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OPTIMIZING METHODS FOR STUDIES OF ADIPOSE TISSUE
FUNCTION IN ATLANTIC SALMON (SALMO SALAR)
-- WITH SPECIFIC FOCUS ON ISOLATION AND CULTURE
CONDITIONS OF ADIPOCYTES AND WHOLE ADIPOSE TISSUE
FIXATION FOR MORPHOLOGICAL STUDIES



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Optimizing Methods for Studies of Adipose Tissue Function in Atlantic Salmon

With specific focus on isolation and culture conditions of adipocytes and whole adipose
tissue fixation for morphological studies

Master Thesis (60 credits)

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Abbreviations

Anti-mIgG	Anti-mouse immunoglobulin G
aSVF	Adipose-derived stromal-vascular fraction
bFGF	Basic fibroblast growth factor human
cAMP	Cyclic adenosine monophosphate
C/EBP(s)	CCAAT/enhancer binding protein(s)
DAPI	4',6-diamidino-2-phenylindole
DEX	Dexamethasone
DHA	Docosahexaenoic acid
DMEM	Dulbecco's modified Eagle's medium
EF1A	Elongation factor 1A
EPA	Eicosapentaenoic acid
FA(s)	Fatty acid(s)
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GM	Growth media
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HUFA	Highly unsaturated fatty acids
IBMX	3-isobutyl-1-methylxantine
L-15	Leibowitz-15
LCFA	Long chain fatty acid
LPL	Lipoprotein lipase
mIgG	Mouse immunoglobulin G
NaCl	Sodium Chloride
NCAM	Neural cell adhesion molecule
OA	Oleic acid

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PPAR	Peroxisome proliferator-activated receptor
PVP	Polyvinylpyrrolidone
REST	Relative Expression Software Tool
RFP	Red fluorescent protein
RT	Reverse transcription
RT-qPCR	Real-time quantitative polymerase chain reaction
T3	Triiodothyronine or 3,3',5-Triiodo-L-thyronine sodium salt
WGA	Wheat germ agglutinin
α	Alpha
β	Beta
γ	Gamma
δ	Delta

Summary

Cell culture of adipocytes is a valuable research tool for studies of adipose tissue functions. The first aim of this thesis was to optimize culture conditions for Atlantic salmon primary cells derived from the adipose-derived stromal vascular fraction (aSVF).

Firstly, we have tested how different levels of fetal bovine serum (FBS) supplemented in the media affect the development of adipocytes, from stem cells (pre-adipocytes), their proliferation and further differentiation to mature adipocytes. Three levels (2.5%, 5% and 10%) of FBS were studied. The result showed that 10% FBS significantly increased the proliferation of pre-adipocytes, while the differentiation degree, was the highest with 5% of FBS. We also tested the performance of cells in two different culture media. Our results suggested that during the proliferation stage, Dulbecco's modified Eagle's medium (DMEM) medium with CO₂, improved the proliferation compared to the Leibowitz-15 (L-15) medium.

We, further, investigated the effects of two doses (0.1mM and 0.6mM) of docosahexaenoic acid (22:6n-3, DHA) and oleic acid (18:1n-9, OA) on one important transcription factor regulating the differentiation process, the CCAAT/enhancer binding protein alpha (C/EBP α) and the expression of fatty acid transport protein (FATP) known to be highly expressed at late differentiation stage. The expressions of both genes (the C/EBP α) and (FATP) 1) were more up-regulated with high dose of fatty acids (FAs) supplementation than with the low dose one, showing that the FA level in the media influence the differentiation degree of adipocytes. More and larger lipid droplets were formed in the cells supplemented with OA than those supplemented with DHA, showing that also the type of FA differently influence the formation of lipid droplets in adipocytes. Both OA and DHA induce the expression of C/EBP α and FATP 1 compared to the control group, however there was a more moderate lipid accumulation with DHA than with OA.

ASVF includes cells of vasculature, immune cells, pre-adipocytes, mature adipocytes and stem

cells. The standard protocol for isolation aSVF cells, removes the mature adipocytes and partly digested tissue. However the cell fractions that are seeded on culture dishes contain adipose cells of many different developmental stages and blood cells. In order to isolate a more pure stem cell and pre-adipocyte fraction, one aim of this thesis was to establish a method for purification of the target cells by Percoll density gradient centrifugation. Density formation was tested by using 36% and 70% Percoll and centrifugation at 30 000g and 60 000g in our study. The results showed that 36% Percoll centrifuged at 60 000g formed a gradient suitable for further centrifugation and purification of stem cells. The verification of the identity of the purified cells was done by gene expression analysis. The results showed that *C/EBP α* , *C/EBP δ* and *FATP 1* were significantly lower expressed in the isolated stem cells compared to mature adipocytes, while *C/EBP β* was higher expressed in mature adipocytes. That implied that the isolated cells were still in an early developmental stage, and they remain viability after the isolation and recover successfully during the cultivation and have high capacity to proliferate. However, we cannot distinguish between stem cells and pre-adipocytes, except based on their differences in gene expression.

CryoWax method was tested for its feasibility in adipose tissue in our laboratory mainly for the purpose of morphological preservation. The process was carried out by freeze substitution and polyester wax embedding procedure. The fine structure and antigenicity of the tissue were well preserved after the above procedure. However, lipids were not well preserved in this high lipid content tissue. Our results indicate a need for further improvement of the method in order to conserve the lipids inside adipose tissue.

1. Introduction

In Atlantic salmon (*Salmo salar*), the primary sites of fat deposition are the visceral adipose tissue (Jeziarska et al. 1982; Morgan et al. 2002; Rowe et al. 1991) and the adipose tissue within connective tissue sheets in muscle (Nanton et al. 2007; Zhou et al. 1995). The amount of visceral fat depots in salmonids has increased with increased lipid levels in fish diets. And adipose tissue is no longer considered only as an inert organ with sole function to store lipids. Today, adipose tissue is also recognized as an important endocrine and secretory organ with numerous functions in regulation of energy homeostasis and immunity in Atlantic salmon (Skugor 2009; Todorcević et al. 2010). Adipocytes, the cells that primarily compose adipose tissue, play a role in determining the adipose biology and regulating adipose tissue homeostasis. Cell culture of adipocytes is therefore a valuable research tool for better understanding of adipose tissue functions. The Nofima laboratory was the first to publish protocols for culture of primary fish adipocytes (Todorcević et al. 2008; Vegusdal et al. 2003). One of the aims of this thesis was to further improve the protocols for cell isolation, purification and culture conditions.

Visceral adipose tissue consists of many different cell types, including mesenchymal stem cells and many different developmental stages of adipocytes (Casteilla & Dani 2006). The previous methods used for isolation of stem cells from adipose tissue in Nofima laboratory results in a relatively heterogeneous cell fraction (Todorcević et al. 2010). A major aim of my thesis was therefore, by the use of Percoll density gradient centrifugation, to isolate a more pure stem cell fraction. The identity of the stem cells in my *in vitro* isolation study, was verified by their capacity to attach to cell vial, to proliferate and by their results of gene expression.

Fetal bovine serum (FBS) is a widely used supplement in culture medium for most cell types. One of the aims of this thesis is to optimize the level of FBS in the media during different developmental stages of adipocytes, from stem cells, their proliferation and further differentiation to mature adipocytes. FBS contains significant amounts of growth factors,

growth inhibitory factors, nutrients and more, which influence the development of fish adipocytes. A number of previous mammalian studies have shown effect of FBS on cell development (Teichert-Kuliszewska et al. 1992), and metabolism, including FA synthesis (Eherton et al. 1987; Vernon & Finley 1988; Walton et al. 1986).

Several studies have also shown that supplementation of culture media with FAs influence the differentiation of both Atlantic salmon adipocytes (Todorčević et al. 2008) and mammalian adipocytes (Ding & Mersmann 2001; Tontonoz et al. 1994; Yamamoto et al. 2005). However, it is not known how different media concentrations of FAs influence the differentiation. A further aim of this thesis was to study how two concentrations of FAs influence the differentiation process. In addition, microscopy technology was used to monitor the adipocytes development and also distinguish the differences among the cells that different treatments yielded.

For morphological studies, adipose tissue is a challenging tissue to perform histological analyses on, due to its high lipid content. One of the aims of this thesis was to test if a newly developed method named CryoWax (Duran et al. 2011) would preserve Atlantic salmon adipose tissue well. CryoWax involves freeze substitution, followed by polyester wax embedding. Fluorescent staining gives a demonstration of a fine structure or component of this tissue with this CryoWax method.

2. Literature review

2.1. Adipose tissue distribution, growth and functions

Adipose tissue is a specialized connective tissue that functions as the major storage site for fat. Different species store various amounts of fat and distribute adipose tissue differently. The majority of lipid storage sites in fish are mesenteric fat, muscle and liver (Sheridan 1988). Visceral adipose tissue develops in certain areas of organism and it is considered as a special loose connective tissue. Visceral fat or abdominal fat also is known as organ fat or intra-abdominal fat, is located inside the abdominal cavity, packed between the organs (stomach, liver, intestines, kidneys, etc.). Visceral fat is different from subcutaneous fat underneath the skin, and intramuscular fat interspersed in skeletal muscles. It was reported that, in the Atlantic salmon, the primary sites of lipid deposition are the visceral adipose tissue (Jeziarska et al. 1982; Morgan et al. 2002; Rowe et al. 1991) and the adipose tissue located together with the connective tissue sheets called myosepta in the muscle (Nanton et al. 2007; Zhou et al. 1995). The major constituent of visceral adipose tissue is the adipocyte (fat cell). The main function of these cells is adapted to store energy in the form of triacylglycerols (TAGs) in the situation of excessive energy and to mobilize this energy for the periods of feed deprivation. When energy is needed, TAGs are broken down into glycerol and FAs, which are used in the beta oxidation to create energy.

It has been shown that individual white adipose tissue (WAT) depots provide distinct molecular and physiological properties. Since individual WAT depots differ broadly in size and show different gene expression profiles, it was suggested to upgrade their status to "mini-organs" (Tchkonia et al. 2006). The excessive energy form of TAGs deposited in white adipocytes can accumulate to a considerable extent. The final mass of adipose tissue is determined by hypertrophy (increase in the cell size) and hyperplasia (increase in the cell number). In the process of hypertrophy, WAT owns the ability to increase its size drastically without an

underlying transformed cellular phenotype (Rajala & Scherer 2003).

As mentioned above, one of the essential functions of adipose tissue is to store and release of fat. In addition, it is also a major protection mechanism against lipotoxicity caused by free fatty acids (FFAs) (Slawik & Vidal-Puig 2007). It is considered to be an active endocrine and secretory organ and is involved in metabolic processes. Adipose tissue is believed to be related to the immune system through the secretion of numerous adipokines, and therefore is recognized as an ancestral immune organ in mammalian studies (Caspar-Bauguil et al. 2005). Adipokines are cytokines with signaling properties in visceral adipose tissue (Lago et al. 2007). The classification is based on their functional roles in regulating appetite and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and homeostasis.

The understanding on the role of adipose tissues has largely expanded for the past decade. And literatures have indicated that development and function of fish adipose tissue may be close to the one in mammalian (Todorčević et al. 2009; Vegusdal et al. 2003).

2.2. Adipose tissue development

Visceral adipose tissue is an heterogeneous organ, made up from a variety of cell types that play significant roles in regulating adipose tissue functions (Casteilla & Dani 2006). The composition of adipose tissue in fish has not been completely investigated. ASVF is comprised of pluripotent stem cells, pre-adipocytes, adipocytes, cells of vasculature (pericytes and endothelial cells), and immune cells (monocytes, macrophages, neutrophils and lymphocytes) (Fig.1) (Casteilla & Dani 2006). The pluripotent stem cells are characterized with their ability to renew themselves through mitotic cell divisions and also the capability to differentiate into various cell types. It has been proposed that pre-adipocytes share a common precursor with osteoblasts, chondrocytes and myocytes (Casteilla & Dani 2006; Gregorieff et al. 1998). For instance, from a number of mammalian species, aSVF are readily induced into several

mesenchymal cell lines *in vitro* when incubated to specific growth conditions, e.g. adipogenic (Zuk et al. 2001; Zuk et al. 2002), osteogenic (Halvorsen et al. 2001; Ogawa et al. 2004), chondrogenic (Awad et al. 2004; Erickson et al. 2002; Huang et al. 2004; Ogawa et al. 2004; Winter et al. 2003), and myogenic lineages (Mizuno et al. 2002; Zuk et al. 2002). This pluripotent function was also demonstrated in Atlantic salmon study (Ytteborg et al. 2010). The findings are important and suggest wide use of these tissue-derived stem cells in further studies.

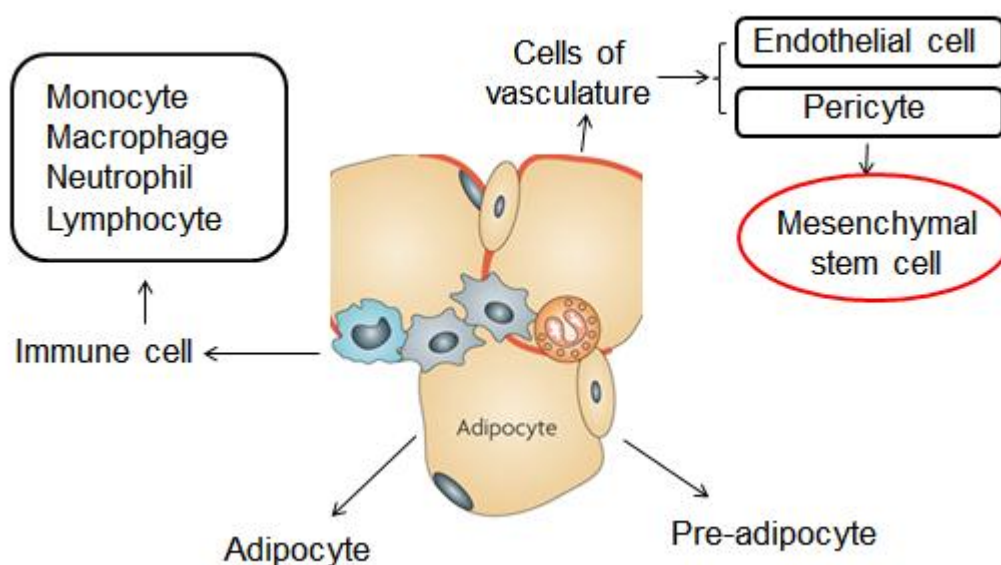


Fig. 1 The composition of adipose tissue.

2.2.1. Adipogenesis

Adipogenesis is one of the main characteristics of the development of fat cells or adipocytes. Cell culture models have been developed to investigate the commitment of a pluripotent stem cell and subsequent differentiation of pre-adipocytes into adipocytes. Adipogenesis can be divided in two major phases: determination and terminal differentiation (Fig.2) (Rosen et al. 2000).

The first determination phase includes the commitment of a pluripotent stem cell to the

adipocyte lineage (Tang et al. 2003). It is impossible to morphologically distinguish committed pre-adipocytes from their precursor cells. In spite of this, at this period, they have lost the potential to differentiate into other cell types. That is the reason why this stage is named as determination. Committed pre-adipocytes first undergo growth arrest through “contact inhibition” at confluence. Then, after hormonal induction, pre-adipocytes re-enter the cell cycle and experience a limited number of cell divisions (Gregoire et al. 1998; Otto & Lane 2005). The stage of re-entry into the cell cycle of growth-arrested pre-adipocytes is known as the “clonal expansion” phase, which is required for the optimal conversion of pre-adipocytes into matured adipocytes (Gregoire 2001).

When clonal expansion is completed, cells become growth arrested again and initiate the expression of adipose-specific genes, involved in the terminal differentiation of adipocytes (Gregoire 2001). Proliferation (growth) and differentiation of pre-adipocytes is controlled by interaction between individual cells or between cells and the extracellular environment (Gregoire et al. 1998). And growth arrest is considered to be a necessity for activating the differentiation of adipocytes (Scott et al. 1982a; Scott et al. 1982b). A salmon adipocyte study indicated that very similar regulation of the determination phase exists in salmon as that described in mammals (Todorcević et al. 2010).

In the second phase of “terminal differentiation”, pre-adipocytes convert to mature adipocytes (Fig.2), including the participate of lipid transport and synthesis, insulin sensitivity and the secretion of adipokines (Rosen & MacDougald 2006). This phase was studied on three fish species: Atlantic salmon (Todorcević et al. 2008; Todorcević et al. 2010; Vegusdal et al. 2003), Red sea bream (Oku & Okumura 2004) and Rainbow trout (Bouraoui et al. 2008).

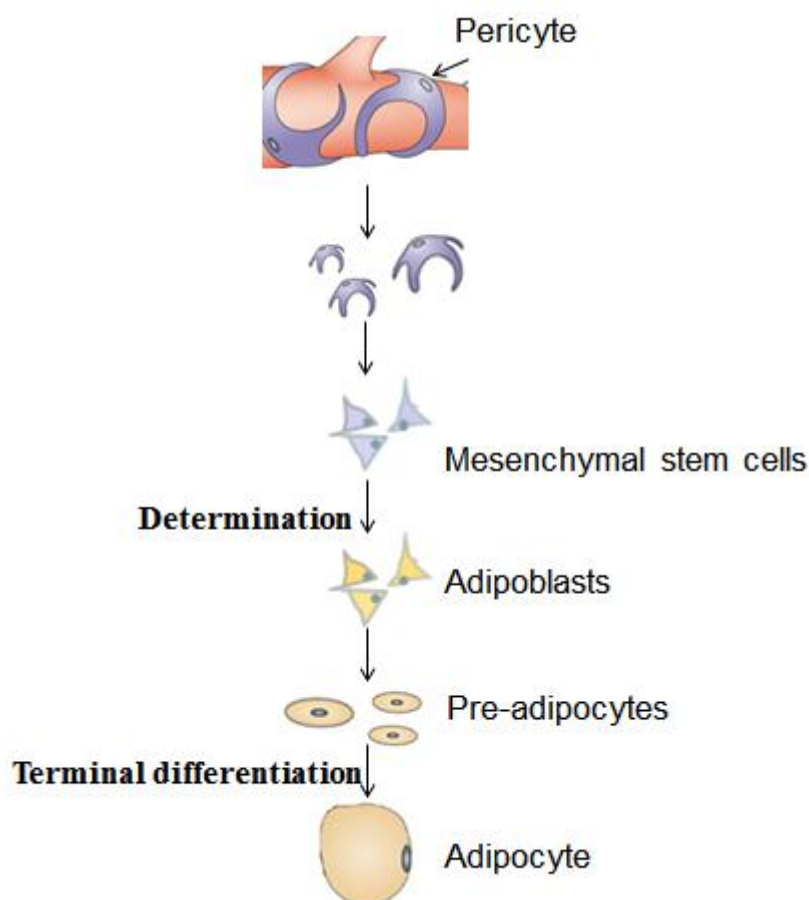


Fig. 2 The different stages of adipogenesis.

In mammalian and fish adipocyte differentiation, the adipocyte phenotype is recognized by changes in the expression of numerous genes. The most important transcription factors involved in this process, include the CCAAT/enhancer binding proteins (C/EBPs), C/EBP α , C/EBP β , C/EBP δ and peroxisome proliferator-activated receptor gamma (PPAR γ) (Ntambi & Kim 2000; Rosen et al. 2000; Todorcević et al. 2010) and each of them will be explained in details below.

2.2.1.1. Main adipogenic transcription factors

PPAR γ is a nuclear transcription factor that activates adipocyte differentiation. It cooperates with C/EBPs, which play a key role on the complex transcriptional regulation during adipogenesis (Rangwala & Lazar 2000; Rosen et al. 2000). PPAR γ is required for adipogenesis and with its absence this process cannot function efficiently (Rosen & Spiegelman 2001; Rosen

et al. 2002). In mammals, C/EBP β and C/EBP δ promote adipogenesis by inducing C/EBP α and PPAR γ . Same importance of these transcription factors in Atlantic salmon adipogenesis, has been reported (Huang et al. 2010; Todorcević et al. 2008; Todorcević et al. 2010).

C/EBP α , a basic leucine zipper transcription factor, is most richly expressed in adipose tissue, placenta, and liver, but is also detected in other various organs, such as lung, kidney, small intestine, brain, and hematopoietic cells (Birkenmeier et al. 1989). Cell culture studies reveal that C/EBP α is able to trigger differentiation of pre-adipocytes into mature adipocytes (Darlington et al. 1998). It regulates adipogenesis by turning on fat-specific genes that are necessary for the lipid synthesis (Madsen et al. 2005) and affects uptake and storage of long chain fatty acids (LCFAs) (Koutnikova & Auwerx 2001). This transcription factor is expressed relatively late during adipogenesis.

C/EBP β , an early adipogenic transcription factor, has been shown to activate the synthesis of insulin-like growth factor I (Pabst et al. 2001). C/EBP β is an integral part of the genetic cascade to activate the activity of adipogenesis (Darlington et al. 1998). Additionally, C/EBP β confers the ability of these cells to be differentiated into adipocytes by hormonal inducers (Wu et al. 1995; Yeh et al. 1995). In addition, an *in vivo* study indicates that C/EBP β is not necessary for differentiation of white adipose tissue (Darlington et al. 1998). However, the absence of C/EBP β leads to a reduced lipid accumulation in adipose tissue (Tanaka et al. 1997). Therefore, C/EBP β is still required in differentiation and plays a dual role as a stimulator of cell determination and differentiation.

C/EBP δ , early transcription factors of adipogenesis, acts in concert with full-length C/EBP β to enhance adipogenesis, decreased with aging during differentiation (Wu et al. 1996). C/EBP δ is highly expressed in adipose tissue, lung and intestine (Ramji & Foka 2002). Accordingly, the expression of C/EBP δ in pre-adipocytes results in the acceleration of the rate of C/EBP α induction (Yeh et al. 1995). It was reported in 3T3-L1 cells that the C/EBP δ expression required

dexamethasone (DEX) to enter a growth-arrested state (Hishida et al. 2009). Further, it may assist in the differentiation of adipose tissue. Therefore, C/EBP δ plays a role in initiating the differentiation cascade.

Fatty acid transport protein (FATP) was firstly discovered in 1994. Since then, it has been investigated for its role in FA transport and regulation. Numerous overexpression systems have implicated FATP in FA uptake (Chiu et al. 2005; Kim et al. 2004). FATP as a membrane-bound protein (Hall et al. 2003), it transports a cellular LCFA across the plasma membrane of cells (Schaffer & Lodish 1994). Since its subcellular localization and demonstration of function, it becomes the most promising candidate for a cellular LCFA transporter (Man et al. 1996). With the lipid transportation, FATP expression regulates the accumulation of lipids during the differentiation (Todorcević et al. 2008).

2.3. Advantages of using primary cells versus immortalized cell lines

In vitro cell culture models have been widely used during the last decade for studies of cellular functions. Cultivated cells from both mammals and fishes can be divided in two categories: immortalized stable cell lines and primary cells (Bols & Lee 1991). Primary cells in culture have a major advantage over immortalized cell lines, since they to a higher extent mimic the *in vivo* state of the particular cell type. Immortalized cell lines are widely used due to its ready availability and no limited cell division (Pan et al. 2009).

Primary cells are isolated from “live” animal tissues. For these kinds of cells, they have relative limited life span and can only be kept in a differentiated state for a relative short period of time (Sultan & Haagsman 2001). Primary cell culture offers a more relevant system to study different cellular functions than cell lines. An important benefit of primary cells, is that they more closely resemble the *in vivo* situation, since they often represent heterogeneous population of cells from the tissue they were isolated. On the other hand, working with primary cells in culture raises

numerous challenges, including the requirement for unique cell culture supplementations and growth conditions should take into consideration (Skugor 2009).

Nevertheless, the use of cell culture does suffer from some limitations. The normal feedback system that operates between the adipose tissue and others organs is not presented in cell culture and this makes it difficult to obtain a combined impact of other tissues and organs in fish.

So far, the best studied model for mammalian adipogenesis is the immortalized murine adipogenic cell line 3T3-L1. 3T3-L1 adipocytes have been introduced 30 years ago by Green and Meuth (1974). And it has been pivotal in advancing the understanding the role of adipocytes in obesity, diabetes, and related disorders in mammals. However, at the moment there is no available adipogenic cell line from fish. The knowledge of the adipocyte development from a fish progenitor cells to mature adipocytes is originated from primary pre-adipocytes. Primary pre-adipocytes are the cells isolated from aSVF grown in *in vitro* cell culture and treated with a combination of growth factors and adipogenic effectors (Todorčević et al. 2008; Todorčević et al. 2009; Vegusdal et al. 2003). Differentiation of pre-adipocytes to mature adipocytes is enhanced with the inducing agents such as DEX, which is used to stimulate the glucocorticoid receptor pathway, and 3-isobutyl-1-methylxantine (IBMX), which is used to stimulate the cAMP (cyclic adenosine monophosphate)-dependent protein kinase pathway, as well as triiodothyronine (T3), which is used to enhance the differentiation-linked expression of lipoprotein lipase (LPL) gene (e.g. PPARs), with the involvement of insulin (Oku et al. 2006; Reusch et al. 2000; Rosen & Spiegelman 2000).

2.4. Supplementation of culture media with FBS

FBS is the most widely used serum in animal cell cultures. It contains higher concentration of growth stimulatory factors and lesser concentration of growth inhibitory factors than other commercial sera, such as human, bovine calf, newborn bovine serum, donor bovine serum and

donor horse serum (Hu 2004). Due to the fact that it provides a very low level of antibodies and more growth factors than other alternative sera, it is recognized as the present standard for most cell culture systems. Additionally, FBS serves several more purposes as a supplement to culture media (van der Valk et al. 2010). Basically, it provides nutrients (proteins, lipids, carbohydrates) that are not presented in basal medium and modulates physiological properties viscosity and colloid osmolality of the culture media. It also contains protease inhibitors which neutralize proteases (like trypsin and collagenase) used during cell isolation. In addition, carrier proteins (for low molecular weight substances), “bulk” proteins (which prevents non-specific adsorption of critical factors to culture vessel) and enzymes (to convert components to utilizable or less toxic form) are presented in FBS. Hormones and factors for cell-substrate attachment and growth are also found in FBS. Furthermore, FBS functions as a binder to neutralize toxic substances and to protect essential nutrients (Hu 2004).

However, the use of FBS in cell culture medium is controversial for a number of reasons. Firstly, the process of collecting serum causes unnecessary suffering for the unborn calf (van der Valk et al. 2004). Subsequently, the fact is that the exact composition of FBS is not known. The components in FBS are not precisely quantified. And seasonal and continental changes in the serum composition, produces batch-to-batch variations. All these, in turn, cause phenotypical differences in the cell cultures, giving rise to variations in the outcomes (van der Valk et al. 2010). FBS contains specific FAs that would influence the FAs effect tested to the cells in the culture trial. Therefore, it is a considerable issue for fish adipocytes to reach a maximal growth with a lower level of FBS.

2.5. The effects of FA supplementation to adipocytes in culture

Energy in fish can be obtained from three major nutrients: proteins, carbohydrates and lipids. The most important energy sources for most fish species are lipids and proteins, since most carnivorous fish species, like Atlantic salmon, have a limited capacity to digest carbohydrates.

To reduce feed cost, a common way adopted is to replace as much protein as possible with lipids (Lee & Putnam 1973). Due to high level of lipids in the diet, Atlantic salmon faces the problem of high lipid stores in flesh and other fat storage organs (visceral adipose tissue), resulting in the reduction of harvest yields and health problems (Sargent et al. 2002). Thereby, lipids, like FAs, have been studied due to their effect on the development of fat deposition in fish trials.

Numerous studies have suggested that FAs are capable of regulating the adipocyte differentiation (Ding & Mersmann 2001; Tontonoz et al. 1994; Yamamoto et al. 2005). In addition, it was reported by Amri et al. (1991) and Distel et al. (1992) that FAs stimulate differentiation of pre-adipocytes. Similar results were proposed by Ding and Mersmann (2001) and Ding et al. (2002), in the differentiation of porcine pre-adipocyte in a cell culture system. All these findings revealed the importance of FAs during the development of adipocytes. Similar findings were found in fish. Different FAs were shown to regulate adipocytes differentiation differently in Atlantic salmon *in vitro* study (Todorcevic et al, 2008). Lower lipid accumulation was obtained during differentiation with n-3 highly unsaturated fatty acids (HUFAs) (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) enriched medium in contrast to oleic acid (OA) (Todorcević et al. 2008). OA (18:1n-9) is classified as a monounsaturated omega-9 FA, the proportion of this FAs can significantly vary among different tissues, such as muscle or adipose (Soriguer et al. 2000). DHA (22:6n-3) is part of HUFA family, is an important primary structural lipid components in the growth and development of biomembranes in mammals (Mann et al. 1994). A study has shown that DHA decreases body fat as well as fat accumulation both in fish (Todorcević et al. 2008; Todorcević et al. 2009) and mammals (Hainault et al. 1993; Parrish et al. 1990).

Adipocyte differentiation is a complex process, regulated by several transcription factors, which promotes cell morphologic conversion, lipogenic gene expression, and TAG accumulation (Rosen & Spiegelman 2000). In another word, the effect of FAs might be related to or due to the regulation of transcription factors. Raclot and Oudart (1999) discovered that in pre-adipocytes

treatment with FAs (especially HUFA), induced the expression of genes encoding for proteins involved in metabolism of FAs. Similarly, several fish studies consistently showed that HUFA induced up-regulation of C/EBP α and FATP 1 gene expression (Huang et al. 2010; Todorcević et al. 2010).

Further, dose of FAs supplied in the culture medium is another important factor in culture condition, which influences the expression of genes involved in development of adipocytes. Excessive uses of FAs not only generate low economic efficiency, but may also lead to toxicity (Hu 2004). Therefore, we seek for the dose effect of each FA during the adipocytes culture.

2.6. Density centrifugation

Isolation of cells from biological fluids and tissues is of importance in basic research. Cell suspensions, isolated from whole tissues, often consist of a mixture of different cells types and cell debris. In order to achieve a pure fraction of the target cell, in our case adipose stem cells, a purification method was required. The purification method must however not compromise the viability and biological function of the cells.

A number of advantages, by the use of density gradient purification of cell suspensions, have been discovered. During centrifugation all cells of same size, shape and density migrate to their corresponding density gradient regions, and remain stable in these positions (Brakke 1953). Moreover, separation of the components in the mixture can be performed by exploiting their differences in size (by rate zonal centrifugation) or density (by isopycnic centrifugation), as well as, by changing sedimentation time, g-force and the period of the density interval, such separation can be achieved (Pertoft 2000). For density gradient centrifugation, it is very important to select a medium that is capable of developing density gradient regions during centrifugation. For heterogeneous cells isolation, the medium called Percoll was successfully applied in stromal vascular fraction (by centrifugation) of inguinal tissue (Bjorntorp et al. 1978).

In our test, we used the same medium to isolate pure stem cells from aSVF. In order to check if we succeeded in isolation of stem cells, several gene markers were measured in the stem cells in comparison to mature adipocytes

Percoll was introduced in 1978 as a gradient medium for density-gradient centrifugation of cells and subcellular organelles (Pertoft et al. 1978). It composes of polyvinylpyrrolidone-coated (PVP-coated) colloidal silica-gel particles, which produce a polydisperse population of approximately spherical particles with an average diameter of 28-30nm when hydrated with 0.15 M-NaCl (Laurent et al. 1980). Good isolation outcome in application of tissues, cells and cell organelles was reported which made the usage of Percoll grow rapidly.

2.7. Methods for studies of adipose tissue morphology

Histology is as a study of microscopic anatomy of cells and tissues, through sectioning and staining to show the improved microscopic structures under microscope. Adipose tissue is a challenging tissue to perform histological analyses on, due to its high content of lipids inside each cell. Fixation of adipocytes requires special adapted fixation and embedding protocols. One of the aims of this thesis was therefore to test a new method in our laboratory for fixation of adipose tissue.

2.7.1. Fixation

Tissue fixation is the first step to prevent tissue from autolysis or putrefaction. To keep an intact native state and to preserve the tissues original structure requires optimal fixation process. It helps to terminate ongoing biochemical reactions in the cells and also increase mechanical strength of the tissue. There are several fixation methods that are commonly used today.

Conventional fixatives, such as formaldehyde or paraformaldehyde (PFA), are very much used

for preservation of biological samples. They show great property in prolonging the decaying process. On the other hand, the chemical fixing method (most common fixative is 10% neutral buffered formalin for light microscopy) leads to degradation of some molecules, like mRNA and DNA in the specimen. However, it functions well for fixation of intracellular lipids and nerve tissue. In tissue, the cross-linking reaction happens between the proteins, which besides the lipids hold them firmly in the original location (Blanchette-Mackie et al. 1995; Brasaemle et al. 2000). Obviously, this cross-linking reaction will not be so effective in lack of protein tissue. As we know, adipose tissue is full of lipids and with the so-called lack of protein situation. Therefore, the fixation of adipose tissue is always regarded as a problem. Most other fixatives like alcohols and glutaraldehyde are poor at preserving lipids due to extraction of lipids in the process. The best fixative for fixation of lipids is osmium tetroxide. However, it shows poor ability to penetrate thick sections. Glutaraldehyde shows low degree of diffusion ability in sections, and does not reveal good fixation of lipids, especially in thick sections. Hence, for immunohistochemistry staining, this fixative is not ideal (Hansen & Olafsen 1999).

Freeze substitution was initially introduced by Simpson (1941) as an efficient and inexpensive means to process tissue. Cryopreservation has its advantage in faster fixation resulting in a better preservation of fine structures in the tissue. As well as the method is simpler or easier to perform than other conventional methods. This technique can be carried out in a routine laboratory, which reflects its convenience compared to other technically demanding alternatives. The process takes place with a cold organic solvent at low temperature to dissolve and substitute the ice inside the cryofixed materials in the presence of a secondary fixative (Porter & Anderson 1982). With the absence of water in the sample, thereby the changes in structure reduced to a minimal range. Not like the conventional fixatives, freeze substitution can keep the sites available for staining, which means the physical nature of proteins remain as original state. And in some studies, it demonstrated that during freeze substitution with some solvents (e.g., acetone), lipids can be well stored (Weibull et al. 1984). Our trial was conducted to test out how the cryopreservation method (freeze substitution -80°C isobutanol for 2 minutes) functions in

preservation of adipose tissue from Atlantic salmon.

2.7.2. Embedding

Embedding is a process of casting or blocking the tissue for the purpose of subsequent sectioning. It involves the enclosure of the tissue in the infiltration medium used for processing, and then allowing the medium to solidify. To maintain a native morphology of tissue is one of the purposes of embedding. Soft tissue specimen will be easy to section with hardened or solidified external with infiltration medium. Adipose tissue, containing considerable amounts of low melting point lipids, is regarded as a challenging tissue during the process of embedding. Usually, paraffin is used as a common embedding material. However with the existence of lipids, the paraffin extracts the lipids out of the adipose tissue, consequently, hampers the effect of lipids fixation (DiDonato & Brasaemle 2003). Therefore, a necessity to find a less harmless fixation to adipose tissue draws our attention.

To achieve a hardened state, frozen section of adipose tissue is another approach instead of chemical fixation. The advantages of frozen section can be declared as following: better preservation of antigenicity and less exposure to the chemical fixative. In general, formalin-fixated paraffin embedded tissues shows bad preservation of RNA and some antigens, nevertheless performs good morphology (Finke et al. 1993). However, frozen section shows its strength in preserve RNA and lipids, despite of poor preservation in morphological detail. That is specifically significant in studying the fixation of adipose tissue.

Infiltration medium is a required component for frozen section to maintain a perfect embedding. Polyester wax as an embedding medium shows its strengths in histology (Steedman 1957). And it is not so widely employed in histology field. FA ester of polyethylene glycol is the main component of polyester wax (Sidman et al. 1961). Its property of low melting point (37-40°C) could avoid the shrinkage of adipose tissues. In paraffin, however, the problems are not only the

high melting point leads to poor morphologic preservation, but also the use of xylene to clear paraffin results in the extraction of fat in adipose tissues. Therefore, polyester wax with its no participant of xylene, as well as the dissolvability in most organic solvents, has its potential in application of adipose tissue. It possesses a good tissue preservation of structure and also allows the application of immunostaining.

2.7.3. CryoWax

The combination of freeze substitution and polyester wax embedding produces a new technology for histological application to those special tissues like adipose tissue. It was named in Duran et al. (2011)'s article as CryoWax. The shortcomings (e.g., structure variation, like shrinkage, antigenicity deterioration) of conventional chemical fixation and embedding processing, have been reported already in other study (Saga 2005). This new method possesses its advantage in faster fixation and stable cellular elements, results in increased interest in preservation of a fine tissue structure. Freeze substitution shows superior benefits, especially in the fluorescent antibody staining due to its low temperature embedding, protecting accurate localizations of antigens available (Balfour 1961). However, no study was reported on adipose tissue with CryoWax. Thereby, we test the feasibility of this method in adipose tissue for the purpose of morphological preservation.

2.7.4. Staining

In histology, staining is used to improve contrast in microscopic images. It involves adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate, to qualify or quantify the presence of a specific compound (Uzairu & Yiase 2010). Staining of adipose tissue serves similar purposes. With the presence of a fluorescent stain molecule, substrates or antibodies in adipose tissue can be distinguished easily. In some cases, specific stain can be used to visualize certain structures like the following stains we used in our adipose tissue study.

In the 1970s, application of 4',6-diamidino-2-phenylindole (DAPI) was used to detect DNA inside cells (Kapusinski 1995). It has the maximum absorption and emission wavelengths of 358nm and 461nm, respectively. Therefore it is excited and detected via a blue filter with ultraviolet light. Meanwhile, phalloidin was first purified in the 1930s (Lynen & Wieland 1938). As a F-actin binding, the distribution of actin can be labeled via this fluorescent imaging tool for light microscopy. In most cases, F-actin as an element of microfilaments (one of three major components of the cytoskeleton), is used to indicate the presence of microfilaments in cytoskeleton and thin filaments in muscle cells. For wheat germ agglutinin (WGA) binds to N-acetyl-D-glucosamine and sialic acid (Peters et al. 1979). The lectin functions on the cell membranes or other subcellular structures with the existence of sialic acid. For cellular lipids staining, LipidTOX is considered as a more selective and flexible microscopy stain to detect. It could be used to visualize lipid droplets during the process of adipogenesis. Furthermore, LipidTOX staining exhibited superior performance over other stains (i.e., Nile red, oil red O) (Ford et al. 1999). Oil red O is a staining used to visualize the lipids and triglycerides on frozen sections (Ramirezzacarias et al. 1992). It demonstrates with a deeper red color, which therefore can be observed easily. It is a dye that belongs to the Sudan staining with high affinity to fats (Zugibe et al. 1959).

Immunofluorescence is generally used to specify the distribution of biological molecules since 1941 (Coons et al. 1941). For the fixed cells, the significant problem is that antibodies exhibit a limited ability to cross the cell membrane. Though, the application of immunofluorescence can be investigated on the presence or absence of a protein, tissue location, sub-cellular distribution, and variation in protein expression or degradation. Neural cell adhesion molecule (NCAM, also as cluster of differentiation CD56) binds the glycoprotein gene-expressed on the neurons, glia, skeletal muscle and natural killer cells (Letourneau et al. 1994). Its role is to induce neurite outgrowth and work upon cell-cell adhesion and a signaling pathway (p59Fyn) (Kolkova et al. 2000).

In Cryowax processed adipose tissue, the preservation of cellular or subcellular components and antigenicity will be verified or examined in our study. The assistance of above stains under the microscopy technology ensures the visualization to test the feasibility of this Cryowax method in adipose tissue.

3. Materials and Methods

3.1. Materials

Atlantic salmon (*Salmo salar*) were farmed in Averøy or Sunndalsøra, Norway (two of the Nofima research stations). Metacain (MS-222) was from Norsk Medisinaldepot (Oslo, Norway). Nunclon Vita MultiDish 6 was obtained from Thermo (Roskilde, Denmark). Cell flasks were obtained from Nalge Nunc International (Rochester, NY, USA). Laminin was from Millipore (Temecula, CA, USA). Isobutanol, Sodium chloride, sodium hydroxide, β -ME was from Merck (Darmstadt, Germany). Ethanol, isopropanol were from Rektifisert spirit (Vestby, Norway). Phosphate buffered saline solution (PBS), Tween-20, Triton-X100, Saponin, oil red O, Dulbecco's modified Eagle's medium (DMEM), Leibowitz-15 (L-15), Fetal bovine serum (FBS), Antibiotic Antimycotic solution (with 10,000 units penicillin, 10mg streptomycin and 25 μ g amphotericin B), 3,3',5-Triiodo-L-thyronine sodium salt (T3), dexamethasone (DEX) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-Isobutyl-1-methylxanthine (IBMX), Basic Fibroblast Growth Factor human (bFGF), Oleic acid (OA) and Docosahexaenoic acid (DHA), albumin, were all supplied from Sigma-Aldrich (St. Louis, MO, USA). Collagenase (type I, 220 U/mg) was obtained from Worthington (Lakewood, NJ, USA). Nylon filters 250/100 were obtained from Sefar AG (Heiden, Switzerland). PFA and formalin were obtained from Electron Microscopy Sciences (Fort Washington, PA, USA). Insulin was obtained from Gibco (Grand Island, NY, USA). Polyester wax was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Dulbecco's modified Eagle's medium (DMEM), Leibowitz-15 (L-15), LipidTOX Neutral Lipid Stains. Alexa Fluor[®] 594 wheat germ agglutinin (WGA), Alexa Fluor[®] 488 Phalloidin, DAPI (4', 6-Diamidino-2-Phenylindole) Nucleic Acid Stain, Ultra Pure[™] LMP Agarose, CD56 (NCAM) Mouse Anti-Human mAb, Alexa Fluor[®] 488 Goat Anti-Mouse IgG antibody were obtained from Invitrogen (Carlsbad, CA, USA). Silica colloid Percoll[™] sterile was purchased from GE Healthcare (Uppsala, Sweden). Glycerol, Microscope slides were supplied by VWR International (West Chester, PA, USA). Fluoromount-G was purchased from Southern Biotech (Birmingham, Alabama,

USA). RNeasy Mini Kit, QIAshredder columns and RNase-free DNase I were purchased from Qiagen (Valencia, CA, USA).

Cells in culture were observed using an inverted phase contrast light microscope (Nikon, Japan). Zeiss Axio Observer Z1 microscope (Carl Zeiss, Germany), was used to view all stained cells. Acquired images were treated by Axio Vision software.

The cells were contained in tube 50 ml and micro tube 1.5 ml (Sarstedt, Germany) during the isolation and the rest of the process. The Avanti[®] J-30I high-performance centrifuge and centrifuge tubes were purchased from Beckman Coulter[™] (Palo Alto, USA). Pump obtained from W-M Alitea AB (Stockholm, Sweden) was applied to pump out each fraction to measure its refractive index. Refractometer was obtained from Mettler Toledo (Tokyo, Japan). Another centrifuge used to centrifuge the different fractions to the bottom of the tube was obtained from Eppendorf (Hamburg, Germany)

In histology experiment, incubator (Thermo, USA) was used to achieve a required temperature. Microtome (Microm, Germany) was employed during the sectioning process. The sections cut from the microtome were attached to Polysine adhesion slides (Thermo, Germany).

3.2. Isolation and culture conditions of pre-adipocytes during the proliferation and differentiation stages

3.2.1. Isolation of pre-adipocytes

Pre-adipocytes were isolated from Atlantic salmon (*Salmo salar*) between the weights of 2 to 4 kg. They were farmed in Averøy or Sunndalsøra, Norway (two of the Nofima Research Stations). Before dissecting the adipose tissue, the arch bow of gills was cut for the fish to bleed out (reduce the amount of blood in adipose tissue). After bleeding for 5 minutes in a

sea-water tank, the abdomen was disinfected with 70-75 % ethanol. The abdomen was cut open so that the abdominal visceral adipose tissue was exposed and easy to excise. To avoid contamination with intestinal contents, the operation was carefully processed. After being excised, the adipose tissue was rinsed in L-15 with 1% antibiotics (100 units penicillin, 0.1mg streptomycin and 0.25 μg amphotericin B per mL) and transferred (approximately 20 g tissue per tube) to the prepared 50 ml sterile tubes. All those tubes with approximately 30 ml L-15 were weighed and kept on ice in advance. With an autoclaved scissor, the tissue was cut into tiny pieces and centrifuged at 300g for 5 minutes at 10°C. The tissue was transferred with an autoclaved spoon to new tubes containing L-15, and then centrifuged again under the same condition. Subsequently, the upper tissue layer was transferred to a bottle with 0.2 % collagenase (type I, 220 U/mg) solution with 1% antibiotics in the ratio of 1 g tissue/5 ml L-15. The digestion was enhanced with stirring gently for one hour at 13°C.

Thereafter, the tissue solution was filtered through 250 μm and 100 μm nylon filters into a new autoclaved bottle to remove large cells and undigested tissue. The filters were rinsed with L-15. Then the cell suspension was transferred into 50 ml tubes and centrifuged for 10 minutes at 800g. After this centrifugation, the supernatant containing mature lipid filled fat cells and the digestion medium were removed by aspiration. The precipitate containing pre-adipocytes was re-suspended in growth media (L-15 (or DMEM, according to the condition), 5% FBS, 10 mM HEPES and 1 % antibiotic) and transferred in a new tubes. After centrifugation for 10 minutes at 800g, the media solution was decanted off, and the cell pellet was resuspended in growth media again to obtain the cell solution.

The final cell solution was seeded out (day 0) into culture flasks pre-coated with Laminin (35 μl Laminin (1mg/mL) spread evenly on 25cm² flask). Most cells were attached to the bottom of the flasks on the next day, and they were carefully washed with L-15 medium every 3 days.

3.2.2. The conditions for optimized cell cultivation

Proliferation media:

Six different growth media were prepared to find out how the different media compositions influence the adipocyte proliferation. There were two culture media applied in this trial, L-15 media without CO₂ and DMEM media with CO₂ (5%) condition. In these two media, three different levels of FBS (2.5%, 5%, and 10% by volume) were provided to figure out how the FBS levels and media type affect the cultivation of adipocytes. All growth media also contained 10 mM HEPES and 1 % antibiotic. Cells with DMEM media were incubated in a CO₂ incubator (ASSAB, Sweden), and cells with L-15 media incubated in an incubator (Thermo, Germany) without CO₂. All those cells were incubated at 13°C circumstance.

Those flasks with cells were cultivated in two distinct conditions as mentioned above, DMEM media with a 5% CO₂ condition, and L-15 media without CO₂ (see more media composition detail in Table 1). The cells were kept in the different growth media for 7 days until the best performing group reached the confluent stage. The growth media was changed every 3 days.

Differentiation media:

We replaced the growth media into a differentiation media when the best performing group had reached the confluent stage at day 7. The differentiation media were performed in DMEM with 5 % CO₂ or L-15 media without CO₂, three different levels of FBS (2.5%, 5%, and 10% by volume) were employed as well, which were the same situation as they were in proliferation stage. All differentiation media included 1μM DEX, 10 nM T3, 12 μM IBMX and 20 μg/ml insulin. The cells were washed every third day until day 12 when they have reached a differentiated stage.

Table 1 Two media composition differences from (Invitrogen)

Components	L-15 (mM)	DMEM (mM)
Amino Acids		
Glycine	2.67	0.4
L-Alanyl-Glutamine		3.97
L-Alanine	2.53	
L-Arginine	2.87	0.398
L-Asparagine	1.89	
L-Cysteine	0.992	
L-Cystine		0.153
L-Glutamine	2.05	
L-Histidine	1.61	0.2
L-Isoleucine	1.91	0.802
L-Leucine	0.954	0.802
L-Lysine	0.514	
L-Lysine hydrochloride		0.798
L-Methionine	0.503	0.201
L-Phenylalanine	0.758	0.4
L-Serine	1.9	0.4
L-Threonine	2.52	0.798
L-Tryptophan	0.098	0.0784
L-Tyrosine	1.66	0.398
L-Valine	0.855	0.803
Vitamins		
Choline chloride	0.00714	0.0286
D-Calcium pantothenate	0.0021	0.00839
Folic Acid	0.00227	0.00907
Niacinamide	0.0082	0.0328

Pyridoxine hydrochloride	0.00485	0.0194
Riboflavin		0.00106
Riboflavin 5'-phosphate Na	0.000209	
Thiamine hydrochloride		0.0119
Thiamine monophosphate	0.00226	
i-Inositol	0.0111	0.04
Inorganic Salts		
Calcium Chloride	1.26	1.8
Ferric Nitrate		0.000248
Magnesium Chloride	0.986	
Magnesium Sulfate	0.814	0.813
Potassium Chloride (KCl)	5.33	5.33
Potassium Phosphate monobasic (KH ₂ PO ₄)	0.441	
Sodium Bicarbonate		44.05
Sodium Chloride (NaCl)	137.93	110.34
Sodium Phosphate monobasic	1.34	0.904
Other Components		
D+ Galactose	5	
D-Glucose (Dextrose)		5.56
Phenol Red	0.0266	0.0399
Sodium Pyruvate	5	1

3.2.3. Fluorescence staining of intracellular lipid droplets

Differentiated adipocytes were firstly washed twice with 0.1M phosphate buffered saline (PBS) solution, and then fixed with 2% PFA for 10 minutes at room temperature. 2% PFA was diluted from 16% PFA with PBS. Subsequently, cells were washed 2-3 times in PBS gently to remove the residual fixative. After washing, cells were incubated with DAPI (200X) nucleic acid stain (2 μ l/ml) and LipidTOX neutral lipid stain (1:200 in PBS) for 15 minutes in dark circumstance. The volume was sufficient to completely cover cells in order to avoid drying out. After the incubation, the slides were mounted with Fluoromount-G. And the cells were visualized using a ZEISS Axio microscope. Slides were placed on an inverted microscope with different wavelengths to detect target signals in a dark circumstance. DAPI signals were detected at 365 nm, Phalloidin and LipidTOX were observed under 488 nm with the green fluorescent protein (GFP) filter. The WGA signals were captured under 594 nm with the red fluorescent protein (RFP) filter. The signals from the same slides were merged together with Axio Vision software to present the distribution of subcellular structures.

3.3. Gradient centrifugation

3.3.1. Formation of Percoll density gradients

As silica colloid Percoll has a very low osmolality (< 25mOsm/kg H₂O), in order to make Percoll isotonic with physiological salt solution, 1 part (v/v) of 1.5 M NaCl was added into 9 parts (v/v) of Percoll to form stock isotonic Percoll. Then this 100 % Percoll* was diluted with different volumes of 0.9% NaCl to form 36% and 70% Percoll working solutions. Both of them were mixed well before centrifugation. Then the solutions were transferred to the 15ml centrifuge tubes separately. To form the gradient, we centrifuged each two tubes (36% and 70%) at 30 000g and 60 000g for 15 minutes in an angle-head rotor in a high speed Beckman centrifuge (Avanti® J-30I). Thereafter, a density gradient was formed during centrifugation.

After the first test run with 36% and 70% Percoll, the centrifugation procedure was stopped here to test the formation of density. All the liquid in the tubes were pumped out by using a peristaltic pump which pumped approximately 2 ml liquid/minute from the bottom of the tube. Fraction of 0.5 ml was transferred to 1 ml eppendorf tubes. One droplet from each fraction was transferred to a refractometer (Mettler Toledo) and the refractive index was measured through the refractometer machine. The density in each fraction was calculated according to the formulae: $y = 6.5458x - 7.7302$ (see Fig. 3, y =density, x =refractive index).

* 100 % Percoll refers to an undiluted Percoll as it came in the bottle in pure water from the manufacturer, or 9/10 prediluted isotonic Percoll solution re-defined as 100%. In the literature about the true concentration of Percoll used in some methods, it was not made clear. In the handbook of Percoll, it pointed out this latter mixture (9/10 prediluted isotonic Percoll solution) is called “100% Percoll”. And in order to make Percoll isotonic with the biological material to be isolated, the osmolality of Percoll from the bottle must first be adjusted with saline or cell culture medium before employing Percoll to prepare a density gradient.

3.3.2. Comparison of density gradient formation with different solvents

From the first trial, we figured out the 36% Percoll and 60 000g achieved a density range of the gradient closest to the target. In this trial it was further tested how two different solvents, to dilute stock Percoll, influence the density gradient formation. 100 % Percoll was respectively diluted with same volume of either 0.9% NaCl or L-15 to form 36% Percoll working solutions. Both of them were mixed well before centrifugation and then centrifuged at 60 000g. After the density gradient was formed, the test tubes were added 1 ml L-15 on top of the gradients in order to mimic application of 1 ml of cell sample on top of the gradient. These tubes were centrifuged again at 1 000g for 25 minutes. After the centrifugation, measurement of refractive index was performed according to the description above in section 3.3.1.

3.3.3. Isolation of mature adipocytes and density gradient isolation of adipose stem cells by using the optimized density gradient centrifugation method

Mature adipocytes were isolated from the top white layer after centrifugation of the cell solution during pre-adipocytes isolation (same protocol in section 3.2.1). The mature adipocytes float on the top of the solution due to their high lipid level giving them very low density. The mature adipocytes were transferred into TRizol for isolation of RNA.

The final test for the optimized gradient centrifugation method was conducted with 36% Percoll with L-15 as a solvent and with formation of a density gradient by centrifugation at 60 000g. After the gradient was formed, 1 ml of adipose cell sample, in growth media, was added on top of the gradient (15 ml). The adipose cell sample in growth media was isolated by following the protocol described in section 3.2.1. The gradient was centrifuged for 25 minutes at 1 000g as explained above. The gradient was fractionated and the densities calculated according to the description in section 3.3.1.

The density ranges was divided in 3 fractions. (Range I: 1.018-1.033 g/ml; Range II: 1.033-1.049 g/ml; Range III: 1.049-1.062 g/ml). The target pre-adipocyte stem cells found in fraction II were spun down at 2 500g for 10 minutes to remove the Percoll medium. The upper suspension containing Percoll medium was decanted off. Small amount of growth media was added into the tubes to resuspend the cells. Thereafter, part of the isolated cells was transferred to TRizol for RNA isolation. And the other part was seeded out in culture dishes and their development was followed by microscopy for 15 days.

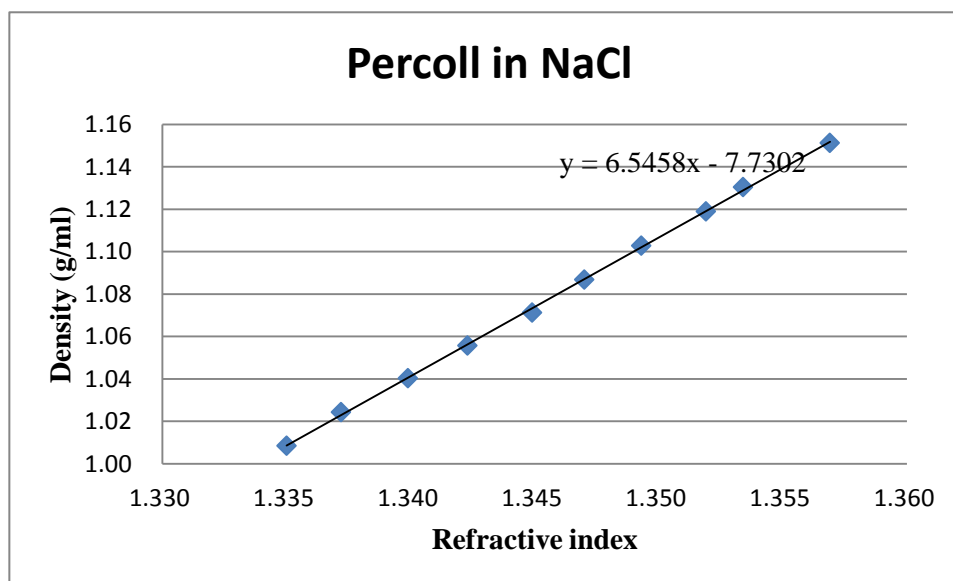


Fig. 3. The correlation between refractive index and density with Percoll in Sodium Chloride (NaCl.) In the equation, y =density, x =refractive index.

3.3.4. Pure stem cells cultivation

The final merged cell solution (Range II) was seeded out (day 0) into 6-well cell culture dishes pre-coated with Laminin, and 25 ng/ml basic fibroblast growth factor (bFGF) was added to stimulate proliferation. Most cells were attached to the bottom of the dishes at the next day, and they were carefully washed with L-15 medium every 3 days. The trial was followed by phase contrast microscopy with images at 10X magnification.

3.4. Adipose tissue morphology

3.4.1. Freeze substitution and polyester wax embedding

Adipose tissue was cut from the abdominal visceral adipose tissue in Atlantic salmons. Before exposing the abdomen part, the outer belly part was disinfected with 70-75% ethanol. Several tubes with pre-cooled 100% isobutanol and 96% ethanol (80 °C) were prepared separately in advance. Adipose tissue was cut into 3 to 4 pieces of small specimens (around 5mm X 5mm X

5mm cubes), and collected by tweezers and immersed into the cool 100% isobutanol for 2 minutes to freeze the sample. The process was handled at around -80 °C surroundings. Then substitution was carried out at the same temperature with transferring the tissue piece to the 96% ethanol tube. After this, all the samples with 96% ethanol were stored in the -80 °C freezer for at least 2 days to allow slow substitution. This fixation and substitution hold out in -80 °C surroundings was part of the method named as CryoWax.

Ethanol-substituted samples were kept in -20 °C freezer for one hour, then at 4 °C fridge for an hour each and at room temperature for another hour. Then specimens were incubated in a 1:1 mixture of 96% ethanol and 100% isobutanol for approximately 40 minutes at 40 °C. Three polyester wax tubes were prepared in advance in a 40 °C incubator. Then the tissue samples were transferred to a polyester wax tube, this process was repeated three times for an hour each at 40 °C. Subsequently, warm wax was poured into a mold and plunged the samples within. Polyester wax blocks solidified at room temperature. The blocks can be set and stored in a fridge for several days.

Sectioning:

A room temperature (below 22 °C) is preferable to avoid the wax blocks melting during the sectioning with Microtome (Microm, Germany). The thickness of the sections was around 7 µm. To stretch the sections to a flat state, distilled water was applied to provide a surface tension to eliminate the curls produced during sectioning. Once the sections were straightened, extra water should be removed and dried out at 4 °C. Then the sections were attached on the slides and sample slides can be stored in cardboard boxes until processing.

3.4.2. Fluorescence staining of tissue sections

Before starting staining, several procedures were processed as following: 96% ethanol was prepared to dewax the specimen sections. And the rehydration procedures was carried out in a series of decreasing concentration of ethanol (96%, 70%, 50% and 25%) for 10 minutes each,

ending up in distilled water. After all these, the sections were incubated in diluted Triton X-100 (1%) or Saponin (0.2%, for lipid staining penetration) with PBS for 10-20 minutes at room temperature, in order to improve penetration. Meanwhile, we prepared the staining solution for each group. One group contained LipidTOX, Neutral Lipid Stains (200X), WGA (100X) and DAPI Nucleic Acid Stain (200X). The other group contained Phalloidin (100X), WGA and DAPI stains. The incubation time lasted for 5 minutes. Washing with PBS was the last step before mounting with Fluoromount-G. The observation was carried out under a microscope, which was similar to the 3.2.3. lipid fluorescence staining. Similar protocol (section 3.2.3. and 3.4.4.) was used for different FAs treated adipocytes for lipid droplets staining and FATP (100X, Alexa Fluor[®] 546, RFP filter) measurement (only one level of FAs was tested).

3.4.3. Lipid observation in oil red O stained adipose tissue before embedding stage

Hand section:

The samples were finished with the freeze substitution procedure already. The whole process started from -80 °C storage in 96% ethanol. Then with a series of increasing temperature, -20 °C, 4 °C and room temperature for an hour each as in section 3.4.1. Then the rehydration reaction was conducted in a series of decreasing concentration of ethanol (96%, 70%, 50% and 25%) for 10 minutes each. The last step was in PBS for 10 minutes. After all this, 4% agarose was prepared with PBS, and the samples were then embedded inside the agarose blocks at room temperature. The hand section was processed until it gelatinized with an adjustable blade. The thickness of each section depended mainly on hand adjustment (roughly 10-20 μm).

Staining

The sections were immersed in prepared 60% isopropanol for 5 minutes. After this, the samples were stained with oil red O for 15 minutes. Oil red O (5 g/L) was dissolved in isopropanol as a stock solution with gentle heating. The working solution was prepared

with dissolving the stocking one with distilled water in the ratio of 3:2 for 10 minutes. Then after the staining was finished, the hand sectioning samples were rinsed with dH₂O, and mounted with glycerol.

3.4.4. Immunofluorescence with adipose tissue sections

The section dewaxing and rehydration protocols were the same as for the fluorescence staining in section 3.4.2. For immunofluorescence, after improved the penetration with diluted Triton X-100 (1%) or Saponin (0.2%, for lipid staining use), blocking solution (5% Molico instant milk powder in PBS, 0.05% Tween 20) was maintained for at least 60 minutes. Here, adipose tissue section slides were stained with specified anti-neural cell adhesion molecule (anti-NCAM) primary antibody (mouse immunoglobulin G, mIgG). After incubating, the diluted primary antibody (1:250) with PBS was incubated in microwave oven at 195 W for 3 minutes. Then they were washed every 5 minutes for 3 times with PBS. After the primary antibody binding, the sections were incubated with secondary antibodies (anti-mouse immunoglobulin G, anti-mIgG) under the same condition inside the microwave oven. After finishing this step, wait for 5 minutes. Slides were covered in PBS with DAPI for 5 minutes. The final step of immunostaining was to wash the slides with PBS with 0.05% Tween 20. After all this, slides were mounted with Fluoromount-G, and then slides were stored in the fridge till visualizing under a microscope was needed.

3.5. FAs (OA and DHA) supplementation to cells in culture for studies of expression of lipid regulatory genes during differentiation of adipocytes

3.5.1. Preparation of stock FAs (OA and DHA)

Two kinds of FAs were supplied in this trial namely OA and DHA. Here is OA as an example how to prepare FA solution, 1 gram of OA powder was dissolved in 5.9 ml chloroform to make a 600mM stock solution. Then 300 µl of 600mM OA stock was transferred to a glass vial and

heated to 50 °C in order to evaporate chloroform out of the tube with the blowing of N₂ to avoid oxidation. Once the evaporation was finished, 5 ml 0.1 M pre-heated NaOH was added into the FAs solution tube. During this procedure, the albumin-PBS solution (185g/l) was prepared in advance. After the albumin was dissolved completely, it was heated to approximately 40 °C; the NaOH/OA solution (5 ml) was mixed with the albumin-PBS solution (20 ml) drop by drop. The final stocking FA concentration was 6mM. DHA solution was prepared in the same way as explained for OA one. And the temperature performed should not be higher than 40 °C to avoid oxidation of FAs by the heating.

3.5.2. Incubation of adipocytes with FAs

Cells were grown in the media containing 10% FBS with DMEM media in 5% CO₂, until they reached confluence (day 7). At this stage, the differentiation media (as explained in section 3.2.2) was employed then. 0.6mM and 0.1mM FAs working solutions were added then separately to each well with 5% FBS previous same differentiation media after 2 days' incubation (day 9). Media was changed every 3 days. The cells were harvested on day 15 for further gene expression studies. Media containing no DHA or OA were regarded as growth media (GM), control.

3.5.3. RNA isolation and cDNA synthesis

RNA isolation with rRNeasy mini kit

Differentiated adipocytes were thoroughly washed twice with PBS. RNA isolation was carried out with an RNeasy[®] Mini Kit. 350 µl RLT buffer (contain 1% β-ME) was added to each well. Furthermore, the cells were scraped off and lysate from each well was transferred to QIAshredder spin columns in a 2 ml collection tube. And then centrifugation at 15 000g for 2 minutes was performed. Thereafter, 1 volume (350 µl) with 70% ethanol was added to the homogenized lysate. The solution was mixed well and transferred to RNeasy spin columns.

After 15 seconds of 8 000g centrifugation, the flow-through was discarded. DNase mix was made by mixing 10 μ l DNase I stock solution with 70 μ l RDD buffer, gently inverting the tube to mix them properly. The same volume (80 μ l) DNase I incubation mix was added to each tube and incubated for 15 minutes in room temperature. 350 μ l RW1 buffer (washing buffer) was added to the RNeasy spin column and centrifuged at 8 000g for 15 seconds. The flow-through was discarded as well. 500 μ l RPE buffer with ethanol (washing buffer) was added to the column. After 15 seconds of 8 000g centrifugation, the flow content in the bottom part was thrown away. Then 500 μ l RPE buffer with ethanol was added again. After 2 minutes of centrifugation at 8,000g, the flow-through was discarded. A new 2 ml tube was replaced with the old one, with the column in a new tube, centrifuged at full speed for 1 minute. Thereafter a new 1.5 ml tube was employed with the spin column inside. Subsequently, 20 μ l RNase free water was added upon the column to elute the RNA centrifugation for 1 minute at 8 000g.

RNA isolation with TRizol for mature cells and stem cells

The sample of stem cells was from fraction of the gradient centrifugation isolation, and matured adipocytes were from the top white layer of digested cell solution in adipocytes isolation (same protocol in section 3.2.1) after centrifugation. 1 ml TRIZol was added into the tube contained stem cells and matured cells separately for 5 minutes at room temperature. Then after the centrifugation at 12 000g for 10 minutes at 4 °C, the supernatant (pink, not the lipid phase on the top) was pipetted to a new eppendorf tube. The centrifugation step was repeated in order to obtain a clean solution. This step depended on the amount of lipids from the previous centrifugation. Thereafter, 200 μ l chloroform was added into the new tube contained pink supernatant and 20 seconds of vortex was performed with 5 minutes incubation afterwards. Another centrifugation was held out with 13 000g for 15 minutes at 4 °C. 3 different phases were observed inside the tubes after this centrifugation. Subsequently, the aqueous phase (colorless phase) was transferred to a new tube. 500 μ l isopropanol was mixed inside to precipitate RNA by inversion for 4-5 times. After the precipitation, centrifugation

was performed at 13 000g for 5 minutes at 4 °C. The supernatant was removed, and 1 ml 75 % ethanol was performed to wash the RNA pellet. Then centrifugation was performed at 7 500g for 5 minutes at 4 °C. The washing process was repeated with another same amount 75 % ethanol at the same condition of centrifugation. The supernatant was decanted off again and air-dry at the room temperature (not too much). The RNA pellet was dissolved by 20 µl RNase-free water.

All RNA samples used in our experiments had A260/280 ratios between 1.80 and 2.30. The total RNA concentration was determined at 260 nm using spectrophotometry (NanoDrop 1000 Spectrophotometer, Germany).

To synthesis cDNA from purified RNA of adipocytes, 0.35 µg of RNA of each sample was reverse-transcribed by using an Affinity Script™ QPCR cDNA Synthesis Kit. All processes were carried out in accordance with following reaction system: 7 µl total RNA with RNase-free water was used in a 20 µl reaction system in each well. 10 µl cDNA Synthesis Master Mix (2×), 1.7 µl Oligo(dT) primer (100 ng/µl), 0.3 µl Random primer, 1 µl AffinityScript RT/ RNase Block Enzyme Mixture was contained in the whole reaction system in each well. The cDNA synthesis was performed with a 5 minutes primer incubation step at 25 °C, a 45 minutes reverse transcription (RT) step at 42 °C, and 5 minutes of RT inactivation at 95 °C.

cDNA was diluted 1:10 before continuing to the real-time quantitative (RT-qPCR) reaction. The reverse transcription products (cDNA) were stored at -20 °C until later use for qPCR of the target genes.

3.5.4. Quantitative real-time RT-PCR (qPCR)

Real-time PCR was performed in duplicates in 96-well optical plates with the Light Cycler®

480 (Roche Diagnostics, Mannheim, Germany), The 10 µl reaction system was consisted with 1 µl primer (0.5 µl reverse and 0.5 µl forward primers), 5 µl SYBR® Green I Master mix, 4 µl tenfold diluted cDNA samples. The conditions of PCR reaction were 95 °C for 5 minutes, 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

The qPCR primers (Table 2) were designed using the Vector NTI software (Invitrogen) and synthesized by Invitrogen. The specificity of PCR amplification was confirmed by melting curve analysis (T_m calling; Light Cycler 480, Roche Diagnostics, Mannheim, Germany).

Table 2 Real-time qPCR primers

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank accession no
CCAAT-enhance-binding protein alpha (C/EBPα)	AGACCTCGGCGAGATTTGT	TGTGGAATAGATCAGCCAG GAA	EU668995
CCAAT-enhance-binding protein beta (C/EBPβ)	CAAACCTACATTACCAGGC	GTTATTGTGTTGCCAGTTG	EU668997
CCAAT-enhance-binding protein delta (C/EBPδ)	TTGGGCGGTGGAGCCTAT	TTTCCTCGCCCGTGTTCAT	EU668996
Fatty acid transport protein (FATP)	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTG G	CA373015/ AF023258
Elongation factor 1A (EF1A)	CACCACCGGCCATCTGATCTAC AA	TCAGCAGCCTCCTTCTCGA ACTTC	AF321836

3.6. Statistical analyses

Statistical analyses of differences among treatment means were carried out by one-way ANOVA (SPSS 16.0, Polar Engineering and Consulting, Nikiski, USA). Differences between control groups and groups in the different treatment groups were assessed with Student's t-test and were considered significant if $P < 0.05$.

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:

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

The gene expression levels were normalized towards the reference gene Elongation factor 1A (EF1A). 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.

The Ct values of test and reference genes were used to calculate the differential gene expression for each group by the $\Delta\Delta\text{Ct}$ method. Differences between the FA group and control group were calculated using Microsoft Excel (2010 Microsoft Corporation, California, USA) with single factor ANOVA ($p < 0.05$).

4. Results

4.1. Optimization of culture conditions for pre-adipocytes during the proliferation stage

This trial was conducted in order to find out how different FBS levels (2.5%, 5% and 10%) and two different types of culture media (DMEM + CO₂ and L-15) influence the proliferation of pre-adipocytes. The proliferation of pre-adipocytes was followed under different culture conditions by phase contrast microscopy over a period of 7 days. It was not easy to observe any difference between the different treatments the first days after seeding (pictures not shown). However after 5 days of cultivation (Fig. 4 and Fig. 5), it was evident that the FBS level affected the proliferation degree. The number of cells was lowest in the 2.5% FBS group, intermediate in the 5% group and with the highest number of cells in the 10% FBS group. Improved proliferation capacity of the cells with increasing FBS level was evident for cells cultivated in both DMEM and L-15. Cells cultivated in the highest level of FBS reached the confluent stage earlier than cells cultivated with lower levels of FBS. While by comparing Fig. 4 and Fig. 5, there seems to be higher proliferation capacity in DMEM than that in L-15 medium. That might be because of some differences in the composition between DMEM and L-15 (see table 1 in the material and method section). The composition in DMEM seems to be more optimal for adipocytes in this stage. In this period, the morphology of the pre-adipocytes was quite similar to that of fibroblasts, with a cytoplasm devoid of lipid droplets. The cells were left for two more days (until day 7) in proliferation media prior to starting of differentiation. There were no major differences in the microscopy images between day 5 and 7, and these images are therefore not shown.

In addition, DAPI staining was conducted to reveal the confluence outcome at the end of this stage. The best performing group in proliferation stage, DMEM medium with 10% FBS, was selected for fluorescence microscopy with staining of nuclei. High confluent cell density can be observed in Fig.6. Once the cell culture reached confluence, proliferation media was replaced with the differentiation media in order to stimulate the differentiation process.

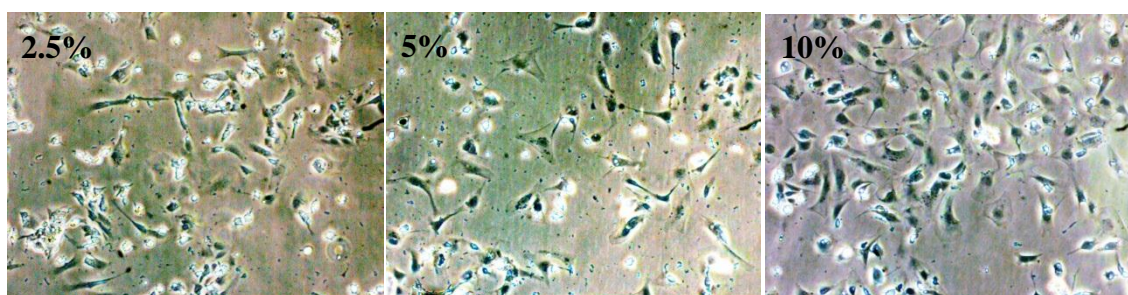


Fig. 4 Phase contrast microscopy images (X 20 magnification) of Atlantic salmon pre-adipocytes on day 5 after seeding. Cells were cultured in DMEM + CO₂ with 3 different levels of FBS, 2.5%, 5% and 10%, respectively.

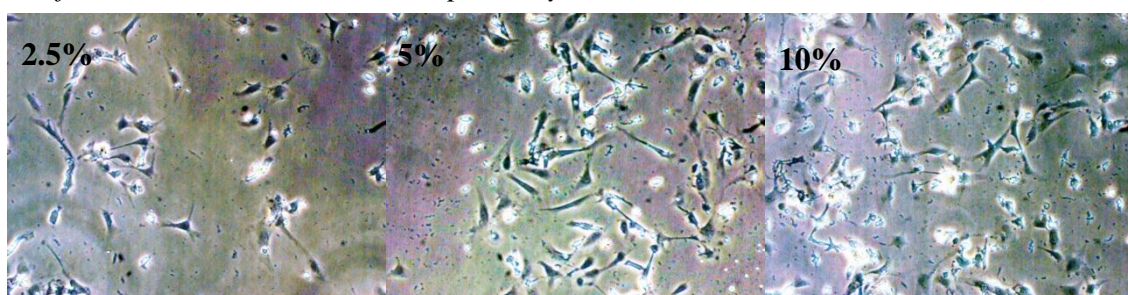


Fig. 5 Phase contrast microscopy images (X 20 magnification) of Atlantic salmon pre-adipocytes on day 5 after seeding. Cells were cultured in L-15 with 3 different levels of FBS, 2.5%, 5% and 10%, respectively.

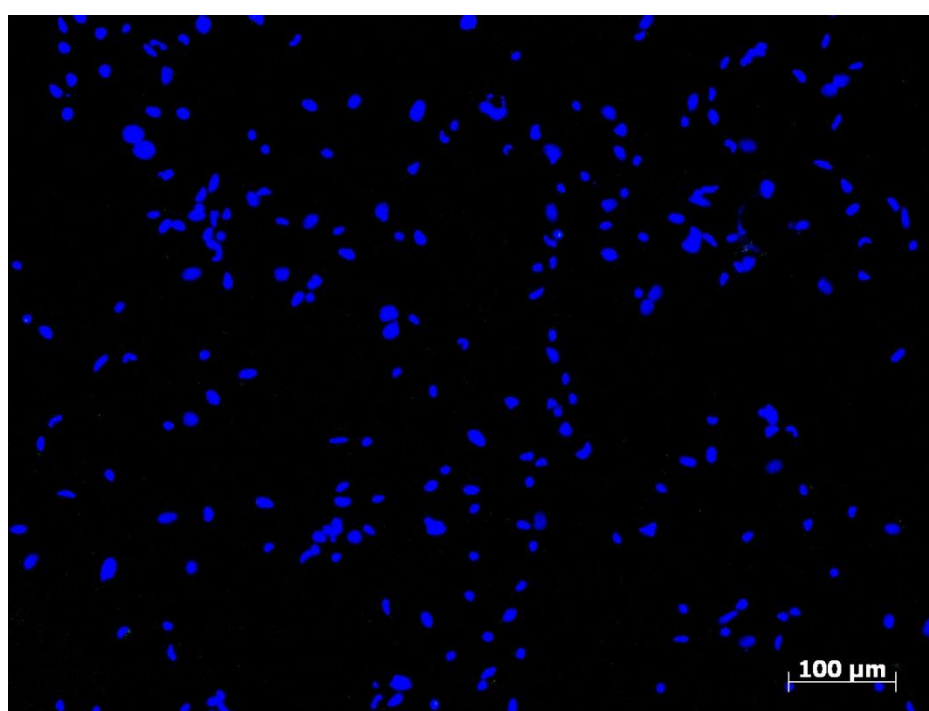


Fig. 6 Micrographs shows the density of DAPI stains (number of nuclei) in Atlantic salmon pre-adipocyte culture at confluence, 10X magnification. Cells were incubated with DMEM medium with 10% FBS, stained with DAPI stain. They were imaged in a Zeiss Axio Observer Z1 microscope, captured monochromatically, and digitally processed to enhance color. Nucleus =blue. Bar=100μm.

4.2. Optimization of culture conditions for pre-adipocytes during the differentiation stage

This trial was conducted in order to study how FBS level and the medium type influence differentiation of pre-adipocytes to more mature lipid filled adipocytes. Differentiation medium (containing insulin, DEX, T3, IBMX) was added at day 7 when the best groups had reached confluence. Cells in the groups with 2.5% and 5% FBS, under the proliferation stage, had not yet reached confluence when they were added differentiation media. We observed that some of these cells were very soon starting to loosen from the bottom of the cells flask after the addition of differentiation media, and after a couple of days there were several empty spots in the culture wells. This was however not observed in the 10% FBS groups that had reached the confluent stage before they were added differentiation media. This demonstrates the necessity for pre-adipocytes to reach confluence before they are added differentiation media. However, there were confluent areas in the wells from all treatment groups (Fig. 7 and Fig. 9), and images are only shown from these areas.

The morphology of undifferentiated pre-adipocytes responded rapidly in the presence of differentiation medium. The cells became larger and more round in shape. Lipid droplets of very small sizes appeared in the cytoplasm of cells already after 24 h in differentiation media (Fig. 7A and Fig. 9A). And with the help of microscope, more and more lipid droplets with different sizes were detected during the subsequent days (Fig. 7B and Fig. 9B). After 3 days in differentiation medium, cells contained many small lipid droplets. As mentioned above, some areas may not have reached confluence before adding differentiation media. And the random figure capture situation, the cells we presented were not ideal enough to compare the quantity of lipid droplets. However the existence of lipid was confirmed or enhanced with the assistance of LipidTOX staining (Fig. 8 and Fig. 10). In the end of our trial, an increase in number of round lipid droplets within the cells can be observed.

There were no major visible differences in morphology between the different treatment groups

that could be observed with phase contrast microscopy. However there was a tendency to more visible lipid droplets in the DMEM and L-15 groups cultivated in 5% FBS than in the groups cultivated in 10% FBS.

As mentioned above, it was quite difficult to observe differences between the treatment groups with phase contrast microscopy alone. Hence, the best performing groups, DMEM with 5% FBS, L-15 with 5% FBS, were selected for fluorescence microscopy with staining of nuclei and lipid droplets. The lipid droplets signals were detected inside the cells after they had been incubated in differentiation medium from confluence until day 11. The signals were captured by the microscope after lipid droplets stained with LipidTOX, which also varies with different FBS levels and media. Less intracellular lipid droplets signals can be observed in L-15 (Fig.10) with the same FBS level than in DMEM one (Fig.8). To adipocytes, hence, there was a tendency to accumulate more lipid droplets with DMEM medium in 5 % CO₂ condition during the differentiation stage.

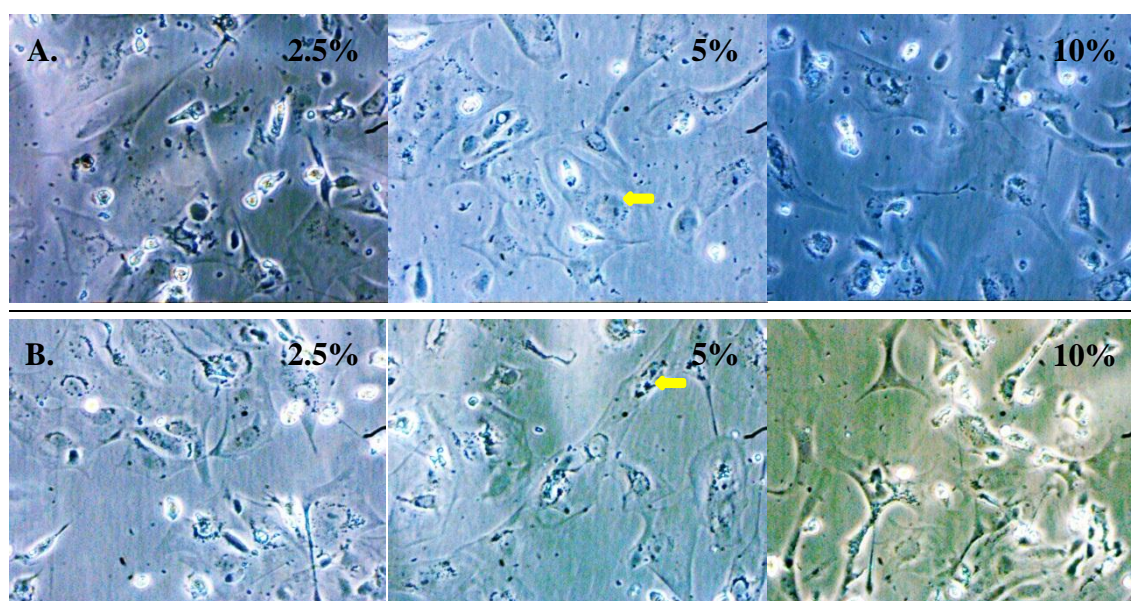


Fig. 7 Phase contrast microscopy images (X 20 magnification) of Atlantic salmon pre-adipocytes on day 8 (A) and 11(B) after seeding. Cells were cultured in DMEM + CO₂ with 3 different levels of FBS, 2.5%, 5% and 10%, respectively. (Yellow arrows= lipid droplets)

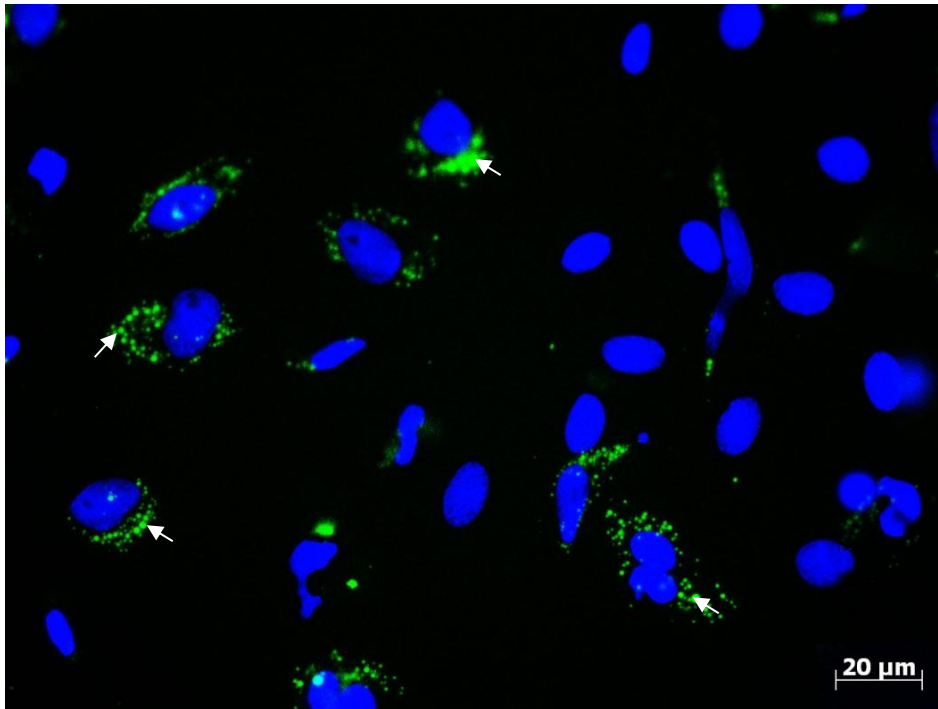


Fig. 8 Micrograph shows LipidTOX stain in differentiated Atlantic salmon adipocytes in primary culture, 40X magnification. Cells were incubated with 5% FBS in DMEM medium, from confluence until day 11, then stained with LipidTOX Green stain (arrowhead) and DAPI stain. They were imaged on a Zeiss Axio Observer Z1 microscope, and digitally processed to enhance color. Nucleus =blue, lipid droplets = green. Bar=20μm.

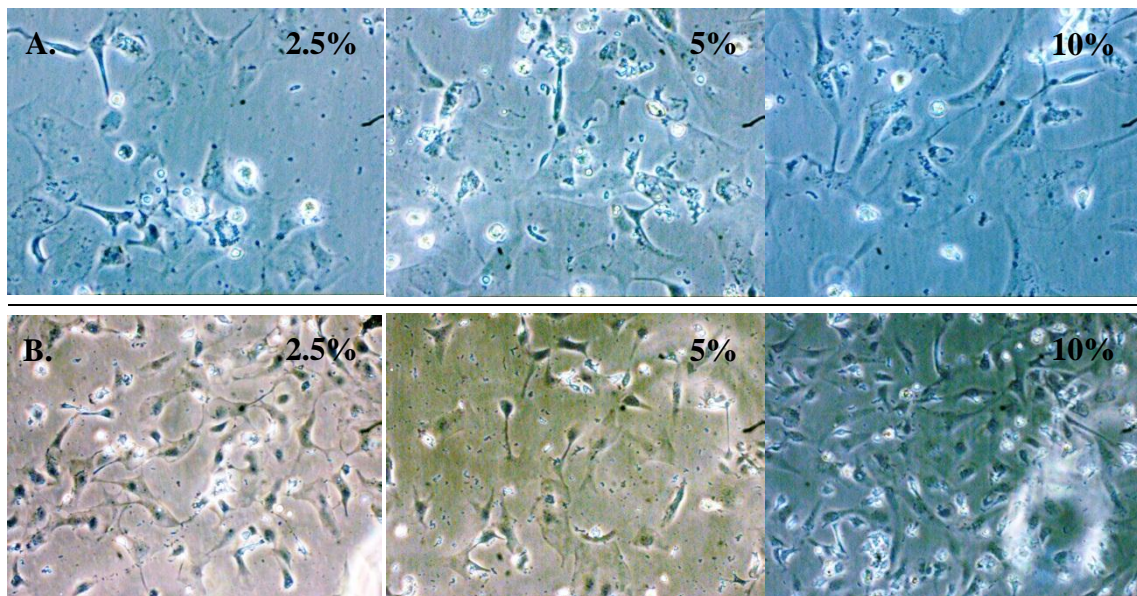


Fig. 9 Phase contrast microscopy images (X 20 magnification) of Atlantic salmon pre-adipocytes on day 8 (A) and 11(B) after seeding. Cells were cultured in L-15 with 3 different levels of FBS, 2.5%, 5% and 10%, respectively.

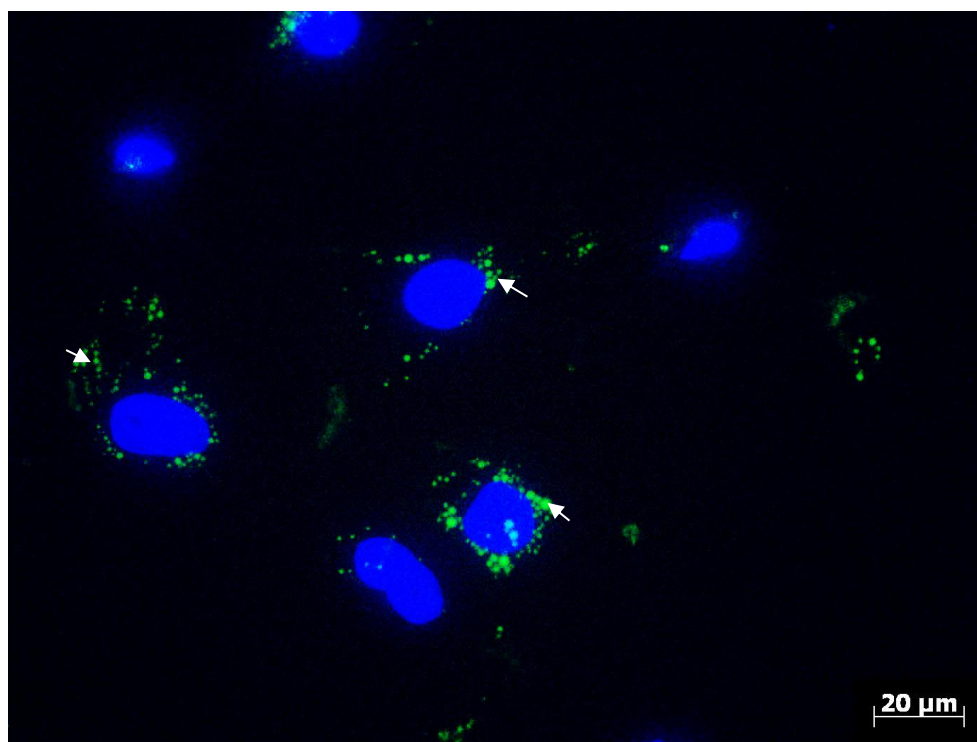


Fig. 10 Micrograph shows LipidTOX stain in differentiated Atlantic salmon adipocytes in primary culture, 40X magnification. Cells were incubated with 5% FBS in L-15 medium, from confluence until day 11, then stained with LipidTOX Green stain (arrowhead) and DAPI stain. They were imaged on a Zeiss Axio Observer Z1 microscope, and digitally processed to enhance color. Nucleus =blue, lipid droplets = green. Bar=20 μ m.

4.3. Cultivation of cells under optimized conditions

In this trial we cultivated cells during proliferation stage with 10% FBS and then we lowered level of FBS to 5% during differentiation in order to study whether this could improve the lipid accumulation in adipocytes cultivated in DMEM. We observed the expected outcome in the cells (Fig.11A), with the high density of confluent cells, the differentiation processed successfully and with a visible necklace of small lipid droplets in the cytoplasm. And strong lipid signals were obtained around the nucleus in the fluorescence microscopy (Fig.11B).

All in all, high lipid droplets accumulation in adipocytes was observed with the process of adipogenesis. The lipid droplets were detected by the help of fluorescence staining. And the optimal condition did improve the lipid droplets accumulation in adipocytes cultivated in DMEM, which was the same conclusion as we assumed in the light microscopy.

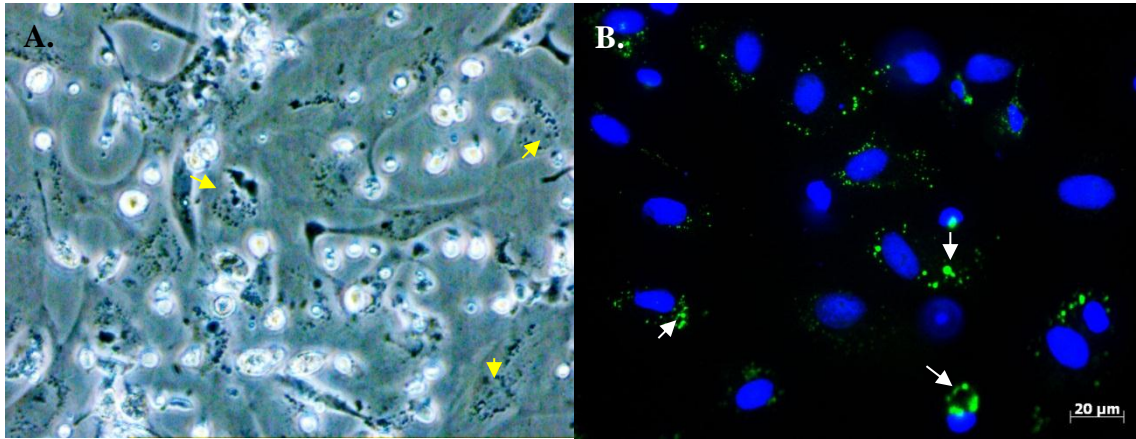


Fig. 11 Micrographs of salmon adipocytes on day 11 under light microscopy (A, 20 X magnification) and fluorescence microscopy (B, 40 X magnification). The cells were cultivated in 10% and 5% FBS DMEM medium in proliferation and differentiation periods, respectively. Then stained with LipidTOX Green stain (white arrowhead) and DAPI stain. The combination culture condition revealed rounded cell phenotype and different sizes of lipid droplets (yellow arrowhead) inside. Nucleus =blue, lipid droplets = green. Bar=20µm.

4.4. The influence of supplementation of the FAs (OA and DHA) on the differentiation of adipocytes

The trials were conducted to reveal the influence of FA supplement (OA and DHA) on the process of differentiation. Cells were cultivated in DMEM proliferation media with 10% FBS until they reached the confluent stage (day 7). At this stage cells were treated with the differentiation media (DMEM with 10% FBS) for 48 h (day 9). Then the differentiation media was replaced with the media containing two different concentrations of FAs until the final harvesting at day 15.

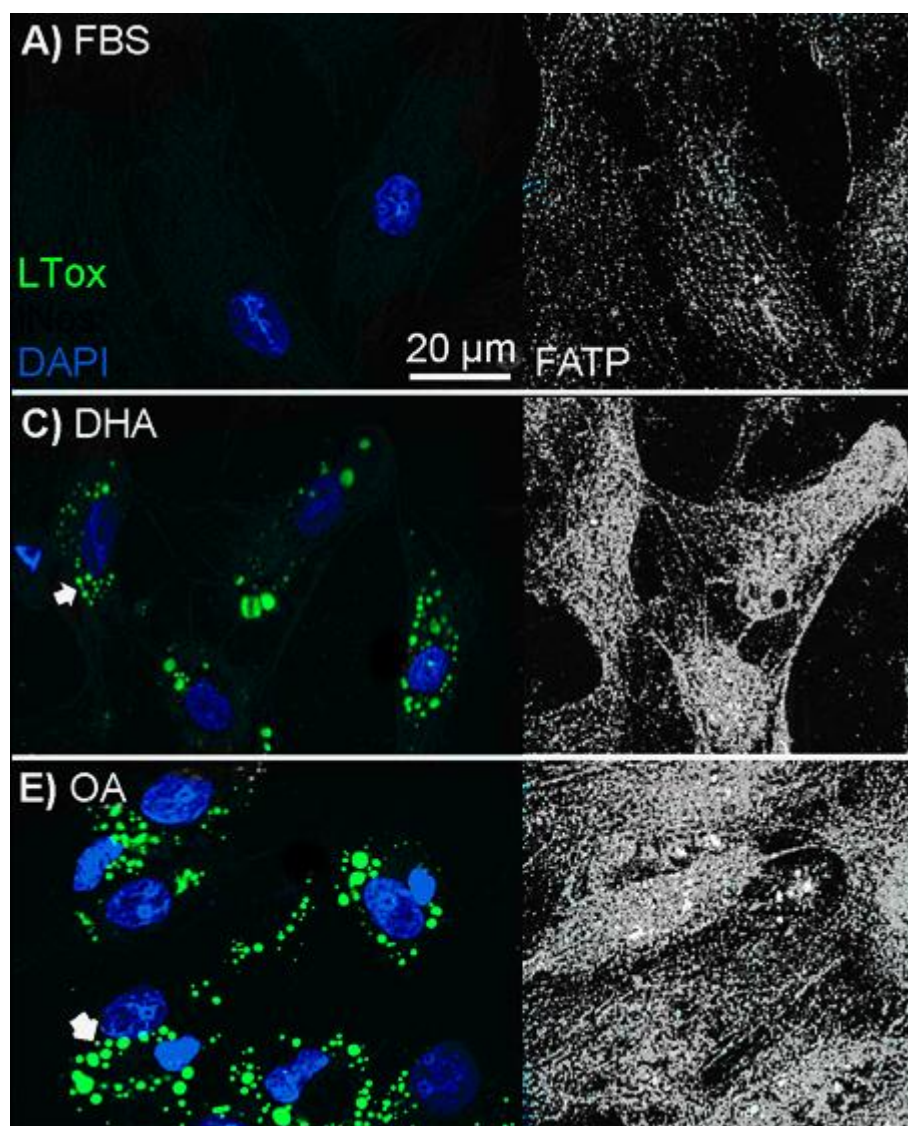


Fig. 12 Lipid droplet staining and immunofluorescence detection of FATP level, the latter shown as LUT images for quantitative comparison. All images are captured using identical settings with the exception of E^* , captured using shorter exposure times for LipidTOX. **A)** FBS control cells describe nearly no signal throughout the cell. FATP is modestly expressed. **C)** DHA treatment increase lipid staining (white arrow). FATP activity is also increased. **E)** OA treatments results in large lipid droplets (white arrow, *) and up regulate FATP activity.

Gene expression:

The C/EBP α gene expression was significantly higher in the cells treated with 0.6mM OA and 0.6mM DHA compare to cells treated with the lower concentration of the same FAs (Fig.13).

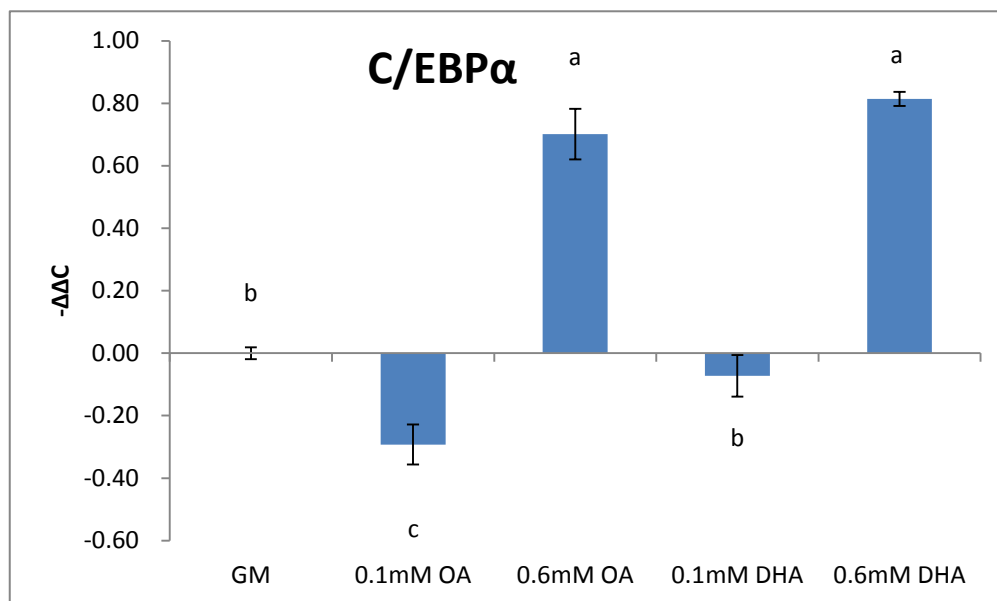


Fig. 13. Relative gene expression in Atlantic salmon adipocytes from adipose tissue after fatty acids treatment. Data were normalized to elongation factor 1A (EF1A). Significant differences in relative expression between groups were analyzed on delta Ct values. Each bar represents the mean \pm SD ($n=4$) and different letters indicates significant differences ($p \leq 0.05$). OA= Oleic acid. DHA= Docosahexaenoic acid. C/EBP α = CCAAT/enhancer binding protein alpha.

The expression of FATP during differentiation stage was significantly different between the different treatment groups. 0.1mM OA and 0.1mM DHA groups showed significantly lower gene expression compared to 0.6mM treatment. With 0.1mM OA group having the lowest expression (Fig. 13). Similar results were presented in lipid droplet staining and immunofluorescence detection of FATP level (Fig. 12). Immunostaining of FATP showed higher expression in cells with the highest level of lipid droplets in the OA group. Relatively lower FATP protein staining was found the DHA group with less lipid droplets. Moderate expression of FATP was observed in the control group which had only been cultivated in growth media. This group had nearly no visible lipid droplets.

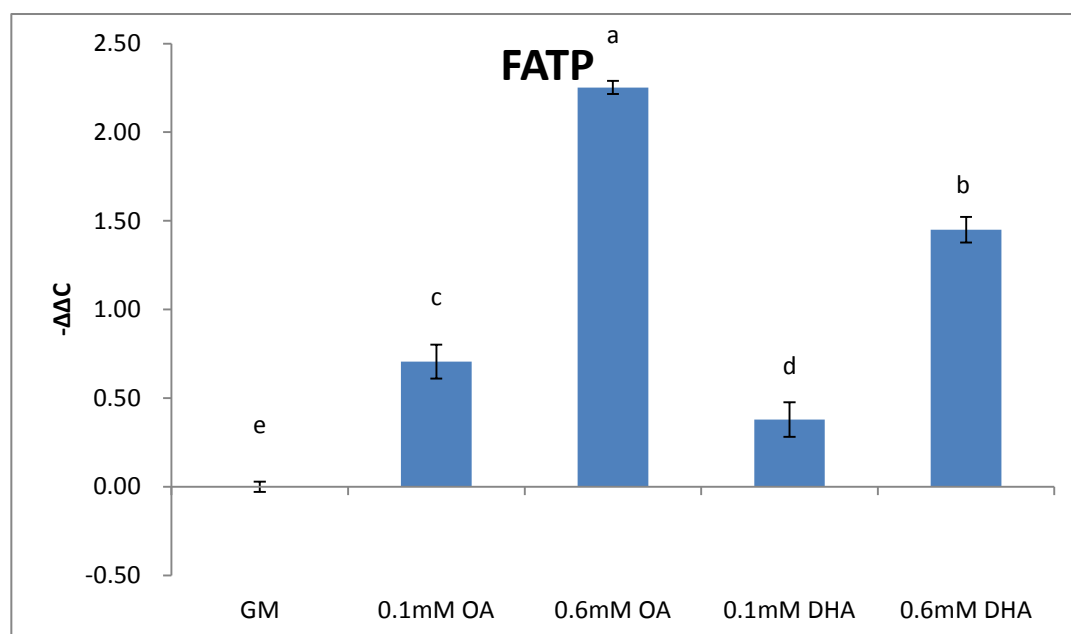


Fig. 15. Relative gene expression in Atlantic salmon adipocytes from adipose tissue after fatty acids treatment. Data were normalized to elongation factor 1A (EF1A). Significant differences in relative expression between groups were analyzed on delta Ct values. Each bar represents the mean \pm SD ($n=4$) and different letters indicates significant differences ($p \leq 0.05$). OA= Oleic acid. DHA= Docosahexaenoic acid. FATP= fatty acid transport protein.

4.5. Establishing a method for purification of stem cells from adipose tissue by Percoll density gradient centrifugation

The density range of stem cells isolated from adipose tissue in Atlantic salmon (*Salmo salar*) is reported to be in the range (1.033-1.049 g/ml) in a Percoll density gradient (Gaben-Cogneville et al. 1983). Stem cells from Atlantic salmon adipose tissue, has to the best of our knowledge, not been purified by gradient density centrifugation previously. In this trial, we wanted to optimize different conditions for formation of a density gradient, both by changing the Percoll concentration, centrifugation speed and different solvents (NaCl and L-15) prior to isolating a stem cell fraction.

The first trials was conducted to compare the density of gradients formed under 4 different conditions, 36% and 70% Percoll solutions centrifuged at 30 000g and 60 000g, respectively. The Fig. 16 shows the density range of 36% Percoll gradients centrifuged at 30 000g and 60

000g. The density ranged from 1.050 to 1.065 g/ml for the 30 000g gradient and from 1.044 to 1.062 g/ml for the 60 000g gradient. Fig. 15 shows the density range of 70% Percoll gradients centrifuged at 30 000g and 60 000g. The density ranged from 1.083 to 1.105 g/ml for 30 000g and 1.079 to 1.1105 g/ml for 60 000g.

These results show that only the 36% gradient centrifuged at 60 000g which gave a density range of 1.044 to 1.062 g/ml, was close to the target density range of 1.033-1.049 g/ml. Both curves presented in the figures (Fig. 16 and 17), demonstrated that the middle part of the density curve was quiet flat, and it is in this middle part of the curve that we wish to locate the target cells. We concluded that the 36% Percoll and 60 000g gave a result closest to the target, but it still needed to be modified in order to extend the lower range of densities to make it suitable to isolate the stem cells.

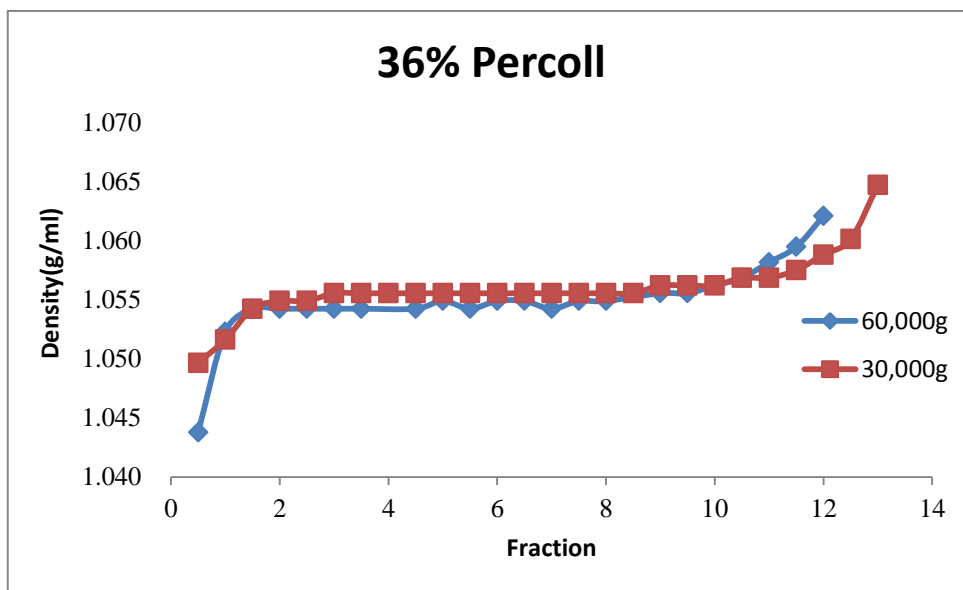


Fig. 16. Two density distribution curves in different conditions (30 000g and 60 000g) of centrifugation with 36% Percoll. The density was determined by measuring refractive index in each fraction of 0.5 ml (of 15 ml total volume of gradient). The lowest density was from the top fraction of the gradient, whereas the heaviest one was from the bottom fraction.

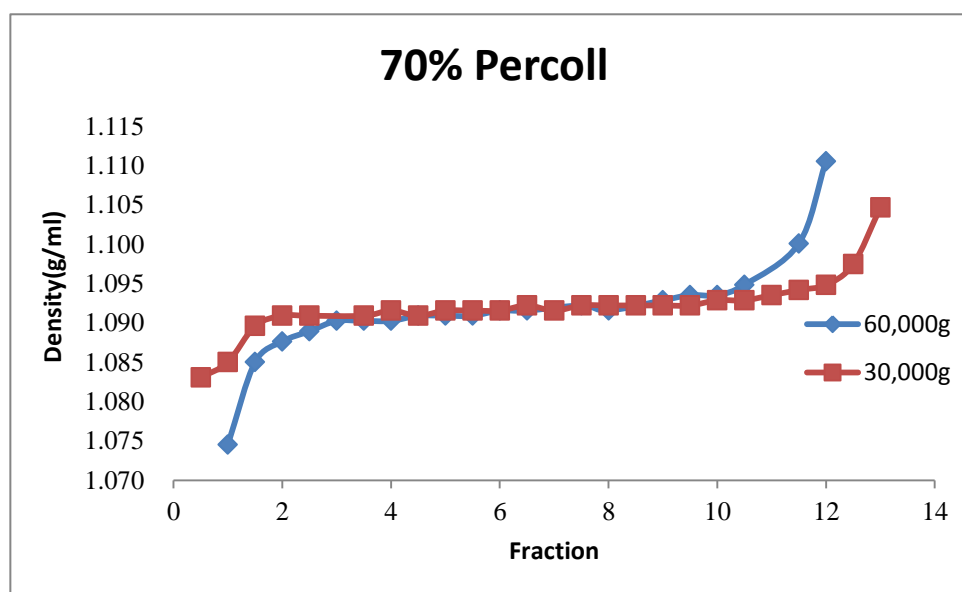


Fig. 17. Two density distribution curve in different conditions (30 000g and 60 000g) of centrifugation with 70% Percoll. The density was determined by measuring refractive index in each fraction of 0.5 ml (of 15 ml total volume of gradient). The lowest density was from the top fraction of the gradient, whereas the heaviest one was from the bottom fraction.

The gradient formation with different solvents to dilute stock Percoll

The next trial was conducted in order to extend the lower densities of the gradient in order to make it more suitable for isolation of stem cells. In addition, the trial was also conducted to reveal how a replacement of a solvent would affect the gradient in a 60 000g circumstance. 0.9% NaCl was recommended to dilute the stock solution, while the cells need nutrition to survive during the isolation as well. Hence, we tested whether an exchange of NaCl with L-15 will change the gradient density formation or not. First the gradient was run at 60 000g, then 1 ml of growth media was added on the top of the formed gradient in order to mimic cell sample application and then the gradient was centrifuged at 1 000g for 25 minutes prior to analyses of density range. Fig. 18 shows that there were no major differences in the density range obtained in the gradients when two different solvents were used. We succeeded in reaching lower density range and the method will therefore be suitable for isolation of stem cells in the density range (1.033-1.049 g/ml). Further, the recommended 0.9% NaCl can be replaced by L-15.

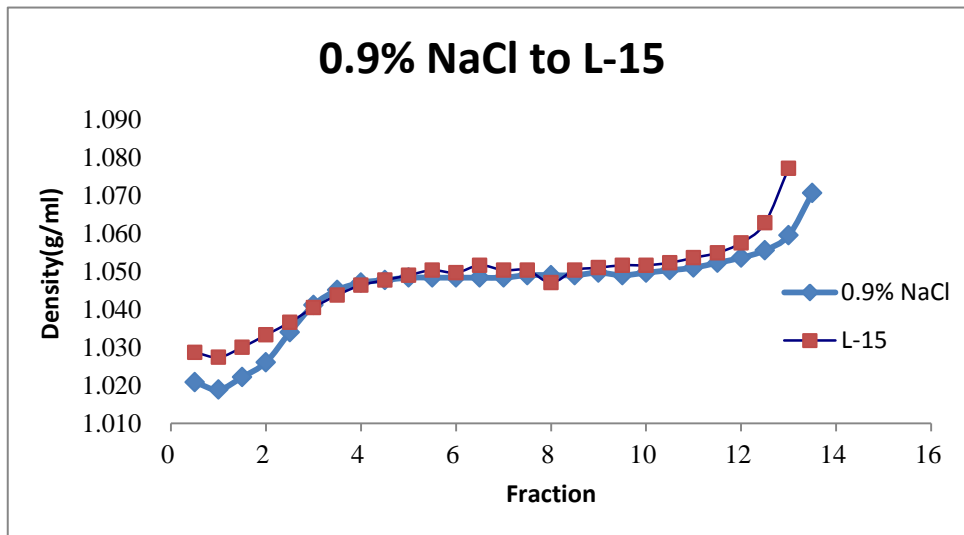


Fig. 18. Two density distribution curve in 60 000g condition of centrifugation with 36% Percoll. The density was determined by measuring refractive index in each fraction of 0.5 ml (of 15 ml total volume of gradient). The lowest density was from the top fraction of the gradient, whereas the heaviest one was from the bottom fraction.

The optimized gradient protocol was tested for isolation of pre-adipocytes.

First, the gradient using 36% Percoll diluted in L-15 and centrifuged at 60 000g was formed. Then 1 ml adipocyte cell suspension was added on the top of the formed gradient and centrifuged at 1 000g for 25 minutes. The density value ranged between 1.027-1.055 g/ml. The density range from 1.027 to 1.033 g/ml (Range I) contained mainly the very buoyant mature adipocytes with high lipid content. The density range from 1.033 to 1.049 g/ml (Range II) contained our target cells, the stem cells. The density range from 1.049 to 1.055 g/ml (Range III) contained cell debris and red blood cells.

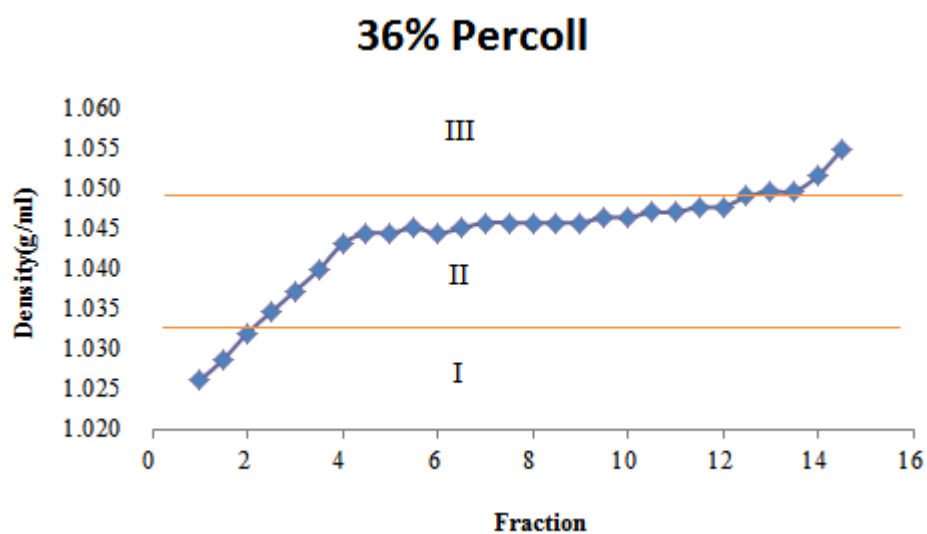


Fig. 19. The density distribution curve in 60 000g condition of centrifugation with 36% Percoll. The density was determined by measuring refractive index in each fraction of 0.5 ml (of 15 ml total volume of gradient). The lowest density was from the top fraction of the gradient, whereas the heaviest one was from the bottom fraction. The density ranges between 1.027-1.055 g/ml. The low density range (1.027 to 1.033 g/ml) was regarded as range I. The intermediate range II was from 1.033 to 1.049 g/ml. Range III was from 1.049 to 1.055 g/ml in this trail.

The cultivation of stem cells after gradient centrifugation

Cells from density fraction II (pre-adipocyte stem cells) were seeded on culture dishes and the proliferation was followed over a period of 10 days. From the Fig. 20, we observed the cells had almost reached confluence stage.

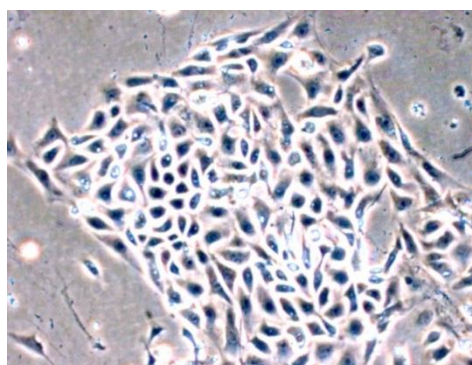


Fig. 20. The cultivation of stem cells in growth media in the proliferation stage at day 10

4.6. Gene expression differences between mature adipocytes and stem cells

This study was conducted in order to compare the gene expression in mature adipocytes with the gene expression in the isolated stem cells from the gradient. The expression of C/EBP α , C/EBP β , C/EBP δ and FATP were examined. C/EBP α , a late adipogenic marker, was significantly higher expressed in mature adipocytes compared to the stem cells (Fig.21). C/EBP β , an early adipogenic marker, was lower expressed in mature adipocytes compared to the stem cells (Fig.22).

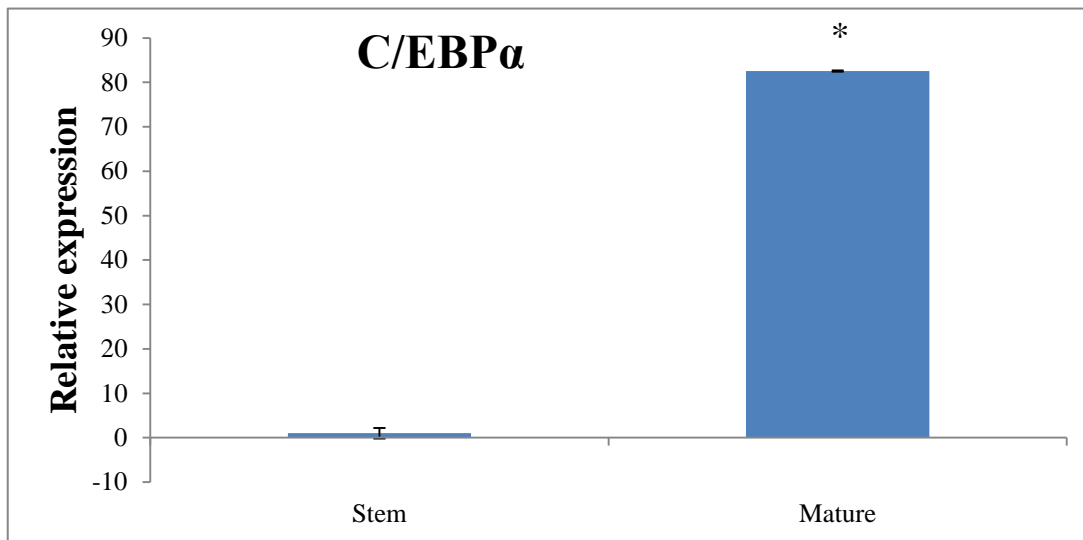


Fig. 21. Relative gene expression between stem cells and mature cells from adipose tissue in Atlantic salmon. Data were normalized to elongation factor 1A (EF1A). The value is set to one in the stem cells group and the expression in mature cells is calculated relative to that. Each bar represents the mean \pm SD ($n=2$ in stem cells & 4 in mature cells) and star indicates significant differences ($p \leq 0.05$). C/EBP α = CCAAT/enhancer binding protein alpha.

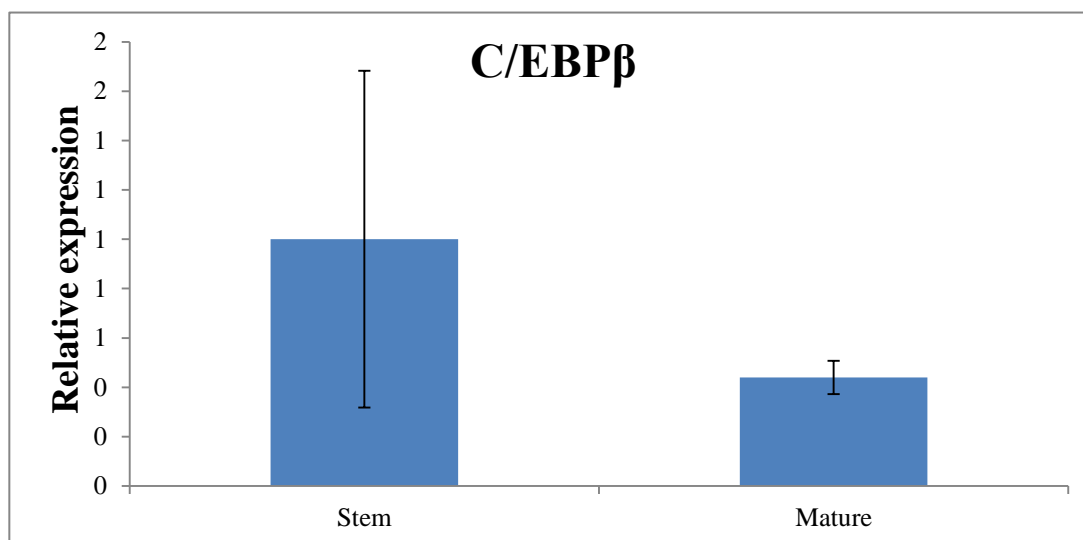


Fig. 22. Relative gene expression between stem cells and mature cells from adipose tissue in Atlantic salmon. Data were normalized to elongation factor 1A (EF1A). The value is set to one in the stem cells group and the expression in mature cells is calculated relative to that. Each bar represents the mean \pm SD ($n=2$ in stem cells & 4 in mature cells). C/EBP β = CCAAT/enhancer binding protein beta.

C/EBP δ gene expression was significantly higher expressed in mature adipocytes compared to the stem cells (Fig.23).

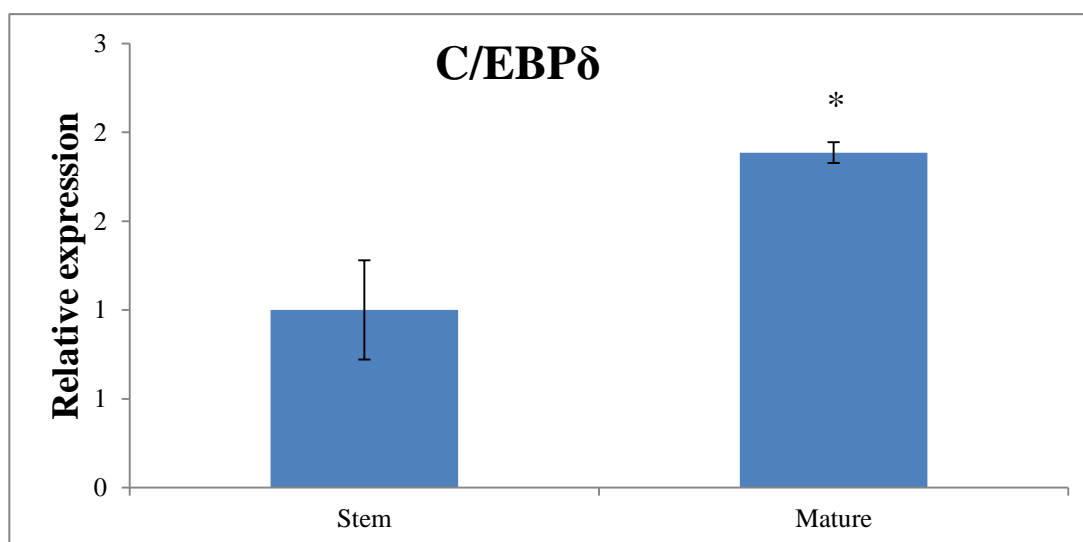


Fig. 23. Relative gene expression between stem cells and mature cells from adipose tissue in Atlantic salmon. Data were normalized to elongation factor 1A (EF1A). The value is set to one in the stem cells group and the expression in mature cells is calculated relative to that. Each bar represents the mean \pm SD ($n=2$ in stem cells & 4 in mature cells) and star indicates significant differences ($p\leq 0.05$). C/EBP δ = CCAAT/enhancer binding protein delta.

FATP gene expression was significantly higher expressed in mature adipocytes than in the stem cells (Fig.24).

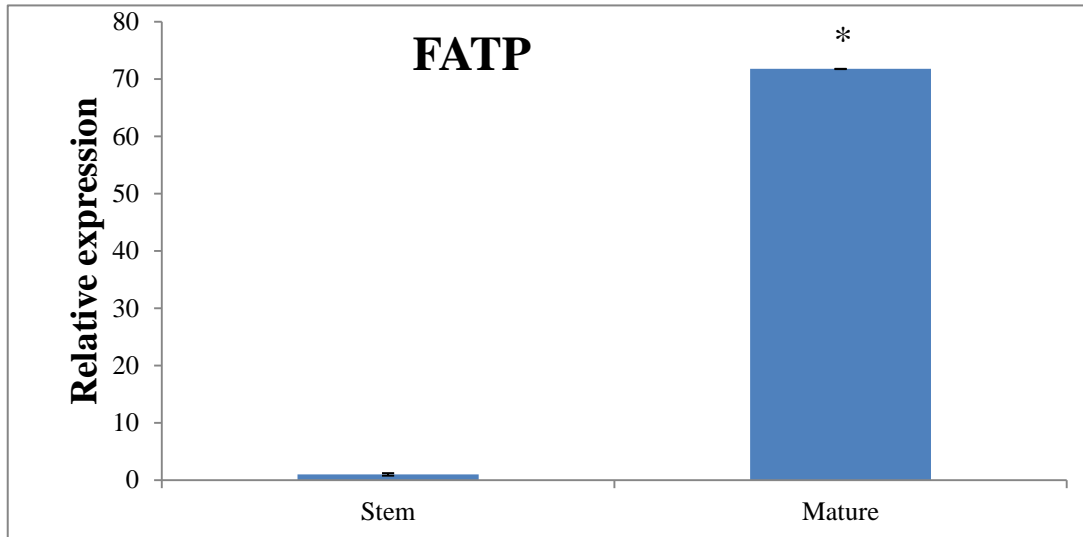


Fig. 24. Relative gene expression between stem cells and mature cells from adipose tissue in Atlantic salmon. Data were normalized to elongation factor 1A (EF1A). The value is set to one in the stem cells group and the expression in mature cells is calculated relative to that. Each bar represents the mean \pm SD ($n=2$ in stem cells & 4 in mature cells) and star indicates significant differences ($p \leq 0.05$). FATP= fatty acid transport protein.

4.7. Evaluating a new method for fixation of visceral adipose tissue sections from Atlantic salmon

Adipose tissue, as a high lipid content tissue, is challenging for histological studies, especially in traditional fixed and embedded specimens (formaldehyde or glutaraldehyde fixation and paraffin embedding). In this trial, we wished to test out a new fixation method, CryoWax, in our laboratory. In other studies with mammalian tissues (Weibull et al. 1984), CryoWax has shown its advantage in adipose tissue preservation as well as the method is simpler or easier to perform than other conventional methods. The trial was therefore conducted to test out how the Cryowax method (freeze substitution -80°C isobutanol for 2 minutes and followed by polyester wax embedding) functions in preservation of adipose tissue from Atlantic salmon.

Quality of tissue sections

The tissue sections were observed by microscopy. The fine structure of the tissue was well preserved after fixation and embedding by the CryoWax procedure (see section 3.4.1.). No shrinkage and distortion could be observed under the microscope. Adipocytes were attached to each other naturally. Nearly no morphological discontinuity or breakage could be observed in the sections. This demonstrates the method is suitable for application on adipose tissue and protects the morphological quality.

4.8. Hand section staining

Hand sectioning was conducted in order to get some information about in which stage the lipids inside the adipocytes start to disappear when using the CryoWax method. Oil red O was used to stain the lipids inside the adipocytes in adipose tissue. The presented outcome demonstrated that the tissue sections still contained high level of lipids inside the adipocytes (black arrows in Fig. 25.) after freeze substitution in -80°C isobutanol for 2 minutes. During polyester wax embedding, however, the lipid was extracted out.

Some lipids were however also unstable after freeze substitution in -80°C isobutanol, since some lipid droplets (yellow arrows in Fig. 25.) were floating outside the sample section (in the glycerol), suggesting that the fixation method need to be improved if someone wish to study the lipid fraction of the adipose tissue.

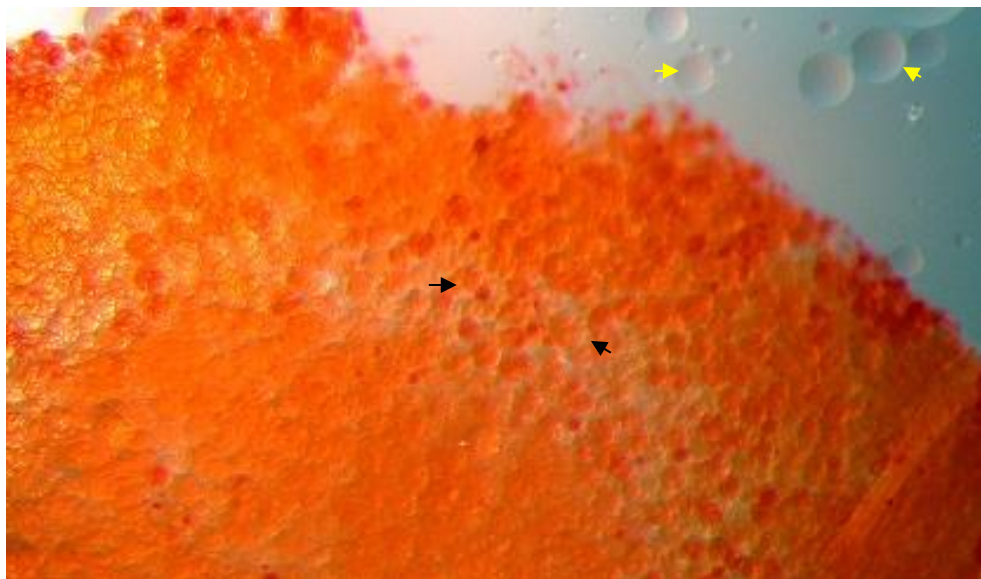


Fig. 25 Light micrographs showing the hand section staining with oil red O to reveal the lipid inside the adipose tissue in Atlantic salmon. Black arrows point to the oil red O stained lipids. Yellow arrows point to the lipids floating outside the tissue in glycerol. Magnification= 6.8 X.

4.9. Fluorescence staining of nuclei, lipids, cell membrane and F-actin.

There was nearly no visible lipid droplets in the adipose tissue slides fixed and embedded by the CryoWax procedure after staining with LipidTOX. Some lipid signals were slightly detected in the edge of cytoplasm (white arrow in Fig. 26 A), lipids that most likely were part of the cell membrane. No lipid signals could be observed inside the “emptied” adipocytes. This result showed that the lipids stored in adipose tissue have been extracted out by the use of the CryoWax procedure. However the integrity of subcellular structures (membranes and blood vessel) was well maintained as shown by the fluorescence microscopy images.

Collagen in adipose tissue

Collagen has important structure functions inside and outside the cell membrane. In order to visualize the collagen and inter-connections between cell membranes, the tissue slides were stained with WGA (Fig. 26 B). The WGA signal was generally located closely to the membrane compartments of each adipocyte and the blood vessels. WGA staining showed that, adipocyte membranes were closely connected to each other (no detachment). In the section slides, we

further discovered a blood vessel that was highlighted by the WGA staining in red. There were several blood cells distributed inside the blood vessel. The nuclei of the red blood cells and adipocytes were stained with DAPI (blue color).

Phalloidin stained for the F-actin (one part of cytoskeleton).

Fig. 26 B showed that the phalloidin enhanced or visualized the actin filaments in blood cells in adipose tissue. The phalloidin signals were strong in blood cells distributed in the vessel of adipose tissue. In contrast, the inner part of adipose tissue did not display any visible signal.

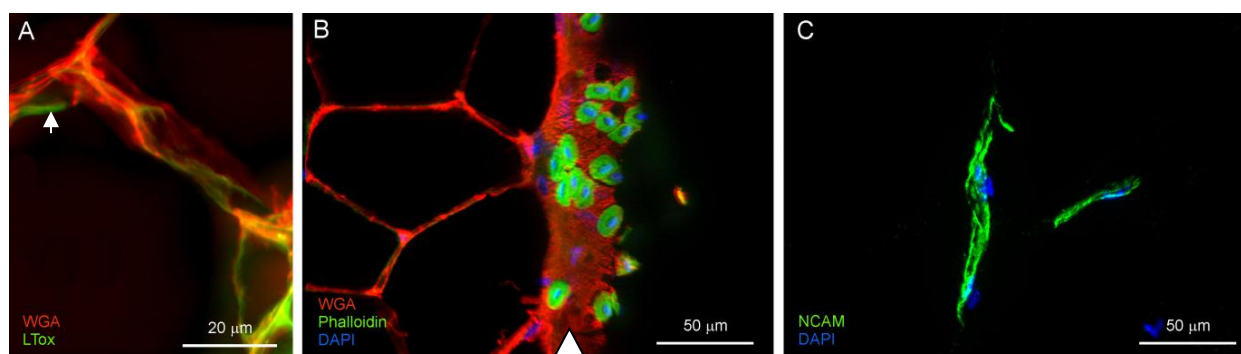


Fig. 26 Histological sections of adipocytes from adipose tissue in Atlantic salmon fluorescence stained for wheat germ agglutinin (red fluorescent signal) and counterstained with LipidTOX (green signal) for **A**, slightly signals were captured (white arrow); Phalloidin (green) and DAPI (blue nuclei) for **B**. Arrowhead points to a blood vessel. Immunostaining for Neural cell adhesion molecule (NCAM), Nuclei have been counterstained in blue with DAPI (**C**). Bars: 20 μm (**A**), 50 μm (**B** and **C**).

Immunofluorescence staining

Immunofluorescence staining was used to evaluate the antigenicity preservation after the CryoWax process.

NCAM in adipose tissue

NCAM was stained to reveal the location of neuron between adipocytes (Fig. 26 C). NCAM as a glycoprotein is expressed on the surface of neurons or glia and binds against anti-neural cell adhesion molecule (anti-NCAM) antibody. From the Fig. 26 C, bindings were mainly located at the neurons with several nuclei inside. The tissue sections implicated a good preservation of

antigenicity was achieved. The nuclei of the neuron between adipocytes were stained with DAPI (blue color). Hence, we believed that CryoWax method functions well in preserving antigenicity.

According to the above tests, we concluded that CryoWax method has its advantage in protecting the morphological quality, as well as for its easy processing and good antigenicity preservation. As unstable lipid droplets inside the adipose tissue during the heat embedding, the outcome therefore was not ideal. The later process gave rise to the lipid extraction in adipose tissue. That demonstrated as the empty holes in the adipose tissue in the slides. Further improvement should be carried out to achieve better lipid conservation in histology for adipose tissue.

5. Discussion

5.1. Optimizing the cultivation conditions for adipocytes

5.1.1. Effect of different FBS levels on proliferation of pre-adipocytes

Primary pre-adipocytes from the visceral adipose tissue of Atlantic salmon proliferate, when they are cultivated in a growth medium containing appropriate levels of nutrients, minerals and growth factors (Hausman et al. 2001). FBS is a good source of nutrients for cultivation of adipocytes. Thereby, in this study, we sought to optimize the level of FBS during the proliferation stage. In previous studies, 10% FBS has been used during the proliferation stage of Atlantic salmon adipocytes in our laboratory (Todorčević et al. 2008; Vegusdal et al. 2003). The 10% FBS, which is quite costly, was chosen based on recommended levels in mammalian studies (Hausman et al. 2001; Suga et al. 2007). The level was not optimized for cultivating fish adipocytes. In this study, we wanted to evaluate if fish cells, which grow at a much lower speed due to low cultivation temperatures than mammalian cells, could cope with lower levels of FBS in the culture medium while still obtaining maximal growth. Our studies did however show that 10% FBS gave higher proliferation capacity than 2.5% and 5% FBS in agreement with mammalian studies (Hausman et al. 2001; Suga et al. 2007).

In our study, there was no significant difference in the number of cells among each treatment groups after the first day's incubation. This outcome might be explained by the fact that the cells were not adapted to the provided conditions, and that isolated pre-adipocytes were not totally adhered to the bottom of the culture wells. The effects of different FBS levels were visible under phase contrast microscopy after 5 days of incubation. Cells incubated with the highest level of FBS (10%) reached confluent stage earlier than cells under other treatments, with both DMEM and L-15 in term of cultivation media. Thus, our results suggest that in the proliferation stage, more nutrition in the growth medium is preferred by pre-adipocytes as it provides a higher level of energy for cell multiplication.

5.1.2. Effect of different FBS levels on adipocytes differentiation

Confluent primary pre-adipocytes in culture start the differentiation process when hormones such as insulin, DEX, IBMX and T3 are added to the culture media (Bouraoui et al. 2008; Todorcević et al. 2008). Ramsay and Rosebrough (2003) and Van Harmelen et al. (2004) found that a medium containing above-mentioned hormones stimulates GPDH activity, which is often used as a differentiation parameter. In salmonid fish, it was found that lipid accumulation was increased with the application of a two-day adipogenic hormonal induction during the differentiation stage (Todorcević et al. 2010). Three levels of FBS (2.5%, 5% and 10%) were also evaluated for effects of different FBS concentrations to the differentiation degree of adipocytes.

Rosen and Spiegelman (2000) referred the differentiation process as the transition of undifferentiated fibroblast-like pre-adipocytes into mature round lipid-filled fat cells. In our study, we observed that the group supplemented with 5% FBS seemed to contain more mature adipocytes with more lipid droplets compared to the 10% FBS group. That suggests that pre-adipocytes prefer moderate level of FBS during differentiation stage. It has previously been reported in mammalian studies that supplementation of growth factors (such as bFGF) stimulates the replication of pre-adipocytes and inhibits differentiation (Teichert-Kuliszewska et al. 1992). FBS has been widely adopted as a standard supplement because of its rich content of growth factors and lesser concentrations of growth inhibitory factors. This indicates that high levels of FBS, containing considerable amount of growth factors, may inhibit the differentiation process towards mature adipocytes. Their conclusion is consistent to our finding showing that 5% level of FBS resulted in the best effect on differentiation with more lipids droplets visible in comparison to the 10% and 2.5% groups. Similar findings were found in studies on other species, e.g. pigs (Walton et al. 1986) and cattle (Etherton et al. 1987). Even though, in previous Atlantic salmon adipocytes research, 10% FBS was used in the medium during the culture (Todorcević et al. 2008; Vegusdal et al. 2003). Result from our study indicates an improvement of

differentiation with less use of FBS. Therefore, we concluded that a moderate level of FBS (5%) is the optimal amount to be used in the medium during differentiation process of Atlantic salmon pre-adipocytes. It reaches a better differentiation while achieving a higher economic saving.

In cell culture biology, confluence refers to the coverage of the well or flask by the cells. It is shown in Ginty et al. (2006) 's study that a cell culture, with above 70% of confluence, spontaneously stimulates differentiation of myoblasts. Similarly, in a recent study, Topman et al. (2011) claims that pre-adipocytes spontaneously differentiate into lipid droplet-containing adipocytes at high levels of culture confluence in mammalian cell lines. In our trial, even after addition of differentiation media, the cells which had not yet reached confluence and the cells which had several open areas with no cells attached, did not differentiate to the same extent as cells which were confluent. In agreement with our observation, another study suggested that NIH/3T3 cells at low levels of confluence, changed their morphology to a flat and elongated more fibroblast like shape after the cultivation in differentiation medium (Rubin 1981). To conclude, a necessity for pre-adipocytes reaching confluence should be considered before differentiation medium is added.

5.1.3. Effect of DMEM and L-15 on adipocytes proliferation

Microscopic images of cells at confluence showed that there seems to be higher proliferation capacity in cells cultivated in DMEM than in L-15 medium. This indicates that the DMEM cultivation media is more preferred by adipocyte than L-15 in the process of proliferation. Previous studies on Atlantic salmon adipocytes have either cultivated cells in DMEM (Vegusdal et al. 2003) or L-15 (Todorčević et al. 2008). Our study is the first one to compare the proliferation efficiency of salmon adipocytes in those two media. DMEM and L-15 differ in some nutritional compositions, which may influence the proliferation capacity. The main carbohydrate source in DMEM is D-glucose (Dextrose), while in L-15 is D-galactose.

Carnivorous fish species like Atlantic salmon have low tolerance for glucose supplement (Moon 2001). A recent study, with salmon adipocytes, has shown that glucose addition to the L-15 culture media improves adipocyte development (Bou et al. 2012). This is consistent with our finding showing that DMEM containing glucose improves the development capacity in adipocytes compared to the L-15 with galactose. It has been shown that glucose is more stable than galactose, which is speculated as the reason of an existing pathway for rapid conversion from galactose to glucose among many species (Blum & Serzedello 1968; Kliegman & Morton 1988). It may be that the DMEM medium, which provides glucose directly, is one of the reasons for higher development capacity of adipocytes grown in this media. In mammalian adipocytes, when glucose appeared to be the sole carbon source from the medium, the contribution of glucose to FA synthesis increased nearly two-fold (Yoo et al. 2004). Another possible reason for differences in proliferation degree between the two media is the differences in amino acid composition. There is a higher arginine content in DMEM than in L-15. Our result is in agreement with the study from Tan et al. (2010), where incubation of intestinal cells with arginine was shown to stimulate proliferation. Further studies are however required to verify the cause of improved proliferation in fish studies.

There were, however, only minor differences in differentiation capacities between cells in these two media, although there was a tendency of having more lipid droplets in cells cultivated in DMEM than in L-15. The tendency of improving differentiation with DMEM, may also be caused by small differences in degree of confluence at the initiation of the differentiation process.

5.1.4. Effect of FAs supplementation on adipocytes differentiation

Previous studies from our group have been testing the effects of different FAs supplementation on development of fish adipocytes in our lab. It was reported that lower lipid accumulation were

obtained in Atlantic salmon during differentiation, when adipocytes were cultivated in n-3 HUFA (DHA) enriched medium was compared to adipocytes supplemented with OA enriched medium (Todorčević et al. 2008). DHA has previously been reported to inhibit the differentiation of adipocytes via the suppression of several regulatory genes (e.g. C/EBP α), which regulates terminal differentiation in both Atlantic salmon (Huang et al. 2010) and mammals (Madsen et al. 2005). In our study, we followed up these findings to further examine the influence of two different doses of FAs on the regulation of C/EBP α and FATP 1 genes expression. The effect of different doses has not been studied before.

Our results showed that the adipocyte differentiation was affected by the FAs supplementation, which is in agreement with Todorčević et al. (2008). By LipidTOX staining of lipid droplets in adipocytes, our study revealed more and larger lipid droplets in the cells supplemented with OA than those supplemented with DHA. There were very few visible lipid droplets in the control group that was not supplemented with any additional FAs. The lower lipid accumulation with DHA than with OA is in agreement with several studies both from fish (Todorčević et al. 2008) and mammals (Hainault et al. 1993; Mann et al. 1994; Parrish et al. 1990). There was also highest degree of immunostaining of FATP in the OA group, compared to the DHA and the control group, which is in agreement with a higher differentiation degree with OA.

In mammalian studies, earlier researches have suggested that FAs are influencing the regulation of adipocyte differentiation (Ding & Mersmann 2001; Tontonoz et al. 1994; Yamamoto et al. 2005). In addition, it is described by Amri et al. (1991) and Distel et al. (1992) that FAs also stimulate differentiation of clonal pre-adipocytes. Therefore, all these findings revealed that FAs stimulate adipogenesis. Furthermore, it was reported by Raclot and Oudart (1999) that the effect of FAs in mammalian adipocytes was related to gene expression. The same authors discovered that pre-adipocytes treated with FAs (especially HUFAs), showed induced expression of genes encoding several FA metabolic proteins. Furthermore, several transcription factors, involving in adipocyte differentiation, are shown to be regulated with FAs on the effects

of cell morphologic conversion, lipogenic gene expression and TAG accumulation (Rosen & Spiegelman 2000).

The expression of C/EBP α and FATP 1 genes were regulated both by the different FAs supplied in the media and their levels. The two types of studied FAs in our experiments also showed slightly different regulatory effects on expression of the genes C/EBP α and FATP 1 during the differentiation of pre-adipocytes. Both FAs induced the expression of C/EBP α and FATP 1 compared to the control group. This result is in agreement with previous results from Atlantic salmon showing up-regulation of C/EBP α and FATP 1 expression with either OA or DHA supplements (Huang et al. 2010). In the previous studies with Atlantic salmon, only one concentration of FAs (0.6mM) was used in the cultivation media, while in our study we wanted to test two different doses (0.1mM and 0.6mM) of both OA and DHA and study whether the dose would influence the gene expression of C/EBP α and FATP 1 differently. Expressions of both genes were more up-regulated with high dose of FAs supplementation than with the low dose.

C/EBP α , is a transcription factor known to be expressed relatively late in the differentiation process where it acts as an inducer of fat-specific genes for the synthesis, uptake and storage of long chain FAs (Koutnikova & Auwerx 2001), which indicate that cells given the highest dose of FAs in our trial is at a more mature stage in the differentiation process than cells given the lower dose of FAs. This is also in agreement with the higher expression of FATP in the high dose FA groups, since FATP is known to increase in expression during differentiation (Todorcević et al. 2010), and the expression of FATP gene is proportional to the lipid accumulation in adipocytes. However, the DHA treated group reveals lower FATP gene expression compared to the OA one. A contrary outcome was observed in C/EBP α expression. The expression was higher in DHA treated group in contrast to the OA one. That suggests that DHA may play a role in stimulating differentiation of adipocytes as well as moderating the lipid accumulation, which is in agreement with mammalian studies (Clarke 2001; Price et al. 2000). In addition, this

finding explains the effect in up-regulation of FATP expression being lower than with the OA one. However, the mechanism of high expression in C/EBP α resulted in a low lipid accumulation in adipocytes is still not clear, which needs to be further investigated. In our finding, DHA treated group resulted in a lower lipid accumulation in the lipid droplets staining outcome and a higher expression of C/EBP α than the OA one.

5.2. Percoll density formation

5.2.1. Use of Percoll density gradient centrifugation to isolate stem cells from adipose tissue

Visceral adipose tissue is a heterogeneous organ (Casteilla & Dani 2006). Primary isolated pre-adipocytes are considered as a mixed fraction containing many cell types. Pluripotent stem cells as one of the components of adipose tissue, are characterized with their ability to renew themselves through mitotic cell divisions and also the capability to differentiate into various cell types (Gregoire et al. 1998). Therefore, to isolate pure stem cells out of multi-component adipose tissue has its significance in further application of those cells.

In accordance with the previous study by Gaben-Cogneville et al. (1983), the Percoll density gradient centrifugation fulfilled the purpose to isolate the stem cells from Atlantic salmon adipose tissue. Usually, separating primary pre-adipocytes is the first step for the further isolation of stem cells. The density range where stem cells from mammalian species can be isolated in a Percoll density gradient is mentioned to be in the range (1.033-1.049 g/ml) (Gaben-Cogneville et al. 1983). The density range for recovery of fish stem cells in percoll gradient had prior to this study not been reported. In order to access the density range of salmon stem cells isolated from adipose tissue, we found that a 36% Percoll gradient covered the wished density range of 1.033-1.049 g/ml better than a 70% Percoll gradient. Moreover, the higher force of gravity (60 000g) compared to lower one (30 000g) revealed a more expanded density in target range in our studies. Apparently, a more expanded density range helps the location and

harvest of the target cells or organelles in formed gradient (Anonymous 2007). Accounting for the inclusion of density range and more expanded target density, therefore, we finally decided that 36% Percoll and 60 000g was suitable condition for isolation of fish adipose-derived stem cells.

By mimicking the addition of cell solution on top of the density gradient, it is shown by Pertoft (2000) that layering the sample on top of a preformed density gradient reduces contamination by cell debris, etc., which retains in the top layer after separation. It was further tested how addition of a cell culture media L-15 on top of the preformed gradient would influence the density gradient after centrifugation at 2500g for 10 minutes. Our study showed that addition of L-15 on top of the gradient, led to an extended density range in the gradient. In addition, a middle target fraction range was maintained for stem cells in such circumstance. This method was therefore further employed to isolate stem cells from a mixed fraction containing many cell types isolated from adipose tissue. Our results are in agreement with the reference article from mammalian cells (Gaben-Cogneville et al. 1983), in which Percoll was diluted with incubation medium (medium 199).

Stem cells isolated by the optimized Percoll gradient centrifugation condition, was seeded on culture dishes in DMEM culture media. The cells easily attached to the cell flask and readily started to proliferate, which is a typical feature of stem cells (Tang et al. 2008). The well growth of stem cells on culture dishes, shows that the cells maintains their viability and capacity to attach to cell flask and proliferate after Percoll density gradient isolation, which shows that the protocol is suitable for isolation of adipose stem cells from Atlantic salmon. Previously, our laboratory has not used density gradient separation of stem cells prior to seeding. The cell fraction that previously has been seeded on cell flasks has contained a mixture of many cell types and it has been a problem with contamination of the cell cultures with red blood cells and mature adipocytes. The red blood cells can sometimes be difficult to get rid of and may influence the growth of the target cells. It has further been a frequent problem with

contamination of mature adipocyte and red blood cells in the culture dishes, which again may influence the differentiation process and therefore not desired. This new Percoll density isolation method, which leads to an isolation of more pure stem cells with no contamination of mature adipocytes and red blood cells, therefore represent a new valuable method for our laboratory. One drawback with Percoll is, once with the presence of salt, it is not possible to autoclave. Also, the Percoll particles are too large to be sterile filtered. However, the sterile problem can be overcome by separately autoclave saline solution and Percoll, and mixing of the two compounds in a sterile environment (Pertoft 2000).

5.2.2. Comparison of gene expression in mature adipocytes and isolated stem cells from adipose tissue

The comparison of gene expression, was taken place in order to verify if the isolated stem cells from gradient centrifugation were different from the more mature cells. Further, we tested if those stem cells were still in an early developmental stage.

C/EBP β is known to precede the expression of C/EBP α (Huang et al. 2010; Rosen & MacDougald 2006). As an early adipogenic transcription factor, C/EBP β has been shown to activate the synthesis of insulin-like growth factor I (Pabst et al. 2001). That suggests a higher expression of C/EBP β during the early stage of cell development. And after a transient induction of C/EBP δ , the level of C/EBP β expression retains low during the rest of adipogenesis. That is consistent with our finding, down-regulation in mature cells compared to up-regulation in stem cells. In addition, higher expression of C/EBP δ in mature cells than in stem cells was observed. The same outcome was found in the study of Todorčević et al. (2010). The authors showed up-regulation of C/EBP δ gene even at day 23 within a whole 30 days culture.

C/EBP α and FATP 1 were two highly up-regulated genes in mature adipocytes compared to stem-cells in our study, revealing high expression of both genes in relatively late stage. C/EBP α

had almost 80 fold higher expression in mature cells than in stem cells, in agreement with the salmonoid study of Todorčević et al. (2010). In addition, earlier studies from mammalian species showed that its high expression is important in regulation of terminal differentiation of adipocytes (Madsen et al. 2005). Similarly, FATP 1 gene had markedly higher expression in mature adipocytes compared to stem cells. This up-regulation during adipocyte differentiation was consistent with the finding by Todorčević et al. (2008) in Atlantic salmon as well as with mammalian cells' studies (Guo et al. 2000). The function of FATP 1 coded proteins is to transport long chain FAs to the cells and in this way lead to a lipid accumulation during the late phase of adipocytes differentiation. In contrast, stem cells remain fairly low expression of FATP 1. That partly explained the differences in shape and lipid content of mature adipocytes and stem cells from the morphological point of view.

Hence, our studies conducted on two adipose-derived cells, which stay in two different developmental stages revealed distinct gene expressions. These differences in respective gene expressions suggested that the isolated stem cells remain viability after the isolation and recover successfully during the cultivation. These findings guarantee the possibility for a further application of Percoll in isolating pure stem cells.

5.3. The benefit of application of CryoWax method

5.3.1. The benefit in protecting quality of tissue sections

The current drawbacks of conventional chemical fixation and embedding techniques are mainly postmortem structural alterations and antigenicity deterioration (Saga 2005). Specially, conventional histology methods are not optimal to use on adipose tissue with high lipid content.

With the development of CryoWax method, it provided another approach for further examining the fine structure of adipose tissue. It consists of freeze substitution of the samples followed by embedding. In earlier studies with the use of CryoWax method (Duran et al. 2011), up to 7 mm

thick specimens was carried out with dissected tissue, while in our study we expected to test its preservation in applying for 3-4 mm thick adipose tissue. Specifically, adipose tissue went through freeze substitution, followed by polyester wax embedment and combined with fluorescent staining to visualize the details of the structure. Some modifications have been carried out in order to improve lipids conservation. Isopentanol was used to reach quick freezing, and methanol was used to slowly freeze substitute tissue water in our reference article (Duran et al. 2011). However, our study replaced isopentanol with isobutanol, and ethanol instead of methanol, to avoid extraction and destruction problem with chemicals to a low possible extent. For instance, methanol fixation process was shown to disrupts the phalloidin-binding site on actin, thereby eliminating staining (Howard & Oresajo 1985).

Our well morphological preservation is due to the advantages of freeze substitution and polyester wax described as following: Freeze substitution was regarded as an efficient and inexpensive means to process tissue. Though not well understood, its low temperature is so far the least harmful established approach to dehydrate a sample (Mobius 2009). Quick freezing immobilizes the sample and prevents ice crystal growth. Subsequently, freeze substitution replaces water with a polar organic solvent (ethanol in our study) (Quintana 1994; Usuda et al. 1990). With the absence of water in the specimen, thereby, changes in the structure reduced to a minimal range; Polyester wax as an embedding medium, its property of low melting point (37-40 °C) could avoid the shrinkage of tissues and help in protecting heat sensitive molecules (Sidman et al. 1961). In addition, being water tolerant and soluble in most organic solvents, prevents specimens from the process of progressive dehydration and clearing agents, and it showed to have no hazard to the tissue immunolocalization in cancer cell lines (Otali et al. 2009). Thereby, a good morphologic structure preservation and ease of immunostaining are the advantages of this wax embedding medium (Merchant et al. 2006).

In general, our modified CryoWax tested on adipose tissue still presents its advantages in preservation of morphology and localizations of antigens. Successful application of freeze

substitution was achieved in adipose tissue specimens. However, CryoWax shows its limitation in the conservation of lipid droplets. To be specific, the protection of lipids in freeze substitution was still acceptable before the embedding section. This was further proved by our hand sectioning study. Our conclusion is in agreement with one study demonstrating that during freeze substitution in some solvents (e.g., acetone), lipids can be well stored in cells of the bacterium (Weibull et al. 1984). Therefore, during the embedding process, it still provided too high temperature for adipose tissue to retain lipids stable inside the tissue sections. However, we still need to point out, its low melting point, remains its benefit in avoiding heat-induced shrinkage and dehydration process. Further researches need to be investigated on for the purpose of the protection of quality in tissue sections.

In our studies, fluorescence staining reveals good preservation in CryoWax applied sections for the following cellular components in adipose tissue. Phalloidin, as a F-actin binding, the distribution of actin can be labeled by this fluorescent imaging tool for microscopy. Usually, F-actin, an element of microfilaments (one of three major components of the cytoskeleton), was used to visualize their presence in cytoskeleton in our adipose tissue specimens. With the enhanced signal of cytoskeleton structure of adipose tissue, stained fine details of blood cells with actins were demonstrated. The main site of cells basally located along with the nuclei situated with DAPI staining. From above description, the fine details of adipose tissue were presented.

Similarly, well preservation in wheat germ agglutinin (WGA) staining was presented in our trails. WGA binds to N-acetyl-D-glucosamine and Sialic acid (Peters et al. 1979). The lectin functions with the existence of sialic acid on the cell membrane or other subcellular structures. Adipose collagen was visualized with the presence of WGA from our study. In adipose tissue, the collagens exhibited among two or three attached adipocytes inside the tissue. The outer layer basically functions as membrane architecture. The merged figure shows all membranes, collagens and inner cytoskeletons of blood cells together, which gives a full frame of adipose

tissue.

Likewise, well protein antigenicity protection is another superiority of these CryoWax treated specimens showed in our study. NCAM as a glycoprotein gene-expressed binder on the neurons, plays roles in inducing neurite outgrowth (Letourneau et al. 1994). It used to recognize certain tumors as well. Back to our finding, the protein has maintained its capacity to bind antibody, suggesting that the protein was well preserved after the CryoWax procedure. The location of the protein was visualized in the section by the staining of NCAM, such as the neuron in between two adipocytes was detected in our section study. Therefore, the CryoWax procedure resulted in a well protection for the quality of adipose tissue section.

5.3.2. The Cryowax method is not suitable for studies of lipid storage in adipocytes

LipidTOX staining is considered to be a good method to visualize lipid droplets (product of adipogenesis) in adipose tissue. Furthermore, it was documented in Ford et al. (1999) that LipidTOX staining exhibited superior performance over other stains (i.e., Nile red, oil red O). That is the reason why this was the method chosen in our study. However, in our test, with the application of CryoWax, the lipids were extracted during the embedding process of adipose tissue. No obvious or weak lipid staining signals in the fat-rich adipose tissue sections was observed after embedding. Hand sectioning further revealed or verified that the extraction of lipids from the tissue sections happened during the embedding process. This might be explained with a low melting point of fish lipids, the embedding temperature (37-40 °C) we provided increased the fluidity of those fixed lipids inside the section, led to the extraction as we observed. Thereby, lower melting point of embedding medium should be tested for the conservation of lipids in adipose tissue. Therefore, the application of this CryoWax method suggested that further improvement should be considered in order to conserve the lipids inside adipose tissue.

6. Conclusion

In the development of adipocytes, 10% FBS significantly increased the proliferation of pre-adipocytes, while differentiation degree was highest with 5% of FBS. DMEM medium with CO₂ improved the proliferation compared to the L-15.

FA level in the media did influence the differentiation degree of adipocytes. Additionally, type of FA differently influences the formation of lipid droplets in adipocytes. That is, a more moderate lipid accumulation with DHA than with OA.

Density gradient centrifugation with 36% Percoll under 60 000g met the demand to form a gradient suitable for further centrifugation and purification of stem cells. Isolated stem cells were further verified based on the result of gene expression compared to those in mature adipocytes.

CryoWax method retained a fine structure and well antigenicity of processed adipose tissue. Lipid conservation is the limitation of this method for adipose tissue, that needs to be considered for an improvement.

This thesis shows improvements to the standard protocols for culture conditions, cell isolation, and purification, as well as fixation and morphological preservation of adipose tissue in Atlantic salmon.

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