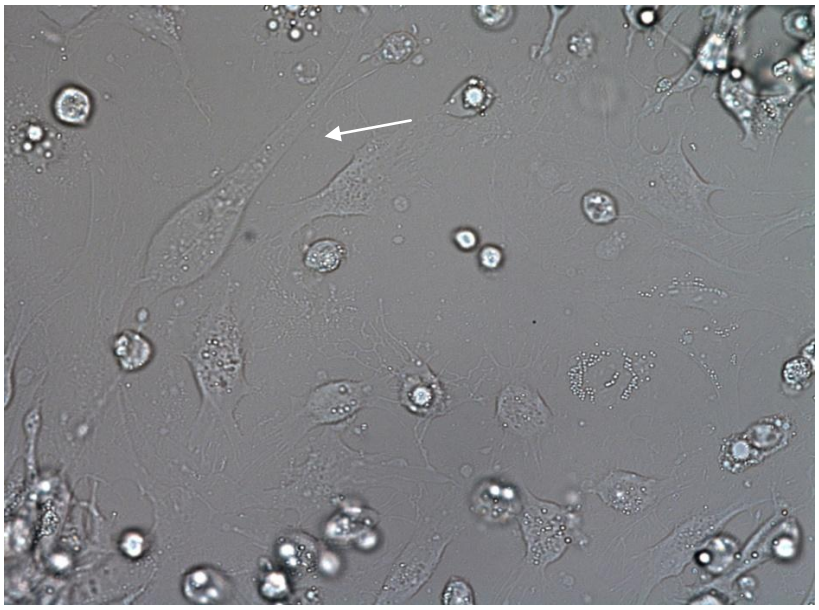


Fig.10. Morphology of cod liver cells. This light micrograph is taken at day 9 after cell seeding (40X magnification) Control cells. The stretched structure of the cells is very clear (white arrow). Lipid droplets are visible in the cells.

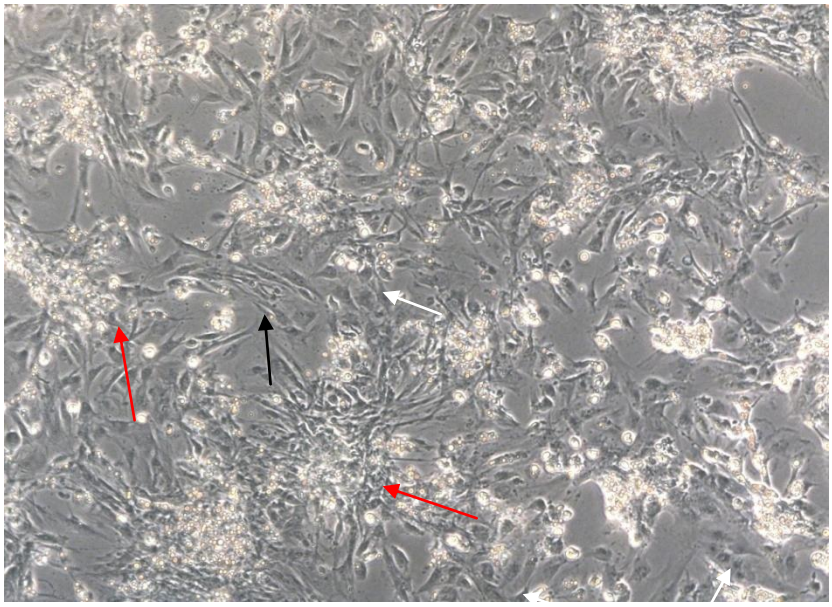


Fig.11. Morphology of cod liver cells. This light micrograph is taken at day 9 after cell seeding, day 2 after differentiation mix were added (10X magnification). Some cells convert back to round in shape (white arrow), the others keep stretching (black arrow). Many stretched cells seem to be formed from the cell clusters with high level of lipid (red arrow).

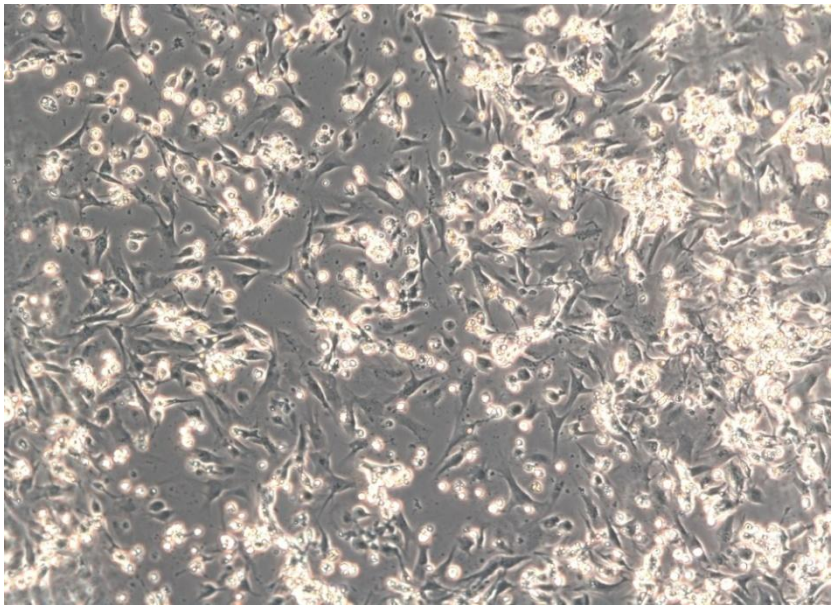


Fig.12. Morphology of cod liver cells. This light micrograph is taken at day 9 after seeding, day 2 after lipid mix were added (10X magnification). Most of the cells keep stretched in shape.

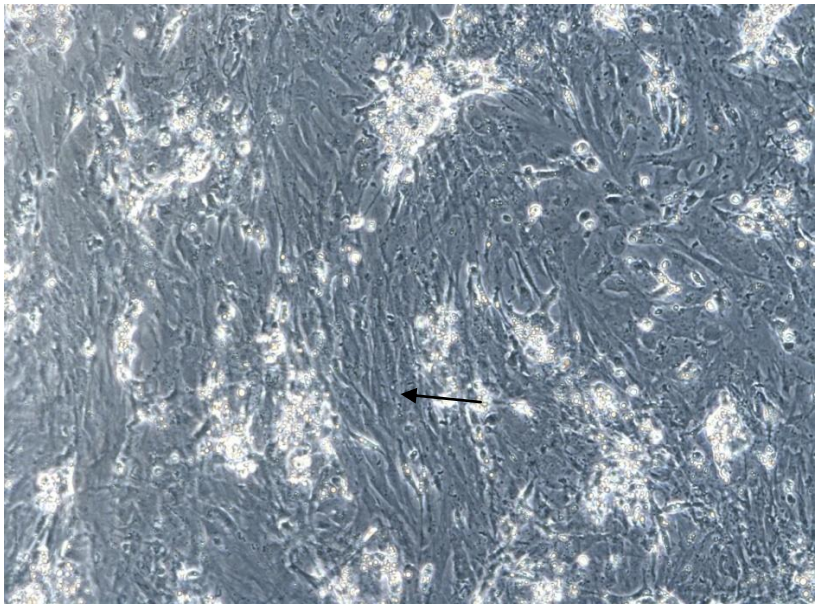


Fig.13. Morphology of cod liver cells. This light micrograph is taken at day 12 after seeding (10X magnification) Control cells. Most of the cells turn to fibroblast-like structure (black arrow).

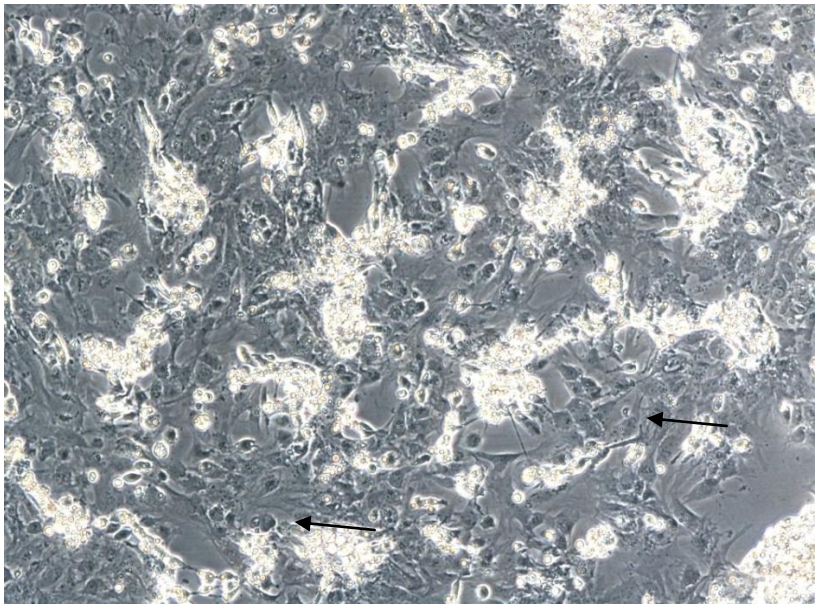


Fig.14. Morphology of cod liver cells. This light micrograph is taken at day 12 after seeding, day 5 after differentiation mix were added (10X magnification). Most of the cells tend to be round and oval in shape (black arrow). They get similar to mature adipocytes in shape.

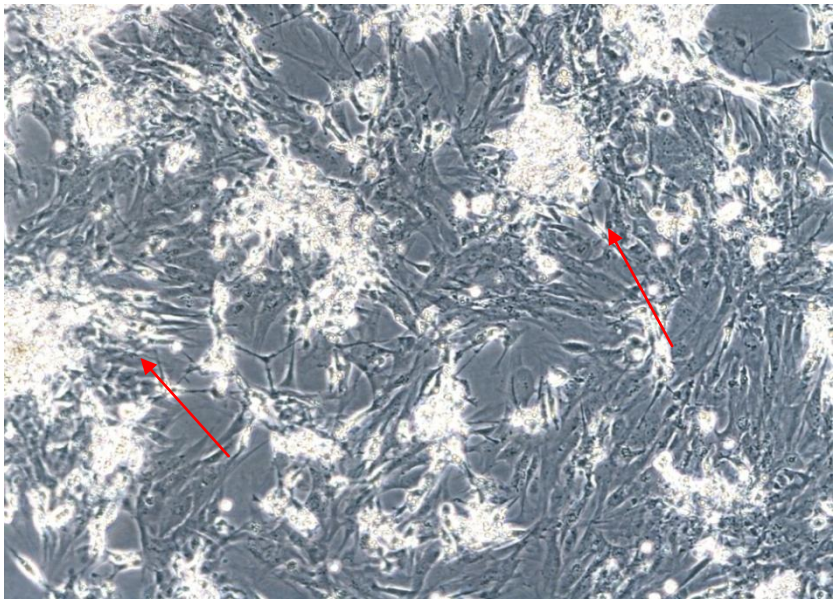


Fig.15. Morphology of cod liver cells. This light micrograph is taken at day 12 after seeding, day 5 after lipid mix were added (10X magnification). Most of the cells are stretched in shape and some seem to be formed from the lipid clusters (red arrow).

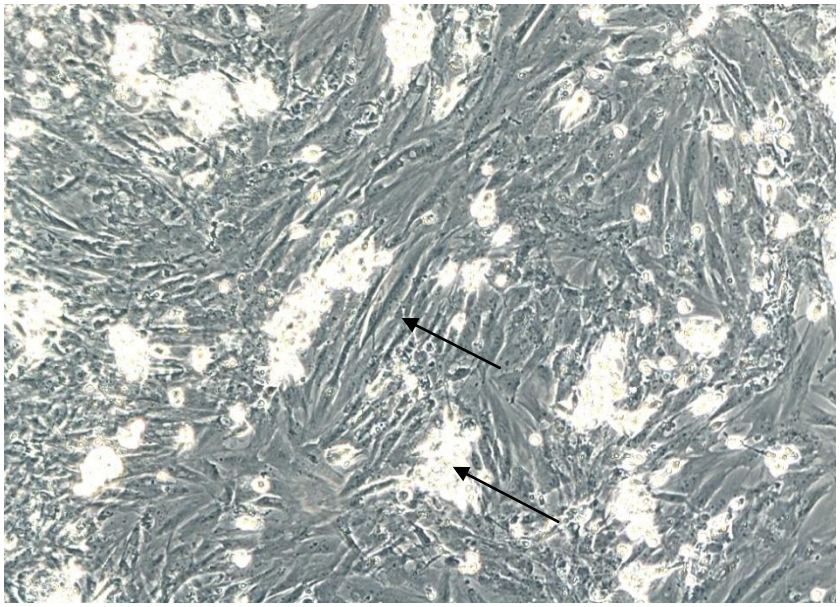


Fig.16. Morphology of cod liver cells. This light micrograph is taken at day 19 after seeding (10X magnification) Control cells. Cells keep stretchingto an extreme spindle shaped structure (black arrow).

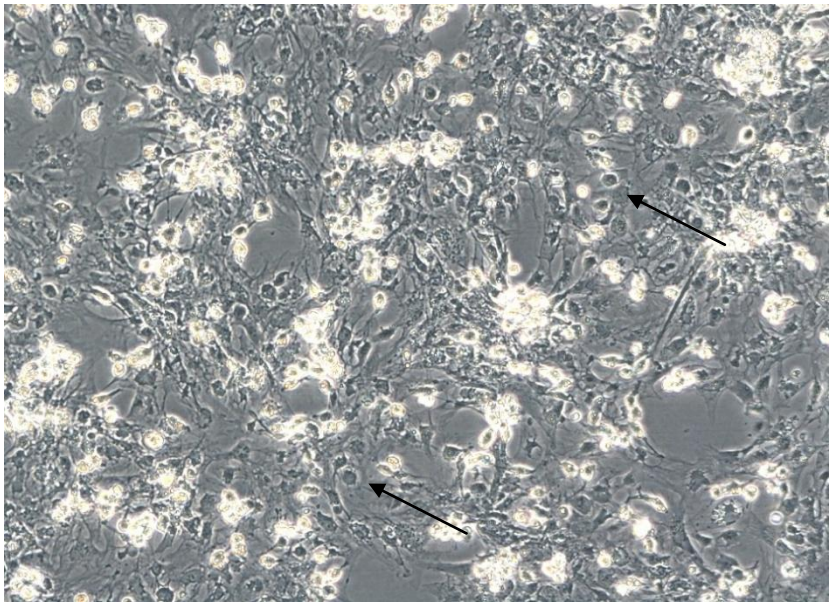


Fig.17. Morphology of cod liver cells. This light micrograph is taken at day 19 after seeding, day 12 after differentiation mix were added (10X magnification). Cells keep round and oval in shape (black arrow).

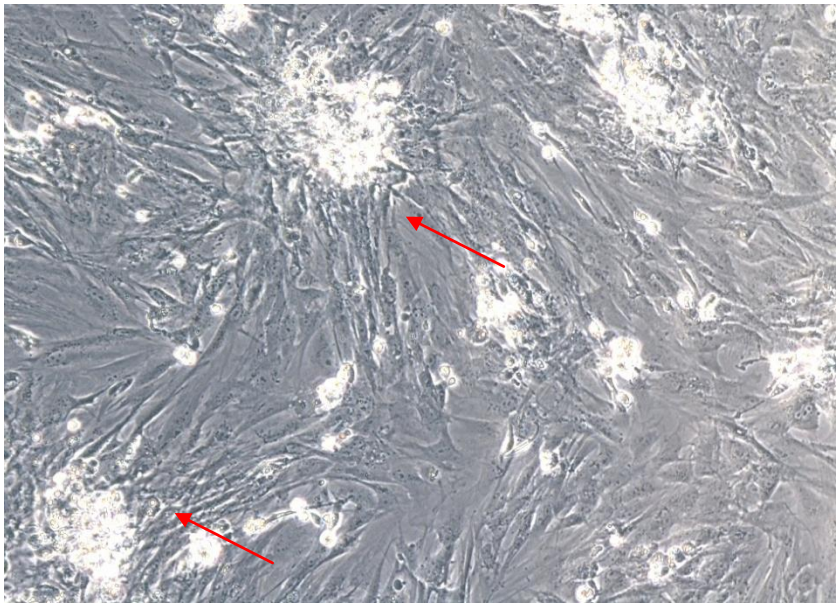


Fig.18. Morphology of cod liver cells. This light micrograph is taken at day 19 after seeding, day 12 after lipid mix were added (10X magnification). Most of the cells are extremely stretched in shape and gather together and stick to those lipid clusters (red arrow).

4.1.2 Cod muscle cells

Cell cultures from muscle tissue in cod were very successful (fig. 19-23). The morphology of the cells varied distinctly between stages. At early stages (day 2), un-mature myosatellite, muscle cells were typically spindle-shaped. We also had more triangular shaped cells that were considered to be fibroblasts. During development, more and more cells get elongated, and some start to fuse with neighboring cells. They seem to continue to elongate, and start to organize. The spindle-shaped un-mature cells gradually disappear. After two weeks in culture (day 15), the muscle cells has reached a mature stage, and almost all the cells are elongated, organized and form myotube structures.

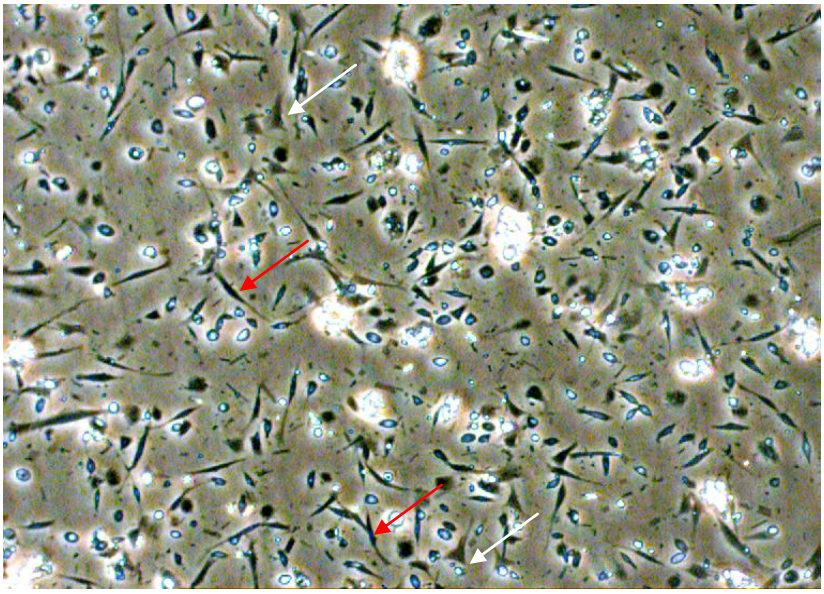


Fig.19. Morphology of cod muscle cells. This light micrograph is taken at day 1 after seeding (10X magnification). Un-mature muscle cells (myosatellites) are spindel-shaped (red arrow), and there are some fibroblastic-like cells show a triangular shape in between (white arrow).

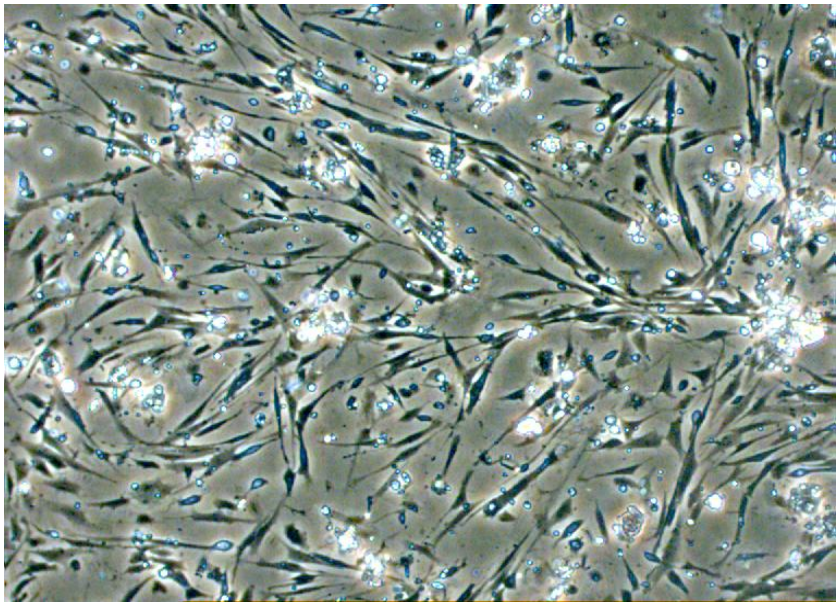


Fig.20. Morphology of cod muscle cells. This light micrograph is taken at day 4 after seeding (10X magnification). More and more “un-mature”, myostallite cells get stretched, elongated and spindle shaped in structure. Some cells seem to fuse with neighboring cells. Still some round cells are found in the culture.

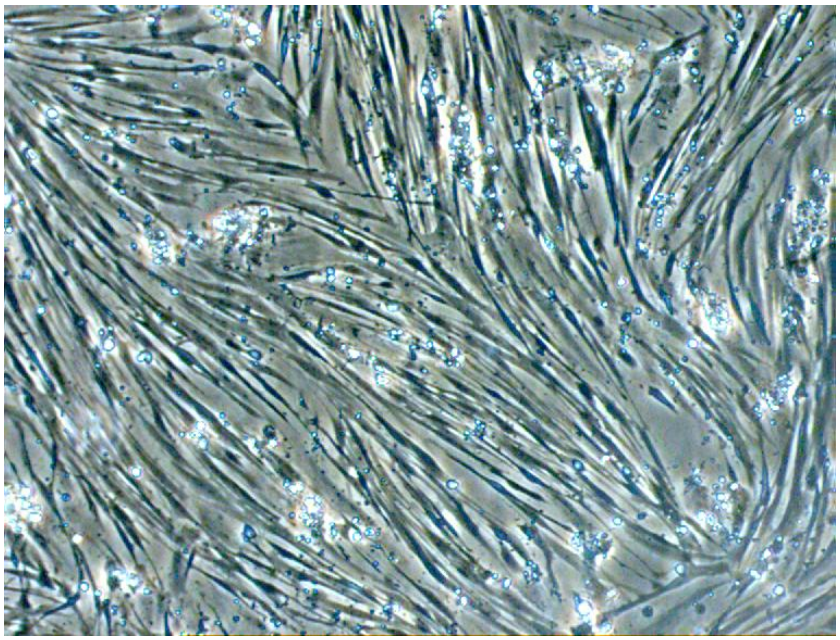


Fig.21. Morphology of cod muscle cells. This light micrograph is taken at day 8 after seeding (10X magnification). Almost all the cells get stretched in structure, looking more like myotubes. The length of the cells increase and the proliferation is also higher.

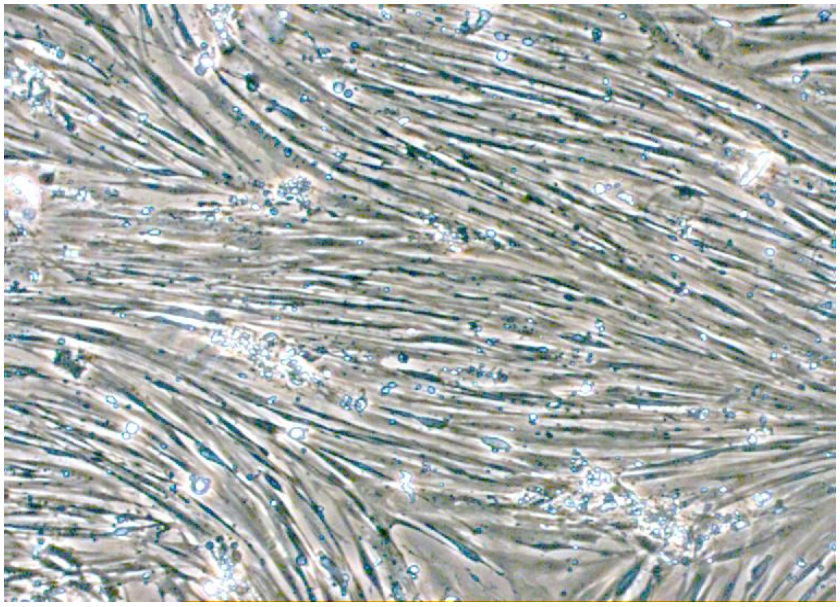


Fig.22. Morphology of cod muscle cells. This light micrograph is taken at day 13 after seeding (10X magnification). All the cells get a stretched structure like myotubes, and the cells get organized (in the same direction).

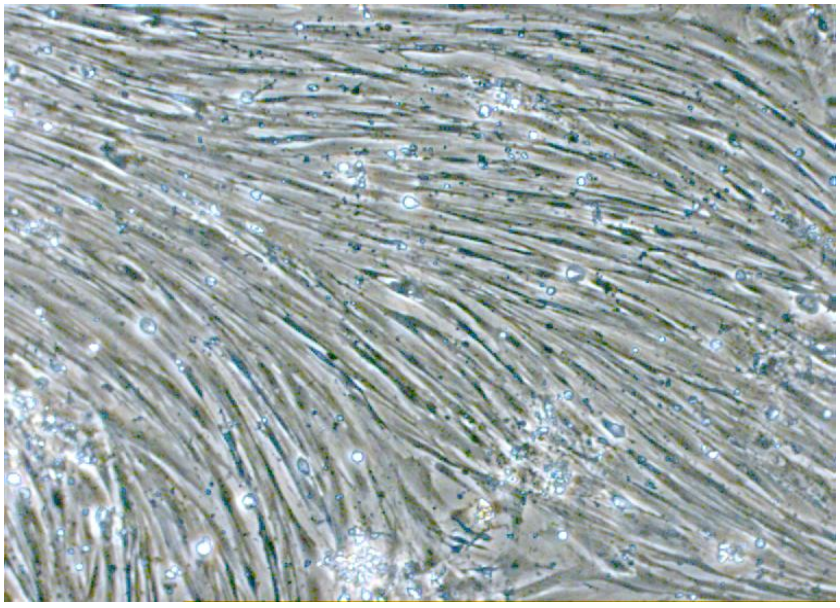


Fig.23. Morphology of cod muscle cells. This light micrograph is taken at day 15 after seeding (10X magnification). Almost all the muscle cells stretch in the same direction and have differentiated into myotubes.In this stage, there are almost no fibroblast-like cells or myosatellite.

4.2 Gene Expression

4.2.1 Gene expression in cod liver cells

The genes tested in the cod liver cells showed few changes in expression, both during development and after treatment. However, some trends were seen. The expression of the GPDH gene showed almost the same trend in control cells as in differentiation mix (followed by lipid mix) and only lipid mix treatment groups. The expression tended to increase between stage 2 and stage 3 and then decrease again until stage 4.

The expression of the adipocyte fatty acid binding protein (AFAP) gene tended to decrease in all groups at early stage of development, from stage 1 until stage 2. The control cells and the cells given differentiation mix (followed by lipid mix) had the same trend further, showing a slight increase in expression until stage 3 and then decrease again until stage 4. In cells given only lipid mix, the expression tended to further decrease after treatment, until stage 3.

The expression of the GLUT2 gene tended to have a small decrease between stage 1 and stage 2 in both the control group and the two treatment groups. Thereafter, the expression did not change.

The expression of the GLUT4 gene tended to have an increased expression between stage3 and stage 4 both in the control cells and in the cells treated with differentiation mix (followed by lipid mix). In only lipid mix group, the expression seemed to increase more rapidly after treatment, between stage 2 and stage 3.

The expression of the PPARβ gene in the control group tended to increase a little bit early during development (between stage 1 and 2). However, in cells treated with both differentiation mix (followed by lipid mix) and only lipid mix, the expression tended to further increase after treatment. In cells treated with only lipid mix the expression of PPARβ was significantly higher at the latest stage than at stage 1.

The expression of the Annexin2 gene in the control group was more or less stabile during development. In cells treated with differentiation mix (followed by lipid mix), the expression tended to decrease a little bit after treatment, while in cells only given lipid mix, the expression tended to increase.

The expression of the Annexin3 gene tended to increase between stage 2 and stage 3 in both the control group and in the two treatment groups. In both the differentiation mix (followed by lipid mix) group and the lipid mix alone group, the expression tended to further increase between stage 3 and stage 4.

The expression of the FABP6 gene showed tendencies to increase between stage 2 and stage 3 in both control cells and in cells only given lipid mix. In these cells, the expression slightly decreased again until stage 4. In cells given differentiation mix (followed by lipid mix), however, the expression tended to decrease from the start until stage 3.

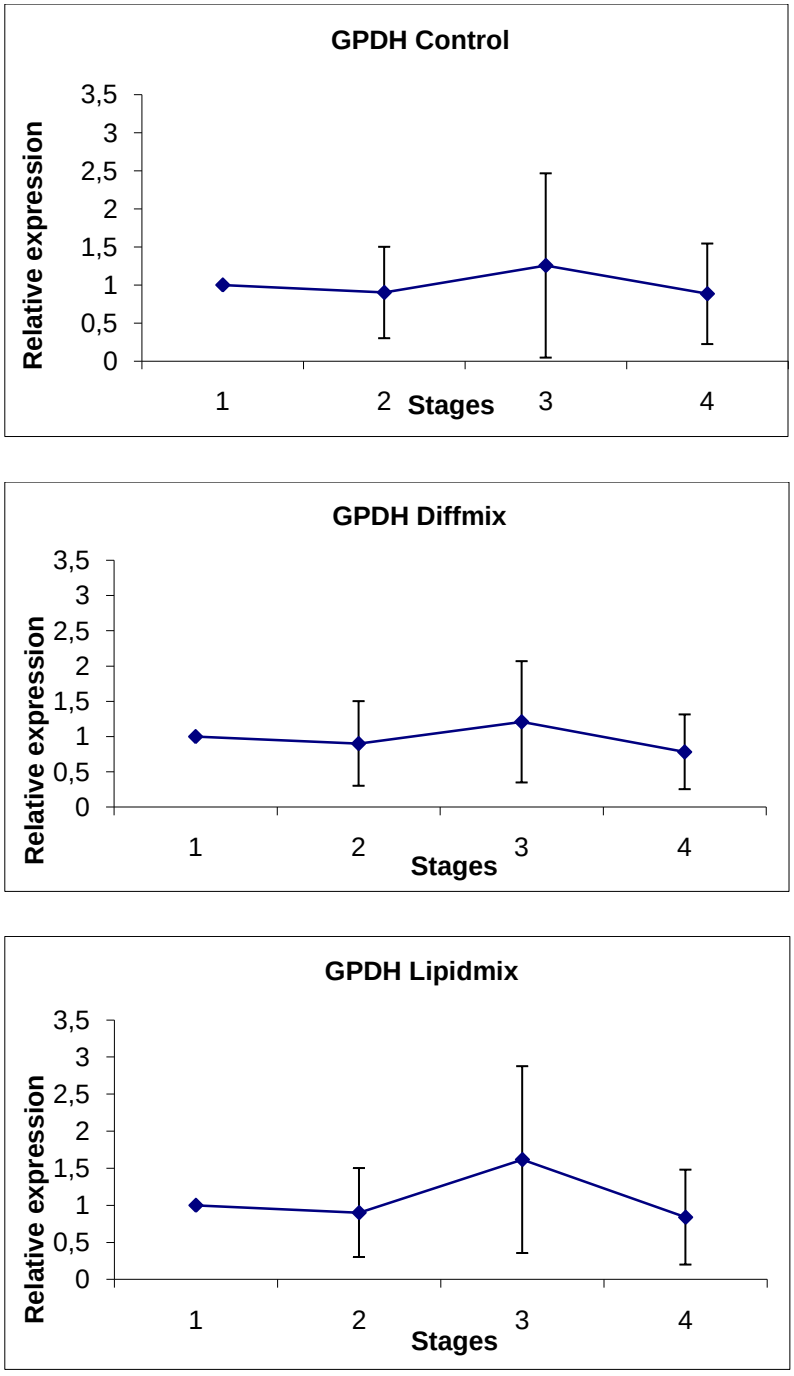


Fig 24. Gene expression of GPDH in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1.Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). GPDH = glycerol-3-phosphate dehydrogenase.

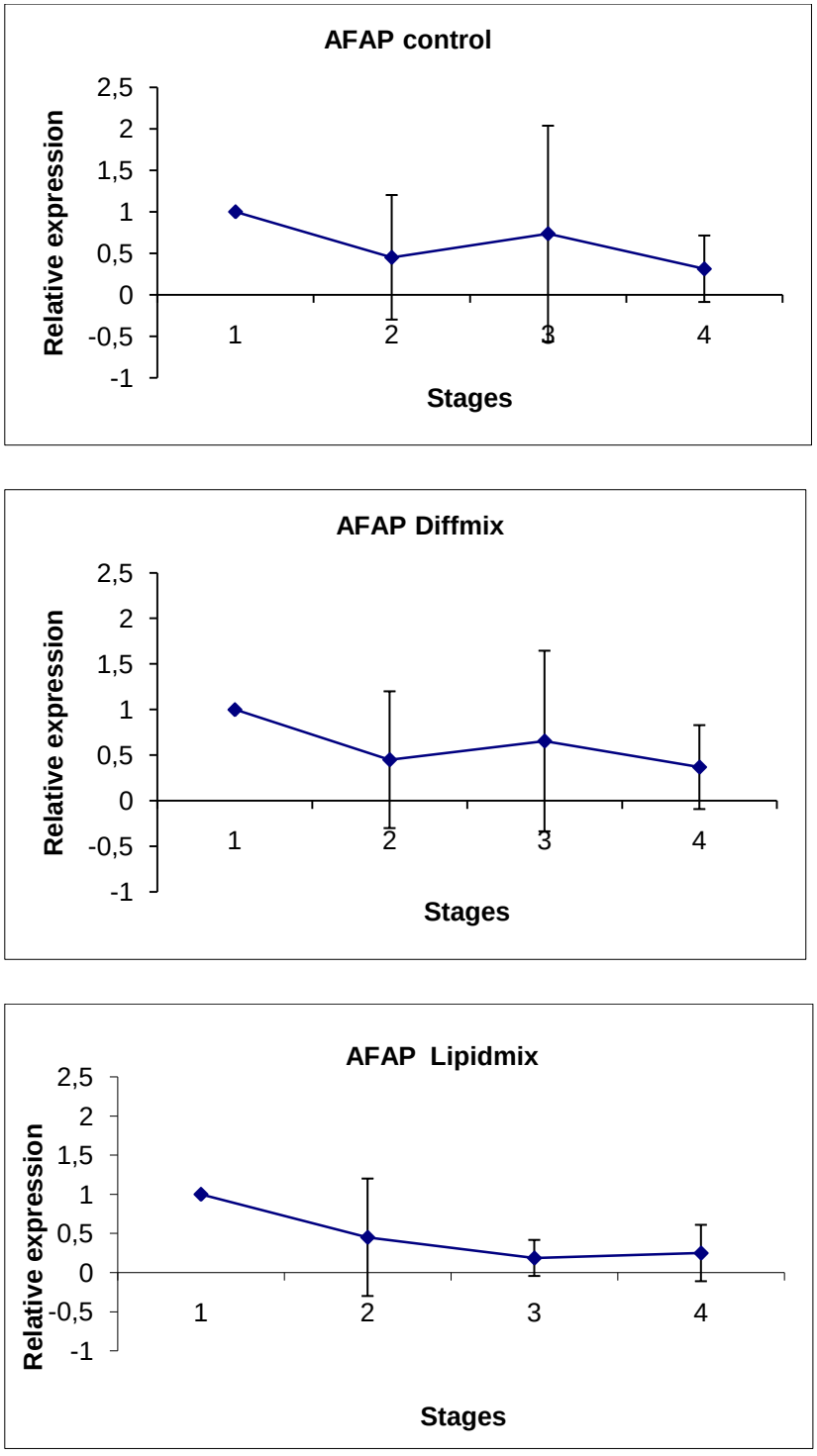
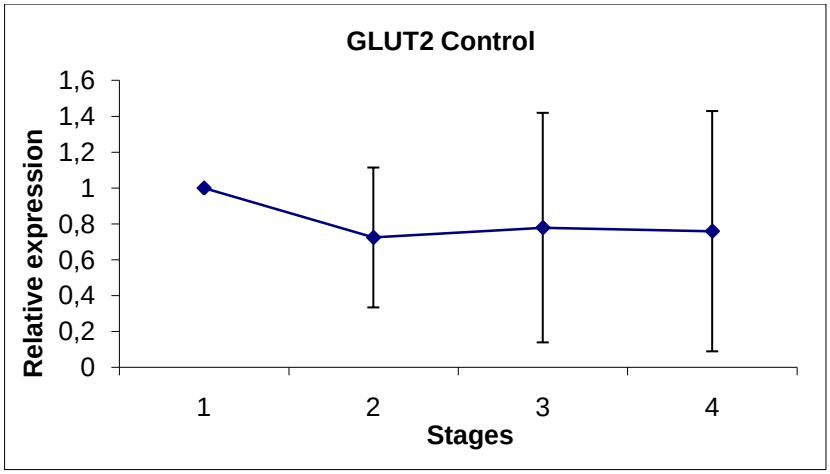
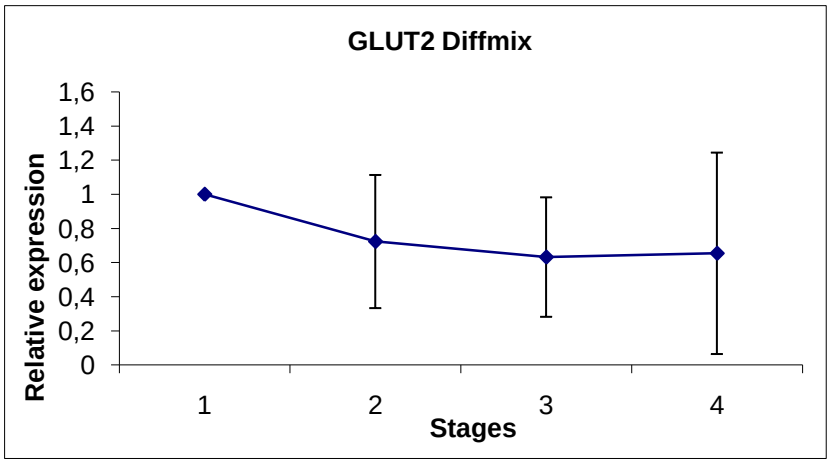


Fig 25. Gene expression of AFAP in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). AFAP = adipocyte fatty acid binding protein.





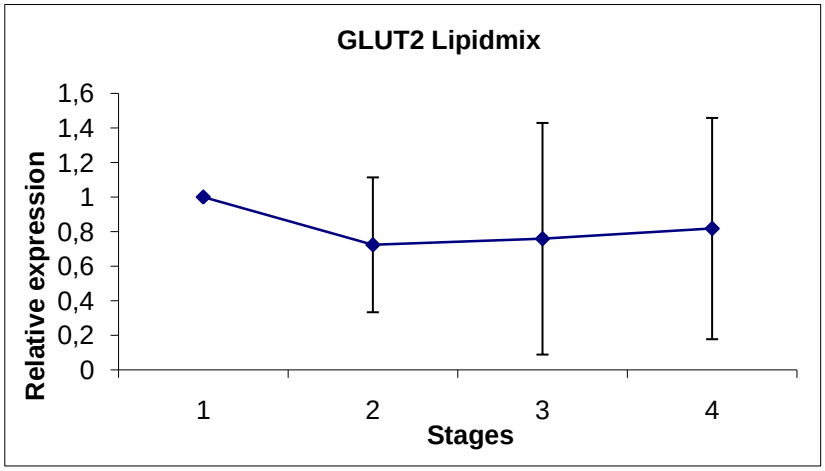
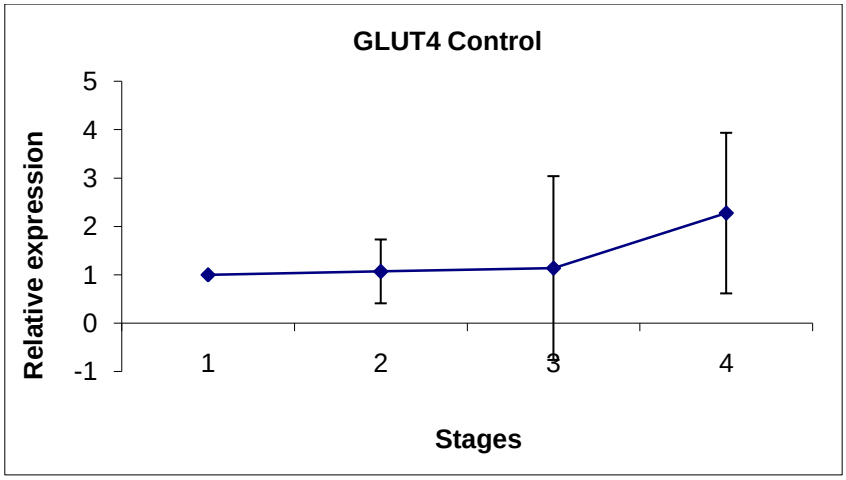
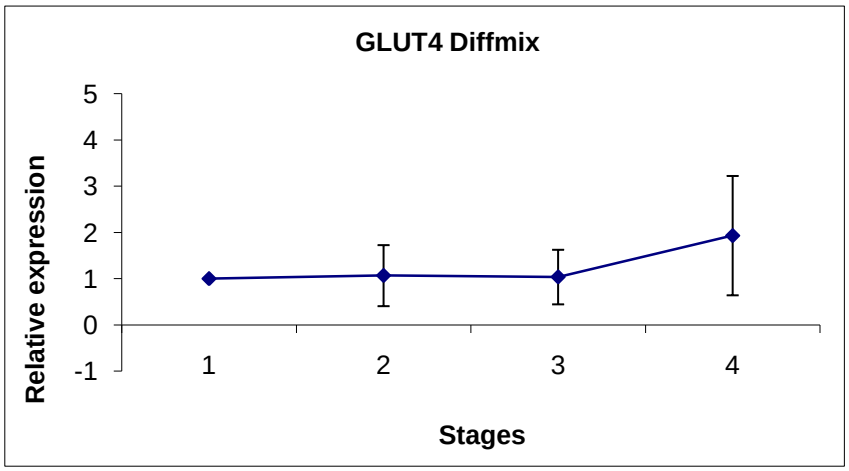


Fig 26. Gene expression of GLUT2 in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1.Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). Glut2 = glucose transporter 2.





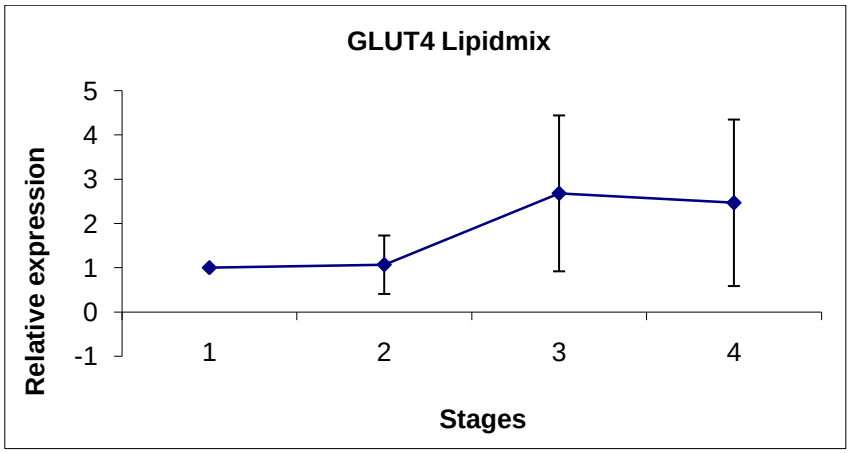
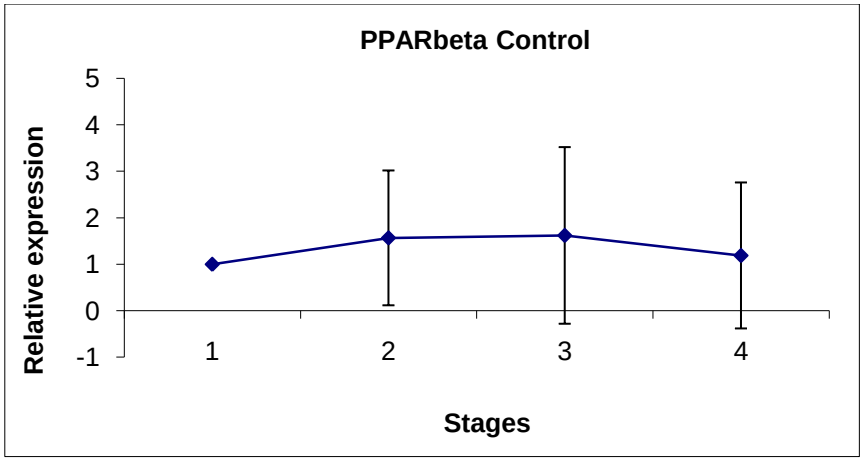
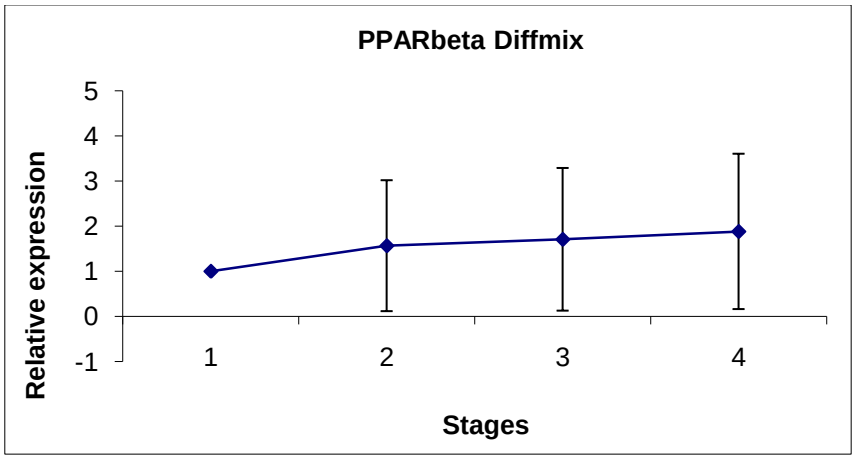


Fig 27. Gene expression of GLUT4 in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). GLUT4 = Glucose transporter type 4





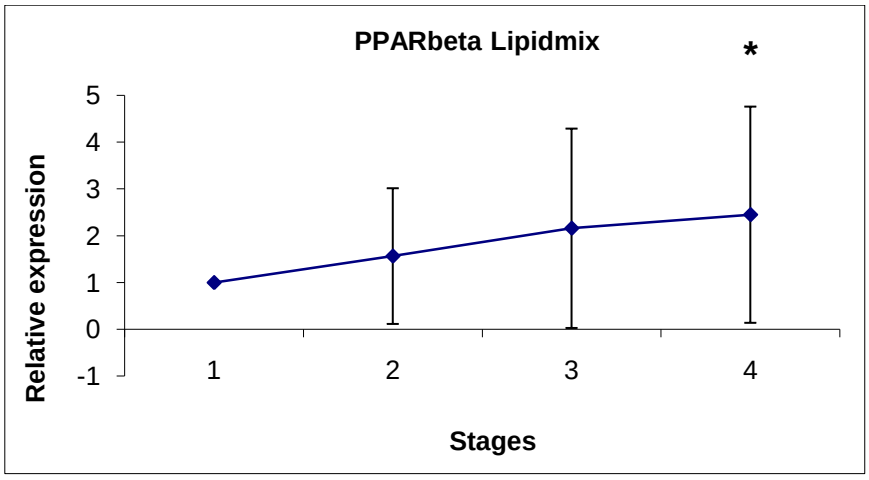


Fig 28. Gene expression of PPARbeta in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). PPARbeta = peroxisome proliferator-activated receptor.

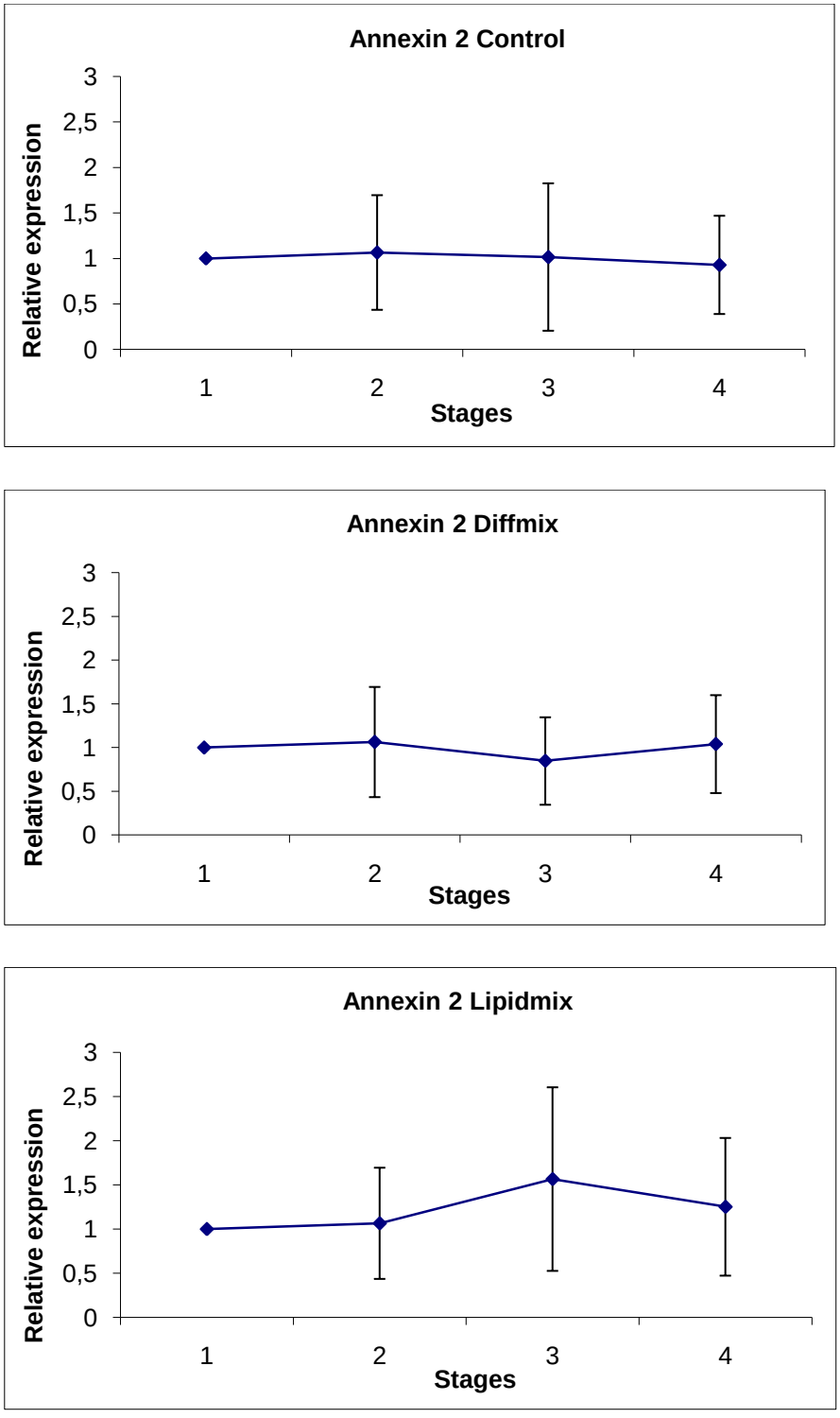
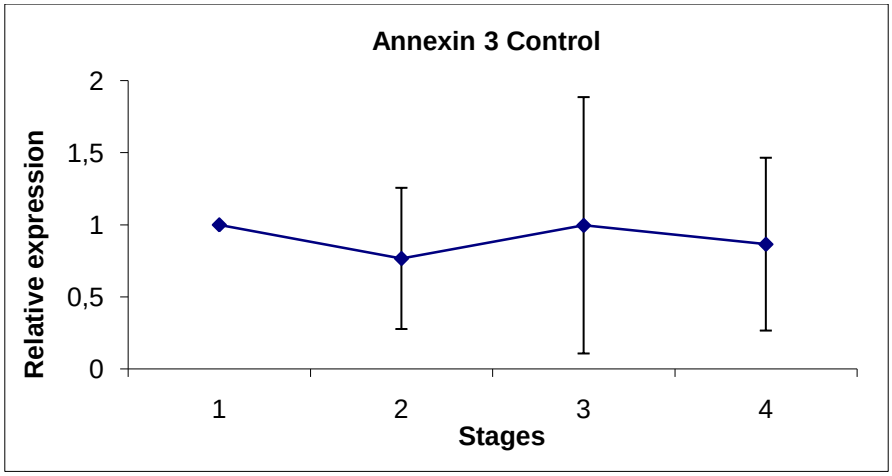
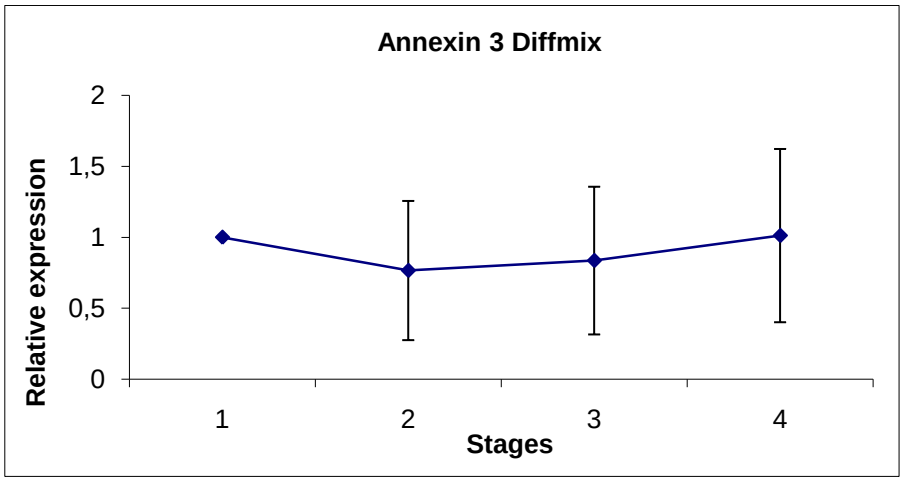


Fig 29. Gene expression of Annexin 2 in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells)





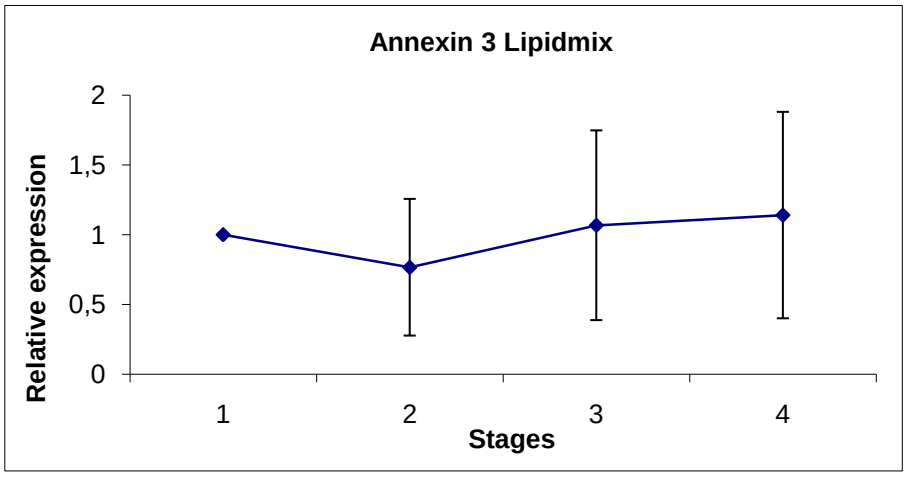
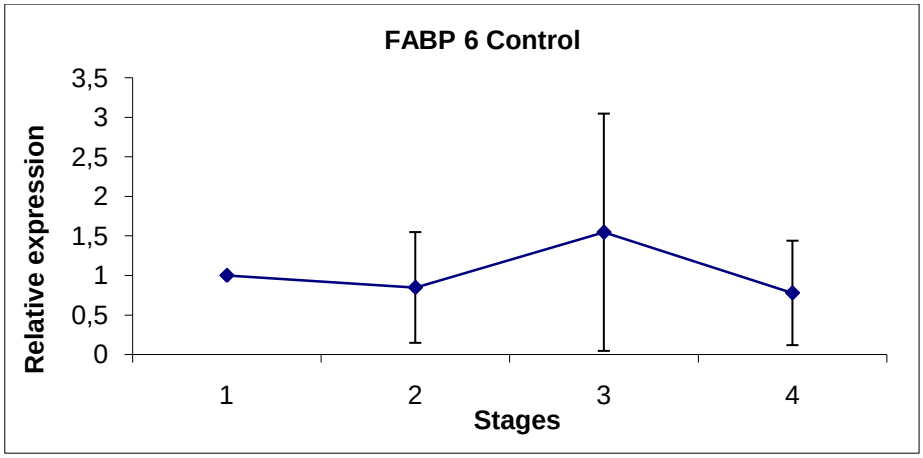
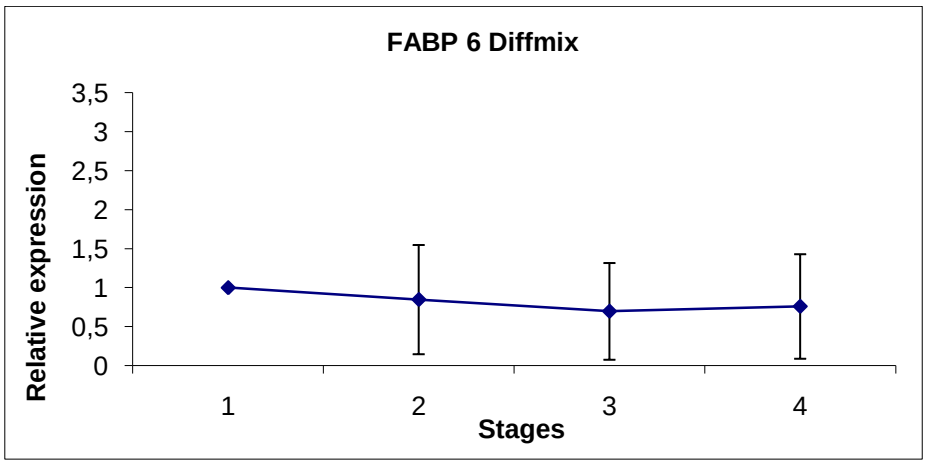


Fig 30. Gene expression of Annexin 3 in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells).





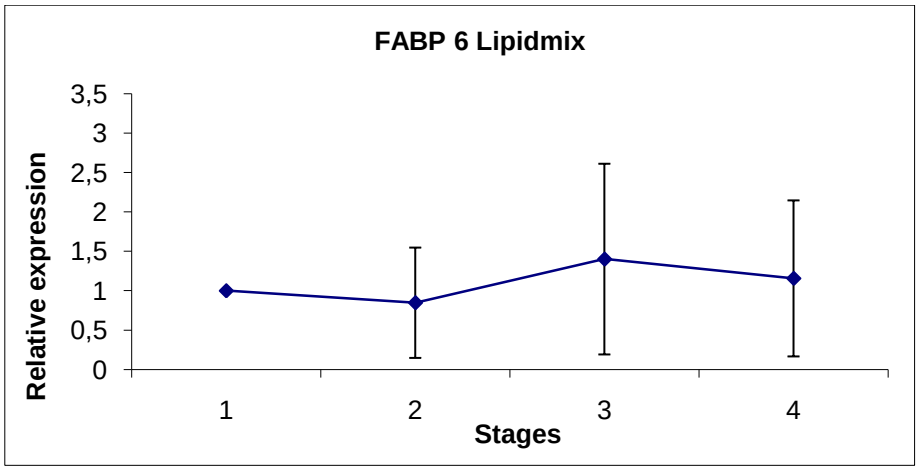


Fig 31. Gene expression of FABP 6 in cod liver cells at four stages (day 2, day 7, day 9, day 19) through development. Stage 1 was set to 1 and the rest data of stage 2, 3, 4 were relative to 1. Cells were treated with either differentiation mix (followed by lipid mix) (B), only lipid mix (C) or left untreated (A). Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). FABP6= fatty acid binding protein 6

4.2.2 Gene expression in cod muscle cell

The expression of the Myogenic Differentiation 1 (MyoD) and Myosin Light Chain (MLC) genes in cod muscle cells both decreased from stage 1 to stage 2. However, this decrease was significant only for the MyoD gene expression. The gene expression of α-actinin increased significantly from stage 1 to stage 2.

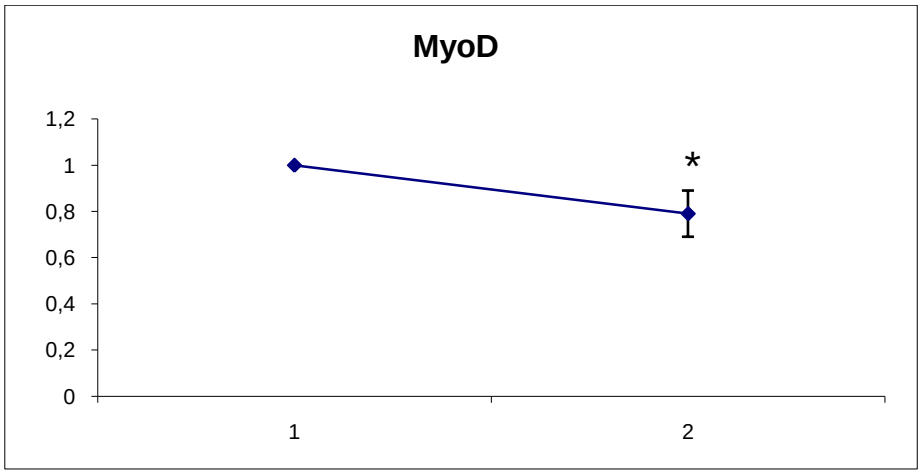


Fig 32. Gene expression of MyoD in cod muscle cells at two stages (day 2, day 9) through development. Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). Stage 1 was set to 1 and the data of stage 2 was relative to 1. MyoD = Myogenic Differentiation 1

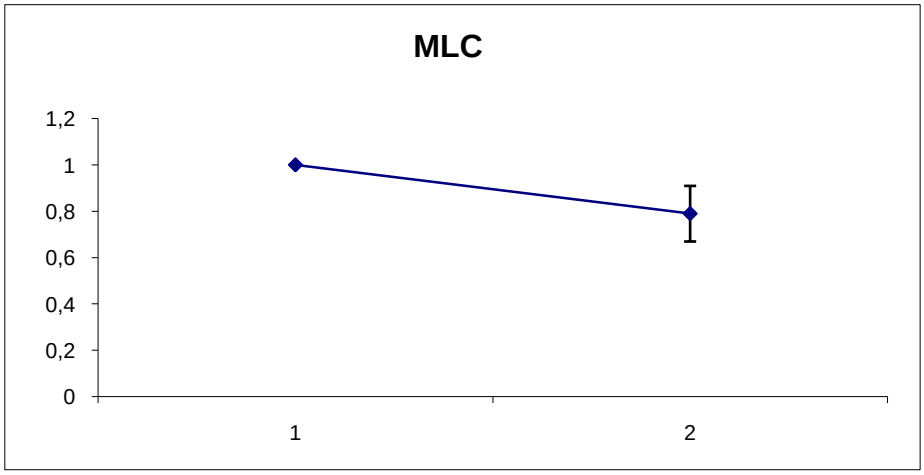


Fig 33. Gene expression of MLC in cod muscle cells at two stages (day 2, day 9) through development. Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). Stage 1 was set to 1 and the data of stage 2 was relative to 1. MLC = Myosin Light Chain

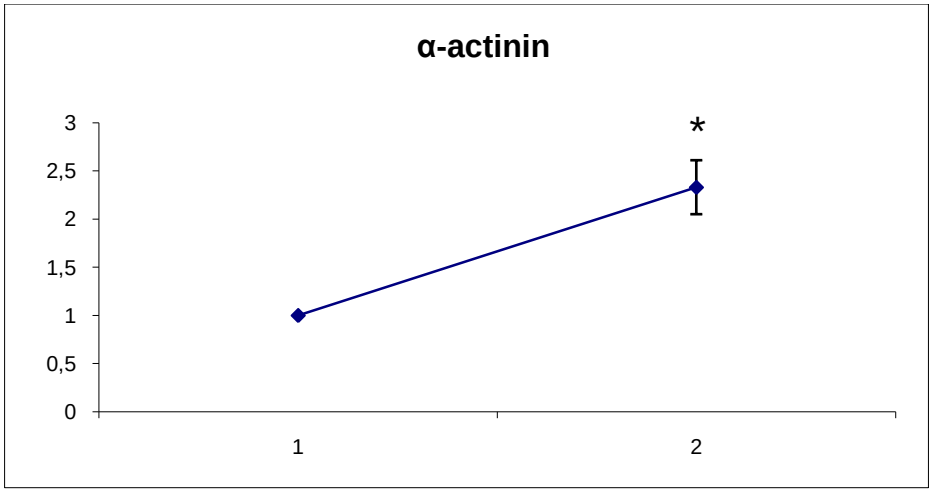


Fig 34. Gene expression of α-actinin in cod muscle cells at 2 stages (day 2, day 9) through development. Each data point represents the mean gene expression value from four RNA samples (made from 6 cell wells). Stage 1 was set to 1 and the data of stage 2 was relative to 1.

4.3 Immunocytochemistry

Result shows that we could see abundant staining for FATP1 in the plasma membrane of cod liver cells.

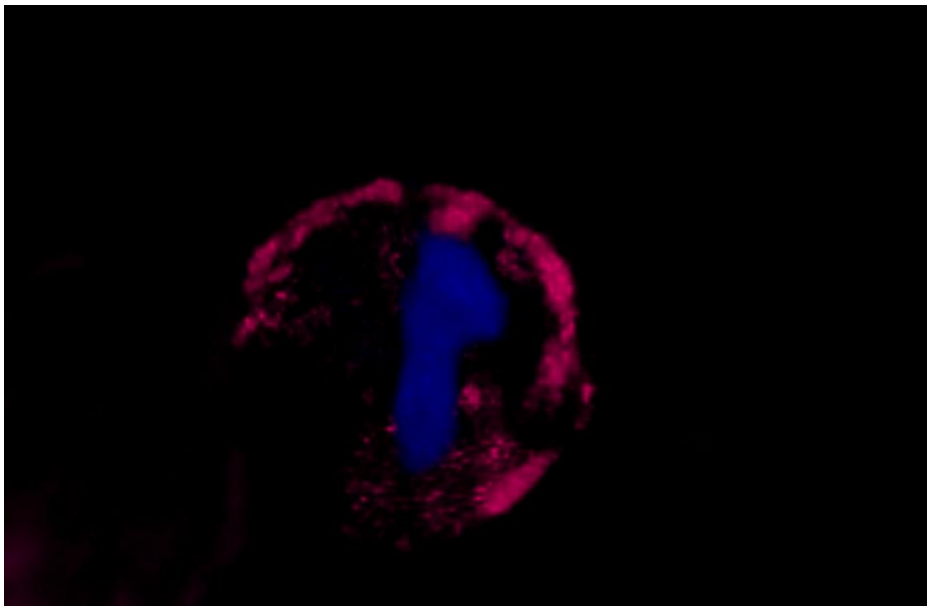


Fig.35. Light micrograph of liver cell immunolabelled for FATP1. The blue color is the nuclear chromosome stained by DAPI. The red color is the stain showing fatty acid transport protein 1 (FATP1) located in the cell membrane. This immunolabelling was done with the control group cells at the confluence stage.

4.4 Lipid Analysis

Table 2 shows the total distribution between different lipid classes found in cod liver cells at the confluent stage. The results show that the liver cells at this stage have 22.5% of its lipids as phospholipids, 5.2% as mono-acylglycerol (MAG), 16.6% di-acylglycerol (DAG), 10.4% as FFA, 24.0% as TAG and 21.4% as cholesterol ester (CE).

Figure 37 shows the incorporation of radiolabelled FAs into different lipid classes in cells given 200 uM PA and in cells given 20 uM PA. In both 20 uM and 200 uM groups, approximately 88% of radioactivity from 1-^{14}C-PA was incorporated into phospholipids. Around 3.5% and 8.1% of radioactivity from 1-^{14}C-PA incorporated into FFA and TAG, respectively. There are no differences in percentage distributions of 1-^{14}C-FAs in different lipid classes between the two treatments. However, when we look at the nmol of radioactive FAs incorporated into the different lipid classes (fig.38), we see that in cells given 20 uM PA, an average of 0.35 nmol from 1-^{14}C-PA incorporates into phospholipids, and 0.01 nmol incorporates into FFA. Around 0.03 nmol are found as TAG. In cells given 200 uM PA, an average of 0.57 nmol of added FAs incorporates into phospholipids, and 0.02 nmol incorporate into FFA. Around 0.05 nmol are found in TAG. According to figure 38, more radiolabelled FA was incorporated into cellular lipids when the cells were incubated with 200 uM PA than with 20 uM PA.

Figure 39 shows that the 1-^{14}C-16:0 (PA) to a major extent got elongated and desaturated to 1-^{14}C-18:1n-9 (Oleic acid, OA). Approximately 90% of the radioactivity was found in this monounsaturated OA, and only 10% of added FA remained as PA. No major differences were seen between the two treatment groups.

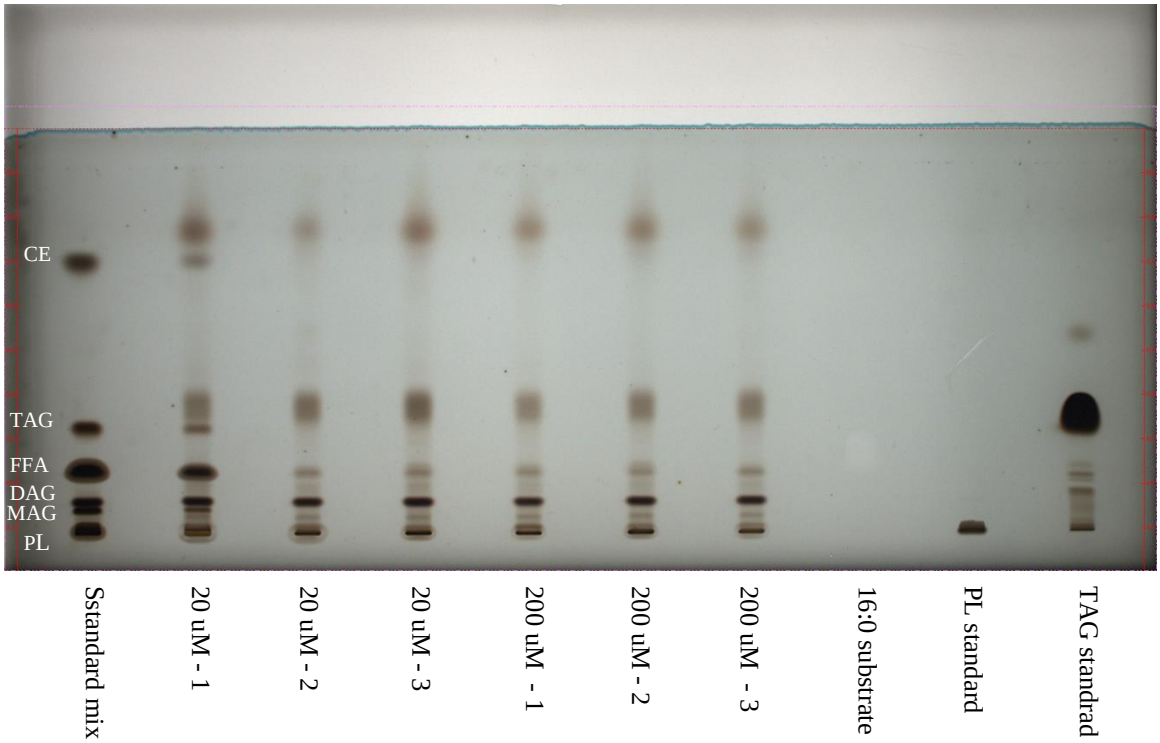


Fig 36. The image of thin-layer chromatography (TLC) of lipid class separation from cod liver cells. PL= phospholipids, MAG= mono-acylglycerols, DAG= Diacylglycerol, FFA= free fatty acids, TAG = triacylglycerols, CE = cholesterol esters

Table 2

Lipid class distribution of cod liver cells in confluent stage.

	Lipid classes					
	PL	MAG	DAG	FFA	TAG	CE
Distribution (%)	22.5±3.99^{d}	5.2±1.49^{a}	16.6±2.70^{c}	10.4±5.03^{b}	24.0±2.59^{d}	21.4±3.59^{d}

The quantity of each lipid class is given as percentage of total lipids. The values given are means ± SD (n = 6). Different letters indicate significant differences ($p<0.05$). PL= phospholipids, MAG= mono-acylglycerols, DAG= di-acylglycerol, FFA= free fatty acids, TAG = triacylglycerols, CE = cholesterol esters.

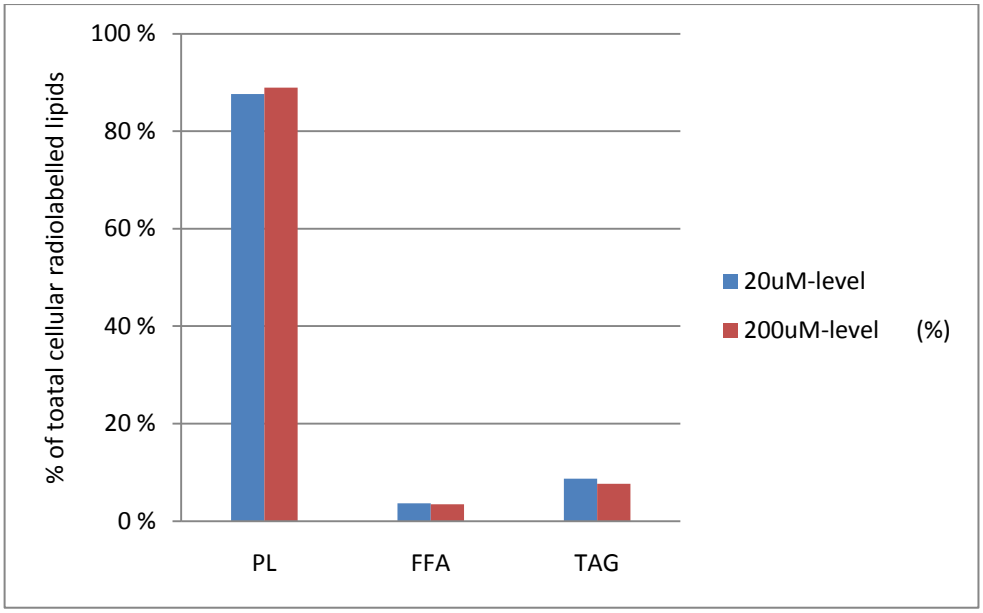


Fig 37. Distribution of 1-^{14}C-FA (%) in different lipid classes. The values given are means ± SD (n = 3). Different letters indicate significant differences ($p<0.05$). PL= phospholipids, FFA= free fatty acids, TAG = tri-acylglycerols.

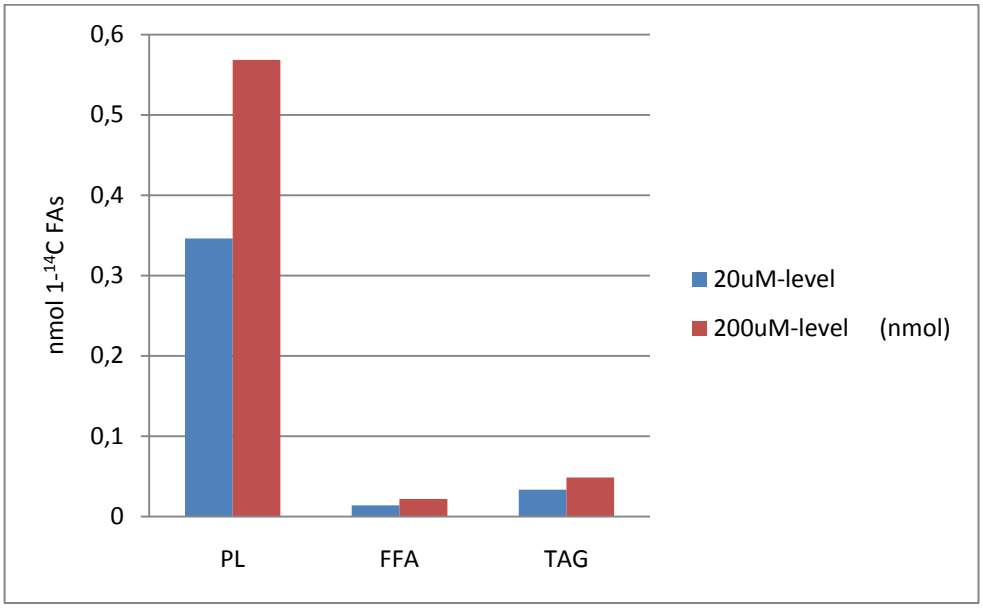


Fig 38. Distribution of 1-^{14}C-FA (nmol) in different lipid classes. The values given are means ± SD (n = 3). Different letters indicate significant differences ($p<0.05$) within each lipid class. PL= phospholipids, FFA= free fatty acids, TAG = tri-acylglycerols.

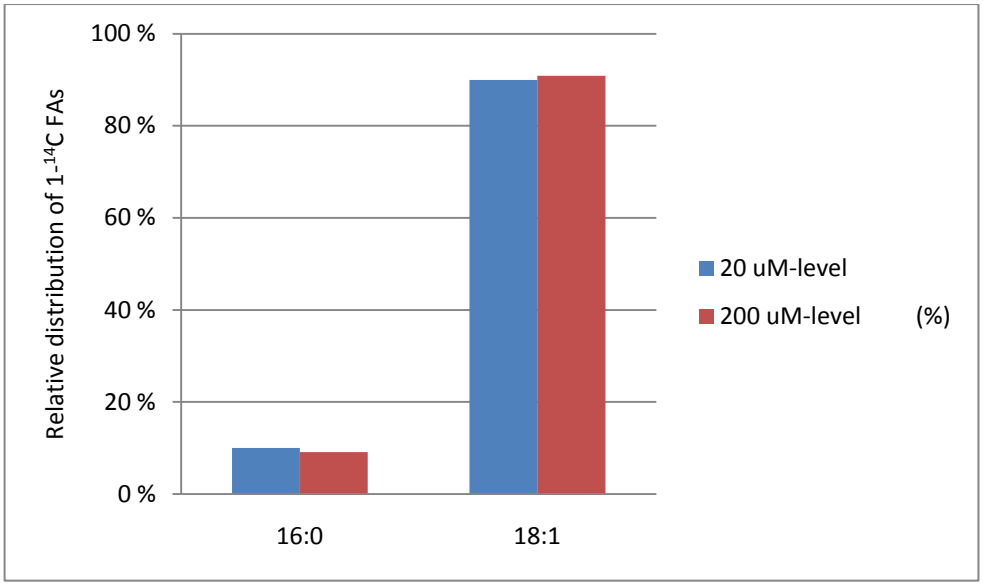


Fig 39. Distribution (%) of radioactivity from the PA substrate between PA and the elongation product 18:1. The values given are means ± SD (n = 3). Different letters indicate significant differences ($p<0.05$).

5 Discussion

5. 1 Cell culturing

For the first time, we have shown that it is possible to isolate, culture, proliferate and differentiate cells from both cod liver and cod muscle tissue. During the in vitro cod liver culture process, the sterility in all steps in the experiment is essential. From sampling, during cell culturing to the final measurements, it is necessary to work strictly under sterile conditions, by using sterile bench, with sterile culture vessels and tools to avoid bacterial contamination. Still, we repeatedly faced problems with bacterial growth, in particular when the cells were close to confluence. The reason why we got bacterial infections was probably due to the fact that the pyloric caeca, which are full of bacteria from the intestinal content, was infiltrated the liver tissue. It is very easy to get the isolated tissue contaminated with bacteria. In order to avoid these contaminations, we tried different strategies, such as picking fish of smaller size to decrease the influence of pyloric caeca infiltration with the liver. We also optimized concentrations of penicillin, streptomycin and amphotericin B in the culture media, and the cells was washed thoroughly every other day during the culture period. We succeeded in getting cultures without bacterial infections. Our experience is that it is essential to use small fish at a stage where the pyloric caeca is still not infiltrated in the liver tissue.

5.2 Differentiation of cells

In our experiment, unspecialised precursor cells from cod liver were used for isolation and culture. That is because the mature differentiated cells in cod liver contain relatively high level of lipids which make the cells fragile and buoyant in culture. These mature

differentiated cells are difficult to culture because they do not easily attach to the bottom of the cell flask.

Compared with mature lipid filled liver cells, unspecialised precursor cells have higher density and attach more easily to the cell flask, which make them easier to culture. Compared with the mammalian liver cell differentiation (Cai et al. 2007; Lavon & Benvenisty 2005), we still have little information about the cod liver cell differentiation process. However, according to the light micrograph, we can infer the rough differentiation process. The microscopy pictures from the first stage show that we do not achieve a homogenous culture of cells. There are probably different cell types, since we find cells with different morphology. Most of the cells, which are round in shape and without visible lipid droplets, are the potential unspecialised precursor cells which are similar to stem cells isolated from adipocytes at the earliest stages (Robin & Roncari 1978). We also find cells with typical fibroblast morphology which is a well known phenomenon when we cultivate primary cells. In addition, we find cells that we expect are more mature/differentiated lipid filled liver cells (probably hepatocytes). From day 5, the round unspecialised precursor cells, started to get stretched to spindle shaped or fibroblastic-like structure, the morphology which was also found in stem cells from visceral adipose tissue, bone marrow, and muscle cells in Atlantic salmon one day after seeding (Friedens.Aj et al. 1970; Todorcevic et al. 2009; Vegusdal et al. 2004). Along with the differentiation process, lots of lipid droplets were produced and gradually formed lipid clusters between the cells. The amount of lipid increased in line with the degree of differentiation. The cell density in the lipid clusters was much higher than the other areas, and the cells seem to be formed from the lipid rich cell clusters. In addition, more and more of the expected mature hepatocytes became filled with lipids with time. We assume that lipids were gradually absorbed from the media by the cells during the differentiation process. However, due to little research related to this area, we cannot be sure about the precise cell type and stage of differentiation only based on our morphology data.

From day 5 to day 9, cell numbers strongly increased, which showed their high proliferation capacity. At this stage we tried to trypsinate the cells, and further perform a

second cell seeding. The cell density increased after very short time, and the cells went from very thin to achieve confluent layer of cells in a couple of days, which showed their huge proliferation capacity. Atlantic salmon adipogenesis has been studied, and at confluent stage cell goes into cell cycle arrest, which is probably necessary for optimizing the adipogenesis process and terminal differentiation (Todorcevic et al. 2010). Similar phenomenon are also found in mammalian species (Blanchet et al. 2009). We are not sure whether the cod cells go in cell cycle arrest, but it most likely happens with cod as well since it happens in most cells studied, and based on the images, the cell number does not seem to increase much after confluence.

It is known that several long-chain FAs, as for instance 18:1 n-9 from rapeseed oil, can trigger trans-differentiation processes in multipotent precursor cells from mammals (Grimaldi et al. 1997), e.g., osteoblasts and myosatellite cells may differentiate towards that of adipocytes (Schiller *et al*., 2001, Grimaldi *et al*., 1997). At confluence, addition of differentiation mix, containing in particular insulin and dexamethasone, is shown to lead the precursor cells from adipose tissue into mature adipocytes (Todorcevic et al. 2010). Similar situation is found in our experiment. Addition of the differentiation mix led to morphological changes of the cells, which again became more rounded and oval in shape, similar to the response previously observed for pre-adipocytes. The lipid mix alone didn't give rise to this change in morphology, instead they were stretched out. Based on our morphology data, we cannot with certainty identify what accurate cell type we have in the culture, but at least we know that all these cells seemed to have the potential as stem cells to attach to the cell flask, proliferate and further to differentiate. They can also produce and deposit lipids. When giving the cells PA, they are very capable of producing both TAGs and phospholipids.

To get a better picture of the differentiation process, it is also attractive to measure the expression of genes known to be highly expressed during different stages of liver cell development. From introduction we know some factors from mammalian studies, such as Wnt2bb, Bmp2b, Fgf, Prox1, Def, Leg1, which have important functions during liver cell differentiation. Unfortunately, none of them are described in cod, so there were no gene probes available for our work. However, a cod genome project was started in 2008, and

the whole genome of cod is now sequenced. We hope it can be used as a valuable tool for further and more precisely research on the differentiation process in the near future.

Despite the lack of genes related to hepatocyte differentiation, several known genes that increased during differentiation of adipocytes in Atlantic salmon (Todorcevic, M et al. 2008) were chosen and tested by Q-PCR in our cod cells. The expression of these genes could give us some indications whether our cells became more differentiated with time. GPDH is known to be involved in lipogenesis, and is a late marker for adipocyte differentiation / terminal differentiation marker. Along with increased GPDH, cells get specialized to produce TAG (Dobson et al. 1987; Gaillard et al. 1989). For example, GPDH activity in adipose tissue from obese human subjects is approximately two times higher than in lean individuals. In our result, the expression of the GPDH gene had a strong tendency to increase after the addition of lipid mix to the cells (from day 7 to 9). This indicates that the exogenous added lipid mix enhance the expression of the GPDH gene. This further indicates that elevated GPDH might contribute to increased TAG synthesis, which is in agreement with a study showing that FAs necessary for glycerol 3-phosphate esterification must be derived from exogenous TAG (Swierczynski et al. 2003). We cannot be sure, based on the above findings, whether the same mechanisms also happens in cod liver cells, which necessitates further research.

FABPs are a family of carrier proteins for FAs and other lipophilic substances (Chmurzynska 2006). The gene expression of FABP 3 is known to increase during adipocyte differentiation (Todorcevic, M et al. 2008). Adipocyte FA–binding protein (AFAP) is a cytosolic FA chaperone expressed in adipocytes. It is known to be highly expressed in adipose tissue. As a main cytosolic protein in mature adipocytes, AFAP accounts for 6% of total cellular proteins (Boord et al. 2002; Makowski & Hotamisligil 2004; Xu et al. 2006). However, our data show that there is a minor tendency of reduced AFAP expression in the group given only lipid mix. It may indicate that the cells have not reached a mature stage, but are still relatively unspecialized. Dexamethasone was able to induce AFAP expression according to a mammalian study (Abdelwahab et al. 2007). However, our differentiation mix also includes IBMX, which has antagonistic effects on AFAP expression (Sun et al. 2003). Therefore, it could be that IBMX had a stronger

effect than dexamethasone in the differentiation mix. Our results also showed a tendency towards a small increased expression of FABP-6 with time (day 7 to day 9) in control cells and cells added only lipid mix.

Glucose uptake is facilitated by a family of GLUT proteins at the plasma membrane by facilitated diffusion (KOKK et al. 2007). GLUT2 catalyzes glucose uptake into the liver (Thorens et al. 1988), but it is not necessary for the hepatic glucose release process (Guillam et al. 1998). GLUT4 is exclusively found in adipocytes and skeletal- and heart muscle. Translocation of GLUT4 from an intracellular pool to the plasma membrane is important for glucose uptake, which is stimulated by insulin (Birnbaum 1989; Cushman & Wardzala 1980; James et al. 1989; Suzuki & Kono 1980). Our results show a tendency to reduced expression of the liver specific GLUT2 and an increased expression of the adipose tissue specific GLUT4. This result may indicate that our cells develop in the direction of adipocyte-like cells. However, this deduction which is only based on tendencies needs to be verified in future studies.

Although most genes studied in the cod liver cells don't show significant changes during development in any of the treatment groups or in control cells, the expression of PPARβ gene in liver cells treated with lipid mix alone was significantly higher at stage 4 than stage 1. PPARβ is a transcription factor known to be induced in the initial stages of adipocyte differentiation (Matsusue et al. 2004). Research show that the function of PPARβ in adipose tissue specifically induces the expression of genes which function in FA oxidation and energy dissipation and finally improves lipid profiles and reduce deposition (Wang et al. 2003). Research has also shown that PPARβ is necessary for adipocyte differentiation (Barak et al. 2002; Peters et al. 2000). In addition, PPARβ is activated by very low-density lipoprotein (VLDL) particles which are rich in TAG. This activation induces key genes in carnitine biosynthesis and lipid mobilization mediated by transport secretion protein 2 (an adipose TAG lipase), resulting in the increase of FA catabolism (Lee et al. 2006). In our experiment, the expression of PPARβ didn't change in control cells during the differentiation. However, there was a tendency to gradual increase with time after addition of differentiation mix (followed by lipid mix). For the cells treated with lipid mix alone, there was a significant increase. These results may

indicate that both differentiation mix and lipid mix can more or less induce the expression of PPARβ.

Annexins are a family of homologous calcium-dependent proteins. They function to annex anionic phospholipid membranes. AnnexinA2 is one of the proteins in this family found on the surface of many cell types (Menell et al. 1999; Waisman 1995). It has been involved in membrane transport and also as a regulator of cell differentiation (Gerke & Moss 1997). Increased expression of annexinA2 corresponds to differentiation rather than to proliferation in normal liver development (Della Gaspera et al. 2001). Annexin3 was expressed in neutrophils and monocytes in humans along with their differentiation (Le Cabec et al. 1992). Recently, it was also indicated that it is expressed in isolated small rat hepatocytes (Niimi et al. 2003). In our study, the addition of lipid mix alone tended to induce a small increase in the expression of both the annexinA2 gene and the Annexin3 gene. This may indicate that lipid mix promote liver cell differentiation.

FATP are a family of proteins implicated in FA uptake and activation (Gimeno 2007). FATP1 is a plasma membrane protein that is expressed in cells and tissues with high level of FA uptake, such as skeletal muscle, adipose tissue, and heart in mammals. Research indicates that the over-expression of mouse FATP1 leads to a significant increase in long-chain FA uptake in cultured fibroblasts (Abumrad et al. 1999; Bonen et al. 1999; Schaffer & Lodish 1994). This corresponds well with the finding of high level of the protein FATP1 in the plasma membrane of cod liver cells at the confluent stage by immunocytochemistry. In our experiment, control cells as well as cells added differentiation mix (followed by lipid mix) or lipid mix alone all accumulated more and more lipid droplets during differentiation. This indicates that these cells all differentiate to a more mature stage as well as getting higher capacity to take up and store lipids. The gene expression results, PPARβ in particular, indicated that the differentiation was more induced in cells added differentiation mix (followed by lipid mix) and/or only lipid mix than in the control cells.

Compared with liver cell, the results from muscle cells were clearer. Our cod muscle cells followed similar differentiation pattern as salmon muscle cells (Vegusdal et al. 2004). The first stage of muscle cells were un-mature myosatellite cells of spindle shape. Triangular shaped cells found in between were considered to be fibroblasts. After getting elongated, some cells started to fuse with neighboring cells and turned to be more and more organized. Finally, the cells achieved mature phase and formed myotube structures. We also tested some genes related to the differentiation of cod muscle cells by Q-PCR. MyoD showed a significant decrease, while the expression of α-actinin gene was significantly increased from stage 1 to stage 2. MyoD is a muscle-specific transcriptional regulator inducing myogenesis in many cell types. The MyoD family have been assigned as myogenic determinants because the expression of these proteins can convert non-myogenic cells into muscle-like fibers (Davis et al. 1987). Our result indicates that MyoD is more highly expressed in stage 1 relative to stage 2, which means that MyoD mainly regulate transcriptional functions in muscle differentiation from uncommitted cells to myosatellite cells in cod muscle. This result fits well with what is known from osteoblast differentiation (Hewitt et al. 2008) and myogenic stem cell differentiation in mouse (Megeney et al. 1996). Also in salmon, MyoD is shown to be expressed early during muscle development (Ostbye et al. 2007). α-actinin is a major actin cross-linking protein both in non-muscle and muscle cells (Blanchard et al. 1989). It mediates the formation of the templates and the organization of adhesions related to actin filaments (Choi et al. 2008). Our result indicates that α-actinin mainly function in later stages of muscle differentiation from myosatellite cells to multinucleated muscle cells (myotubes). This also correspond well with what is known from salmon (Andersen et al. 2004) as well as mammals (Pomies et al. 1997; Pomies et al. 1999). Recent research suggests that MLCs are a series of sarcomere-related proteins that function as traditional regulators of muscle contraction. MLCs can negatively impact myoblast proliferation by promoting myoblast withdrawal from cell differentiation process (Zhang et al. 2009). Our result indicates that MLC has a tendency to reduced expression in stage 2 relative to stage 1, which means it reduces when cells change from uncommitted cells to myosatellite cells.

5.3 Lipid analysis

The result of lipid class distribution shows that cod liver cells at confluence are still unspecialized precursor cells that store relatively little lipid compared to mature liver cells. Our data show that the relative distribution between phospholipids, CE and TAGs are almost equal. It is becoming increasingly clear that human adipocytes are capable of synthesizing FAs and TAGs from non-lipid precursors, the process known as *de novo* lipogenesis (DNL). PA is the product of DNL. Functionally, around 20% of PA in adipocyte TAGs arise from DNL in mammals. During hypercaloric, high-carbohydrate feeding, DNL increases considerably both in liver and adipose tissue (Aarsland et al. 1997; Pasquet et al. 1992). We do not know however, whether DNL are important for lipid accumulation in cod liver cells. It is shown that differentiating mammalian adipocytes might defend themselves against adverse accumulation of saturated FAs produced from DNL, by close linkage of DNL with further modification of FAs (Collins et al. 2010). There is evidence that hepatic DNL is regulated in parallel with elongation of FAs and desaturation by the enzyme stearoyl-CoA desaturase (SCD, or delta-9 desaturase) (Chong et al. 2008). In this study we have therefore added radiolabelled PA to the cod liver cells at confluence (at the initiation of the differentiation process) in order to study the cells capacity to perform elongation and Δ-9 desaturation of 16:0 to 18:1. Further we wanted to look at how these FA incorporated into different lipid classes in the cell. Our results in cod are well in agreement with what is known from mammalian studies of cells in the differentiation process, showing that more than 80% of the radiolabelled 16:0 was very rapidly converted to the elongated and Δ-9 desaturated products 18:1n-9, thereby protecting the cells from adverse effects of high levels of saturated FAs. There were no big differences between the 20uM and 200uM treatments, which mean that the percentage of 16:0 elongated and desaturated to 18:1 will remain stable at different concentrations. Our data definitely show that the cod liver cells possess the protection mechanisms necessary for cells that are destined to store huge levels of lipids. However an important aspect of future studies would be to study the cells capacity for DNL, by for instance adding radiolabelled glucose and determine the production of PA.

Most of the radiolabelled FA incorporates into phospholipids, showing that more than 80% goes to phospholipids irrespective of FA concentration. This may indicate that different concentration of 16:0 added to the liver cells will not change their regularity of distribution at this differentiation stage. However, this may be changed at later stages of differentiation, when more probably will go into TAG. When we look at the nmol FA incorporated, we see that the amount of FA from the 1-^{14}C-PA substrate that incorporates into phospholipids, FFA and TAG did increase in the cells given 200 uM PA compared to the cells given 20 uM PA. However, the increment is not in step with the concentration of 1-^{14}C-PA. That means, the quantity of 1-^{14}C-PA incorporation into phospholipids, FFA and TAG didn't increase 10 times as the concentration change between 20 uM and 200 uM. Instead, the incorporation only increased around 1.5-2 times. This indicates that the incorporation is not a free diffusion process, but is likely to be operated by receptors being regulated. Regulation of receptors might be a slow process and hence, it is important to note that we do the measurement solely after 48 hours. Maybe a longer incubation time would reveal bigger differences in PA uptake between the two treatment groups. And maybe more 1-^{14}C-PA probably will incorporate into TAGs at later differentiation stages. The uptake of PA is also most probably dependent on the numbers of receptors. Slow or reduced uptake of PA could imply few receptors available, however, we found a high level of FATP in the plasma membrane of the cod liver cells already at confluence.

In conclusion, we have shown that the cod liver contains unspecialised precursor cells which can proliferate and differentiate into a more mature cell type with time upon nutritional and hormonal stimuli. The cells induced by differentiation mix (followed by lipid mix) seem to differentiate into cells looking like adipocytes. However, the control group and the cells induced by lipid mix alone may not have the right stimuli to develop in the direction of adipocyte-like cells. We cannot however, state with certainty whether the different treatments make the cells differentiate towards hepatocyte-like or adipocyte-like cells.

The lipid analysis shows that the relative distribution between phospholipids, CE and TAGs are almost equal in the liver cells at confluence stage. 80% radioactivity from 16:0

FA incorporate into phospholipids as the desaturated and elongated product 18:1n-9 and the regularity of distribution don’t change in different concentration. Our data definitely show that the cod liver cells possess the protection mechanisms necessary for cells that are destined to store huge levels of lipids.

Precursor cells isolated from cod muscle are able to proliferate and differentiate into mature multinucleated myotubes in culture.

References

Aarsland, A., Chinkes, D. & Wolfe, R. R. (1997). Hepatic and whole-body fat synthesis in humans during carbohydrate overfeeding. *American Journal of Clinical Nutrition*, 65 (6): 1774-1782.

Abdelwahab, S., Owada, Y., Kitanaka, N., Adida, A., Sakagami, H., Ono, M., Watanabe, M., Spener, F. & Kondo, H. (2007). Enhanced expression of adipocyte-type fatty acid binding protein in murine lymphocytes in response to dexamethasone treatment. *Molecular and Cellular Biochemistry*, 299 (1): 99-107.

Abumrad, N., Coburn, C. & Ibrahimi, A. (1999). Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1441 (1): 4-13.

Andersen, Ø., Østbye, T.-K., Gabestad, I., Nielsen, C., Bardal, T. & Galloway, T. (2004). Molecular characterization of a PDZ–LIM protein in Atlantic salmon (Salmo salar): a fish ortholog of the α-actinin-associated LIM-protein (ALP). *Journal of Muscle Research and Cell Motility*, 25 (1): 61-68.

Arnason, J., Bjornsdottir, R., Arnarsson, I., Arnadottir, G. S. & Thorarensen, H. (2010). Protein requirements of Atlantic cod Gadus morhua L. *Aquaculture Research*, 41 (3): 385-393.

Auwerx, J., Leroy, P. & Schoonjans, K. (1992). Lipoprotein Lipase: Recent Contributions from Molecular Biology. *Critical Reviews in Clinical Laboratory Sciences*, 29 (3-4): 243-268.

Barak, Y., Liao, D., He, W. M., Ong, E. S., Nelson, M. C., Olefsky, J. M., Boland, R. & Evans, R. M. (2002). Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proceedings of the National Academy of Sciences of the United States of America*, 99 (1): 303-308.

Birnbaum, M., Schultz, J. & Fain, J. (1976). Hormone-stimulated glycogenolysis in isolated goldfish hepatocytes. *Am J Physiol*, 231 (1): 191-197.

Birnbaum, M. J. (1989). IDENTIFICATION OF A NOVEL GENE ENCODING AN INSULIN-RESPONSIVE GLUCOSE TRANSPORTER PROTEIN. *Cell*, 57 (2): 305-315.

Blanchard, A., Ohanian, V. & Critchley, D. (1989). THE STRUCTURE AND FUNCTION OF ALPHA-ACTININ. *Journal of Muscle Research and Cell Motility*, 10 (4): 280-289.

Blanchet, E., Annicotte, J. S. & Fajas, L. (2009). Cell cycle regulators in the control of metabolism. *Cell Cycle*, 8 (24): 4029-4031.

Blouin, A., Bolender, R. P. & Weibel, E. R. (1977). Distribution of Organelles and Membranes between Hepatocytes and Nonhepatocytes in the Rat Liver Parenchyma. A Stereological Study. *The Journal of Cell Biology*, 72 (2): 441-455.

Bonen, A., Miskovic, D. & Kiens, B. (1999). Fatty acid transporters (FABPpm, FAT, FATP) in human muscle. *Canadian Journal of Applied Physiology-Revue Canadienne De Physiologie Appliquee*, 24 (6): 515-523.

Boord, J. B., Fazio, S. & Linton, M. F. (2002). Cytoplasmic fatty acid-binding proteins: emerging roles in metabolism and atherosclerosis. *Current Opinion in Lipidology*, 13 (2): 141-147.

Burtle, G. J. & Liu, Q. (1994). Dietary Carnitine and Lysine Affect Channel Catfish Lipid and Protein Composition. *Journal of the World Aquaculture Society*, 25 (2): 169-174.

Cai, J., Zhao, Y., Liu, Y. X., Ye, F., Song, Z. H., Qin, H., Meng, S., Chen, Y. Z., Zhou, R. D., Song, X. J., et al. (2007). Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology*, 45 (5): 1229-1239.

Capilla, E., Médale, F., Navarro, I., Panserat, S., Vachot, C., Kaushik, S. & Gutiérrez, J. (2003). Muscle insulin binding and plasma levels in relation to liver glucokinase activity, glucose metabolism and dietary carbohydrates in rainbow trout. *Regulatory Peptides*, 110 (2): 123-132.

Caseras, A., Metón, I., Vives, C., Egea, M., Fernández, F. & Baanante, I. V. (2002). Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (Sparus aurata). *British Journal of Nutrition*, 88 (06): 607-614.

Chatzifotis, S., Takeuchi, T. & Seikai, T. (1996). The effect of dietary carnitine supplementation on growth of red sea bream (Pagrus major) fingerlings at two levels of dietary lysine. *Aquaculture*, 147 (3-4): 235-248.

Chmurzynska, A. (2006). The multigene family of fatty acid-binding proteins (FABPs): Function, structure and polymorphism. *Journal of Applied Genetics*, 47 (1): 39-48.

Choi, C. K., Vicente-Manzanares, M., Zareno, J., Whitmore, L. A., Mogilner, A. & Horwitz, A. R. (2008). Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nature Cell Biology*, 10 (9): 1039-U36.

Chong, M. F. F., Hodson, L., Bickerton, A. S., Roberts, R., Neville, M., Karpe, F., Frayn, K. N. & Fielding, B. A. (2008). Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *American Journal of Clinical Nutrition*, 87 (4): 817-823.

Clarke, S. D. (1993). REGULATION OF FATTY-ACID SYNTHASE GENE-EXPRESSION - AN APPROACH FOR REDUCING FAT ACCUMULATION. *Journal of Animal Science*, 71 (7): 1957-1965.

Collins, J. M., Neville, M. J., Hoppa, M. B. & Frayn, K. N. (2010). De Novo Lipogenesis and Stearoyl-CoA Desaturase Are Coordinately Regulated in the Human Adipocyte and Protect against Palmitate-induced Cell Injury. *Journal of Biological Chemistry*, 285 (9): 6044-6052.

Coutteau, P., Geurden, I., Camara, M. R., Bergot, P. & Sorgeloos, P. (1997). Review on the dietary effects of phospholipids in fish and crustacean larviculture. *Aquaculture*, 155 (1-4): 149-164.

Craig, S. R. & Gatlin, D. M. (1997). Growth and body composition of juvenile red drum (Sciaenops ocellatus) fed diets containing lecithin and supplemental choline. *Aquaculture*, 151 (1-4): 259-267.

Cushman, S. W. & Wardzala, L. J. (1980). POTENTIAL MECHANISM OF INSULIN ACTION ON GLUCOSE-TRANSPORT IN THE ISOLATED RAT ADIPOSE CELL-APPARENT TRANSLOCATION OF INTRACELLULAR-TRANSPORT SYSTEMS TO THE PLASMA-MEMBRANE. *Journal of Biological Chemistry*, 255 (10): 4758-4762.

Dani, C., Smith, A. G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C. & Ailhaud, G. (1997). Differentiation of embryonic stem cells into adipocytes in vitro. *Journal of Cell Science*, 110: 1279-1285.

Davis, R. L., Weintraub, H. & Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, 51 (6): 987-1000.

Della Gaspera, B., Braut-Boucher, F., Bomsel, M., Chatelet, F., Guguen-Guillouzo, C., Font, J., Weinman, J. & Weinman, S. (2001). Annexin expressions are temporally and spatially regulated during rat hepatocyte differentiation. *Developmental Dynamics*, 222 (2): 206-217.

Dobson, D. E., Groves, D. L. & Spiegelman, B. M. (1987). NUCLEOTIDE-SEQUENCE AND HORMONAL-REGULATION OF MOUSE GLYCEROPHOSPHATE DEHYDROGENASE MESSENGER-RNA DURING ADIPOCYTE AND MUSCLE-CELL DIFFERENTIATION. *Journal of Biological Chemistry*, 262 (4): 1804-1809.

Dossantos, J., Burkow, I. C. & Jobling, M. (1993). PATTERNS OF GROWTH AND LIPID DEPOSITION IN COD (GADUS-MORHUA L) FED NATURAL PREY AND FISH-BASED FEEDS. *Aquaculture*, 110 (2): 173-189.

FAO. (2008). The State of World Fisheries and Aquaculture 2008. *FAO,Rome*.

Folch, J., Lees, M. & Stanley, G. H. S. (1957). A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry*, 226 (1): 497-509.

Freshney, R. b. R. I. (2005). Culture of animal cells: A manual of basic technique, fifth edition.

Freshney, R. I. (1987). Culture of Animal Cells. A Manual of Basic Technique. (New York, Alan R. Liss, Inc.).

Friedens.Aj, Chailakh.Rk & Lalykina, K. S. (1970). DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS. *Cell and Tissue Kinetics*, 3 (4): 393-&.

Fryer, J. L. & Lannan, C. N. (1994). Three decades of fish cell culture: A current listing of cell lines derived from fishes. *Methods in Cell Science*, 16 (2): 87-94.

Gaillard, D., Negrel, R., Lagarde, M. & Ailhaud, G. (1989). REQUIREMENT AND ROLE OF ARACHIDONIC-ACID IN THE DIFFERENTIATION OF PRE-ADIPOSE CELLS. *Biochemical Journal*, 257 (2): 389-397.

Gerke, V. & Moss, S. E. (1997). Annexins and membrane dynamics. *Biochimica Et Biophysica Acta-Molecular Cell Research*, 1357 (2): 129-154.

Gimeno, R. E. (2007). Fatty acid transport proteins. *Current Opinion in Lipidology*, 18 (3): 271-276.

Gregoire, F. M., Smas, C. M. & Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews*, 78 (3): 783-809.

Grimaldi, P. A., Teboul, L., Inadera, H., Gaillard, D. & Amri, E. Z. (1997). Trans-differentiation of myoblasts to adipoblasts: triggering effects of fatty acids and thiazolidinediones. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 57 (1): 71-75.

Guillam, M. T., Burcelin, R. & Thorens, B. (1998). Normal hepatic glucose production in the absence of GLUT2 reveals an alternative pathway for glucose release from hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 95 (21): 12317-12321.

Hansen, J. O., Berge, G. M., Hillestad, M., Krogdahl, A., Galloway, T. F., Holm, H., Holm, J. & Ruyter, B. (2008). Apparent digestion and apparent retention of lipid and fatty acids in Atlantic cod (Gadus morhua) fed increasing dietary lipid levels. *Aquaculture*, 284 (1-4): 159-166.

Harmelen, V., Skurk, T. & Hauner, H. (2005). Primary Culture and Differentiation of Human Adipocyte Precursor Cells. In, pp. 125-135.

Harrison, R. G., Greenman, M. J., Mall, F. P. & Jackson, C. M. (1907). Observations of the living developing nerve fiber. *The Anatomical Record*, 1 (5): 116-128.

Hatlen, B., Helland, S. J. & Grisdale-Helland, B. (2007). Energy and nitrogen partitioning in 250 g Atlantic cod (Gadus morhua L.) given graded levels of feed with different protein and lipid content. *Aquaculture*, 270 (1-4): 167-177.

Hewitt, J., Lu, X., Gilbert, L. & Nanes, M. S. (2008). The Muscle Transcription Factor MyoD Promotes Osteoblast Differentiation by Stimulation of the Osterix Promoter. *Endocrinology*, 149 (7): 3698-3707.

Hillestad, M. & Johnsen, F. (1994). High-energy/low-protein diets for Atlantic salmon: effects on growth, nutrient retention and slaughter quality. *Aquaculture*, 124 (1-4): 109-116.

Hoshi, M., Williams, M. & Kishimot.Y. (1973). ESTERIFICATION OF FATTY-ACIDS AT ROOM-TEMPERATURE BY CHLOROFORM-METHANOLIC HCL-CUPRIC ACETATE. *Journal of Lipid Research*, 14 (5): 599-601.

James, D. E., Strube, M. & Mueckler, M. (1989). MOLECULAR-CLONING AND CHARACTERIZATION OF AN INSULIN-REGULATABLE GLUCOSE TRANSPORTER. *Nature*, 338 (6210): 83-87.

Jobling, M. (1988). A review of the physiological and nutritional energetics of cod, Gadus morhua L., with particular reference to growth under farmed conditions. *Aquaculture*, 70 (1-2): 1-19.

Jobling, M., Knudsen, R., Pedersen, P. S. & Dos Santos, J. (1991). Effects of dietary composition and energy content on the nutritional energetics of cod, Gadus morhua. *Aquaculture*, 92: 243-257.

Jobling, M., Meløy, O. H., Santos, J. & Christiansen, B. (1994). The compensatory growth response of the Atlantic cod: effects of nutritional history. *Aquaculture International*, 2 (2): 75-90.

Jobling, M. & Pedersen, T. (1995). Cultivation of atlantic cod *in production of aquatic animals*: 347-356.

Karlsen, Ø., Hemre, G. I., Tveit, K. & Rosenlund, G. (2006). Effect of varying levels of macro-nutrients and continuous light on growth, energy deposits and maturation in farmed Atlantic cod (Gadus morhua L.). *Aquaculture*, 255 (1-4): 242-254.

Khaitlina, S. Y. (2001). Functional specificity of actin isoforms. In vol. Volume 202 *International Review of Cytology*, pp. 35-98: Academic Press.

Kim, J.-D. & Lall, S. P. (2001). Effects of dietary protein level on growth and utilization of protein and energy by juvenile haddock (Melanogrammus aeglefinus). *Aquaculture*, 195 (3-4): 311-319.

Kjær, M. A., Vegusdal, A., Berge, G. M., Galloway, T. F., Hillestad, M., Krogdahl, Å., Holm, H. & Ruyter, B. (2009). Characterisation of lipid transport in Atlantic cod (Gadus morhua) when fasted and fed high or low fat diets. *Aquaculture*, 288 (3-4): 325-336.

KOKK, K., VERÄJÄNKORVA, E., WU, X.-K., TAPFER, H., PÕLDOJA, E., SIMOVART, H.-E. & PÖLLÄNEN, P. (2007). Expression of Insulin Signaling

Transmitters and Glucose Transporters at the Protein Level in the Rat Testis. *Annals of the New York Academy of Sciences*, 1095 (Signal Transduction Pathways, Part C: Cell Signaling in Health and Disease): 262-273.

Koumans, J. T. M., Akster, H. A., Dulos, G. J. & Osse, J. W. M. (1990). MYOSATELLITE CELLS OF CYPRINUS-CARPIO (TELEOSTEI) INVITRO - ISOLATION, RECOGNITION AND DIFFERENTIATION. *Cell and Tissue Research*, 261 (1): 173-181.

Lavon, N. & Benvenisty, N. (2005). Study of hepatocyte differentiation using embryonic stem cells. *Journal of Cellular Biochemistry*, 96 (6): 1193-1202.

Le Cabec, V., Russo-Marie, F. & Maridonneau-Parini, I. (1992). Differential expression of two forms of annexin 3 in human neutrophils and monocytes and along their differentiation. *Biochemical and Biophysical Research Communications*, 189 (3): 1471-1476.

Leaver, M. J., Bautista, J. M., Bjornsson, B. T., Jonsson, E., Krey, G., Tocher, D. R. & Torstensen, B. E. (2008). Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture. *Reviews in Fisheries Science*, 16: 73-94.

Lee, C. H., Kang, K. W., Mehl, I. R., Nofsinger, R., Alaynick, W. A., Chong, L. W., Rosenfeld, J. M. & Evans, R. M. (2006). Peroxisome proliferator-activated receptor delta promotes very low-density lipoprotein-derived fatty acid catabolism in the macrophage. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (7): 2434-2439.

Lemaigre, F. & Zaret, K. S. (2004). Liver development update: new embryo models, cell lineage control, and morphogenesis. *Current Opinion in Genetics & Development*, 14 (5): 582-590.

Liang, X.-F., Ogata, H. Y. & Oku, H. (2002). Effect of dietary fatty acids on lipoprotein lipase gene expression in the liver and visceral adipose tissue of fed and starved red sea bream Pagrus major. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 132 (4): 913-919.

Lie, Ø., Lied, E. & Lambertsen, G. (1986). Liver retention of fat and of fatty acids in cod (Gadus morhua) fed different oils. *Aquaculture*, 59 (3-4): 187-196.

Makowski, L. & Hotamisligil, G. S. (2004). Fatty acid binding proteins - The evolutionary crossroads of inflammatory and metabolic responses. *Journal of Nutrition*, 134 (9): 2464S-2468S.

Mason, M. E. & Waller, G. R. (1964). DIMETHOXYPROPANE INDUCED TRANSESTERIFICATION OF FATS+OILS IN PREPARATION OF METHYL ESTERS FOR GAS CHROMATOGRAPHIC ANALYSIS. *Analytical Chemistry*, 36 (3): 583-&.

Matschak, T. W. & Stickland, N. C. (1995). The growth of Atlantic salmon (Salmo salar L.) myosatellite cells in culture at two different temperatures. *Cellular and Molecular Life Sciences*, 51 (3): 260-266.

Matsusue, K., Peters, J. M. & Gonzalez, F. J. (2004). PPARβ/δ potentiates PPARγ-stimulated adipocyte differentiation. *Faseb Journal*: 04-1944fje.

Megeney, L. A. & Rudnicki, M. A. (1995). etermination versus differentiation and the MyoD-family of transcription factors. *Biochem. Cell Biol.*, 73 (3): 723-732.

Megeney, L. A., Kablar, B., Garrett, K., Anderson, J. E. & Rudnicki, M. A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes & Development*, 10 (10): 1173-1183.

Menell, J. S., Cesarman, G. M., Jacovina, A. T., McLaughlin, M. A., Lev, E. A. & Hajjar, K. A. (1999). Annexin II and Bleeding in Acute Promyelocytic Leukemia. *N Engl J Med*, 340 (13): 994-1004.

Meton, I., Caseras, A., Fernandez, F. & Baanante, I. V. (2004). Molecular cloning of hepatic glucose-6-phosphatase catalytic subunit from gilthead sea bream (Sparus aurata): response of its mRNA levels and glucokinase expression to refeeding and diet composition. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 138 (2): 145-153.

Morais, S., Bell, J. G., Robertson, D. A., Roy, W. J. & Morris, P. C. (2001). Protein/lipid ratios in extruded diets for Atlantic cod (Gadus morhua L.): effects on growth, feed utilisation, muscle composition and liver histology. *Aquaculture*, 203 (1-2): 101-119.

Nanton, D. A., Lall, S. P. & McNiven, M. A. (2001). Effects of dietary lipid level on liver and muscle lipid deposition in juvenile haddock. *Aquaculture Research*, 32 (s1): 225-234.

Niimi, S., Oshizawa, T., Yamaguchi, T., Harashima, M., Seki, T., Ariga, T., Kawanishi, T. & Hayakawa, T. (2003). Specific expression of annexin III in rat-small-hepatocytes. *Biochemical and Biophysical Research Communications*, 300 (3): 770-774.

Nordlie, R. C., Foster, J. D. & Lange, A. J. (1999). Regulation of glucose production by the liver. *Annual Review of Nutrition*, 19: 379-406.

Ostbye, T. K. K., Wetten, O. F., Tooming-Klunderud, A., Jakobsen, K. S., Yafe, A., Etzioni, S., Moen, T. & Andersen, O. (2007). Myostatin (MSTN) gene duplications in Atlantic salmon (Salmo salar): Evidence for different selective pressure on teleost MSTN-1 and -2. *Gene*, 403 (1-2): 159-169.

Paisley, L. G., Ariel, E., Lyngstad, T., Jónsson, G., Vennerström, P., Hellström, A. & Østergaard, P. (2010). An Overview of Aquaculture in the Nordic Countries. *Journal of the World Aquaculture Society*, 41 (1): 1-17.

Pasquet, P., Brigant, L., Froment, A., Koppert, G. A., Bard, D., Degarine, I. & Apfelbaum, M. (1992). MASSIVE OVERFEEDING AND ENERGY-BALANCE IN MEN - THE GURU-WALLA MODEL. *American Journal of Clinical Nutrition*, 56 (3): 483-490.

Peters, J. M., Lee, S. S. T., Li, W., Ward, J. M., Gavrilova, O., Everett, C., Reitman, M. L., Hudson, L. D. & Gonzalez, F. J. (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). *Molecular and Cellular Biology*, 20 (14): 5119-5128.

Pfaffl, M. W., Horgan, G. W. & Dempfle, L. (2002). Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*, 30 (9).

Pierleoni, C., Verdenelli, F., Castellucci, M. & Cinti, S. (1998). Fibronectins and basal lamina molecules expression in human subcutaneous white adipose tissue. *European Journal of Histochemistry*, 42 (3): 183-188.

Poliard, A., Nifuji, A., Lamblin, D., Plee, E., Forest, C. & Kellermann, O. (1995). CONTROLLED CONVERSION OF AN IMMORTALIZED MESODERMAL PROGENITOR-CELL TOWARDS OSTEOGENIC, CHONDROGENIC, OR ADIPOGENIC PATHWAYS. *Journal of Cell Biology*, 130 (6): 1461-1472.

Polvi, S. M. & Ackman, R. G. (1992). Atlantic salmon (Salmo salar) muscle lipids and their response to alternative dietary fatty acid sources. *Journal of Agricultural and Food Chemistry*, 40 (6): 1001-1007.

Pomies, P., Louis, H. A. & Beckerle, M. C. (1997). CRP1, a LIM domain protein implicated in muscle differentiation, interacts with alpha-actinin. *Molecular Biology of the Cell*, 8: 2414-2414.

Pomies, P., Macalma, T. & Beckerle, M. C. (1999). Purification and characterization of an alpha-actinin-binding PDZ-LIM protein that is up-regulated during muscle differentiation. *Journal of Biological Chemistry*, 274 (41): 29242-29250.

Poston, H. A. (1991). Response of Rainbow Trout to Soy Lecithin, Choline, and Autoclaved Isolated Soy Protein. *The Progressive Fish-Culturist*, 53 (2): 85-90.

Robin, L. R. & Roncari, D. A. K. (1978). Complete differentiation of adipocyte precursors. *Cell and Tissue Research*, 195 (2): 317-329.

Rodríguez, C., Pérez, J. A., Díaz, M., Izquierdo, M. S., Fernández-Palacios, H. & Lorenzo, A. (1997). Influence of the EPA/DHA ratio in rotifers on gilthead seabream (Sparus aurata) larval development. *Aquaculture*, 150 (1-2): 77-89.

Rosenlund, G., Karlsen, Ø., Tveit, K., Mangor-Jensen, A. & Hemre, G.-I. (2004). Effect of feed composition and feeding frequency on growth, feed utilization and nutrient retention in juvenile Atlantic cod, Gadus morhua L. *Aquaculture Nutrition*, 10 (6): 371-378.

Rudnicki, M. A. & Jaenisch, R. (1995). The MyoD family of transcription factors and skeletal myogenesis. *BioEssays*, 17 (3): 203-209.

Rumsey, G. L. (1991). Choline-betaine requirements of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, 95 (1-2): 107-116.

Russell, P. M., Davies, S. J., Gouveia, A. & Tekinay, A. A. (2001). Influence of dietary starch source on liver morphology in juvenile cultured European sea bass (Dicentrarchus labrax L.). *Aquaculture Research*, 32: 306-314.

Santulli, A., Modica, A., Curatolo, A. & D'Amelio, V. (1988). Carnitine administration to sea bass (Dicentrarchus labrax (L.)) during feeding on a fat diet: Modification of plasma lipid levels and lipoprotein pattern. *Aquaculture*, 68 (4): 345-351.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. & Estevez, A. (1999). Recent developments in the essential fatty acid nutrition of fish. *Aquaculture*, 177 (1-4): 191-199.

Schaffer, J. E. & Lodish, H. F. (1994). Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell*, 79 (3): 427-436.

Segner, H. (1998). Isolation and primary culture of teleost hepatocytes. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, 120 (1): 71-81.

Soto-Gutierrez, A., Navarro-Alvarez, N., Rivas-Carrillo, J. D., Chen, Y., Yamatsuji, T., Tanaka, N. & Kobayashi, N. (2006). Differentiation of Human Embryonic Stem Cells to Hepatocytes Using Deleted Variant of HGF and Poly-amino-urethane-Coated Nonwoven Polytetrafluoroethylene Fabric. *Cell Transplantation*, 15: 335-342.

Spisni, E., Tugnoli, M., Ponticelli, A., Mordenti, T. & Tomasi, V. (1998). Hepatic steatosis in artificially fed marine teleosts. *Journal of Fish Diseases*, 21 (3): 177-184.

Sun, L., Nicholson, A. C., Hajjar, D. P., Gotto, A. M. & Hau, J. H. (2003). Adipogenic differentiating agents regulate expression of fatty acid binding protein and CD36 in the J744 macrophage cell line. *Journal of Lipid Research*, 44 (10): 1877-1886.

Suzuki, K. & Kono, T. (1980). EVIDENCE THAT INSULIN CAUSES TRANSLOCATION OF GLUCOSE-TRANSPORT ACTIVITY TO THE PLASMA-MEMBRANE FROM AN INTRACELLULAR STORAGE SITE. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 77 (5): 2542-2545.

Swierczynski, J., Zabrocka, L., Goyke, E., Raczynska, S., Adamonis, W. & Sledzinski, Z. (2003). Enhanced glycerol 3-phosphate dehydrogenase activity in adipose tissue of obese humans. *Molecular and Cellular Biochemistry*, 254 (1): 55-59.

Takeuchi, T., Feng, Z., Yoseda, K., Hirokawa, J. & Watanabe, T. (1994). NUTRITIVE-VALUE OF DHA-ENRICHED ROTIFER FOR LARVAL COD. *Nippon Suisan Gakkaishi*, 60 (5): 641-652.

Thorens, B., Sarkar, H. K., Kaback, H. R. & Lodish, H. F. (1988). CLONING AND FUNCTIONAL EXPRESSION IN BACTERIA OF A NOVEL GLUCOSE TRANSPORTER PRESENT IN LIVER, INTESTINE, KIDNEY, AND BETA-PANCREATIC ISLET CELLS. *Cell*, 55 (2): 281-290.

Ting, T. & Peng, J. R. (2009). Liver development in zebrafish (Danio rerio). *Journal of Genetics and Genomics*, 36 (6): 325-334.

Tocher, D. R. (2010). Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research*, 41 (5): 717-732.

Todorcevic, M., Vegusdal, A., Gjoen, T., Sundvold, H., Torstensen, B. & Kjaer, M. (2008). Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids*, 1781: 326 - 335.

Todorcevic, M., Vegusdal, A., Gjoen, T., Sundvold, H., Torstensen, B. E., Kjaer, M. A. & Ruyter, B. (2008). Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 1781 (6-7): 326-335.

Todorcevic, M., Kjaer, M. A., Djakovic, N., Vegusdal, A., Torstensen, B. E. & Ruyter, B. (2009). N-3 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon visceral adipose tissue. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 152 (2): 135-143.

Todorcevic, M., Skugor, S., Krasnov, A. & Ruyter, B. (2010). Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. *BMC Genomics*, 11 (1): 39.

Vegusdal, A., Ostbye, T. K., Tran, T. N., Gjoen, T. & Ruyter, B. (2004). beta-oxidation, esterification, and secretion of radiolabeled fatty acids in cultivated Atlantic salmon skeletal muscle cells. *Lipids*, 39 (7): 649-658.

Waisman, D. M. (1995). ANNEXIN II TETRAMER - STRUCTURE AND FUNCTION. *Molecular and Cellular Biochemistry*, 149: 301-322.

Wang, Y.-X., Lee, C.-H., Tiep, S., Yu, R. T., Ham, J., Kang, H. & Evans, R. M. (2003). Peroxisome-Proliferator-Activated Receptor [delta] Activates Fat Metabolism to Prevent Obesity. *Cell*, 113 (2): 159-170.

Wilson, R. P. & Poe, W. E. (1988). Choline nutrition of fingerling channel catfish. *Aquaculture*, 68 (1): 65-71.

Wolf, K. & Quimby, M. C. (1962). ESTABLISHED EURYTHERMIC LINE OF FISH CELLS IN VITRO. *Science*, 135 (3508): 1065-&.

Xu, A., Wang, Y., Xu, J. Y., Stejskal, D., Tam, S., Zhang, J., Wat, N. M. S., Wong, W. K. & Lam, K. S. L. (2006). Adipocyte Fatty Acid-Binding Protein Is a Plasma Biomarker Closely Associated with Obesity and Metabolic Syndrome. *Clin Chem*, 52 (3): 405-413.

Yang, S. D., Lin, T. S., Liou, C. H. & Peng, H. K. (2003). Influence of dietary protein levels on growth performance, carcass composition and liver lipid classes of juvenile Spinibarbus hollandi (Oshima). *Aquaculture Research*, 34 (8): 661-666.

Zhang, S. Z., Xu, Y., Xie, H. Q., Li, X. Q., Wei, Y. Q. & Yang, Z. M. (2009). The possible role of Myosin light chain in myoblast proliferation. *Biological Research*, 42 (1): 121-132.

Zheng, F., Takeuchi, T., Yoseda, K., Kobayashi, M., Hirokawa, J. & Watanabe, T. (1996). Requirement of larval cod for arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid using by their enriched Artemia Nauplii. *Nippon Suisan Gakkaishi*, 62 (4): 669-676.