

# Polar bear adipose tissue-derived stem cells as an *in vitro* model for effects of environmental contaminants on adipogenesis

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Master thesis in biology – environmental toxicology

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Bergen, September 2015

Lene Øy garden



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**ABSTRACT**

The polar bear (*Ursus maritimus*) is currently listed as a vulnerable species by the IUCN red list, mainly due to the predicted decline in sea ice caused by global warming. Polar bears are dependent on sea ice for hunting their main prey, the ringed seal (*Phoca hispida*). Shrinkage of sea ice can result in energetic challenges and increased mortality rates in polar bear populations, as their access to prey declines and the seasonal fasting period gets prolonged. Also affecting polar bears is the exposure and accumulation of persistent organic pollutants (POPs) to unusually high levels due to their lipid rich diet. Some POPs act as endocrine disrupting chemicals (EDCs), leading to metabolic disruption. EDCs may interact with nuclear receptors (NRs), including NRs involved in regulation of metabolism and adipogenesis, e.g. peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ). Interference by environmental contaminants on NRs regulating adipogenesis in polar bear may affect lipid metabolism and thereby decrease their ability to respond to climate change.

The aim of this thesis was to develop an *in vitro* method for studying effects of environmental contaminants on adipogenesis in polar bear by using adipose tissue-derived stem cells derived from polar bear (pbASCs). PbASCs were successfully maintained and propagated in culture and shown to have adipogenic potential when cultivated with adipogenic inducers. Lipid content (Oil red O staining) and transcript levels of the adipocyte specific genes PPAR $\gamma$ , fatty acid-binding protein 4 (FABP4) and leptin (LEP) were used to monitor differentiation of pbASCs. While lipid accumulation and transcript levels of PPAR $\gamma$  and FABP4 reflected the degree of adipogenic differentiation, transcript levels of LEP apparently did not reflect adipogenic differentiation, indicating that LEP may not be a suitable target gene for investigating adipogenic induction in pbASCs.

Exposure of pbASCs with known EDCs demonstrated that mono(2-ethylhexyl)phtalate (MEHP) induced adipogenesis, while bisphenol A (BPA) did not.

This study has established an *in vitro* method for investigating effects of environmental contaminants on adipocyte differentiation of pbASCs, by quantification of lipid content and transcript level analysis. The use of pbASCs as an *in vitro* model for effects of environmental contaminants on adipogenesis can provide valuable information on how contaminant exposure can affect energy homeostasis in the polar bear.

**ABBREAVTIONS**

<b>Abbreviations</b>	<b>Full name</b>
A	Adenosine
AGE	Agarose gel electrophoresis
AIM	Adipogenic induction medium
ASCs	Adipose tissue-derived stem cells
BFR	Brominated flame retardant
BMSC	Bone marrow-derived stem cells
BPA	Bisphenol A
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer-binding protein
cAMP	Cyclic AMP
cDNA	Complementary DNA
ddNTPs	Dideoxynucleotides
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl)phthalate
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EDC	Endocrine disrupting chemical
EtBr	Ethidium bromide
FABP4	Fatty acid-binding protein 4
GF1	Insulin-like growth factor 1
GR	Glucocorticoid receptor
IBMX	3-Isobutyl-1-methylxanthine
INDO	Indomethacin
ISCT	The International Society for Cellular Therapy
LB	Lysogeny broth
LBD	Ligand binding domain
LEP	Leptin
MSC	Mesenchymal stem cells
NR	Nuclear receptor
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator-activated receptor
PPIA	Cyclophilin A
PREF-1	Preadipocyte factor 1
PVC	Polyvinyl chloride
RNA	Ribonucleic acid

## ABBREVIATIONS

ROSI	Rosiglitazone
rRNA	Ribosomal RNA
RT	Room temperature
SD	Standard deviation
SVF	Stromal vascular fraction
TBE	Tris-borate-EDTA
U	Uridine
UV	Ultraviolet
V	Volt
WAT	White adipose tissue
YWHAZ	Tyrosine 3/tryptophan 5-monooxygenase activation protein

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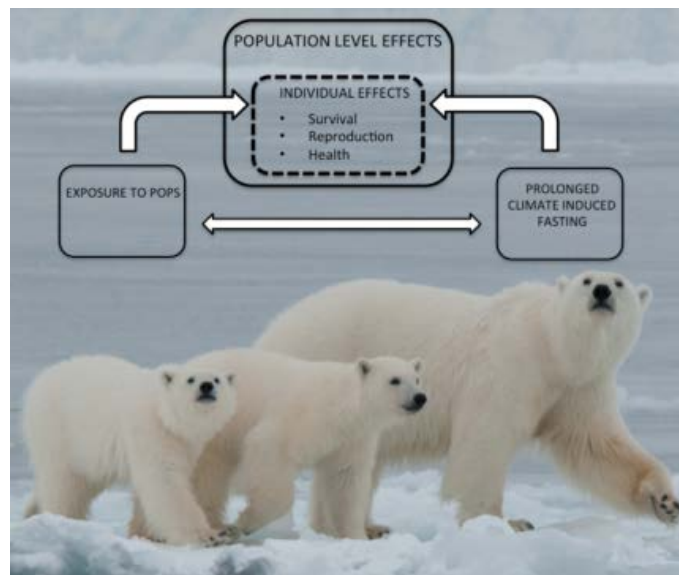
# 1 INTRODUCTION

## 1.1 POLAR BEARS FACE ANTHROPOGENIC CHALLENGES

Wilcove *et al.* (1998) suggested that “habitat loss is the single greatest threat to biodiversity”. In the Arctic the sea ice constitutes the habitat of several arctic animals, but it is currently rapidly declining due to global warming (Comiso *et al.*, 2008).

The polar bear (*Ursus maritimus*) is highly adapted to the arctic environment and is currently listed as a vulnerable species by the IUCN Red List ([www.iucnredlist.org](http://www.iucnredlist.org)), due to predictions which estimate a minimum 30 % reduction in the polar bear population within 45 years. This reduction is mainly due to the predicted decline in sea ice, but other stress factors include environmental pollutants, shipping, and oil and gas exploration (Schliebe *et al.*, 2008, Amstrup *et al.*, 2008, Durner *et al.*, 2009). As stress factors can affect polar bear health, reproduction and survival, and in turn population levels, it is important to gain detailed knowledge about the effect of these factors, and how they may interact with each other (Fig. 1.1.) (Jenssen *et al.*, 2015).

An introduction to how climate warming and environmental pollution can affect the vitality of polar bears is provided, as these are the two factors relevant to understand the background of this study.



**Figure 1.1. Anthropogenic stressors may affect polar bears on population level.** The figure illustrates how climate warming and environmental pollution can interact to affect polar bear survival, reproduction and health, on both individual and population level (figure from Jenssen *et al.*, 2015).

### 1.1.1 DECLINING SEA ICE POSES A THREAT TO POLAR BEARS

The polar bear is especially sensitive to climate warming as it is dependent on sea ice for hunting seals, and performing seasonal migration between summer and winter habitats (Laidre *et al.*, 2008). Vast areas of sea ice melts during summer, leaving polar bears with scarce access to food. To survive they must go through a seasonal fasting period which may last for up to eight months, by which the polar bears can lose up to 43 % of their body mass (Atkinson and Ramsay, 1995). During spring polar bears consume vast amounts of blubber by feasting on their main prey, the ringed seal (*Phoca hispida*), which they capture on land-fast ice or drifting pack-ice (Derocher *et al.*, 2002). The consumed energy is stored as fat reserves in white adipose tissue (WAT), and utilized during the seasonal fasting period. To avoid potentially fatal protein loss it is crucial that the polar bear has enough fat stored to cover the energy expenses, as over 90 % of the energy used during the fasting period originates from stored fat (Atkinson and Ramsay, 1995, Atkinson *et al.*, 1996). Previous studies have shown that pregnant polar bears and their cubs are especially at risk during the seasonal fasting, and that the reproductive capability decreases with lower body fat content in female polar bears (Robbins *et al.*, 2012, Rode *et al.*, 2010).

As a consequence of decline in sea ice, polar bears' food availability is reduced and thus they may not be able to store enough fat reserves to survive the fasting season. Another consequence of the shrinking sea ice is that polar bears may walk and swim more to access their prey, which results in a higher energy expenditure for capturing prey (Derocher *et al.*, 2004).

Climate warming is predicted to result in increased energetic challenges and mortality rates in polar bear populations, as winter habitats where the polar bear can hunt will decline, while the seasonal fasting period during summer will increase (Durner *et al.*, 2009, Amstrup *et al.*, 2008, Molnar *et al.*, 2010). Also, a reduced reproductive success due to climate change has already been shown in some subpopulations (Stirling and Derocher, 2012). Fasting is physiologically stressful, and an optimal control of metabolism is needed to maintain a sufficient supply of energy to all organs. Exposure to contaminants has been shown to interfere with energy metabolism, for example through the nuclear receptor (NR) peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (Casals-Casas and Desvergne, 2011, Feige *et al.*, 2006). Thus, it is possible that exposure to contaminants can add to the increased mortality rates and decreased reproductive success induced by the prolonged fasting period, as hypothesized by Jenssen *et al.* (2015).

### 1.1.2 PERSISTENT ORGANIC POLLUTANTS IN POLAR BEARS

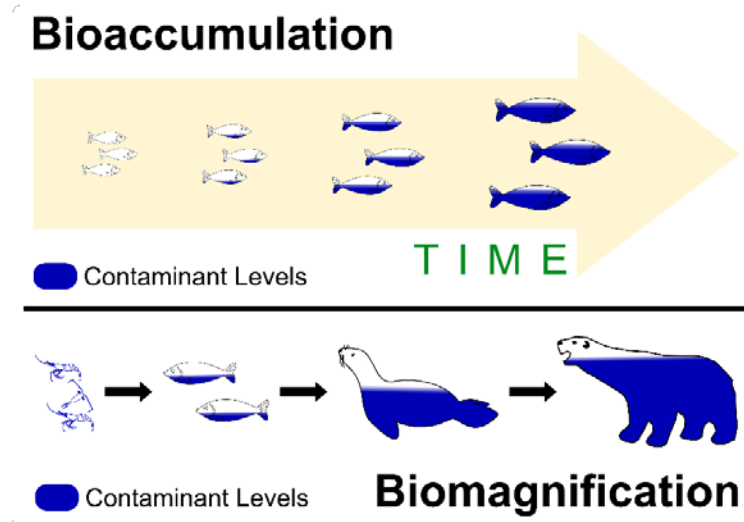
Persistent organic pollutants (POPs) are organic chemicals characterized by a distinct set of properties, such as having a long degradation time in the environment. These chemicals can be transported over long distances by wind or ocean currents. They are also lipophilic and hence able to bioaccumulate, and are toxic to living organisms. Sources of POPs are agriculture, industry and unintentionally generated by-products of incomplete reactions (Miniero and Iamiceli, 2008, AMAP, 2009) At present 21 substances are classified as POPs by the Stockholm Convention, and twelve of these constitute the legacy POPs. These organochlorine substances, such as polychlorinated biphenyls (PCBs), dioxins, and dichlorodiphenyltrichloroethane (DDT), were the first POPs to be recognized as hazardous to human and wildlife health and environment, and the use them are now banned or restricted ([www.pops.int](http://www.pops.int)).

Even though vast areas of the Arctic are remote from industry and human activity many environmental pollutants are found in these regions, due to long-range transport (Macdonal et al., 2000). The cold environment favors the deposition of POPs from the atmosphere, and the contaminants are retained in the Arctic by entering the marine food web (AMAP, 2009). Because arctic animals store high amounts of energy as fat in white adipose tissue (WAT), for periods when food availability is scarce, they have an unusually high potential of accumulating and storing POPs, as POPs generally are lipophilic chemicals (Gebbinck *et al.*, 2008).

POPs can affect the health of human and wildlife, for example by disturbing the development and function of the endocrine system. This can lead to developmental abnormalities, reduced reproductive success, alterations in the immune system, and metabolic disruption, such as obesity and type 2 diabetes (Colborn *et al.*, 1993, Casals-Casas and Desvergne, 2011, Ruzzin *et al.*, 2010). In polar bears effects on both on the reproductive and immune system have been reported (Sonne, 2010). Therefore, it is important to characterize, monitor, and regulate dangerous pollutants by international monitoring programs, such as the arctic monitoring and assessment programme (AMAP) ([www.amap.no](http://www.amap.no)), and by global treaties, such as the Stockholm Convention ([www.pops.int](http://www.pops.int)).

As a top predator in the Arctic the polar bear accumulates high levels of POPs, and polar bears living in Svalbard, Hudson Bay (Canada) and East Greenland are considered to be especially at risk for POP exposure and associated effects (Letcher *et al.*, 2010). The chemical concentration of POPs increases upwards in the food chain by the process of biomagnification, and apex predators, such as polar bears, are therefore exposed to higher

levels of persistent pollutants (Fig. 1.2.) (Hop *et al.*, 2002, Letcher *et al.*, 2010). Another factor that greatly influences the contaminant load that polar bears are subjected to, is that polar bears feed almost exclusively on seal blubber, and therefore accumulates unusually high amounts of POPs (Derocher *et al.*, 2002, Jenssen, 2006).



**Figure 1.2. Bioaccumulation and biomagnification.** (Figure from Olenick, 2013).

Due to the bans and restrictions on uses of legacy POPs the levels of these chemicals have declined significantly in arctic biota over the last decades (Riget *et al.*, 2010). However, the levels in polar bears are still high enough to exceed the toxic effect threshold, with PCBs as the main contributors, and this may lead to health risks (Dietz *et al.*, 2015). Levels of hydroxylated and methyl sulfone metabolites of PCBs in polar bears are also over the toxic effect threshold, and therefore contributes to the health risk posed by PCB congeners (Sonne, 2010, Sandala *et al.*, 2004). Even though the use of many POPs are prohibited or restricted, new chemicals emerge to replace banned chemicals, or to fulfill new ranges of application. New chemicals, including organohalogenes such as brominated flame retardants (BFRs) and fluorinated compounds, have been found in the arctic biota, and there are concerns about how they may affect the arctic animals (Braune *et al.*, 2005, Verreault *et al.*, 2005, Bytingsvik, 2012). It is not only the high contamination levels of distinct compounds in polar bears that cause concern, but also what the consequences of this combined chemical cocktail may be (Sonne, 2010).

Since most POPs are lipophilic, WAT functions as storage site for these chemicals. A study by Gebbink *et al.* (2008) found that over 91 % of the total body burden of organohalogenes was accounted for by adipose tissue in polar bears from East Greenland. There is an increasing concern about how POPs can affect energy homeostasis by altering the function of WAT (Muellerova and Kopecky, 2007). Also, when polar bears perform their



seasonal fasting, stored fat is utilized for energy and this leads to an increased release of POPs from the WAT into the blood stream. This subsequently leads to an increased concentration of POPs in other organs and tissues, which can lead to hazardous health effects (La Merrill *et al.*, 2013).

## **1.2 WHITE ADIPOSE TISSUE**

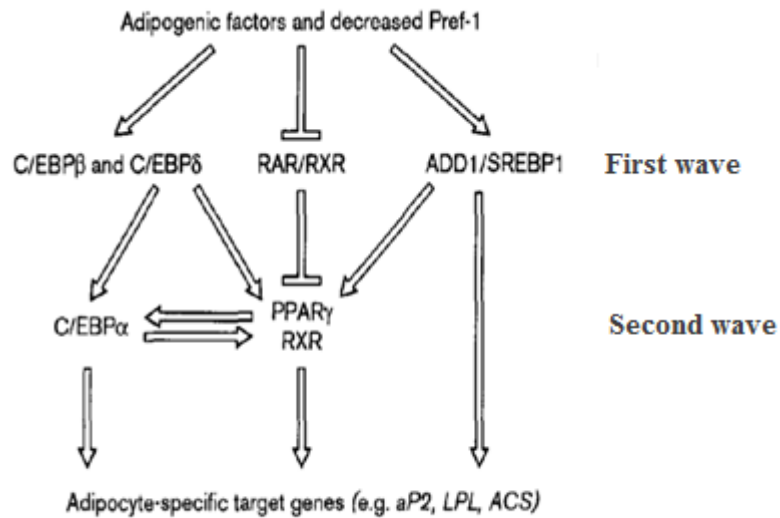
WAT is found all over the body, as subcutaneous fat, under the skin, or as visceral fat, surrounding the organs (Ibrahim, 2010). For a long time WAT was considered as a passive organ that only functioned as storage for energy. This view has changed, and WAT is now recognized as a highly active metabolic and endocrine organ, which responds to hormonal signals, but also secretes signaling factors, such as leptin (Kershaw and Flier, 2004). The secreted factors are known to play a central role in regulating energy expenditure, satiety, and insulin sensitivity; and dysfunction of the regulation of the secreted factors can lead to metabolic diseases, such as diabetes, and inflammatory responses (Galic *et al.*, 2010). Adipocytes, or fat cells, are the main cell type in WAT, but the tissue also contains stromal vascular cells, which include endothelial cells, fibroblasts, immune cells, committed preadipocytes, and stem cells (Katz, 2002). The quantity of WAT in the body varies with energy intake and depletion. Previously WAT was believed to have a fixed number of adipocytes which varied in size, but now it is established that that the change in mass is a result of change in both size and numbers of adipocytes (Prins and O'Rahilly, 1997).

## **1.3 FORMATION OF FAT CELLS BY ADIPOCYTE DIFFERENTIATION**

The increase in number of adipocytes during expansion of WAT, is due to the differentiation of preadipocytes into mature adipocytes, known as adipogenesis (Gregoire, 2001). During adipogenesis sequential changes in the expression of specific adipocyte genes occur, which leads to changes in cell appearance and function, as it starts to accumulate triglycerides in large vacuoles (Niemelä *et al.*, 2008, Fan *et al.*, 1983).

Adipocyte differentiation has been extensively studied for the past 20 years using cell culture models of various preadipocyte cell lines, such as 3T3-L1, and primary cultures of mesenchymal stem cells (MSCs), from sources such as humans, rats, rabbits and pigs (Gregoire *et al.*, 1998). For MSCs to be able to differentiate into mature adipocytes the cells must first commit to the preadipocyte lineage, meaning that the cells are restricted to differentiate into adipocytes, succeeded by terminal differentiation, which is induced by adipogenic stimuli (Cristancho and Lazar, 2011). The differentiation from preadipocytes into mature adipocytes is a complex process which involves changes in the expression of over

2000 genes (Guo and Liao, 2000). Understanding the molecular events that takes place during this process is a major task and there are still numerous gaps in our knowledge, especially concerning the early stages of adipogenic commitment and differentiation (Gregoire, 2001). The process, and some of the key transcription factors (TFs) (Fig. 1.3.) will be briefly explained.



**Figure 1.3. Regulation of adipogenesis by transcription factors (TFs).** Adipogenesis is regulated through two waves of TFs (figure modified from Fajas, 1998).

### 1.3.1 Induction of adipocyte differentiation

The mechanisms by which MSCs, *in vitro* and *in vivo*, commits to the preadipocyte lineage is still a poorly understood process (Cawthorn *et al.*, 2012), and will not be discussed here. Cultured preadipocytes have to enter growth arrest before adipocyte differentiation is possible. This is normally achieved through contact inhibition, by culturing the cells until confluence is reached, which leads to growth arrest. However, confluence in itself is not required for adipocyte differentiation, as it is possible to enter cells into growth arrest by using certain serum-free media. Both peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and CCAAT-enhancer-binding protein alpha (C/EBP $\alpha$ ) is found to play a role in inducing growth arrest (Gregoire *et al.*, 1998).

After growth arrest is achieved, adipogenic differentiation can be initiated, if the cells receive appropriate adipogenic stimuli. Through studies using cell models, it has become clear that adipogenesis is a process that occurs through the activation of two waves of transcription factors (TFs). During *in vitro* studies the first wave is induced directly by an adipogenic induction medium (AIM) (Lefterova *et al.*, 2014). The AIM may contain several

adipogenic stimulating compounds, such as dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), indomethacin (INDO) and insulin, which induces different TFs. DEX induces the glucocorticoid receptor (GR) and C/EBP $\delta$ , and inhibits preadipocyte factor 1 (PREP-1), an inhibitor of adipocyte differentiation (Wu *et al.*, 1996, Smas *et al.*, 1999). IBMX elevates the cyclic AMP (cAMP) levels in the cells, which stimulates the cAMP response element-binding protein (CREB). CREB is considered to be a primary regulator of adipogenesis and promote the transcription of several adipocyte-specific genes, such as C/EBP $\beta$  (Reusch *et al.*, 2000, Niehof *et al.*, 1997). INDO increases the expression of both PPAR $\gamma$  and C/EBP $\beta$  (Lehmann *et al.*, 1997, Styner *et al.*, 2010).

The first wave TFs in turn activate TFs of the second wave, such as PPAR $\gamma$  and C/EBP $\alpha$ . These two TFs have binding sites at most genes associated with adipogenesis, and have been shown to crosstalk with each other (Siersbaek *et al.*, 2010). The molecular basis by which the two TFs cooperate to regulate adipogenesis is still not known, but many adipocyte specific genes have binding sites for both C/EBP proteins and PPAR $\gamma$  (Rosen *et al.*, 2000). Binding of the TFs leads to the activation and regulation of the adipocyte specific genes needed for the terminal differentiation of preadipocytes, such as fatty acid-binding protein 4 (*FABP4*) (Lefterova *et al.*, 2014). Insulin activates the insulin-like growth factor 1 (IGF1) receptor and induces several downstream transduction pathways, such as the activation of adipocyte determination and differentiation factor 1 (ADD1) (Mur *et al.*, 2003, Niemelä *et al.*, 2008). The mechanisms by which ADD1 induces adipogenic differentiation remains unknown, but ADD1 is known to promote adipocyte differentiation and gene expression linked to triglyceride synthesis (Kim and Spiegelman, 1996, Rosen *et al.*, 2000)

### 1.3.2 The CCAAT-enhancer-binding proteins

Three of six members of the C/EBP family are known to play a part during induction and regulation of adipocyte differentiation. These are the TFs C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$  (Mandrup and Lane, 1997). One of the main functions of C/EBP $\beta$  and C/EBP $\delta$  during adipogenesis is to induce the expression of C/EBP $\alpha$  and PPAR $\gamma$  (Rosen and MacDougald, 2006). During the early phase of adipogenic differentiation there are expressed high levels of C/EBP $\beta$  and C/EBP $\delta$ , induced by IBMX and DEX. During the terminal phase of differentiation the levels of these TFs diminish, while the level of C/EBP $\alpha$  increases (Yeh *et al.*, 1995). Slightly after the increase in expression of C/EBP $\alpha$  most of the adipocyte specific genes are being activated (Rosen *et al.*, 2000).

The C/EBPs are absolutely required to accomplish adipogenic differentiation, but the presence of PPAR $\gamma$  is needed for these TFs to function efficiently (Lin and Lane, 1992, Rosen and MacDougald, 2006). For instance, C/EBP $\beta$  cannot induce the expression of C/EBP $\alpha$  in the absence of PPAR $\gamma$  (Zuo *et al.*, 2006). C/EBP $\alpha$  cooperates with PPAR $\gamma$  towards terminal differentiation, and also sustains a high level of PPAR $\gamma$  in the mature adipocyte through a feed-forward loop (Fajas *et al.*, 1998).

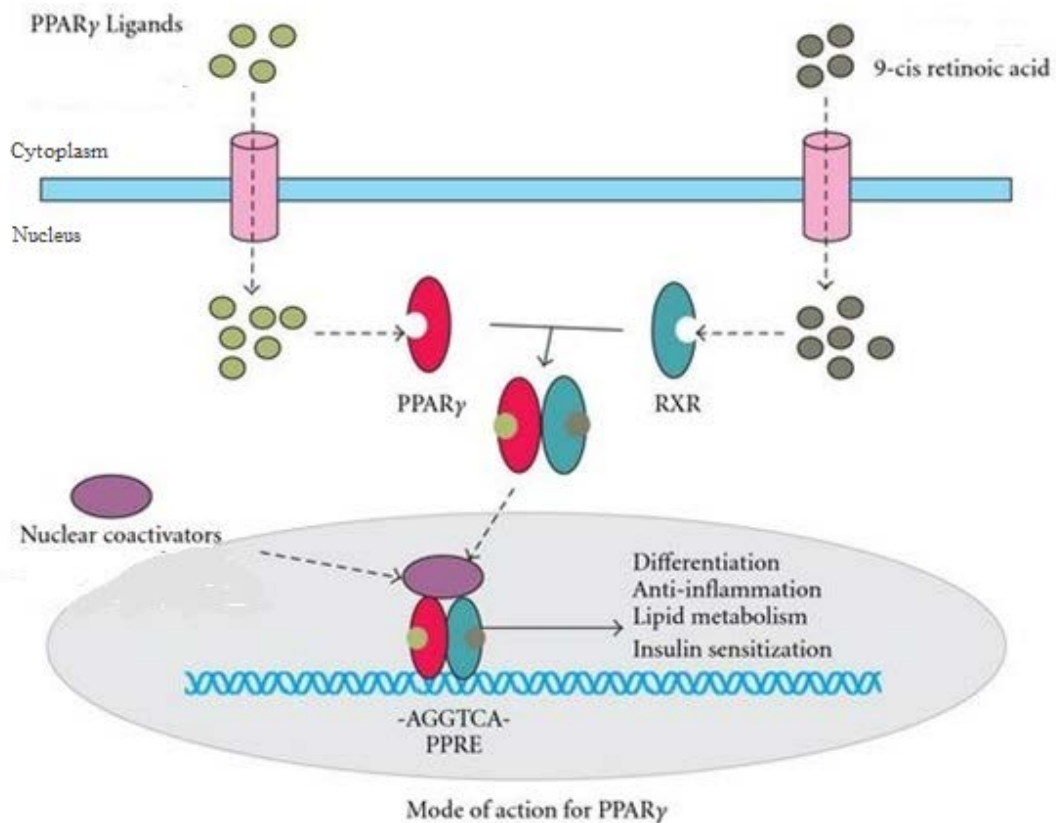
### 1.3.3 Peroxisome proliferator-activated receptor gamma

Rosen *et al.* (1999) showed that PPAR $\gamma$  is required for adipogenic differentiation, both *in vitro* and *in vivo*, and today PPAR $\gamma$  is considered as the master regulator of adipogenesis.

The PPARs are TFs that belong to the nuclear receptor (NR) superfamily (Mangelsdorf *et al.*, 1995). The PPARs include PPAR $\alpha$ , PPAR $\beta$  and PPAR $\gamma$ , and they function as lipid sensors that adapt gene expression to regulate lipid metabolism. Fatty acids and eicosanoids are examples of natural PPAR ligands, but PPARs also bind to a variety of xenobiotics such as plasticizers, dioxins and pesticides (Berger and Wagner, 2002, Casals-Casas and Desvergne, 2011). In addition, synthetic PPAR $\gamma$  ligands, such as rosiglitazone (ROSI), are used in medicine for their antidiabetic effects (Kersten *et al.*, 2000).

PPAR $\alpha$  is mainly expressed in tissues with a high rate of fatty acid catabolism, such as muscles, brown adipose tissue and liver, where it is involved in fatty acid oxidation, and control of lipoprotein metabolism and inflammation limitation. PPAR $\beta$  has been detected in all tested tissues, and have an overlapping role with PPAR $\alpha$  in addition to play a part in cell differentiation and survival. PPAR $\gamma$  is predominately expressed in adipose tissue, where it is involved in controlling adipogenesis, lipid storage, and insulin sensitivity (Casals-Casas and Desvergne, 2011). Due to the major role of PPAR $\gamma$  in adipogenesis we will focus on the function of this isoform.

All PPARs must form heterodimers with retinoid X receptor (RXR), another nuclear receptor, before activation of their target genes is possible (Fig. 1.4.) (Wagner *et al.*, 2010). Ligand-bound PPAR $\gamma$  alters its conformation and forms a heterodimer with RXR, which has 9-cis retinoic acid as its natural ligand. Nuclear coactivators binds to the PPAR $\gamma$ -RXR heterodimer, and the transcriptional machinery binds to specific DNA sequence elements, designated PPAR response element (PPRE), and controls the expression of numerous target genes, such as lipoprotein lipase (*LPL*), fatty acid-binding protein 4 (*FABP4*), and leptin (*LEP*) (Cho *et al.*, 2008, Tontonoz *et al.*, 1994, Hollenberg *et al.*, 1997).



**Figure 1.4. Mode of action of PPAR $\gamma$ .** Ligand bound PPAR $\gamma$  forms a heterodimer with RXR, which translocate into the nucleus and binds to the PPAR response element (PPRE) and controls expression of numerous target genes (Figure modified from Wagner *et al.*, 2010)

PPAR $\gamma$  target genes are involved in regulating processes such as initiating growth arrest, triglyceride synthesis, accumulation of fat in cells and insulin sensitivity (Tontonoz and Spiegelman, 2008).

### 1.3.4 Fatty acid-binding protein 4

The FABPs belong to the lipid binding protein superfamily, and 9 different FABPs are identified in mammals. The primary role of all FABPs is to regulate fatty acid uptake and metabolism, and facilitate intracellular transport of fatty acids. The different FABPs are tissue specific, and adipocytes express high levels of FABP4 (Storch and Thumser, 2010). FABP4 bind various hydrophobic ligands, and many of them are of the same character as the ligands that bind to PPAR $\gamma$ . FABP4 increases the aqueous solubility of its bound ligands, and aids in their intracellular transportation (Chmurzynska, 2006). One route of transportation is when FABP4 delivers ligands from the cytosol into the nucleus to PPAR $\gamma$ , and thereby enhance the activity of this important transcription factor (Ayers *et al.*, 2007).

### 1.3.5 Leptin

Leptin is a protein hormone that is expressed in, and secreted by, mature adipocytes and has been extensively studied due to its direct link to obesity (Auwerx and Staels, 1998). The hormone has been associated with regulating food intake, energy expenditure, and whole-body energy balance in mammals, such as rodents and humans (Houseknecht *et al.*, 1998). Secreted leptin enters the circulation system and interacts with the central nervous system by binding to the leptin receptor, for example in the hypothalamus, which leads to a cascade of biological responses (Campfield *et al.*, 1996, Tartaglia *et al.*, 1995).

The secretion of leptin is positively correlated to the adipose tissue mass, but the expression is also regulated by factors such as nutritional status and signaling molecules, such as insulin, and estrogens, which elevates the levels of leptin (Kershaw and Flier, 2004). TFs, such as C/EBP $\alpha$  and PPAR $\gamma$ , are known to control the transcriptional regulation of leptin (Houseknecht *et al.*, 1998).

## 1.4 ADIPOSE TISSUE-DERIVED STEM CELLS

Stem cells are divided into two types; embryonic stem cells, and adult stem cells, which are known as mesenchymal stem cells (MSCs). MSCs are stromal cells that are able to self-renew and to differentiate into different cell types, such as osteoblasts, chondrocytes, or adipocytes (Ding *et al.*, 2011, Minguell *et al.*, 2001). Most of the studies conducted on MSCs have used stem cells isolated from bone marrow (BMSC). However, isolating cells from bone marrow can result in pain and risk of infections in the donor, as well as in yielding low cell numbers upon harvest. Recent advances in stem cell research have led to the discovery that MSCs can be isolated from a variety of tissues, such as adipose tissue (Zuk *et al.*, 2002, Ding *et al.*, 2011).

The first report on the isolation of MSCs from adipose tissue came in 2001. Human adipose tissue was obtained by liposuction, and the stromal vascular fraction (SVF) was isolated. The majority of cells in the SVF were of mesenchymal origin with low levels of other cell types, such as endothelial cells. The cells could be cultured over time with stable population doublings and low levels of senescence, and were able to differentiate *in vitro* into adipogenic, chondrogenic, myogenic and osteogenic cells, when introduced to the appropriate induction factors (Zuk *et al.*, 2001). Since, the interest in adipose tissue-derived stem cells (ASCs) has increased, especially in the field of tissue engineering (Bunnell *et al.*, 2008). ASCs have some clear advantages over BMSCs, as adipose tissue is abundant and readily accessible, and it is easy to harvest samples from a donor with minor discomfort

(Bunnell *et al.*, 2008, Nakagami *et al.*, 2006). Also, the cell number yields are higher, as the adipose tissue is a rich source of stem cells, and they can be more heavily expanded and maintain the ability for differentiation at higher passages (Zhu *et al.*, 2008, Zachar *et al.*, 2011).

As cultured ASCs are primary cultures and not established cell lines, there are some aspects to keep in mind when working with such cultures, such as from where, and from whom the cells are obtained. In rats, ASCs isolated from subcutaneous fat had a higher differentiation capacity than ASCs isolated from visceral fat (Gregoire *et al.*, 1990). Cells isolated from different donors have been shown to display varying degree of differentiation ability (Sen *et al.*, 2001). One of the reasons for this may be that ASCs from older donors have a reduced ability to replicate and differentiate (Choudhery *et al.*, 2014). Also, cell density and time in culture can affect cell characteristics such as morphology, differentiation ability and gene expression patterns (Neuhuber *et al.*, 2008).

## 1.5 ENDOCRINE DISRUPTING CHEMICALS

Endocrine disrupting chemicals (EDCs) are exogenous chemicals, such as POPs, which can disturb the function of the endocrine system, by affecting various hormone systems, and consequently affect health aspects and reproduction in an organism, or its offspring (Damstra *et al.*, 2002, Casals-Casas and Desvergne, 2011, UNEP/WHO, 2013). Several EDCs have been linked to metabolic disruption, leading to obesity, metabolic syndrome, and type 2 diabetes (Casals-Casas and Desvergne, 2011). EDCs which can influence adipogenesis and cause obesity are often referred to as environmental obesogens (Grun and Blumberg, 2006).

Hormones regulate the endocrine system via transcription factors (TFs), including membrane bound receptors, which respond to peptide hormones, or nuclear receptors (NRs). Small lipophilic compounds, such as steroid hormones as well as other endogenous compounds and xenobiotics, including POPs, activate NRs (Casals-Casas and Desvergne, 2011).

EDCs are able to interact with one or more NRs, such as pregnane X receptors (PXR) and the peroxisome proliferator-activated receptor (PPAR) family, and in turn influence the expression of genes regulated by these TFs (Lille-Langoy *et al.*, 2015, Grun and Blumberg, 2009). PCBs, dioxins, organotins, fluorinated compounds, phthalates, and bisphenol A (BPA) have been categorized as environmental obesogens, by causing metabolic disruption through interactions with several NRs, such as PPAR $\gamma$  or the glucocorticoid receptor (GR) (Casals-Casas and Desvergne, 2011). In this study the effect of the phthalate mono(2-

ethylhexyl)phthalate (MEHP) and bisphenol A (BPA) on adipocyte differentiation of polar bear adipose tissue-derived stem cells (pbASCs) have been investigated.

### 1.5.1 Mono(2-ethylhexyl)phthalate

Phatalates are a group of industrial chemicals which are commonly used as plasticizers to soften polymer products, mainly polyvinyl chloride (PVC) products. The most widely used phatalate is bis(2-ethylhexyl)phthalate (DEHP). DEHP is found in a range of products, such as flooring, cables, toys and clothes (ECHA, 2009). Upon digestion DEHP is metabolized into MEHP. MEHP has been shown to activate PPAR $\gamma$ , and thereby contributes to adipocyte differentiation by activating this NR pathway (Feige *et al.*, 2006, Hurst and Waxman, 2003, Taxvig *et al.*, 2012).

### 1.5.2 Bisphenol A

BPA is also an industrial chemical used in plastics. It is used to make polycarbonate, a hard, clear plastic. Polycarbonate is used in many consumer products, such as bottles and food containers (fda.gov, 2014). Previous studies have found that BPA induce adipocyte differentiation in 3T3-L1 cells (Taxvig *et al.*, 2012, Masuno *et al.*, 2002, Bastos Sales *et al.*, 2013), but by a PPAR $\gamma$  independent mechanism (Chamorro-Garcia *et al.*, 2012). Several mechanisms have been proposed for the induction of BPA on adipogenesis, such as activation of the glucocorticoid receptor (GR), or through the phosphatidylinositol 3-kinese pathway (Sargis *et al.*, 2010, Masuno *et al.*, 2005). Interestingly, findings have indicated that BPA does not induce adipocyte differentiation in BMSCs, from human or mouse (Chamorro-Garcia *et al.*, 2012)

## 1.6 BACKGROUD OF THE STUDY

This study is a part of the project “Contaminant effects on energetics” in the Fram Centre Hazardous Substances – program. The project aims to investigate the impact of contaminant exposure on energy homeostasis in arctic wildlife, such as polar bears. One of the projects’ main focuse is to investigate the effects of contaminants on endpoints that are stressed by climate change effects, such as energy metabolism, and the project hypothesizes that “contaminant exposure in arctic wildlife may affect their energy homeostasis and thus decrease their ability to respond to climate change”. As the polar bear endures a physiological stressful seasonal fasting period, it is important that polar bears possess an optimal control of their metabolism. Exposure to contaminants may interfere with regulation



of metabolism by disturbing the function of nuclear receptors (NRs), such as the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) (Grun and Blumberg, 2007).

As a part of the “Contaminant effects on energetics” project the effect of contaminant mixtures, consisting of contaminants at the composition and concentration found in polar bear adipose tissue, on adipogenesis in a cell line of preadipocytes derived from mouse (3T3-L1 cells) have been studied (Routti et al, unpublished). But it is possible that contaminant exposure may affect polar bears differently. A reason for this is because polar bears exhibit molecular adaptations in genes associated with bioenergetics, due to adaptation to the arctic environment (Welch et al., 2014). Among other factors the polar bear endure cold temperatures, feed on a marine lipid rich diet, store high amounts of energy as fat and endure a seasonal fasting period. Also, it has been found that the ligand binding domain of PPAR $\gamma$  differs between mouse and polar bear (Routti et al, unpublished).

This study will focus on developing methods for using polar bear adipose-tissue derived stem cells (pbASCs) to gain more knowledge on how contaminant exposure may affect energy homeostasis in polar bears.

## 1.7 AIMS

The main goal of this thesis was to develop an *in vitro* method for studying effects of environmental contaminants on adipogenesis in polar bears using pbASCs. This was addressed through the following sub-goals:

1. Establish a method for successfully maintaining and propagate pbASCs in culture.
2. Investigate the pbASCs’ capability of adipogenic differentiation, when induced by adipogenic stimuli.
3. Establish conditions which allowed for low and high level of adipogenic differentiation
4. Assess the effect of known endocrine disrupting chemicals (EDCs) on adipogenic differentiation in polar bears.

## 2 MATERIALS

### 2.1 GENERAL CHEMICALS AND REAGENTS

**Table 2.1. General chemicals and reagents**

<b>Chemical</b>	<b>Cat. No.</b>	<b>Supplier</b>
10X loading buffer	9157	TaKaRa
2-Log DNA Ladder (0.1-10.0 kb)	N3200L	New England Biolabs
3-Isobutyl-1-methylxanthine (IBMX)	I7018	Sigma-Aldrich
Agar-Agar	1.01614.1000	Merck
Agarose (SeaKem <sup>®</sup> LE)	50004	Lonza
Ampicillin sodium salt	A9518	Sigma-Aldrich
$\beta$ -mercaptoethanol	M6250	Aldrich
Bisphenol A (BPA)	239658	Sigma-Aldrich
Boric Acid	1.00165.1000	Merck
Bovine Serum Albumin (BSA) (0.1%)		TaKaRa
Dexamethasone (DEX)	D4902	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	D2650	Sigma-Aldrich
dNTPs (dATP, dCTP, dGTP, dTTP)	201912	Qiagen
Ethanol (EtOH)	32221	Sigma-Aldrich
Ethidium bromide (EtBr)	E1510	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	1.08418.10000	Merck
Fetal bovine serum (FBS)	F7524	Sigma-Aldrich
Formalin solution, neutral buffered, 10 %	HT501128	Sigma-Aldrich
Formamide	F9037	Sigma-Aldrich
Gentamicin solution	G1397	Sigma-Aldrich
Hanks balanced salt solution	H6648	Sigma-Aldrich
Indomethacin	I7378	Sigma-Aldrich
Insulin	I2643	Sigma-Aldrich
Isopropanol	E41333	Kemetyl
L-Glutamine	G7513	Sigma-Aldrich

Minimum essential medium eagle, alpha modification ( $\alpha$ -MEM)	M4526	Sigma-Aldrich
Oil Red O	O0625	Sigma-Aldrich
Penicillin-Streptomycin solution	P4333	Sigma-Aldrich
Mono-2-ethylhexyl ester phthalate (MEHP)	CDS010608	Sigma-Aldrich
Potassium chloride	P9541	Sigma-Aldrich
Potassium dihydrogen phosphate	4873.1	Merck
Rosiglitazone	R2408	Sigma-Aldrich
SOC Outgrowth Medium	B9020S	BioLabs Inc.
Sodium chloride	1.06404.1000	Merck
Sodium phosphate dibasic dihydrate	30435	Sigma-Aldrich
Trizma® base	T1503	Sigma-Aldrich
Trypan blue solution	93595	BioWhittaker™
Trypsin + EDTA solution (0.25 %)	T4049	Sigma-Aldrich
Tryptone	1.11931.1000	Merck
Yeast Extract	92144	Fluka

## 2.2 GENERAL BUFFERS AND SOLUTIONS

**Table 2.2. 10X Phosphate buffered saline (PBS), pH 7.4**

Compound	Concentration
NaCl	80 g/L
KCl	2 g/L
Na <sub>2</sub> HPO <sub>4</sub>	14.4 g/L
KH <sub>2</sub> PO <sub>4</sub>	2.4 g/L

**Table 2.3. 5X Tris-borate-EDTA (TBE) buffer, pH 8.3**

Compound	Concentration
Tris base	54 g/L
Boric acid	27.5 g/L
EDTA	4.65 g/L

**Table 2.4. Oil Red O Stock Solution**

Compound	Concentration
Oil Red O	3.5 mg/mL
Isopropanol	100 %

-Stir 2 hours, filter (0.2  $\mu$ m), store protected from light at RT  
 - Work solution: mix two parts stock solution and one part distilled water, stir 20 min, filter (0.2  $\mu$ m)

## 2.3 GROWTH MEDIA

### 2.3.1 Growth media used for pbASCs

**Table 2.5. Composition of growth medium for pbASCs**

<b>Compound</b>	<b>Concentration</b>
Minimum essential medium eagle, alpha modification ( $\alpha$ -MEM)	1X
Fetal bovine serum (FBS)	10% v/v
Penicillin-Streptomycin	100 U/mL
L-glutamine	4 mM
Gentamicin	50 $\mu$ g/mL

-Freezing medium: 30% v/v FBS, 10% v/v DMSO

### 2.3.2 Bacterial growth media

**Table 2.6. Lysogeny broth (LB) medium**

<b>Compound</b>	<b>Concentration</b>
Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

-LB-Ampicillin medium: 0.1 mg/mL Ampicillin added

**Table 2.7. LB-ampicillin agar plates**

<b>Compound</b>	<b>Concentration</b>
LB medium (Table 2.5.)	
Agar	15 g/L
Ampicillin	0.1 mg/mL

## 2.4 PLASMIDS AND PRIMERS

**Table 2.8. Previously linearized pSC-A vectors containing gene fragment insert used for Q-PCR standard curve**

<b>Gene fragment insert</b>	<b>Origin</b>
Pb_PPARY	Roger Lille-Langøy, University of Bergen,
Pb_FABP4	Norway
Pb_PPIA	

**Table 2.9. Primers used for amplification of fragments of selected polar bear genes**

<b>Name</b>	<b>Sequence (5'-3')</b>
Pb_PPARy_fwd	CACAATGGCATCAGGTTTGG
Pb_PPARy_rev	GGGGGTGATGTGGTGTTTGAAC
Pb_FABP4_fwd	GAAGTAGGAGTGGGCTTTGC
Pb_FABP4_rev	AGGACACCTCCATCTAAGGTT
Pb_LEP fwd	TGCATTCCAGAAATGTGGTC
Pb_LEP rev	ACCACCTCCGTGGAGTACAG
Pb_YWHAZ_fwd	ACTTTTGGTACATTGTGGCTTCAC
Pb_YWHAZ_rev	CCGCCAGGACAAACCAATAT
Pb_PPIA_fwd	GTCTCCTTTGAGCTGTTTGC
Pb_PPIA_rev	AGTCTTGGCAGTGCAGATGA

**Table 2.10. Primers specific for the pSC-A vector**

<b>Name</b>	<b>Sequence (5'-3')</b>
M13-20	ACTGGCCGTCGTTTTAC
T3	AATTAACCCTCACTAAAGGGAA

## 2.5 KITS

**Table 2.11. Kits**

<b>Name</b>	<b>Cat. No</b>	<b>Supplier</b>
Aurum™ Total RNA Mini Kit	732-6820	Bio-Rad
MicroElute® Gel Extraction Kit	D6294	Omega bio-tek
iScript™ cDNA synthesis kit	170-8891	Bio-Rad
StrataClone PCR Cloning Kit	240205	Agilent Technologies
E.Z.N.A® Plasmid DNA Mini Kit 1	D6942	Omega bio-tek
GenElute™ PCR Clean-Up Kit	NA1020	Sigma-Aldrich
iQ™ SYBR® Green Supermix	170-8882	Bio-Rad

## 2.6 APPARATUS AND SOFTWARE

Table 2.12. Apparatus and software

Application	Name	Manufacturer
Centrifugation	Himac CT15RE	VWR
	Galaxy ministar	VWR
	Heraeus Multifuge X3R	Thermo Scientific
	Avanti J-26 XP	Bekman Coulter
Electrophoresis	PowerPac HC	Bio-Rad
Heating	Thermomixer compact	Eppendorf
	VorTemp™ 56EVC	Labnet
	AMW 232	Whirlpool
	GD100	Grant
Imaging	Gel Doc™ EZ Imager + Image lab 3.0	Bio-Rad
Incubation	Galaxy 170 R	New Brunswick
	Infors HT Multitron Standard	VWR
	INCU-Line	VWR
Microscopy	DMIL LED	LEICA Microsystems
	SMZ-645	Nikon
Mixing	Magnetic stirrer, VS-C7	VWR
	Lab Dancer	IKA®
	Vortex genie 2	Scientific Industries
pH measurement	pHM210 Standard pH Meter	MeterLab®
Plate reading	EnSpire™ 2300 Multilabel reader	PerkinElmer
	+ EnSpire manager v 4.0.3006.25	
Shaking	HS501 Digital	IKA®-Werke
	4710 Series Ultrasonic	Cole-Pharmer Instrument
Sonication	Homogenizer	Co.
Spectrophotometry	NanoDrop® ND-1000	Saveen Werner
	+ NanoDrop 1000 3.81	
Sterile environment	Scanlaf Mars	LaboGene
Thermocycling	DOPPIO	VWR
	LightCycler® 480	Roche
	+ LightCycler® 480 software	
Water supply	Advantage A10	Millipore
Weighing	SI-64	Denver Instrument
	EK-300i	A&D

## 2.7 ENZYMES AND CORRESPONDING BUFFERS

**Table 2.13. Enzymes and corresponding buffers**

Compound	Concentration	Cat. No	Supplier
<i>Polymerase</i>			
DreamTaq DNA Polymerase	5 U/ $\mu$ L	EP0702	Thermo Scientific
+ DreamTaq Green Buffer	10X	B71	
<i>Restriction endonucleases</i>			
Not I	10 U/ $\mu$ L	1166A	TaKaRa
+ Buffer H	10X		
Xba I	15 U/ $\mu$ L	1093A	TaKaRa
+ Buffer M	10X		
<i>Sequencing</i>			
BigDye® Terminator v3.1		4337455	Applied Biosystems
+ BigDye sequencing Buffer			

### **3 METHODS**

#### **3.1 COLLECTION OF POLAR BEAR ADIPOSE TISSUE SAMPLES AND SUBSEQUENT ISOLATION OF ADIPOSE TISSUE-DERIVED STEM CELLS**

Adipose tissue samples from a 10 year old male polar bear were collected by the Norwegian Polar Institute, as a part of the BearEnergy project, at Svalbard in September 2012. The polar bears were immobilized by remote injection of a dart containing the drug Zoletil ® 100 (Virbac, France), fired from a helicopter. Adipose tissue samples were collected using an 8 mm sterile biopsy punch. Samples were transferred in phosphate buffered saline (PBS) and kept at room temperature (RT). The stromal vascular fraction (SVF), containing the polar bear adipose tissue-derived stem cells (pbASCs), was isolated from tissue samples at Alborg University by Trine Fink as previously described (Fink *et al.*, 2011), within 48 hours of harvest. Cells in passage two were frozen in aliquots of approximately  $1 \times 10^6$  cells, transported to and stored in the vapor phase of liquid nitrogen at the University of Bergen.

#### **3.2 HANDLING POLAR BEAR ADIPOSE TISSUE-DERIVED STEM CELLS (pbASCs)**

##### **3.2.1 Cryopreservation and thawing**

Cryopreservation is widely used to preserve cells long term, and all pbASCs used have been cryogenically frozen. ASCs can be frozen and thawed with minimal loss of viability, and without loss of differentiation potential (Minonzio *et al.*, 2014). However, thawing is a stressful process for the cells, and needs to be done quickly to ensure that the cells remain viable and able to recover afterwards.

When cultured pbASCs needed to be cryogenically frozen for storage, the cells were distributed in aliquots of approximately  $1 \times 10^6$  cells in cold freezing medium, containing 10 % DMSO (Table 2.5.), frozen at -80 °C for 24 hours and transferred to the vapor phase of nitrogen for long term storage.

PbASCs were thawed in lukewarm water bath and resuspended in 5 mL growth medium (GM, Table 2.5.). The freezing medium was removed by centrifugation ( $500 \times g$  for 5 minutes), before cells were resuspended in fresh GM and seeded at a density of 3000



cells/cm<sup>2</sup> in 300 cm<sup>3</sup> cell culture flasks<sup>1</sup>. Cells were cultivated at 37 °C and 5 % CO<sub>2</sub>, and GM was replaced twice a week during expansion.

### 3.2.2 Subculturing

During culturing the cells were observed visually, by using an inverted microscope, to monitor for changes in appearance, and to ensure that no contamination by bacteria etc., had occurred.

At approximately 70 % confluency pbASCs were subcultured (i.e. passaged) using the proteolytic enzyme trypsin to dissociate the adherent cells from the culture vessel. The cells were washed twice with 1 x PBS, pH 7.4, covered with 0.125 % Trypsin/0.1 % EDTA (0.5 mL per 10 cm<sup>3</sup>) and incubated at 37 °C for 3 minutes. Dissociation was confirmed under the microscope. Trypsinization was stopped by addition of growth medium. To remove trypsin from the medium, the cell suspension was centrifuged (500 x g, 5 minutes), and the pellet resuspended in growth medium (Table 2.5.). Cell density was determined (3.2.3) and cells were reseeded as previously described.

In addition, the effect of reaching confluence during subculturing on contact inhibition was investigated. PbASCs, in passage 5, which had not been above 70 % confluent, and cells, in passage 7, who had been confluent during subculturing were cultured to confluency in 6-well plates and maintained in culture for 37 days. The experiment was executed once, without parallels.

PbASCs were generally subcultured when approximately 70 % confluence was reached, but we wanted to see if reaching 100 % confluence before subculturing would have any effect on contact inhibition. Cells, in passage 5, that had never been above 70 % confluent, and cells, in passage 7, that had been 100 % confluent during subculturing were cultured to 100 % confluence in 6-well plates and maintained in that culture for 37 days.

### 3.2.3 Determining cell density and viability

Cell density was determined using a haemocytometer and viability evaluated by the trypan blue exclusion test. The haemocytometer determines cell density by counting the number of cells in a specific volume of fluid. The trypan blue stain passes through the cell

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<sup>1</sup> The decision to seed pbASCs at a density of 3000 cells/cm<sup>2</sup> in 300 cm<sup>3</sup> cell culture flasks as the standard method was based on experience acquired during the early stages of this study. Initially, 10 cm culture dishes and a seeding density of 5000 cells/cm<sup>2</sup> were used.

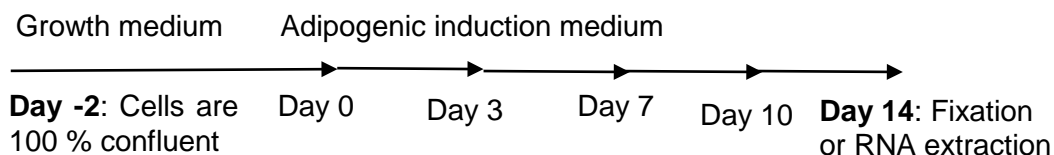
membrane of dead cells, but is excluded from live cells. Using phase contrast microscopy, dead cells appear blue, while living cells appear colorless.

Equal volumes of cell suspension and 0.4 % trypan blue stain was mixed. Cells in the four corner squares of the haemocytometer were counted, and the concentration (cells/mL) was calculated by multiplying the average number of cells per square with 2 (dilution factor) and  $10^4$  (volume conversion factor). Percent viability was calculated by dividing viable cells on the total number of cells, multiplied by 100.

### 3.3 ADIPOGENIC DIFFERENTIATION

#### 3.3.1 General adipogenic differentiation procedure and method establishment

As a starting point for this study we took basis in an adipogenic differentiation method previously used for both human and brown bear (*Ursus arctos*) ASCs (Fink *et al.*, 2008, Fink *et al.*, 2011). The following principles for adipogenic differentiation of pbASCs was used during experiments: Cells in passage 5 were seeded in 6-well or 12-well cell culture plates at a density of 20,000 cells/cm<sup>3</sup> in growth medium (Table 2.5) and cultivated to 100 % confluency, designated as day -2. After two days (day 0) adipogenic differentiation was initiated by using an adipogenic induction medium (AIM). The AIM was changed twice a week, as illustrated below (Fig. 3.1.) On day 14 cells were either fixed for quantification of lipid content (3.4), or RNA was extracted for subsequent analysis of transcript levels (3.5.1).



**Figure 3.1. Differentiation protocol for pbASCs.**

Because adipose tissue-derived stem cells from polar bears have not previously been studied, to our knowledge, suitable conditions for adipogenic differentiation needed to be established (4.2.1). To establish the duration of the induction period needed for the cells to go through adipogenic differentiation, cells were initially grown in AIM for 1, 2 or 3 weeks. Seven different AIMS, which consisted of GM (Table 2.5.) supplemented with various concentrations of the adipogenic inducers 3-isobutyl-1-methylxanthine (IBMX), indomethacin (INDO), dexamethasone (DEX), rosiglitazone (ROSI) and insulin (Table 3.1.) were tested to find the AIM most suitable for the pbASCs. Non-induced control cells were maintained in basal medium (BM) (Table 3.1.) The experiment was conducted once, in exposures in triplicates.

Also, the effect of ROSI on adipogenic conversion was evaluated by inducing cells in MDI+ROSI, or MDI+ 0.1 % DMSO (4.2.2).

Part of the experiments were repeated in both 6- and 12-well plates, to see if similar results were obtained with different well sizes, and which well size would be most suitable to use in experiments, both for quantification of lipid content and measuring transcript levels (Appendix A).

**Table 3.1. Concentration of adipogenic inducers in adipogenic induction media (AIMs).**

Concentration of components in AIMs					
AIM	IBMX ( $\mu\text{M}$ )	Insulin ( $\mu\text{g/mL}$ )	INDO ( $\mu\text{M}$ )	DEX ( $\mu\text{M}$ )	ROSI ( $\mu\text{M}$ )
MII200	450	1	200		
MDII200	450	1	200	0.1	
MDII200R	450	1	200	0.1	1
MII50	450	1	50		
MDII50	450	1	50	0.1	
MDII50R	450	1	50	0.1	1
MDI+ROSI	450	1		0.1	1
BM		1			

### 3.3.2 Test of adipogenic potential of known EDCs in exposure experiments

To test the adipogenic potential of selected EDCs cells were seeded in 12-well plates, and the general procedure (3.3.1) was followed. On day 0 the growth medium was changed to basal medium (BM) or MDI (Table 3.2.), supplemented with DMSO, ROSI, or test chemicals, at concentrations denoted in Table 3.3. BM + DMSO was used as an undifferentiated control, while MDI + DMSO were used as a differentiated control with a low level of differentiation, which allowed for the discrimination of the effects of the test chemicals. Cells subjected to quantification of lipid content by Oil Red O (ORO) staining were seeded in triplicates, while cells used for real time Q-PCR were seeded in duplicates. Experiments were repeated twice.

**Table 3.2. Concentration of adipogenic inducers in media used during exposure experiments**

Adipogenic inducers			
Media	IBMX ( $\mu\text{M}$ )	Insulin ( $\mu\text{g/mL}$ )	DEX ( $\mu\text{M}$ )
BM		1	
MDI	450	1	0.1

**Table 3.3. Concentration of solvent and test chemicals used in exposure experiments**

Chemical	Concentration
DMSO	0.10 %
Rosiglitazone (ROSI)	1 $\mu\text{M}$
Mono(2-ethylhexyl)phthalate (MEHP)	50 $\mu\text{M}$ or 10 $\mu\text{M}$
Bisphenol A (BPA)	50 $\mu\text{M}$ or 10 $\mu\text{M}$

### 3.4 QUANTIFICATION OF LIPID CONTENT BY OIL RED O STAINING

During adipogenesis lipid droplets accumulate in the cell (Gregoire *et al.*, 1998). These droplets can be visualized using Oil Red O (ORO), a lipid soluble dye. By extracting ORO from the cells, adipogenic differentiation, measured by increase in lipid content, can be quantified by spectrophotometry, as described by Ramirez-Zacarias *et al.* (1992).

pbASCs were gently washed twice in 1X PBS (Table 2.2.) and fixed in 10 % formalin at 4° for minimum 1 hour while protected from light. Excess formalin was removed, and the cells washed twice with 1X PBS before staining. The cells were covered with freshly prepared ORO working solution (Table 2.4.), and incubated for 15 minutes at room temperature before excess ORO was removed. The cells were washed repeatedly with deionized water until the washing solution was clear. Following washing, excess water was removed, and plates were left to dry. ORO was eluted from the cells by adding 0.5 mL 100 % isopropanol to each well of the 12-well plate. After 10 minutes of incubation the isopropanol was carefully collected.

To quantify the amount of ORO in exposed and non-exposed cells, 150  $\mu\text{L}$  isopropanol eluate, and a blank sample containing 100 % isopropanol, were transferred to a 96-well plate, and the absorbance of light at 500 nm wavelengths was measured. Measurements were conducted in duplicates.

### 3.5 MOLECULAR CLONING OF GENE SEQUENCE FRAGMENTS

Molecular cloning is process used for replicating DNA fragments of interest by inserting recombinant DNA, consisting of the desired DNA sequence and a cloning vector, into a host organism for replication (Primrose *et al.*, 2006). Results from this part can be found in Appendix B.

#### 3.5.1 RNA extraction, purification and quality assessment

RNA needed to be extracted from the cells for subsequent molecular cloning and real-time Q-PCR analysis. Total RNA was extracted with Aurum<sup>TM</sup> Total RNA Mini Kit (Bio-Rad), according to the manufacturer's protocol. The kit uses guanidine isothiocyanate and  $\beta$ -mercaptoethanol to lyse samples and inactivate RNase. As real-time Q-PCR is a very sensitive technique of mRNA detection, it is crucial to obtain RNA free from residual genomic DNA, as this can result in false positives and background. DNase 1 digest was included in the kit. The extracted RNA was stored at -80 °C.

The concentration and purity of extracted total RNA was measured using a Nanodrop® ND-1000 spectrophotometer, which takes advantage of the fact that nucleic acids absorbs at 260 nm and proteins at 280 nm. The 260/280 ratio was used to indicate the purity of RNA and DNA, at ratios of 2.0 and 1.8, respectively, even though the validity of using this ratio in regards to evaluating purity of nucleic acid preparations have been questioned (Glasel, 1995).

RNA integrity was assessed using denaturing agarose gel electrophoresis (denaturing AGE). Most RNA forms secondary structures, which can influence the mobility of RNA in the gel. These structures can be denatured by heat or denaturing agents, such as formamide, which keeps RNA denatured by preventing reformation of secondary structures (Masek *et al.*, 2005). The presence and quantity of 28S and 18S ribosomal RNA (rRNA) was used to assess RNA integrity.

One hundred ng of total RNA in 50 % formamide containing 1 x loading buffer was heated at 65 °C for 10 minutes to denature RNA secondary structures. The denatured RNA, and 2-log DNA ladder, was subjected to electrophoresis (0.7 % agarose, 0.5 x TBE buffer (Table 2.3.)) for 40 minutes at 100 V. Before visualization of RNA under UV light, gels were incubated on shaker for 10 minutes in 0.5 x TBE buffer with 0.4 mg/mL EtBr.

#### 3.5.2 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesized from extracted RNA using the enzyme reverse transcriptase, oligo(dT) and hexamer primers, provided by iScript<sup>TM</sup> cDNA synthesis

kit (Bio-Rad). 0.5 µg total RNA was used for each reaction and the cDNA synthesis was performed according to the suppliers protocol. A no reverse transcriptase control was prepared, to ensure that all genomic DNA had been digested during purification.

### 3.5.3 Amplification of DNA by polymerase chain reaction

Polymerase chain reaction (PCR) is used to exponentially amplify DNA segments (Mullis and Faloona, 1987). There are three different steps in a PCR: Denaturation of DNA, annealing of sequence specific primers, and extension of DNA strands by a thermostable DNA polymerase, such as *Taq* polymerase.

A fragment of polar bear leptin (*LEP*) and tyrosine 3/tryptophan 5-monooxygenase activation protein (*YWHAZ*) was amplified using sequence specific primers (Table 2.9.). For each reaction a no template control (NTC) was prepared, to ensure that there was no contamination or non-specific amplification. A no reverse transcriptase control was also loaded, to verify that the samples were free from genomic DNA. PCR was performed on cDNA according to Tables 3.4. and 3.5.

**Table 3.4. Reaction solution for PCR**

Reagent	Quantity
10X Green DreamTaq Buffer	1x
dNTP Mix	0.2 mM
Forward primer	0.5 µM
Reverse primer	0.5 µM
DreamTaq DNA Polymerase	0.63 U
cDNA (from 0.5 g RNA)	0.5 µL
Deionized water	
Total	25 µL

**Table 3.5. PCR thermal cycling conditions**

Step	Temperature °C	Time	Cycles
Initial denaturation	95	2 min	1
Denaturation	95	30 s	30
Annealing	55/50*	30 s	
Extension	72	1 min	
Final Extension	72	5 min	1
Store	4	∞	

\* Annealing temperature *YWHAZ*: 55 °C; annealing temperature *LEP*: 50 °C

### 3.5.4 Agarose gel electrophoresis (AGE) – DNA/PCR products

PCR products, and 2-log DNA ladder, were loaded on 1 % agarose gel in in 0.5 x TBE buffer (Table 2.3.). Gels ran for 40 minutes at 100 V. Before visualization of PCR products under UV light, the gels incubated on shaker for 10 minutes in 0.5 x TBE buffer with 0.4 mg/mL EtBr.

### 3.5.5 Gel extraction of PCR products

PCR products were subjected to AGE as previously described (3.5.4). Products of the expected sizes were excised from the gel and PCR products were extracted using MicroElute® Gel Extraction Kit according to the manufacturers' protocol.

### 3.5.6 Subcloning of PCR products

*Taq* polymerase creates PCR products with 3'-end A-overhangs. The pSC-A cloning vectors have U-overhangs at the 5'-end, which enables base pairing. The recombinant DNA, consisting of cloning vector and DNA segment, is subsequently incorporated into a host cell for replication.

Purified PCR products were subcloned using the StrataClone PCR Cloning Kit (Agilent Technologies). The supplier's protocol was followed, with some modifications:

The ligation reaction was performed as recommended by the manufacturer. 2 µL of the ligation reaction was gently mixed with 16-25 µL StrataClone SoloPack competent cells, and incubated on ice for 20 minutes. The cells were heat-shocked at 42 °C for 45 seconds, and then placed on ice for 2 minutes. The heat-shocked bacteria were added 250 µl pre-warmed SOC medium and carefully mixed, following one hour incubation with agitation at 37 °C. Two different volumes of preculture, 15 and 150 µL, were seeded to LB-ager plates containing ampicillin for selection (Table 2.7.) and incubated over night at 37 °C.

### 3.5.7 Identification of transformed bacteria by colony PCR

Ampicillin resistant bacteria colonies were screened for recombinant DNA by using the pSC-A vector specific primers T3 and M13-20 (Table 2.10.), which annealed up- and downstream of the PCR product insertion site, respectively.

Colonies were picked, smeared onto new LB-ager plates containing ampicillin (Table 2.7.), and added to the reaction solution for PCR (Table 3.2.). A NTC was prepared for each PCR. Also, pSC-A cloning vector containing a gene sequence fragment of the *ADIPOQ* gene (previously prepared by Roger Lille-Langøy, University of Bergen) was used as a positive control. PCR products were evaluated by AGE (3.5.4).

### 3.5.8 Plasmid purification

It was necessary to extract plasmids from host cells for further use. Colonies suspected to contain recombinant DNA, based on colony PCR results, were selected and inoculated in 2 mL LB-ampicillin, (Table 2.6.) overnight on shaker at 250 rpm and 37 °C.

Small-scale purification of plasmid DNA from bacteria was performed using E.Z.N.A® Plasmid DNA Mini Kit 1 (Omega bio-tek), according to suppliers' protocol. Genomic DNA and proteins were denatured and precipitated in lysed bacteria, while plasmids stayed in the solution. Plasmid DNA was transferred and bound to a DNA binding column during washing steps, and subsequently eluted. Concentration and purity of eluted DNA was measured spectrophotometrically using Nanodrop® (3.5.1).

### 3.5.9 Sequencing of plasmids using Sanger sequencing

Sanger sequencing is a DNA sequencing method which makes use of dideoxynucleotides (ddNTPs) as chain-terminating inhibitors of DNA polymerase (Sanger *et al.*, 1977). When using dye-terminator sequencing each of the four ddNTPs contain a different fluorescent dye, which permits sequencing in a single reaction.

The sequencing reaction was carried out using BigDye version 3.1, according Table 3.6. and 3.7. After thermal cycling, the sequencing reaction was added 10 µL of deionized water. The DNA Sequencing Facility at the Department of Molecular Biology at the University of Bergen conducted the separation of sequencing reaction products and base calling.

**Table 3.6. Sequencing reaction solution for Big-Dye version 3.1.**

Reagent	Quantity
BigDye v.3.1	1 µL
Sequencing buffer	1 µL
Template	200 ng
M13-20 primer	3.2 pmol
Deionized water	
Total	10 µL

**Table 3.7. Thermal cycling conditions for Big-Dye version 3.1.**

Step	Temperature °C	Time	Cycles
Initial denaturation	96	5 min	1
Denaturation	96	10 s	35
Annealing	50	5 s	
Extension	60	4 min	
Store	4	∞	



### 3.5.10 Restriction endonuclease digestion

Plasmids needed to be linearized before use in real-time Q-PCR. Restriction enzymes, which recognize and cut specific sequences in double stranded DNA, performs restriction digestions. The digestion can be evaluated using AGE, as plasmids have different shapes depending on the number of intact DNA strands. Uncut plasmids have the shape of a supercoiled helix, and therefore runs faster through the gel than linearized plasmids. Plasmids with one nicked DNA strand have the shape of an open circle, and thereby runs slower through the gel (Primrose *et al.*, 2006).

Restriction digestions were composed according to Table 3.8., and carried out at 37 °C for 2 hours, and subsequently evaluated by AGE (0.4 % agarose (3.5.4)). Linearized plasmids were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), according to the suppliers' protocol. Concentration and purity of linearized plasmids were measured using Nanodrop® (3.5.1). The digestion success and integrity of the plasmids were evaluated with AGE as previously described.

**Table 3.8. Restriction digestion reaction for *YWHAZ* and *LEP* plasmids (pSC-A)**

Reagent	Volume (µL)	[Final]	Reagent	Volume (µL)	[Final]
Buffer M	7	1X	Buffer H	7.5	1X
BSA	7	0.01 %	BSA	7.5	0.01 %
pSC-A <i>YWHAZ</i>	50	4.3 µg	Triton X-100	7.5	0.01 %
Xba I	0.6	9 U	pSC-A <i>LEP</i>	45	5.8 µg
Deionized water	5.4		Not I	1	10 U
			Deionized water	6.5	
Total volume	70		Total volume	75	

## 3.6 MEASURING TRANSCRIPT LEVELS USING REAL-TIME QUANTITATIVE PCR

Real time Q-PCR is a sensitive method used for quantifying the transcript levels of desired genes present in a sample (Bustin, 2000). In this study we investigated the transcript levels of the adipocyte specific genes *PPAR $\gamma$* , *FABP4*, and *LEP*. SYBR green binds to double stranded DNA, and after each cycle of amplification the fluorescence of the bound dye is measured. Ideally the PCR product doubles during the exponential phase of PCR, and eventually the fluorescence from the amplification exceeds the background, which is known

as the detection threshold ( $C_T$ ) value. At which cycle  $C_T$  is reached, depends on the initial transcript copy number (Heid *et al.*, 1996). Two reference genes were used to normalize samples for relative quantification. Reference genes have stable transcript levels, which should not be affected by experimental conditions. We chose to use tyrosine 3/tryptophan 5-monoxygenase activation protein (*YWHAZ*) and cyclophilin A (*PPIA*) as reference genes, as they have been verified as stable reference genes in ASCs (Fink *et al.*, 2008). We chose to use two reference genes for normalization, as this measures expression levels more accurately, and calculate the normalization values as a geometric mean, as recommended by Vandesompele *et al.* (2002). The use of geometric mean adjust for the fact that housekeeping genes are expressed at different levels. Melting curves was used to evaluate the specificity of the amplification products.

All real-time Q-PCR samples were prepared using iQ<sup>TM</sup> SYBR® Green Supermix (Table 3.9.). The real-time Q-PCR was performed using LightCycler® 480, according to Table 3.10. For all assays there was a no template control (NTC) and a calibrator sample. Measurements were conducted in triplicates. The results were analyzed using the LightCycler® 480 software.

Standard curves were created by serial dilutions of linearized pSC-A plasmids containing the selected gene sequences (*PPAR $\gamma$* , *FABP4*, *LEP*, *YWHAZ* or *PPIA*). From these the amplification efficiencies for the reactions were calculated. These values were used during normalization of samples. Also, 1:10, 1:20 and 1:40 dilutions of pooled cDNA were measured, to evaluate which dilution factor was most suitable. Detailed procedure and results from these assays can be found in Appendix C.

To examine the transcript levels of the three target genes during different stages of adipocyte differentiation, RNA was extracted (3.5.1) from cells 2, 6, 10 and 14 days after induction by BM or MDI medium, supplemented with DMSO or ROSI (Table 3.2. and 3.3.). Otherwise the general adipogenic procedure was followed (3.3.1). cDNA was synthesized (3.5.2) and real-time Q-PCR was performed as previously described. The experiment was conducted once, in duplicates.

Reference gene stability was evaluated by inserting the measured  $C_T$  values of *YWHAZ* and *PPIA* from the above experiment into RefFinder ([leonxie.com/referencegene.php](http://leonxie.com/referencegene.php)) (Appendix C).

**Table 3.9. Reaction solution for real-time Q-PCR**

<b>Reagent</b>	<b>Quantity</b>
2x iQ™ SYBR® Green Supermix	1x
Forward primer	300 nM
Reverse primer	300 nM
cDNA (1:10 dilution)	5 µL
Ultrapure water	
<b>Total</b>	<b>20 µL</b>

**Table 3.10. Real-time Q-PCR thermal cycling conditions**

<b>Step</b>	<b>Temperature (°C)</b>	<b>Hold time</b>	<b>Ramp Rate (°C/s)</b>	<b>Cycles</b>	<b>Acquisitions (per °C)</b>
<b>Pre-incubation</b>	95	3 min	4.40	1	
<b>Amplification</b>					
Denaturation	95	10 s	4.40	30	
Annealing	50/57/60*	30 s	2.20		
Extension	72	30 s	4.40		
<b>Melting curve</b>					
Denaturation	95	5 s	4.40	1	5
Lowest	65	1 min	2.20		
Highest	97		0.11		
<b>Cooling</b>	40	30 s	2.20		

\* Annealing temperatures: *LEP*: 50 °C; *PPIA*, *PPAR $\gamma$*  and *FABP4*: 57 °C; *YWHAZ*: 60 °C.

### 3.7 STATISTICAL ANALYSIS

To evaluate the effect of media or test compounds on adipogenesis the results were made relative to control samples, and the statistical validity evaluated using a paired, two-tailed t-test.

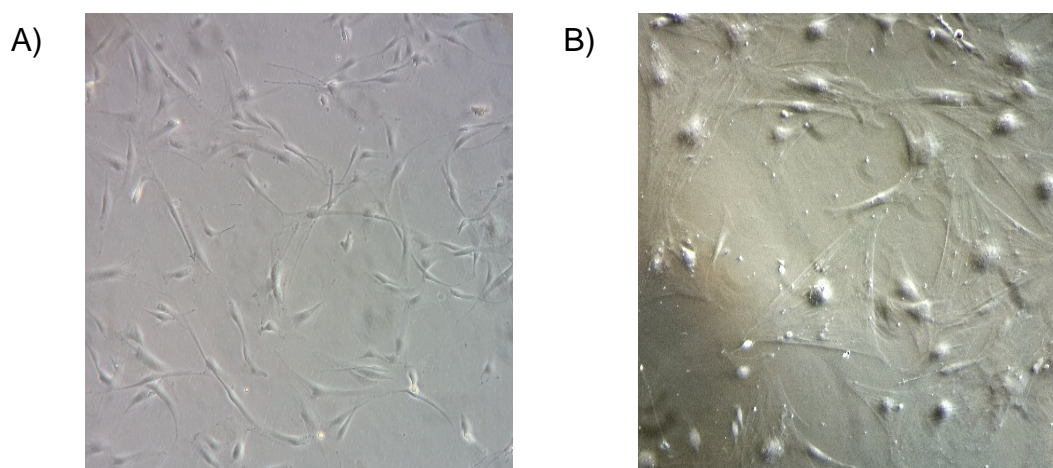
## 4 RESULTS

### 4.1 GENERAL OBSERVATIONS REGARDING CULTIVATION OF pbASCs

#### 4.1.1 Proliferation, shape, and size of pbASCs

PbASCs were expanded in GM (Table 2.5.) and subcultured at approximately 70 % confluence (3.2.2). It took approximately one to one and a half week for cells seeded at a density of 3000 cells/cm<sup>2</sup> (i.e. approximately 15 % confluence) to reach 70 % confluence. During this period the number of cells could increase with a factor of 3 to 6, mainly depending on the shape of the cells.

Cultured pbASCs grew in an adherent monolayer (Fig. 4.1.). In early passages the cells were uniform in shape, based on visual observation by an inverted microscope. They were thin, small, and spindle-shaped (Fig 4.1.A.). Wider, larger and triangular-shaped cells started to appear (Fig 4.1.B.), when subcultured into higher passages, and the culture dishes contained areas dominated by one of the two cell shapes, in varying degrees. When initial seeding density was kept constant, it was observed that cultures containing mainly the larger cell type used longer time to reach 70 % confluence compared to cultures that primarily contained the smaller cell type. This could suggest that the larger cells were proliferating more slowly than the smaller cells.

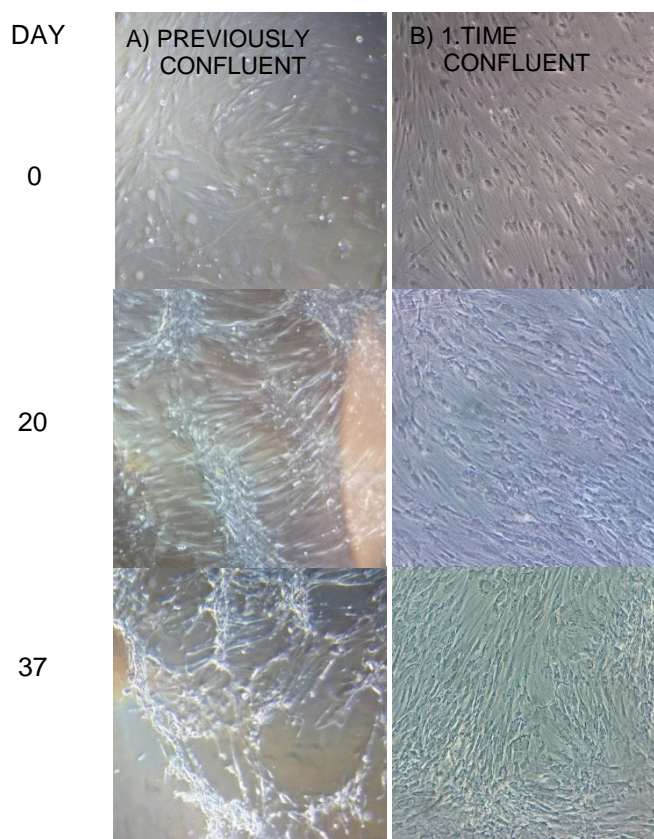


**Figure 4.1. Cell shape and size of pbASCs.** PbASCs were cultured in GM and subcultured when approximately 70 % confluence was reached. Cells were visualized by inverted microscopy with a 10x objective. A) spindle-shaped pbASCs in passage 2. B) Triangular-shaped pbASCs in passage 7.

#### 4.1.2 Confluence and cell contact inhibition

PbASCs were generally subcultured at approximately 70 % confluent, but we wanted to investigate if reaching confluence during subculturing affected the cells contact inhibition. Cells that had been subcultured at 70 % confluence and cells that had reached confluence during subculturing, were cultured to confluency and maintained in culture for 37 days (3.2.2)

It was observed that cells that previously had been confluent, did not stop growing, but started to grow on top of each other (Fig 4.2.A.). Also, 37 days after 100 % confluence the cells were dying, observed as empty fields in the well. Cells that had not previously been confluent remained in a monolayer for a long period of time. At day 37 the cells still remained viable, but overgrowing was observed (Fig 4.2.B.). This may indicate that reaching confluency during subculturing leads to loss of contact inhibition.



**Figure 4.2. Effect of confluence level of pbASCs during cultivation on contact inhibition.** Cells, in passage 7, that had been 100 % confluent during subculturing (A), and cells, in passage 5, that had never been above 70 % confluent (B), were cultured to 100 % confluence in 6-well plates and maintained in that culture for 37 days. Cells were visualized by inverted microscopy with a 10x objective.

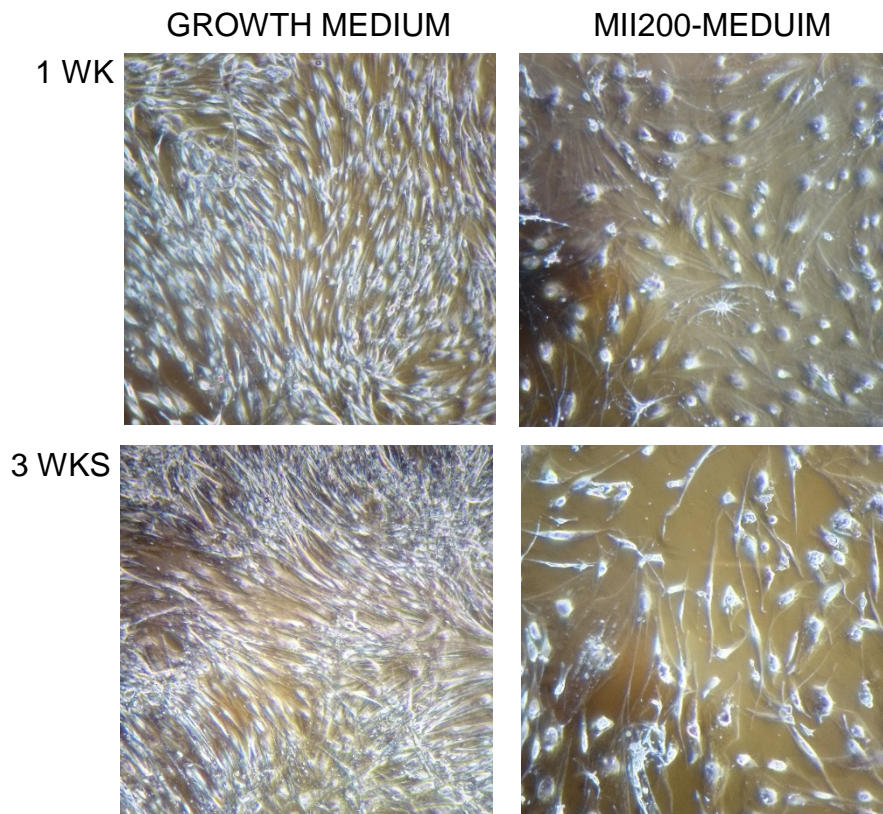
## **4.2 ESTABLISHMENT OF CONDITIONS FOR ADIPOGENIC DIFFERENTIATION OF POLAR BEAR ADIPOSE TISSUE-DERIVED STEM CELLS (pbASCs)**

As adipose tissue-derived stem cells from polar bears have not previously been studied to our knowledge, suitable conditions for adipogenic differentiation needed to be established. This was done by testing seven adipogenic induction media which differed in concentrations of adipogenic inducers (Table 3.1.).

### **4.2.1 Conditions allowing adipogenic differentiation of pbASCs**

Attempts to induce adipogenic differentiation in pbASCs essentially as described by Fink *et al* (2008) proved unsuccessful. Using this method, cells were induced by MII200 (Table 3.1.), following the general procedure (3.3.1). As it was uncertain how long the cells needed to be induced before the adipogenic conversion was apparent, cells were induced for two different periods of time. Half of the cells were induced for one week, while the other half was induced for three weeks, as no adipogenic differentiation was apparent after two weeks.

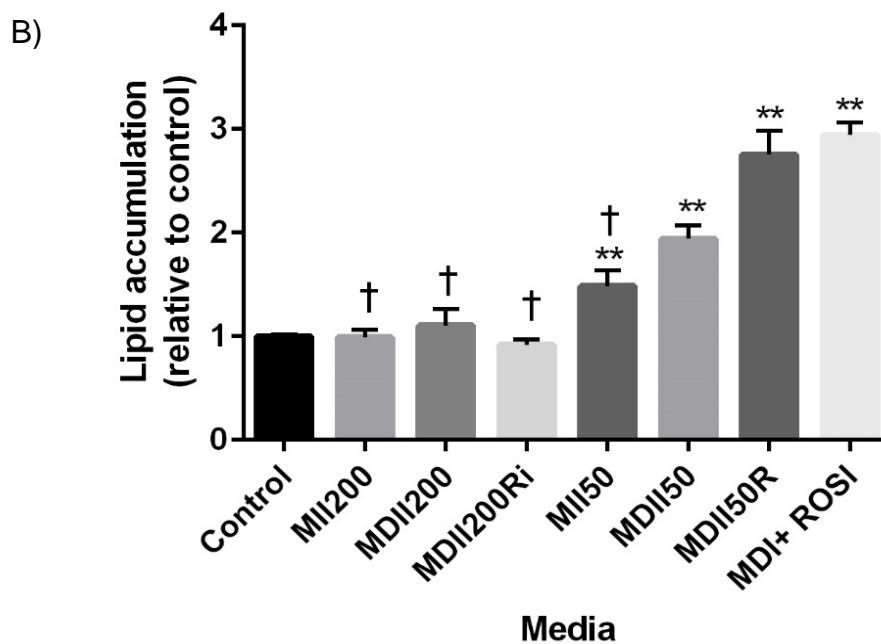
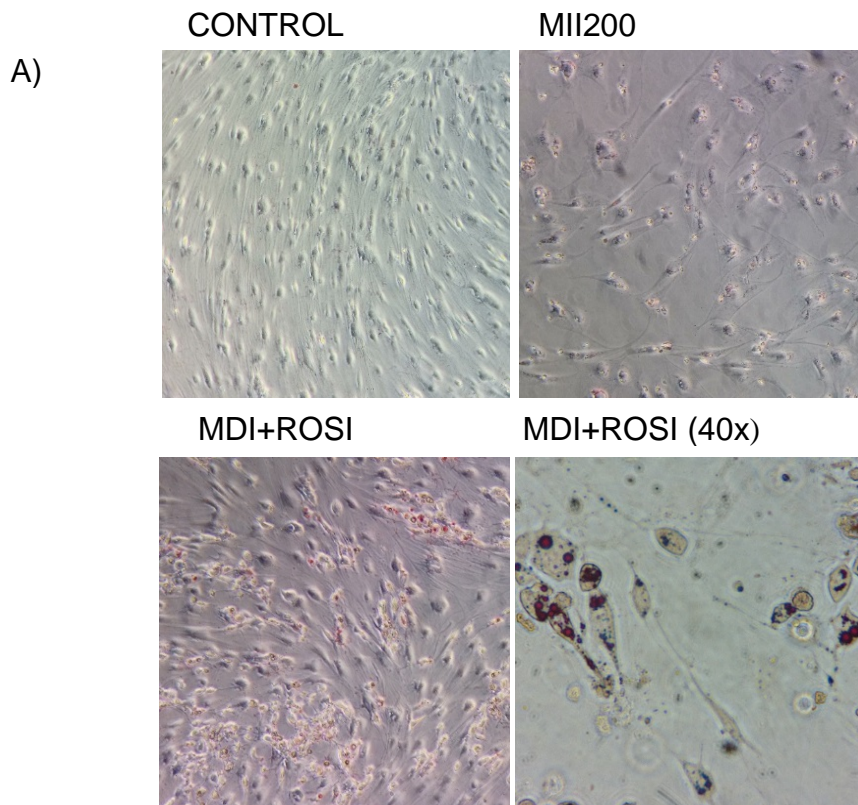
A high level of cell death in MDII200 induced cells was observed visually, by using an inverted microscope, as the presence of detached, floating cells, which resulted in a reduction in confluence during cultivation. This could indicate that the pbASCs responded negatively to the MII200-medium. Also, no staining of the cells by Oil red O (ORO) could be observed, indicating a lack of triglyceride accumulation and thereby absence of adipogenic differentiation (Fig. 4.3). It was also observed that cells not stimulated for differentiation (cultivated in GM) did not stop to proliferate after confluency was reached, as cells were growing on top of each other in week three (Fig. 4.3.). A possible reason for this could be that the cells had lost the property of contact inhibition during cultivation, as the cells used in this experiment previously had been confluent (4.1.2). This could subsequently affect the cells capability to undergo adipogenic differentiation as growth arrest is a requirement for adipogenesis to occur.



**Figure 4.3. PbASCs cultured in growth medium or MII200-medium.** Confluent pbASCs in passage 8 was cultured in 6-well plates in GM, or in the adipogenic induction medium MII200, for 1 or 3 weeks, and subsequently fixed and stained with Oil Red O. Cells were visualized by inverted microscopy with a 10x objective.

Because of the suspected negative response of the pbASCs to one or more components in the MDII200-medium, and the possible interference of using cells that had previously been confluent, a new experiment was conducted (3.3.1). PbASCs, which were constantly kept at < 70 % confluence during subculturing at all passages, were induced for 14 days to seven various AIMS (Table 3.1.) containing different adipogenic inducing compounds, including IBMX, INDO, DEX and ROSI. Non-induced control cells were cultured in BM.

Visual inspection of ORO-stained cells revealed that all sample wells with cells receiving AIMS contained cells who had accumulated lipids in vacuoles, to varying extents, while no lipid accumulation was observed in wells containing non-induced control cells (Fig. 4.4.A.). However, cell death were observed in all wells receiving media containing 200  $\mu$ M INDO (MII200, MDII200 and MDII200R) (Fig. 4.4.B.), indicating that 200  $\mu$ M INDO might be toxic for pbASCs.



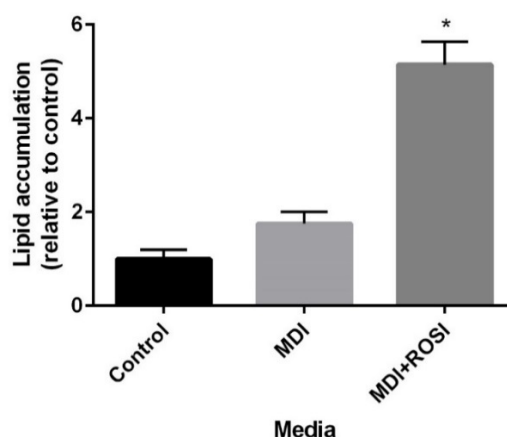
**Figure 4.4. Effect of various adipogenic induction media on lipid accumulation in pbASCs.** PbASCs were cultured in AIMs for two weeks, and subsequently fixed and stained with Oil Red O (ORO). A) Cells were visualized by inverted microscopy with 10x and 40x (bottom right) objectives. The red droplets are lipids stained by ORO. B) The absorbance ( $\lambda=500$  nm) in isopropanol extracts was measured to quantify lipid content. Cells were seeded in 6-well culture plates and in triplicates ( $n=3$ ). † = high level of cell death (observed by inverted microscopy as detached cells floating in the medium and a decrease in confluence to below approximately 70 % or less during cultivation). \*\* = significantly different from undifferentiated control cells,  $P<0.01$ .



Cells receiving media containing 50  $\mu\text{M}$  INDO (MII50, MDII50, and MDII50R) showed less signs of distress, and lipid content measured by eluted ORO from the cells were significantly higher than ( $P < 0.01$ ) in non-induced control cells. Cells receiving IBMX, DEX, and ROSI, but no INDO (MDI+ROSI), had the highest lipid content with an approximately three-fold increase in lipid content compared to the control cells ( $P < 0.01$ ). Comparable lipid content was observed in cells receiving MDII50R-medium, while the lipid content in cells cultured in MDII50- and MII50-medium was approximately twice as high as in the control cells ( $P < 0.01$ ).

#### 4.2.2 Effect of rosiglitazone on adipogenic differentiation in MDI medium

The effect of ROSI on adipogenic differentiation was investigated by inducing cells in MDI+ROSI (Table 3.1.), or MDI+ 0.1 % DMSO for two weeks, following the general induction protocol (3.3.1). Lipid content, measured using ORO-staining, was almost three times higher in cells receiving MDI+ROSI compared to the cells receiving MDI ( $P < 0.01$ ), indicating that 1  $\mu\text{M}$  ROSI strongly induce adipogenic differentiation in pbASCs (Fig. 4.5.). The lipid content in cells induced by MDI was double the measured content in the control, indicating that the MDI medium induces adipocyte differentiation, but in a less extent than MDI+ROSI. Due to this, it was decided that MDI-medium could be used as a differentiated control with a low level of differentiation, which allows for discrimination of effects of the test chemicals, in future experiments.



**Figure 4.5. Effect of rosiglitazone on lipid accumulation in pbASCs.** PbASCs were cultured in basal medium (GM supplemented with 1  $\mu\text{g}/\text{ml}$  insulin, and 0.2% DMSO), MDI (0.2 % DMSO) or MDI+ 1  $\mu\text{M}$  ROSI for two weeks, and subsequently fixed and stained with Oil Red O (ORO). The absorbance ( $\lambda=500$  nm) in isopropanol extracts was measured to quantify lipid content. Cells were seeded in duplicates ( $n=2$ ) in 6-well culture plates ( $n=2$ ). \* = significantly different from MDI,  $P < 0.05$ .

### **4.3 TRANSCRIPT LEVELS OF ADIPOCYTE SPECIFIC GENES DURING DIFFERENT STAGES OF ADIPOGENIC DIFFERENTIATION**

Polar bear ASCs were induced for 2, 6, 10, or 14 days in MDI-medium, supplemented with 1  $\mu$ M ROSI or DMSO, or maintained in BM, as non-induced controls (Table 3.2), before RNA was extracted from the cells (3.5.1). cDNA was synthesized (3.5.2) and expression levels of adipogenic genes were measured. The transcript levels of all target genes, *PPAR $\gamma$* , *FABP4*, and *LEP*, were normalized against the reference genes, before relative quantification against control samples were executed.

#### **4.3.1 MDI and ROSI induce transcription of *PPAR $\gamma$* in pbASCs**

*PPAR $\gamma$*  transcript levels in non-induced cells cultivated in BM remained relatively stable throughout the 14 day cultivation period (Fig. 4.6.A.), as an 1.1-fold increase in transcript levels were measured the on day 6, 10 and 14, compared to transcript levels on day 2. This indicates that BM did not affect the expression of *PPAR $\gamma$*  in the non-induced cells. Cells induced by MDI, without ROSI, had an increased expression of *PPAR $\gamma$*  from day 2 to day 6, and remained stable from day 6-14, and the expression of *PPAR $\gamma$*  were lower than in cells induced by MDI+ROSI. Six days after induction the transcription levels of *PPAR $\gamma$*  in cells induced by MDI+ROSI were over 30 times higher than in non-induced cells, and 3 times higher than in cells induced by MDI alone, at day 6. The *PPAR $\gamma$*  levels remained unchanged until day 10, but had decreased by 30 % by day 14. This shows that exposure to ROSI induces *PPAR $\gamma$*  transcription in pbASCs.

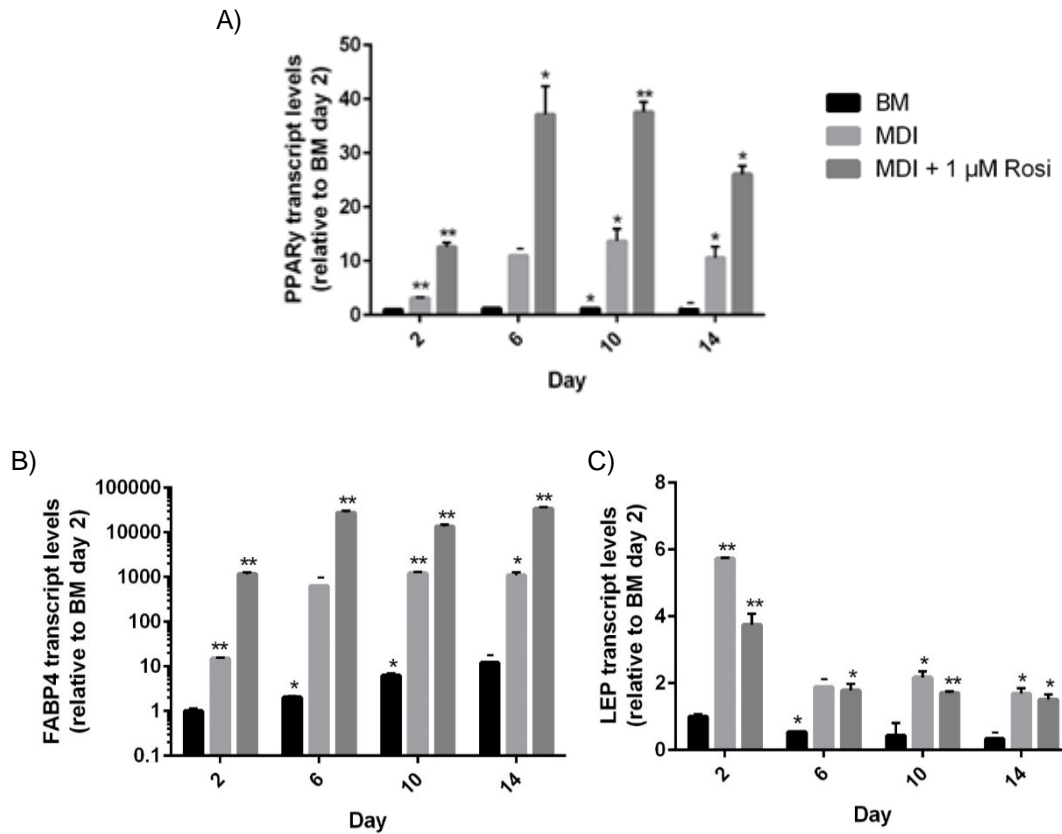
#### **4.3.2 Transcription levels of *FABP4* are elevated during adipogenesis in pbASCs**

The mRNA expression of *FABP4* was elevated in cells induced by MDI and MDI+ROSI compared to the non-induced cells cultured in BM throughout the 14 day induction period (Fig. 4.6.B.). Transcription levels of *FABP4* were 84 and 2692 timer higher in MDI and MDI+ROSI induced cells compared to the cells cultured in BM at day 14. *FABP4* transcript levels were also higher in cells induced by MDI+ROSI than in cells induced by MDI (30 times higher on day 14). The mRNA expression of *FABP4* in cells cultured in BM were increasing during the culturing period, and at day 14 the transcript levels were 12 times higher compared to day 2.

#### **4.3.3 Transcription of *LEP* is elevated by MDI induction**

The transcript levels of *LEP* were highest two days after induction (5.6 and 3.6 times higher in MDI and MDI+ROSI induced cells, respectively) and remained stable from day 6

until the end of the experiment (Fig. 4.6.C.). Except from on day 2, *LEP* levels appeared to be independent of ROSI, and the *LEP* gene expression was continuously higher in MDI/MDI+ROSI exposed cells than in BM cultured cells (average of 3 times higher from day 6 to day 14).



**Figure. 4.6. Relative transcript levels of *PPAR $\gamma$* , *FABP4* and *LEP* during different stages of adipogenic differentiation of pbASCs.** RNA was extracted from pbASCs cultured in BM or MDI medium, supplied with 0.1% DMSO or 1  $\mu$ M ROSI, for 2, 6, 10 or 14 days. Subsequently cDNA synthesis was performed and real-time Q-PCR conducted. Transcript levels were normalized against the reference genes *WYHAZ* and *PPIA*, and the expression made relative to that of the control cells (cells cultivated in BM for 2 days). Cells were seeded in duplicates (n=2). Two samples were omitted from the analysis (MDI Day 6, and BM day 14) due to lack of a valid  $C_T$  value, or extreme aberration. \* = significantly different from control cells,  $P < 0.05$ ; \*\* = significantly different from control cells,  $P < 0.01$ .

#### **4.4 EFFECTS OF EXPOSURE TO ENDOCRINE DISRUPTING CHEMICALS ON ADIPOGENIC DIFFERENTIATION IN pbASCs**

PbASCs were exposed to two known EDCs, mono(2-ethylhexyl)phthalate (MEHP) and bisphenol A (BPA), supplemented in BM or MDI medium for 14 days (3.3.2). The experiments were repeated twice (experiments A and B). Cells were stained with Oil red O to quantify lipid content and mRNA expression was studied by real-time Q-PCR in parallel experiments, using cells from the same culture flasks, and the same prepared media, to obtain the most similar conditions possible.

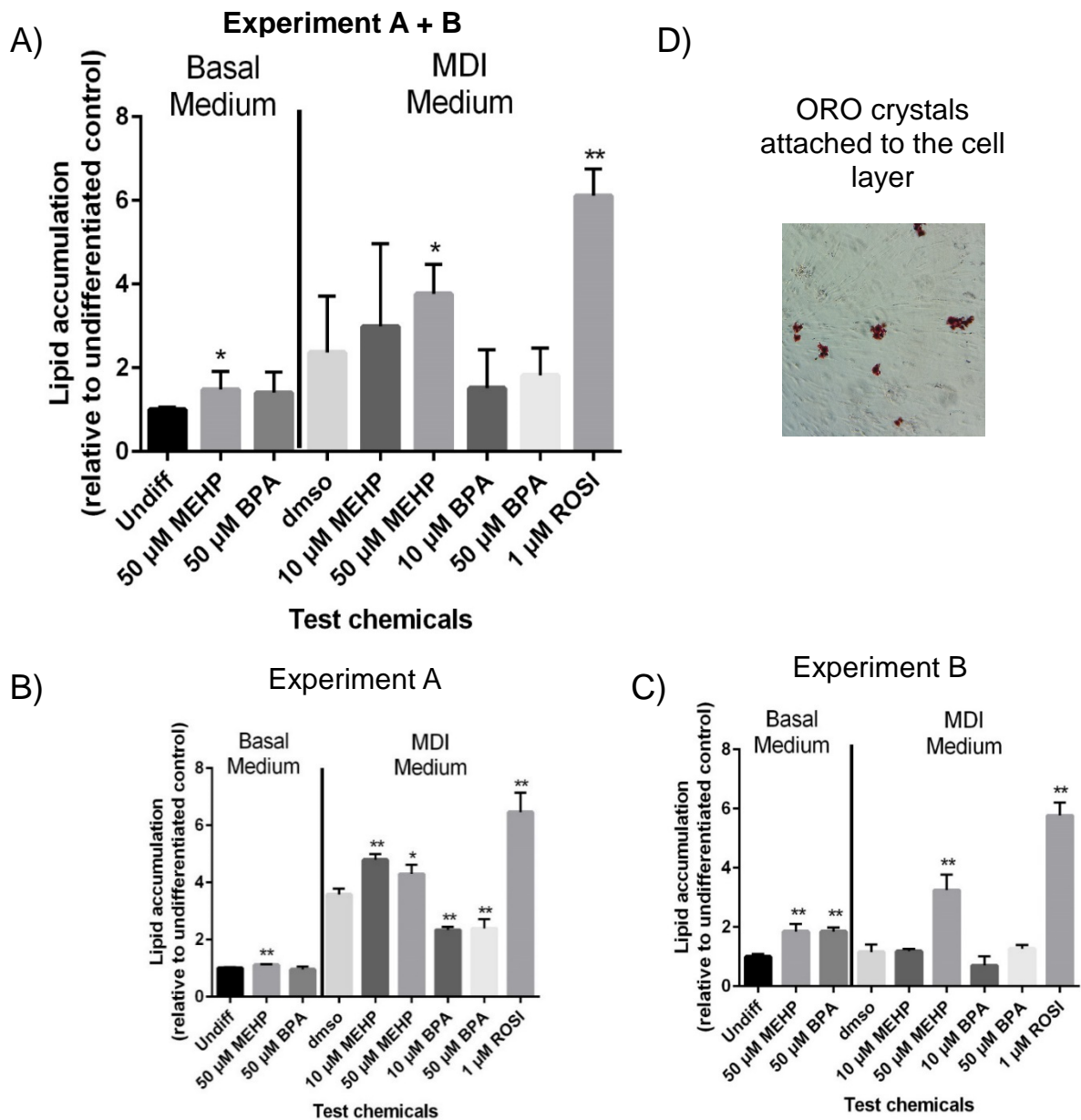
##### **4.4.1 Quantification of lipid content by Oil Red O staining**

When evaluating experiment A and B together (Fig. 4.7.A.) a significant ( $p < 0.05$ ) increase in lipid content was observed in cells exposed to 50  $\mu\text{M}$  MEHP, both in BM and MDI medium, compared to non-exposed control cells. Further, results from experiment A alone showed that MDI medium supplemented with 10  $\mu\text{M}$  MEHP also increased lipid content, while 50  $\mu\text{M}$  MEHP was needed to increase lipid content in the cells in experiment B (Fig. 4.7.B and C.).

Exposure to BPA did not result in any change in lipid content compared to the control cells in the overall results (A+B). Although, in experiment A, both 10 and 50  $\mu\text{M}$  BPA (in MDI-medium) reduced lipid accumulation compared non-induced control cells.

The highest lipid content was measured in cells cultured in MDI + ROSI. In these cells the lipid content was three times higher compared to the MDI + DMSO control cells.

This inter-experimental variation might have a technical explanation. Some problems were encountered during ORO staining of experiment B (Fig. 4.7.C.). During the procedure differentiated cells detached, observed in the microscope as areas without cells in the wells. This leads to an uneven amount of differentiated cells to stain with ORO, and subsequently quantify, compared to experiment A. Also, small crystals of ORO had attached to the cell layer, as observed in the microscope (Fig. 4.7.D.). Through the microscope one could clearly see that the crystals were stuck on top of the cells, and not absorbed inside the cells in lipid vacuoles. After elution of ORO these crystals can lead to a higher measured absorbance, and successively a higher measured lipid content. These two factors have likely contributed to some of the variance in results from the experiments.



**Figure 4.7. Effect of MEHP or BPA on lipid accumulation in pbASCs.** PbASCs were cultured in for two weeks in BM or MDI, supplemented with BPA, MEHP, ROSI, or DMSO (0.1%), and subsequently fixed and stained with Oil Red O (ORO). The absorbance ( $\lambda=500$  nm) in isopropanol extracts was measured to quantify lipid content. A) Graph showing the overall results (n=6). B) and C) Results from each individual experiment (n=3). D) ORO crystals attached to the cell layer (inverted microscope, 10x objective). \* = significantly different from control cells (undiff or DMSO),  $P < 0.05$ ; \*\* = significantly different from control cells (undiff or DMSO),  $P < 0.01$ .

#### 4.4.2 Transcript levels of adipocyte specific genes measured by real-time Q-PCR

Due to time constraints, transcript levels were only measured from one of the two experiments aimed to study changes in the expression of selected adipogenic genes after exposure to EDCs (4.4.1, experiment A). The transcript levels of all target genes, *PPAR $\gamma$* , *FABP4*, and *LEP*, were normalized against the reference genes, *YWHAZ* and *PPIA*, before relative quantification against the undifferentiated control samples was executed.

##### 4.4.2.1 MDI medium and ROSI induce the transcription of adipogenic genes in pbASCs

Results showed that the transcript levels of all target genes were noticeably higher in differentiated control cells cultured in MDI+DMSO compared to undifferentiated control cells cultured in BM+DMSO ( $p < 0.01$ ) (Fig. 4.8.). Especially the transcript levels of *PPAR $\gamma$*  and *FABP4* were increased (by 19- and 18-fold, respectively). This was expected as the MDI-medium contains the adipogenic inducers DEX and IBMX, in addition to insulin. Cells cultured in MDI+ROSI resulted in an even higher transcript level of all target genes ( $P < 0.01$ ), also here especially of *PPAR $\gamma$*  and *FABP4* (36- and 400-fold, respectively). This indicates that ROSI is a *PPAR $\gamma$*  agonist and adipogenic inducer also in pbASCs.

##### 4.4.2.2 MEHP increases transcription of *PPAR $\gamma$* , *FABP4* and *LEP* in pbASCs

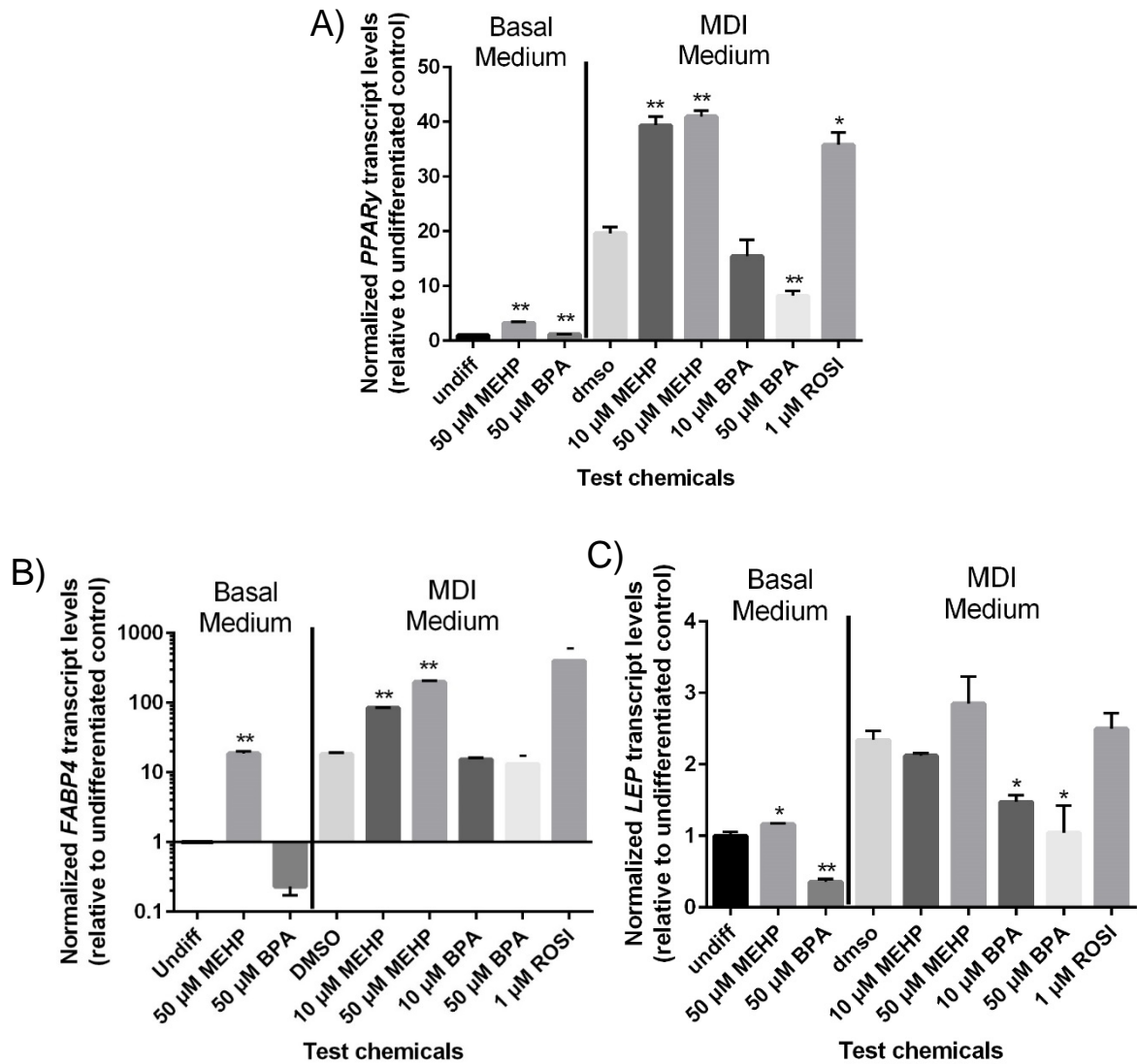
All three MEHP treatments (BM+50  $\mu$ M, MDI+10  $\mu$ M and MDI+50  $\mu$ M) resulted in significantly ( $P < 0.01$ ) higher transcript levels of *PPAR $\gamma$*  and *FABP4*, compared to non-exposed control cells (undiff and DMSO) (Fig. 4.8.A and B.). A 1.2-fold increase in transcript levels of *LEP* in cells subjected to BM+50 $\mu$ M MEHP was measured, while no change was detected in cells subjected to MEHP in MDI medium on *LEP* gene expression, compared to control cells (Fig. 4.8.C.).

##### 4.4.2.3 Effect of BPA on *PPAR $\gamma$* dependent on MDI-induction

Cells exposed to BPA had an increase of 1.2-fold on transcript level of *PPAR $\gamma$*  when subjected to BM+50  $\mu$ M BPA, while a 2.4-fold decrease was measured in cells subjected to MDI+ 50  $\mu$ M BPA (Fig. 4.8.A.). Exposure to BPA did not result in change of transcription levels of *FABP4* (Fig. 4.8.B.).

##### 4.4.2.4 BPA inhibits mRNA expression of *LEP* in pbASCs

All three BPA treatments (BM+50  $\mu$ M, MDI+10  $\mu$ M and MDI+50  $\mu$ M) resulted in lower transcript levels of *LEP*, compared to the controls (Fig. 4.8.C). In cells not induced by MDI, BPA reduced *LEP* transcripts by 1.35 fold, while in MDI-induced cells *LEP* was inhibited by 1.6- and 2.3-fold when exposed to 10 and 50  $\mu$ M BPA, respectively.



**Figure 4.8. Effect of MEHP or BPA on transcript levels of *PPAR $\gamma$* , *FABP4* and *LEP* in pbASCs.** PbASCs were seeded in duplicates (n=2) and cultured in for two weeks in BM or MDI, supplemented with BPA, MEHP, ROSI, or DMSO (0.1%), before RNA was extracted and synthesis of cDNA was conducted. Transcript levels were measured by real-time Q-PCR, and normalized against reference genes (*WYHAZ* and *PPIA*). Two samples were omitted from the *FABP4* results (MDI+50  $\mu$ M BPA, and MDI+1  $\mu$ M ROSI) due to lack of a valid  $C_T$  value, or extreme aberration. \* = significantly different from control cells (undiff or DMSO),  $P < 0.05$ ; \*\* = significantly different from control cells (undiff or DMSO),  $P < 0.01$ .

## 5 DISCUSSION

In this thesis, I have demonstrated that adipose tissue-derived stem cells from polar bear (pbASCs) can be successfully maintained and propagated in culture. In addition, the cells were shown able to differentiate to adipocytes when induced by adipogenic stimuli. In this work effects of test chemicals (EDCs) on adipogenic conversion were also shown. This was done by measuring changes in lipid content and transcript levels of adipocyte specific genes (*PPAR $\gamma$* , *FABP4* and *LEP*) in pbASCs. Exposure to the known EDCs mono(2-ethylhexyl)phthalate (MEHP) and bisphenol A (BPA) indicated that adipogenic differentiation is induced by MEHP, but not by BPA in pbASCs, at the tested concentrations. In this chapter, these findings will be discussed in relation to relevant literature.

### 5.1 ISSUES REGARDING CULTIVATION OF POLAR BEAR ADIPOSE-TISSUE DERIVED STEM CELLS

#### 5.1.1 Cell isolates from polar bear adipose tissue possess several characteristics of mesenchymal stem cells

Mesenchymal stem cells (MSCs) are stromal cells that are able to self-renew and to differentiate into several different cell types. MSCs can be obtained from sources such as bone marrow and adipose tissue, and MSCs derived from these two tissues are denoted as BMSCs and ASCs, respectively. The International Society for Cellular Therapy (ISCT) have formulated three minimum criteria to define multipotent human MSCs (and for the following discussion it is assumed that these criteria also are valid for polar bear MSCs); the cells must adhere to plastic when maintained in culture, they must have a specific surface antigen expression, and be able to differentiate into adipocytes, osteoblasts, and chondroblasts *in vitro* (Dominici *et al.*, 2006).

In this study polar bear MSCs isolated from adipose tissue (pbASCs) was shown to adhere to plastic (4.1.1), thus possessing the first of three MSC characteristics. Furthermore, it was shown that the cells were capable of adipogenic differentiation, through Oil Red O staining (4.2.1) and transcriptional analysis of adipocyte specific genes (4.4). This confirms that the pbASCs could differentiate to adipocytes. However, to fully characterize the isolated polar bear cells as multipotent ASCs, their capabilities of differentiating towards the



osteogenic and chondrogenic lineages would need to be confirmed. It should also be investigated if the cells have the stem cell specific expression of surface antigens (Dominici *et al.*, 2006), for example by immunostaining assays.

### 5.1.2 Initial seeding density of pbASCs

As pbASCs are costly and laborious to obtain, it was important to utilize the cells in the best possible manner. To conduct the experiments in this study a large amount of cells was needed, therefore a substantial expansion of the cells was conducted.

Several studies have shown that MSCs proliferate faster at low seeding densities (Bartmann *et al.*, 2007, Lode *et al.*, 2008). A study by Mochizuki *et al.* (2006) found that the optimal initial seeding density for maximizing cell yields per dish were 1000 cells/cm<sup>3</sup>, while others recommend to seed MSCs at 100 cells/cm<sup>3</sup> (Lund *et al.*, 2009, Both *et al.*, 2007). Because we had limited time to use for cell expansion an initial seeding density of 3000 cells/cm<sup>2</sup> was used for the most part of this study, as this allows for the expansion of more cells at the same time than lower densities. Seeding cells at a higher density reduces the time needed to obtain the necessary amount of cells, at the expense of a poorer utilization of the isolated pbASCs. When seeded at 3000 cells/cm<sup>2</sup>, the number of cells could increase up to six times before subculturing at 70% confluence (4.1.1). Seeding cells at this density presumably do not affect the potential of the cells to differentiate, as it previously have been shown that seeding cells at 5000 cells/cm<sup>3</sup> or 100 cells/cm<sup>3</sup> did not affect the differentiation potential of human BMSC (Both *et al.*, 2007).

### 5.1.3 Cell density during expansion

A more crucial factor regarding cell density and differentiation potential is cell density during expansion. It has been found that human ASCs harvested at 50% confluence or 90% confluence have different gene expression patterns of genes involved in processes such as proliferation, cell communication, signal transduction and motility (Kim *et al.*, 2014). As reaching confluence during culturing can modify expression patterns of ASCs, it is important to maintain the cells at a subconfluent level. However, it has been found that changes in the expression of genes involved in contact inhibition did not affect the ability to differentiate in ASCs derived from mouse (Jeon *et al.*, 2012).

In this study it was observed that pbASCs allowed to reach 100% confluency during culturing behaved differently from cells that had been maintained at subconfluency (4.2.2). The previously confluent cells did not stop growing after reaching confluence, perhaps due to loss of contact inhibition. It is possible that this could affect the cells' capability to undergo

adipogenic differentiation as growth arrest is a requirement for adipogenesis to occur. The mechanisms of how growth arrest is regulated still remains unclear, but both peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and CCAAT-enhancer-binding protein alpha (C/EBP $\alpha$ ) are involved in the regulation (Gregoire *et al.*, 1998). In adipogenic induction media (AIM), the adipogenic inducers dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and rosiglitazone (ROSI) induce the expression of PPAR $\gamma$  and C/EBP $\alpha$ , directly or indirectly. With this in mind, it could be interesting to study if confluence during subculturing leads to loss of contact inhibition, and if this in turn affects the cells' capability to undergo differentiation.

#### **5.1.4 Time spent in culture**

A previous study using rat BMSCs has shown that the amount of time cells spend in culture can affect morphology and lead to loss of differentiation ability (Neuhuber *et al.*, 2008). In our study it was observed that the cultured pbASCs had a small and spindle shaped appearance in early passages, but in higher passages larger, triangular shaped cells were observed (4.1.1). These larger cells seemed to proliferate at a slower rate than the smaller cells. These observations are in accordance with several other studies (Sekiya *et al.*, 2002, DiGirolamo *et al.*, 1999, Bruder *et al.*, 1997, Wagner *et al.*, 2008). The results from those studies indicated that the large, wider cells have a reduced differentiation potential, and also that this appearance is a sign of senescence, but this was not investigated in our study. To avoid variation in cell shape and possibly differentiation potential, cells in same low passage numbers should be used to study adipogenic potentials of pbASCs.

## **5.2 CHOICE OF ADIPOGENIC MEDIUM AND INDUCTION PERIOD**

To our knowledge, this is the first study on ASCs from polar bear (pbASCs). Thus, conditions for induction of adipogenic differentiation of pbASCs needed to be established. However, methods for inducing adipogenic differentiation of ASCs from other species, such as human, mouse and brown bear, have been described but the conditions used often vary (reviewed by Scott *et al.*, 2011). In this study, seven different AIMs, which contained various concentrations of the adipogenic inducers IBMX, DEX, ROSI and indomethacin (INDO), (Table 3.1.), were tested to find the most effective AIM for the differentiation of pbASCs. Visual inspection of Oil Red O (ORO) stained cells revealed that all seven AIMs had resulted in triglyceride accumulation, but to varying degrees (4.2.1). However, pbASCs may be more sensitive to INDO than human and brown bear ASCs (Fink *et al.*, 2008, Fink *et al.*, 2011), as a high level of cell death was observed for cells receiving media containing 200  $\mu$ M

INDO, while cells induced by 50  $\mu\text{M}$  INDO showed less sign of distress. No previously published data on toxic effect of INDO could be found, but a study by Scott *et al* (2011) found differences in the concentration of adipogenic inducers used for optimal differentiation of human and mouse MSCs. This could indicate that there are species differences in sensitivity to adipogenic inducers. The highest lipid content was found in cells induced by MDI+ROSI which contained 1  $\mu\text{M}$  of the PPAR $\gamma$  ligand ROSI and no INDO. The contribution of ROSI on adipogenic differentiation was investigated by comparing lipid content of cells induced by MDI+ROSI or MDI (0.1% DMSO) (4.2.2). The lipid content in MDI+ROSI induced cells were three times higher than in cells induced by MDI, indicating that ROSI strongly induce adipogenic differentiation of pbASCs. This is in accordance with literature and the induction is probably due to the fact that ROSI is a ligand of PPAR $\gamma$ , which is required for adipogenic differentiation (Benvenuti *et al.*, 2007, Rosen *et al.*, 1999)

As the highest concentration (200  $\mu\text{M}$ ) of INDO appeared to be toxic for the pbASCs, and the cells induced by MDI+ROSI had accumulated the most lipids, the MDI+ROSI medium composition was used as a positive control medium in the remain of the study, assuming that this medium induced a high level of adipogenic differentiation in the pbASCs. The MDI medium, without ROSI, induced a low level of adipogenic differentiation, and therefore could be used as a differentiated control, to allow for the discrimination of effects of test chemicals.

In this study cells were continuously stimulated for 14 days, which is common for ASCs. Visual signs of triglyceride accumulation were however observed as early as 7 days after induction (results not shown). The possibility of shortening the induction period should be investigated, as a shorter induction period would save both time and materials.

It could also be very interesting to investigate if it is enough to induce the cells by AIM rather than to maintain them in it, as both DEX and IBMX induce first wave transcription factors (TFs) of adipogenesis. This could be done by inducing the cells for a short period (2-3 days) with AIM followed by maintaining the cells in basal medium (consisting of growth medium supplemented with insulin) similar to the protocols commonly used for 3T3-L1 cells.

### **5.3 TRANSCRIPT LEVELS OF ADIPOCYTE SPECIFIC GENES IN pbASCs CHANGE DURING THE INDUCTION PERIOD IN RESPONSE TO ADIPOGENIC INDUCTION MEDIA**

Human BMSCs have been shown capable of accumulating lipids without transcribing adipocyte specific genes, which could indicate that the lipid accumulation occurred independently of adipogenic differentiation (Fink *et al.*, 2004). Thus, it was necessary to confirm that the accumulation of lipids in the pbASCs was a result of adipogenic differentiation by measuring the transcript levels of the adipocyte specific genes peroxisome proliferator-activated receptor gamma (*PPAR $\gamma$* ), fatty acid-binding protein 4 (*FABP4*), and leptin (*LEP*) by real-time quantitative-PCR (real-time Q-PCR), after culturing the cells in three different media (BM, MDI or MDI+ROSI). RNA was extracted 2, 6, 10 and 14 days after induction, and the transcript levels were measured to investigate changes in expression during different stages of adipogenesis.

#### **5.3.1 MDI and MDI+ROSI induce transcription of *PPAR $\gamma$* in pbASCs**

*PPAR $\gamma$*  is commonly used as an early marker of adipogenic differentiation, as the expression is turned on early during adipogenesis, and it is required for adipocyte differentiation (Chawla *et al.*, 1994, Rosen *et al.*, 1999). In the present study the expression of *PPAR $\gamma$*  remained relatively unchanged in non-induced cells cultured in BM, indicating that BM poorly induce expression of *PPAR $\gamma$*  in pbASCs (Fig. 4.6.A.). Cells cultured in MDI or MDI+ROSI medium had a higher expression of *PPAR $\gamma$*  compared to the non-induced control cells, and highest expression levels were measured on day 6 and 10 after induction. The expression was higher in cells induced by MDI+ROSI than in cells induced by MDI medium (3 times higher on day 6), indicating that ROSI also is a *PPAR $\gamma$*  ligand in polar bears. On day 14 the transcript levels in cells induced by MDI+ROSI had declined by 30 %, compared to day 10. This is in accordance with a previous study using 3T3-L1 cells, which found that the presence of *PPAR $\gamma$*  ligands can lead to a down-regulation of *PPAR $\gamma$*  expression during later stages of differentiation (Camp *et al.*, 1999). Thus, it is possible that the presence of ROSI leads to similar effects in the pbASCs at this late stage of differentiation.

#### **5.3.2 Transcription levels of *FABP4* are elevated during adipogenesis in pbASCs**

Transcript levels of *FABP4*, a target of *PPAR $\gamma$* , were elevated in cells induced by MDI and MDI+ROSI, compared to non-induced control cells, during the 14 day induction period (Fig 4.6.B.). Also, transcript levels were substantially higher in cells induced by MDI+ROSI

than in cells induced by MDI (30 times higher on day 14), which was to be expected as ROSI activate PPAR $\gamma$ , which subsequently induces transcription of *FABP4* (Tontonoz *et al.*, 1994). This indicates that ROSI activates PPAR $\gamma$  in polar bear and this activation may induce the expression of *FABP4*.

An increase of *FABP4* transcript levels was observed in non-induced control cells, cultured in BM during the induction period. A possible reason for this increase could be that the cells were cultured to 100% confluence, and confluence is known to change gene expression patterns in human ASCs (Kim *et al.*, 2014). Alternatively, a small portion of the cells could have spontaneously started to differentiate towards the adipocyte lineage, as several types of MSCs have been shown capable of spontaneous differentiation (Adamzyk *et al.*, 2013, Zhang and Kilian, 2013).

### 5.3.3 Transcription of *LEP* is elevated by MDI induction

The measured transcript levels of *LEP* were highest two days after induction, and remained stable from day 6 until the end of the experiment (Fig 4.6.C.). The transcription levels of *LEP* were continuously higher in MDI and MDI+ROSI exposed cells than in BM cultured cells (average of 3 times higher from day 6 to day 14) indicating that MDI induction elevates transcription of *LEP*. From day six and throughout the experiment levels of *LEP* appeared to be independent of ROSI. These results were contradictory to results in a previous study, where the transcript levels of *LEP* in 3T3-L1 cells increased over time during induction by MDI and further increased by MDI supplemented with troglitazone, another PPAR $\gamma$  ligand (Kamstra *et al.*, 2014).

To get a better understanding of how the expression of leptin change during adipogenesis it would be interesting to measure the amount of leptin released into the media during adipogenesis, which have been done in several other studies, using 3T3-L1 cells or human ASCs (Taxvig *et al.*, 2012, Arsenescu *et al.*, 2008, Rodriguez *et al.*, 2005). An interesting observation is that it has been shown that the amount of secreted leptin can increase even though transcript levels remain unchanged (Howell and Mangum, 2011).

## 5.4 EXPOSURE TO CONTAMINANTS SHOWED THAT pbASCs CAN BE USED TO STUDY ADIPOGENESIS

One of the main goals of this study was to develop an *in vitro* method to study effects of contaminants on adipogenesis in polar bears. We chose to use known endocrine disrupting chemicals (EDCs), mono(2-ethylhexyl)phthalate (MEHP) and bisphenol A (BPA), in order to evaluate the results in comparison with previous findings.

PbASCs were exposed to MEHP or BPA for 14 days in BM (50  $\mu$ M MEHP or BPA) or MDI medium (10 or 50  $\mu$ M MEHP, or 10 or 50  $\mu$ M BPA), before analysis of lipid content and gene expression were conducted. Results from ORO staining and real-time Q-PCR were consistent with each other.

#### **5.4.1 EXPOSURE TO MEHP INDUCED ADIPOCYTE DIFFERENTIATION OF pbASCs**

In the pooled results of the two conducted experiments the lipid content of pbASCs exposed to 50  $\mu$ M MEHP, both in BM and MDI medium, were significantly higher ( $P < 0.05$ ) compared to non-exposed control cells (Fig 4.7.A.). 10  $\mu$ M MEHP in MDI medium led to an increased lipid content in experiment A (Fig 4.7.B.), but not in experiment B (Fig. 4.7.C.).

This is noteworthy because there were encountered some problems during the Oil Red O staining (as elaborated in 5.5.2) of experiment B, which may have led to unrepresentative results.

From the transcript level analysis it was found that the transcript levels of PPAR $\gamma$  and *FABP4* in all cells exposed to MEHP (BM+50 M MEHP, MDI+10 M MEHP and MDI+50  $\mu$ M MEHP) were significantly higher compared to the non-exposed control cells (Fig. 4.8.A and B.). This could indicate that MEHP is a PPAR $\gamma$  ligand also in polar bears. MEHP had no effect on transcript levels of *LEP* (Fig. 4.8.C.). A possible explanation for this is that transcript levels of *LEP* in polar bear do not reflect the degree of adipogenic conversion taken place. This is based on the findings from the experiment were transcript levels of *LEP* were measured at different stages of adipogenic differentiation (4.4, 5.3). Here we found that the gene expression of *LEP* were independent of ROSI from day 6 and throughout the experiment, hence it is possible that *LEP* expression is independent of MEHP exposure as well.

Exposure to MEHP, both at 50 and 10  $\mu$ M, led to an increase in both lipid content and transcription levels of PPAR $\gamma$  and *FABP4*. Thus indicating that exposure to MEHP at these concentrations may induce adipogenesis in polar bear ASCs, which is in accordance with previous findings using 3T3-L1 cells (Feige *et al.*, 2007, Hurst and Waxman, 2003, Taxvig *et al.*, 2012). However, it was not possible to compare if the induction was at a higher, lower or similar level as in these studies, due to difference in differentiation protocol and exposure concentrations.

A previous study where 3T3-L1 cells were induced by another PPAR $\gamma$  ligand (troglitazone, 1  $\mu$ M), found that this led to an induction of non-induced cells after exposure

of troglitazone for one week (Grun et al., 2006). As MEHP induced adipogenesis in non-induced cells, this could be another indication of MEHP as a PPAR $\gamma$  ligand also in polar bears.

#### **5.4.2 EXPOSURE TO BPA DID NOT INDUCE ADIPOCYTE DIFFERENTIATION OF pbASCs**

No change in lipid content by exposure to BPA was detected in the pooled results of the two conducted experiments (Fig. 4.7.A.). But, results from Experiment A indicated that cells induced by MDI medium supplemented with BPA (10 or 50  $\mu$ M) had a lower lipid content than cells non-exposed cells in MDI medium (Fig 4.7.B.). This is noteworthy for the same reasons as explained in the previous section.

The transcript level analysis indicated that cells subjected to BM+50  $\mu$ M BPA had an increased transcript level (1.24-fold) of PPAR $\gamma$ , while cells subjected to MDI+50  $\mu$ M BPA had a decreased transcript level (2.3-fold), compared to non-exposed controls (Fig. 4.8.A.). No significant change in transcript levels of *FABP4* was found (Fig. 4.8.B.). Transcript levels of *LEP* were lower in cells exposed to BPA, compared to non-exposed control cells (Fig. 4.8.C.), indicating that BPA exposure decrease transcription of *LEP*.

Exposure to BPA did not affect lipid content in the pooled results from the Oil Red O staining, nor result in any strongly marked changes in transcript levels of PPAR $\gamma$  or *FABP4*, indicating that BPA did not increase adipogenic differentiation of pbASCs. This is in accordance with previous study by Chamorro-Garcia which found that BPA induced adipocyte differentiation of 3T3-L1 cells, but not in BMSCs derived from human or mouse (Chamorro-Garcia *et al.*, 2012).

However, results from the lipid quantification of experiment A indicated that BPA decreased adipocyte differentiation of cells induced by MDI (Fig. 4.7.B.). An interesting study by Biemann *et al.* (2012) found that there were a decrease in adipogenic differentiation in BMSCs derived from mouse, when the cells were exposed to BPA during undifferentiated growth, , i.e. before confluence. This indicated that the effect of BPA depends on when the cells are exposed, and that BPA affects the level of adipogenic differentiation by reducing commitment of BMSCs to the adipogenic lineage (Biemann et al., 2012). It would be interesting to see if this is the case in pbASCs as well.

## **5.5 FACTORS THAT MAY HAVE AFFECTED THE REPRODUCIBILITY AND RELIABILITY OF THE FINDINGS IN THIS STUDY**

### **5.5.1 Primary cultures of MSCs are heterogeneous and donor dependent**

There are some advantages and disadvantages of using primary cultures of mesenchymal stem cells (MSCs) or cell lines (such as 3T3-L1) to study adipogenesis and effects of pollutants on this process. Cell lines can be better controlled, as they contain a homogenous cell population at the same stage of differentiation, while cultures of MSCs are heterogeneous, containing other cell types, and cells at different stages of their life span. An advantage of using stem cells isolated from tissue, such as adipose, is that they may reflect more accurately the conditions *in vivo* (Butterwith, 1994).

In this study all the pbASCs used are derived from the same polar bear, a ten year old male. It is known that the differentiation capacity of primary cultures is donor dependent and decreases with age (Choudhery *et al.*, 2014, Gregoire *et al.*, 1995, Kirkland *et al.*, 1990).

Also, the polar bear originated from Svalbard. Polar bears from this area are considered to be especially at risk for POP exposure and associated effects (Letcher *et al.*, 2010). Hence, it is possible that exposure to contaminants already have affected the isolated cells in some manner. To properly evaluate effects of contaminants on adipogenesis in pbASCs, experiment should be conducted on pbASCs derived from multiple polar bears with different contaminant exposure.

### **5.5.2 The protocol used for Oil red O quantification of lipid content can lead to several sources of error**

Adipogenic differentiation of pbASCs was quantified by measuring the lipid content in pbASCs, as measured absorbance in eluates from Oil Red O (ORO) staining. Several problems were encountered using this method.

First, it was observed that when the cells undergo adipogenic differentiation the cells had an increased tendency to detach from the culture plate. This observation is in accordance with literature (Zebisch *et al.*, 2012, Fink and Zachar, 2011). A potential loss of cells makes comparison of lipid content in different wells challenging, due to different number of cells eluting Oil Red O to the lysates. Also, this phenomenon can strongly influence the reproducibility of the experiments, because a loss of differentiated cells will affect the amount of ORO staining, and subsequently quantification of lipid content by absorbance measurements. This can lead to results that do not reflect the amount of adipogenic conversion taking place during the experiment accurately.



Second, during some experiments, small crystals of ORO were attached to the cell layer (Fig 4.7.D.)). The reason for these crystals remains unknown, as it did not help to prepare fresh stock solution, nor working solution. It was possible to remove some of the crystals from the samples by repetitive washing with water. But the presence of these crystals could lead to falsely high absorbance measurements, and subsequently to unrepresentative results.

In addition, this method can produce a high background staining, by the presence of residual stain in the culture plate. High background staining is unfavorable, as it can reduce the ability of this method to detect differences in lipid content between different cell treatments. To avoid background staining it is crucial to work fast, steady and accurately, to limit the amount of stain attached on the culture plate. An alternative could be to quantify the lipid content directly (e.g. by using an adipogenesis detection kit). Direct quantification is faster and requires few washing steps, thus reducing the risk of loss of differentiated cells. Due to time constraints a planned assessment of using an adipogenesis detection kit (ab102513, abcam.com) compared to ORO staining was not conducted in this study, but this is an option that could be considered in the future.

### **5.5.3 Small sample size and no repeated experiments**

Several of the findings in this study originate from experiments that have only been conducted once. Cells used for lipid quantification were seeded in triplicates, while cells used for gene expression analysis were seeded in duplicates. For biological assays the general rule is that a minimum of three replicates is needed, and that the experiments should be repeated two to three times. Hence, to verify the findings in this study the experiments must be repeated.

## 6 CONCLUSIONS

This study represents, to our knowledge, the first study to describe cultivation and adipogenic stimulation of adipose-tissue derived stem cells from polar bear (pbASCs). It was demonstrated that the pbASCs were adherent to plastic when maintained in culture, and was shown to be capable of adipogenic differentiation, thus fulfilling some of the requirements formulated by the International Society for Cellular Therapy (ISCT) for defining cells as multipotent mesenchymal stem cells (MSCs).

In the present study conditions for inducing different levels of adipogenic differentiation of pbASCs were established. To be able to detect effects of test chemicals, an adipogenic induction medium (AIM) inducing a low level of adipogenic differentiation was needed. MDI medium, consisting of growth medium supplemented with 0.45 mM IBMX, 0.1  $\mu$ M DEX and 1  $\mu$ g/mL insulin, induced a low level of adipogenic differentiation, while addition of 1  $\mu$ M ROSI to the MDI medium induced a high level of adipogenic differentiation in the pbASCs. Hence, MDI medium can be used as differentiated control with a low level of differentiation, which allows for discrimination of effects on adipogenesis by test chemicals, while MDI-ROSI can be used as a positive control in adipocyte differentiation experiments using pbASCs.

The degree of adipogenic conversion in the pbASCs was reflected by the amount of accumulated lipids, as well as transcript levels of the adipocyte specific genes *PPAR $\gamma$*  and *FABP4*. Lipid content and transcript levels of *PPAR $\gamma$*  and *FABP4* were higher in cells induced by MDI+ROSI than in cells induced by MDI medium, which were in turn higher than in non-induced cells cultured in BM. In contrast to lipid quantification measurement and transcript analysis *PPAR $\gamma$*  and *FABP4*, the transcription levels of *LEP* did not reflect the degree of adipocyte conversion. This indicates that *LEP* may not be a suitable target gene for investigating adipogenic induction in pbASCs.

Exposure to a known EDC, MEHP, induced adipocyte differentiation of pbASCs, both in non-induced cells cultured in BM and in cells induced by MDI medium. MEHP is known to be a *PPAR $\gamma$*  ligand, and the results indicated that MEHP induces adipogenesis also in pbASCs through *PPAR $\gamma$*  activation.

No increase in adipogenic differentiation was found by exposure of pbASCs to BPA, which is in contrast with previous studies on 3T3-L1 cells from mouse. This highlights the necessity of using primary cells derived from the species in question

In conclusion, this study has shown that it is possible to detect effects of environmental contaminants on adipocyte differentiation of pbASCs by quantification of lipid content and transcript level analysis, although the experiments needs to be repeated to verify the findings.

### 7 FUTURE PERSPECTIVES

In this thesis a method for detecting effects of environmental contaminants on adipocyte differentiation of pbASCs has been established. Using pbASCs to study effects of contaminants in polar bears avoids extrapolation between species, and may reflect more accurately the adipose tissue *in vivo*. Also, results from contamination studies using pbASCs can be of relevance to human health, as the ligand binding domain (LBD) of PPAR $\gamma$  is identical in human and polar bear (Routti *et al.*, unpublished).

As a continuation of the work conducted in this study, the next step would be to further develop the *in vitro* method established. Further work suggestions include:

- Investigating the possibility of shortening the induction period of pbASCs. In this study visual sign of triglyceride accumulation was visible after only seven days of induction, and levels of PPAR $\gamma$  and FABP4 were highly elevated six days after induction.
- Develop a method for measuring levels of secreted leptin from pbASCs in the media, as this would probably reflect the expression of leptin more accurately. Also, many studies have measured the amount of leptin in media during exposure studies, allowing for comparison with previous findings.
- Repeat the experiments on pbASCs derived from other polar bears.
- Assess if measuring lipid content directly can provide more reliable and reproducible results than Oil red O staining.

When this method has been validated and optimized, a natural continuation of the work would be to expose pbASCs to environmental contaminants at the levels found in polar bears, in single exposures and in mixtures, to evaluate their effect on adipogenesis. Also, to increase the understanding of how environmental contaminants affect adipogenesis in polar bear it could be interesting to conduct experiments which investigate the mechanic effects

## CONCLUSIONS AND FUTURE PERSPECTIVES

of the contaminants. One possibility could be to use microarray assays, as these can measure the expression levels of a large number of genes simultaneously.

Further, one of the characteristics of mesenchymal stem cells (MSCs) is the ability to differentiate adipocytes, osteoblasts, and chondroblasts. Thus, the ability of pbASCs of osteogenic and chondrogenic differentiation should be investigated, and perhaps pbASCs can function as an *in vitro* model for effects of environmental contamination on osteogenic and chondrogenic differentiation as well.

As a part of the “Contaminant effects on energetics” project the effect of contaminant mixtures, consisting of contaminants at the composition and concentration found in polar bear adipose tissue, on adipogenesis in 3T3-L1 cells have been conducted. Although 3T3-L1 cells are a well-studied model of adipogenesis they may not be able to accurately reflect the effects of contaminants on adipocyte differentiation in MSCs. The 3T3-L1 cells are a continuous cell line, and differentiation properties may differ from that of stem cells isolated from tissue (Bjorntorp et al., 1980, Qian et al., 2010). For example, in the present study BPA did not induce adipocyte differentiation, although it is known to do so in 3T3-L1 cells. It is possible that BPA is not the only EDC that affects pbASCs differently than 3T3-L1 cells. This could be investigated by comparing results from exposure studies on pbASCs and 3T3-L1 cells, or by comparing to previously conducted studies using 3T3-L1 cells.

PbASCs as an *in vitro* model for effects of environmental contaminants on adipogenesis can provide valuable information on how contaminant exposure can affect energy homeostasis in polar bears. Exposure studies using *in vitro* models allow us to evaluate mechanisms of contaminants and examine dose-response relationships (DeRaso, 1993). Thus, the use of *in vitro* studies can provide information that cannot be obtained by field or correlation studies of polar bears. By combining findings from *in vitro* and field studies it is possible to gain a more comprehensive picture of effects of environmental contaminants and climate warming on polar bear health and survival.

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## APPENDIX A

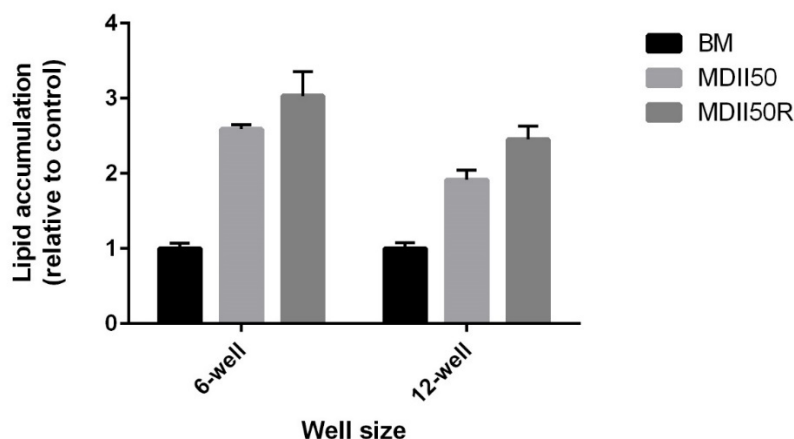
### SUITABLE CULTURE PLATE SIZES FOR EXPERIMENTS

It was desirable to look at the possibility of using 12-well culturing plates instead of 6-well culturing plates, which is very commonly used for both Oil Red O staining and RNA extraction, as this will require less than half the amount of cells needed for each well. We have a limited quantity of polar bear ASCs available, and it is therefore desirable to reduce the number of cells needed for each experiment.

#### Oil red O staining

Confluent cells were cultured in 6- or 12-well culturing plates for two weeks in BM, MDII50 or MDII50R medium (Table 3.1), and subsequently stained with Oil Red O (ORO). ORO was eluted by incubating the 6-well plates in 1 mL of 100 % isopropanol for 10 minutes, while 12-well plates were incubated in 0.5 mL 100 % isopropanol. The absorbance was measured at 500 nm.

The results showed the same trends in both the 6- and 12- well samples, but the measured lipid content was a bit lower in the cells cultivated in 12-well plates (Fig. A.1.). Based on these results it was decided to use 12- well culturing plates for exposure experiments.



**Figure A.1 Effect of well size on measured fold induction**

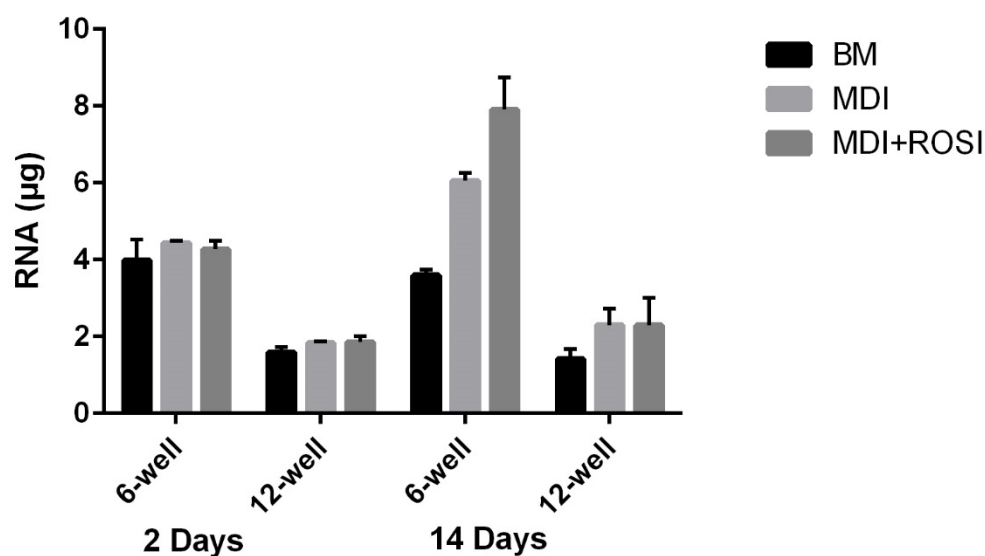
PbASCs in 6- or 12-well culturing plates were incubated with MDII50 (Table 3.1.) supplemented with 0.2 % DMSO or MDII50R for two weeks, and subsequently fixated and stained with Oil Red O (ORO). The absorbance of eluted ORO was measured at 500nm to quantify lipid content. The experiment was conducted in duplicates.



### Real time Q-PCR analysis

Cells were cultured in 6- or 12-well culturing plates for two and 14 days in BM, MDI or MDI+1  $\mu$ M ROSI (Table 3.2.). RNA was extracted, and the concentration measured using Nanodrop <sup>®</sup>. Over twice as much RNA could be extracted from 6-well plates compared to 12-well plates (Fig. A.2.).

For cDNA synthesis 0.5  $\mu$ g of RNA is needed, and the lowest amount of RNA extracted in any of the 12-well plates were 1.4  $\mu$ g (BM, 14 days), which is almost three times the amount needed. Also here it was decided that 12- well culturing plates would be used for exposure experiments, as this would require much fewer cells.



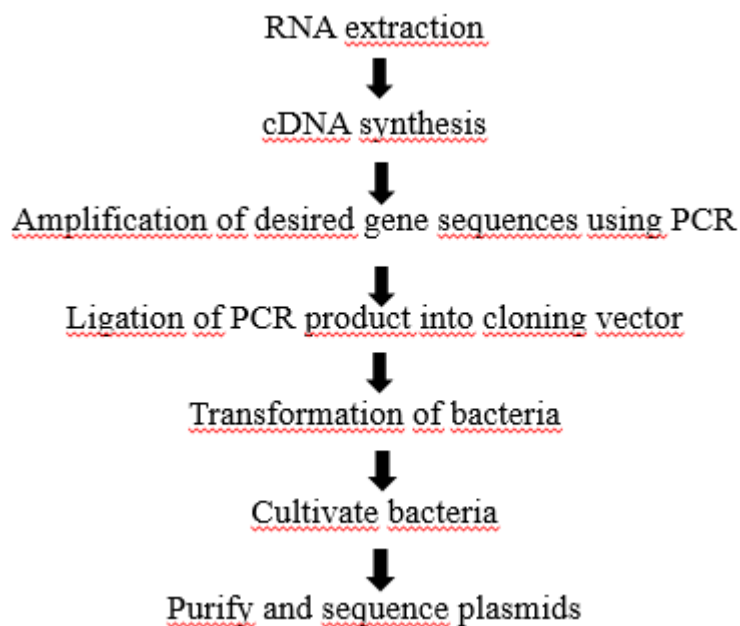
**Figure A.2 Effect of well size on measured fold induction**

PbASCs were incubated with MDI (Table 3.2.) supplemented with 0.2 % DMSO or 1  $\mu$ M ROSI for two days, and subsequently RNA was extracted. The absorbance of eluted ORO was measured at 500nm to quantify lipid content. The experiment was conducted in 6-well duplicates. Abbreviations:

## APPENDIX B

### PREPARATION OF STANDARD MATERIAL FOR REAL-TIME Q-PCR

PCR products consisting of *LEP* or *YWHAZ* gene sequence fragments were ligated into cloning vectors, transformed into bacteria for cloning, and subsequently purified and sequenced (Fig. B.1.). Only the molecular cloning results for the *LEP* PCR products will be shown, as the results for obtaining plasmids containing the *YWHAZ* gene sequence was equivalent to these results.



**Figure B.1 Strategy for obtaining plasmids containing selected gene sequence fragments.**

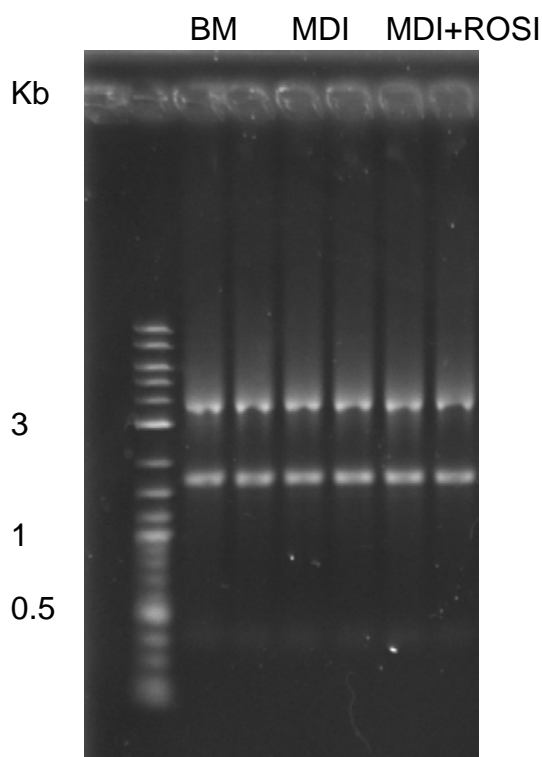
#### RNA extraction, purification and quality assessment

PbASCs was cultured in BM, MDI or MDI+1  $\mu$ M ROSI (Table 3.2.), following the general adipogenic induction protocol (3.3.1). Total RNA was extracted from the cells, and spectrophotometric measurements and denaturing AGE was conducted (3.5.1)

The A260/280 ratio suggested that the RNA were without contamination of proteins (Table B.1.). Denaturing AGE indicated that the integrity of the total RNA was conserved, as 28S and 18S ribosomal RNA was clearly visible as two strong bands (Fig. B.2.)

**Table B.1. Spectrophotometric measurements of concentration and purity of extracted total RNA from duplicate samples using NanoDrop.**

Media	Sample		Sample			
	No.	[ng/uL]	A <sub>260/280</sub>	No.	[ng/uL]	A <sub>260/280</sub>
BM	1	43.2	2.1	2	46.0	2.1
MDI	1	74.2	2.1	2	77.8	2.1
MDI+ROSI	1	91.0	2.1	2	106.3	2.1

**Figure B.2. Evaluation of extracted and purified total RNA by denaturing AGE.**

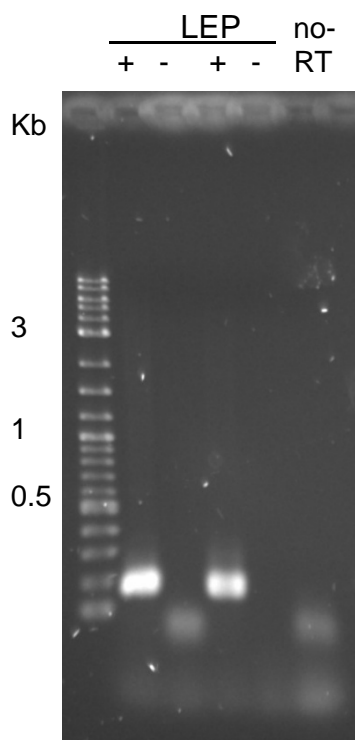
One hundred ng of total RNA extracted from pbASCs, which had been cultures for two weeks in media (BM, MDI or MDI+ROSI), were subjected to denaturing AGE (0.7 % agarose, 0.5 x TBE buffer), along with 100 ng 2-log DNA ladder.

### Synthesis of complementary DNA and amplification of the *LEP* fragment

Complementary DNA (cDNA) was synthesized from RNA (3.5.2), originating from MDI+ROSI sample number 1 (Table B.1.). Subsequently the *LEP* gene sequence fragment was amplified by PCR (3.5.3), using *LEP* specific primers (Table 2.9.).

The amplification product was approximately 200 bp (Fig. B.3), which coincide with the theoretical size of 171 bp. The non template control (NTC) yielded no such product, indicating that no contamination had occurred. A control reaction, where no reverse transcriptase (no-RT) was added during cDNA synthesis, yielded no PCR product at 200 bp,

indicating that the total RNA was free from genomic DNA contamination. The PCR seemingly yielded one product, as only one single product band was present on the agarose gel. This is an indication of high primer specificity.



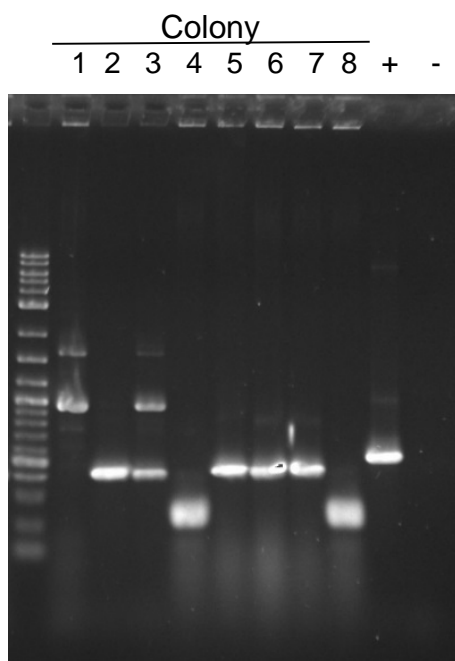
**Figure B.3 Evaluation of amplification of the *LEP* fragment.**

PCR, using cDNA as a template, was performed with *LEP* gene sequence specific primers. For each sample reaction a no template control was prepared. A no reverse transcriptase (no-RT), sample was also prepared. The samples were subjected to AGE (1 % agarose, 0.5 x TBE buffer), along with 100 ng 2-log DNA ladder.

#### **Preparation of plasmids containing the amplified *LEP* fragment**

The *LEP* fragment was ligated into a cloning vector, and the recombinant DNA was inserted into bacteria by transformation (3.5.6). To confirm that ampicillin resistant colonies contained the recombinant DNA a colony PCR was conducted (3.5.7).

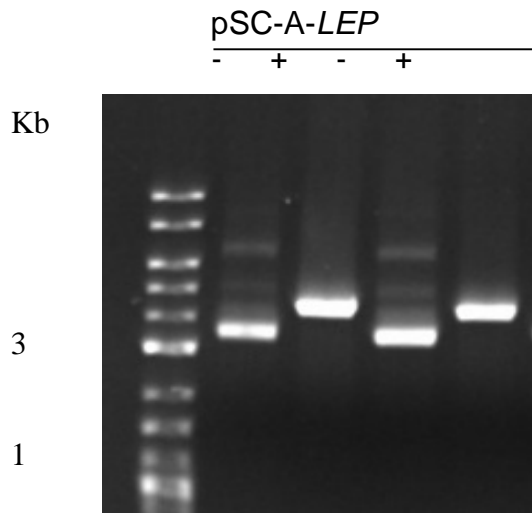
PCR products of approximately 400 bp indicated that the plasmids used for template in the reaction contained the *LEP* fragment, as the theoretical size of the amplification product was 390 bp (Fig B.4.). By comparing the sequencing results from Sanger sequencing (3.5.9) to the theoretical sequence, it was confirmed that the amplified sequence was the intended *LEP* sequence.



**Figure B.4. Identification of transformed bacteria containing plasmids with the *LEP* gene sequence.** Eight ampicillin resistant bacteria colonies were subjected to colony PCR to identify which of the colonies that contained plasmids ligated with the amplified *LEP* fragment, using vector specific primers. A control plasmid (pSC\_A-ADIPOQ) was used as a positive control, and a no template control was also prepared. The samples were subjected to AGE (1 % agarose, 0.5 x TBE buffer), along with 100 ng 2-log DNA ladder.

#### **Linearization of plasmids (pSC-A-*LEP*)**

Plasmids containing the *LEP* gene sequence fragment were linearized using NotI restriction enzyme (3.5.10). By subjecting the samples to AGE it was confirmed that the digestion was complete (Fig. B.5.). The theoretical size of the plasmids were 4441 bp, and the digested samples produced only one band at approximately 4500 bp, indicating that all of the plasmids had been linearized. Undigested samples yielded three distinct bands, one for each possible shape of the plasmid. The strong band at approximately 3500 bp represents the intact plasmids, as these are in a supercoiled state and therefore runs faster through the gel. Plasmids with one nicked DNA strand are located in the upper band, at approximately 7000 bp, as these run slower through the gel due to their circular shape.



**Figure B.5. Plasmids linearized by restriction enzymes.**

pSC-A-LEP plasmids were linearized using the restriction enzyme Not I. The restriction digestion samples, and undigested control samples, were subjected to AGE (0.4 % agarose, 0.5 x TBE buffer), along with 100 ng 2-log DNA ladder.

## APPENDIX C

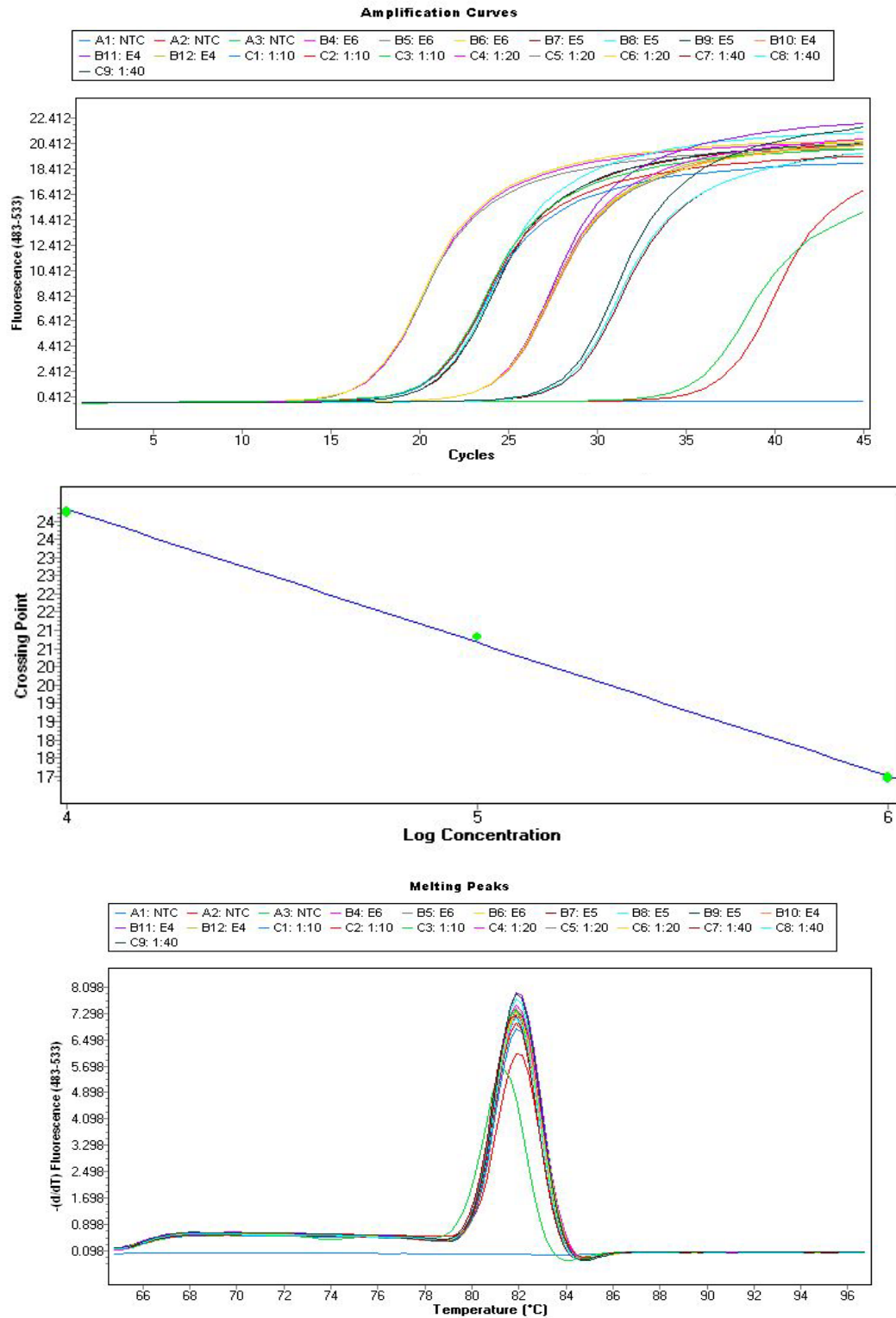
### EVALUATION OF STABILITY OF THE REFERENCE GENES *YWHAZ* AND *PPIA* ACROSS EXPERIMENTAL CONDITIONS

The stability of the reference genes tyrosine 3/tryptophan 5-monooxygenase activation protein (*YWHAZ*) and cyclophilin A (*PPIA*) were evaluated by evaluating variance in the  $C_T$  values for all *YWHAZ* and *PPIA* samples originating from the experiment where the gene expression of selected target genes during adipogenic differentiation were investigated (n=23, one sample was omitted as no  $C_T$  value was obtained for that sample) using RefFinder. RefFinder uses software from four of the most common methods used to evaluate gene stability, including Genorm, NormFinder, and BestKeeper. All methods showed similar results. The results from BestKeeper were as follows: Mean of  $C_T$  of *YWHAZ* samples was 20.35, with a SD of 0.26, while the mean  $C_T$  and SD for *PPIA* samples were 18.78 and 0.19. The Pearson correlation coefficient and P-value were 0.857 and 0.001 for *YWHAZ* samples, and 0.730 and 0.001 for *PPIA* samples, respectively. These results indicate that the reference genes were stable across experimental conditions (Pfaffl *et al.*, 2004). Thus, *YWHAZ* and *PPIA* were considered suitable reference genes for studying adipogenesis in pbASCs.

### STANDARD CURVES USED IN REAL-TIME Q-PCR

Standard curves were created by serial dilutions of linearized pSC-A plasmids containing the selected gene sequences (*PPAR $\gamma$* , *FABP4*, *LEP*, *YWHAZ* or *PPIA*). The biomath calculator from Promega was used to convert the spectrophotometrically measured plasmid concentration (Nanodrop®) from ng/ $\mu$ L to pmol/ $\mu$ L. From this the number of molecules per  $\mu$ L was calculated, and subsequently dilutions containing the desired start concentration (e.g.  $2 \times 10^8$  molecules/ $\mu$ L) were made. Serial dilutions were carried out with a dilution factor of ten, using ultra-pure water. 1:10, 1:20 and 1:40 dilutions of pooled cDNA was also measured, to evaluate which dilution factor that was most suitable to use. Samples were run as previously described (3.6).

It was decided that a 1:10 dilution of cDNA samples could be used for all real-time Q-PCR analysis.



**Figure C.1. Amplification, standard and melting curves of the pSC-A plasmid containing the pbYWHAZ gene fragment in a dilution series of  $10^6$ - $10^4$  molecules. Amplification efficiency was 1.877.**



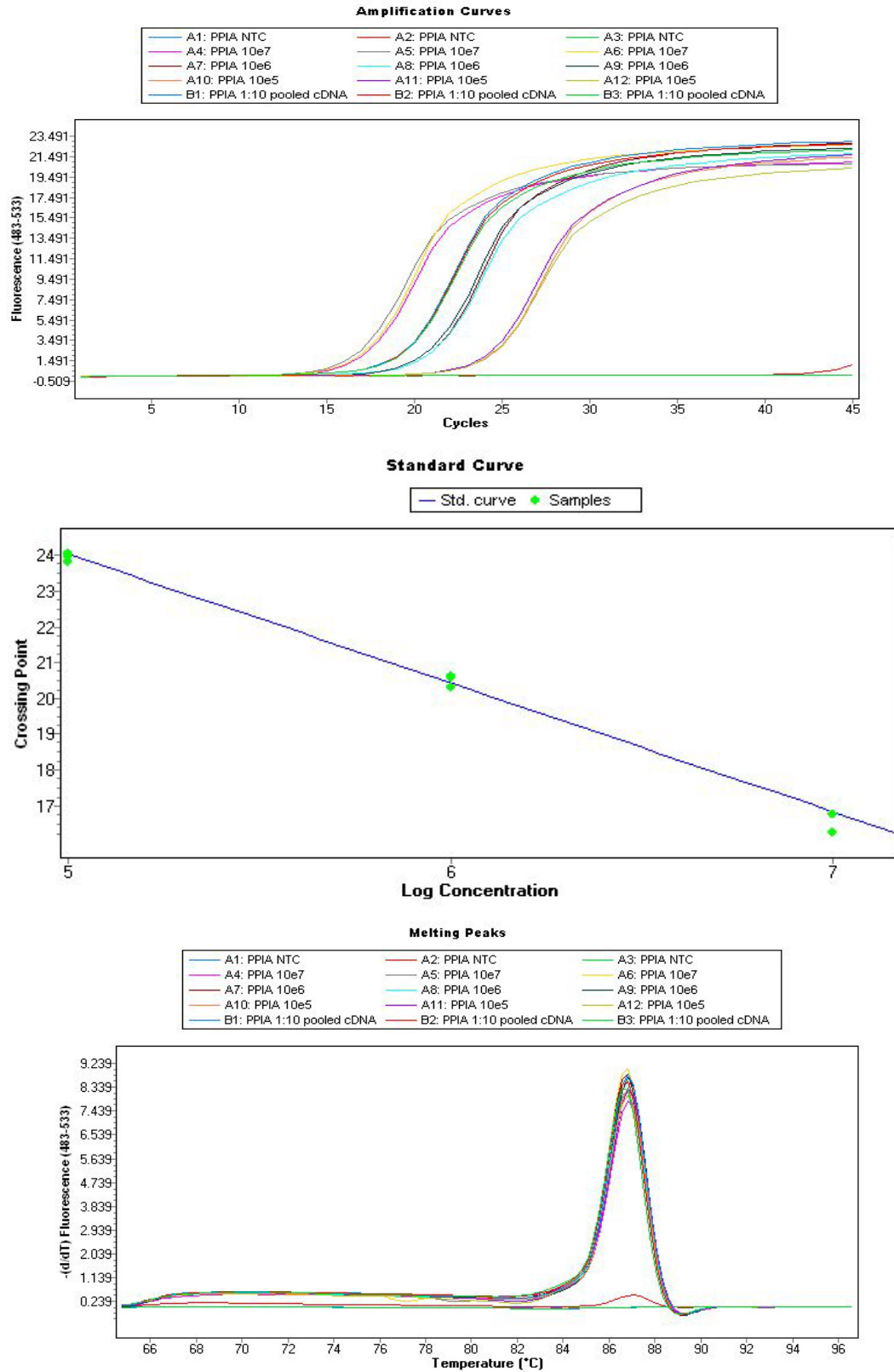
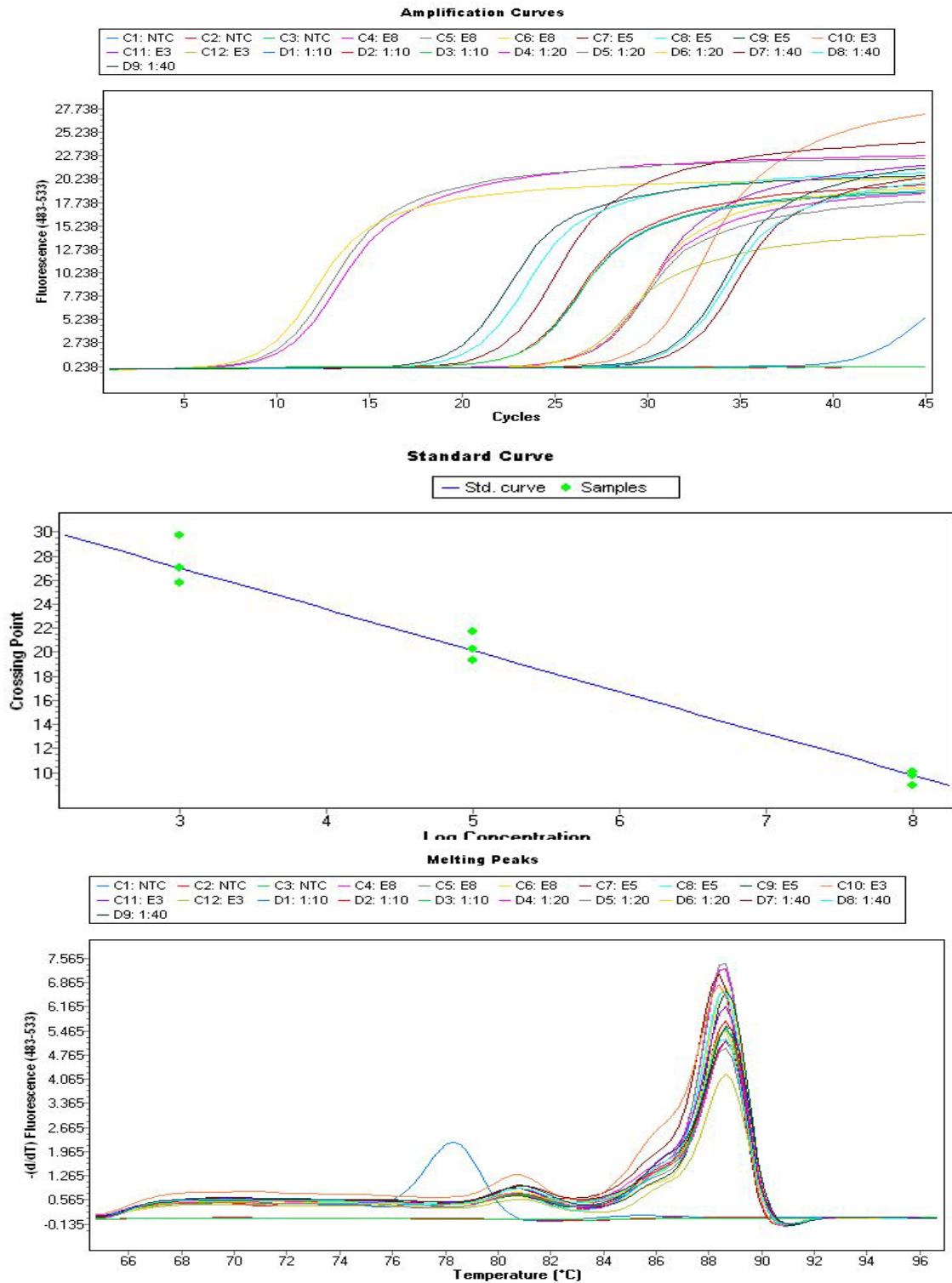
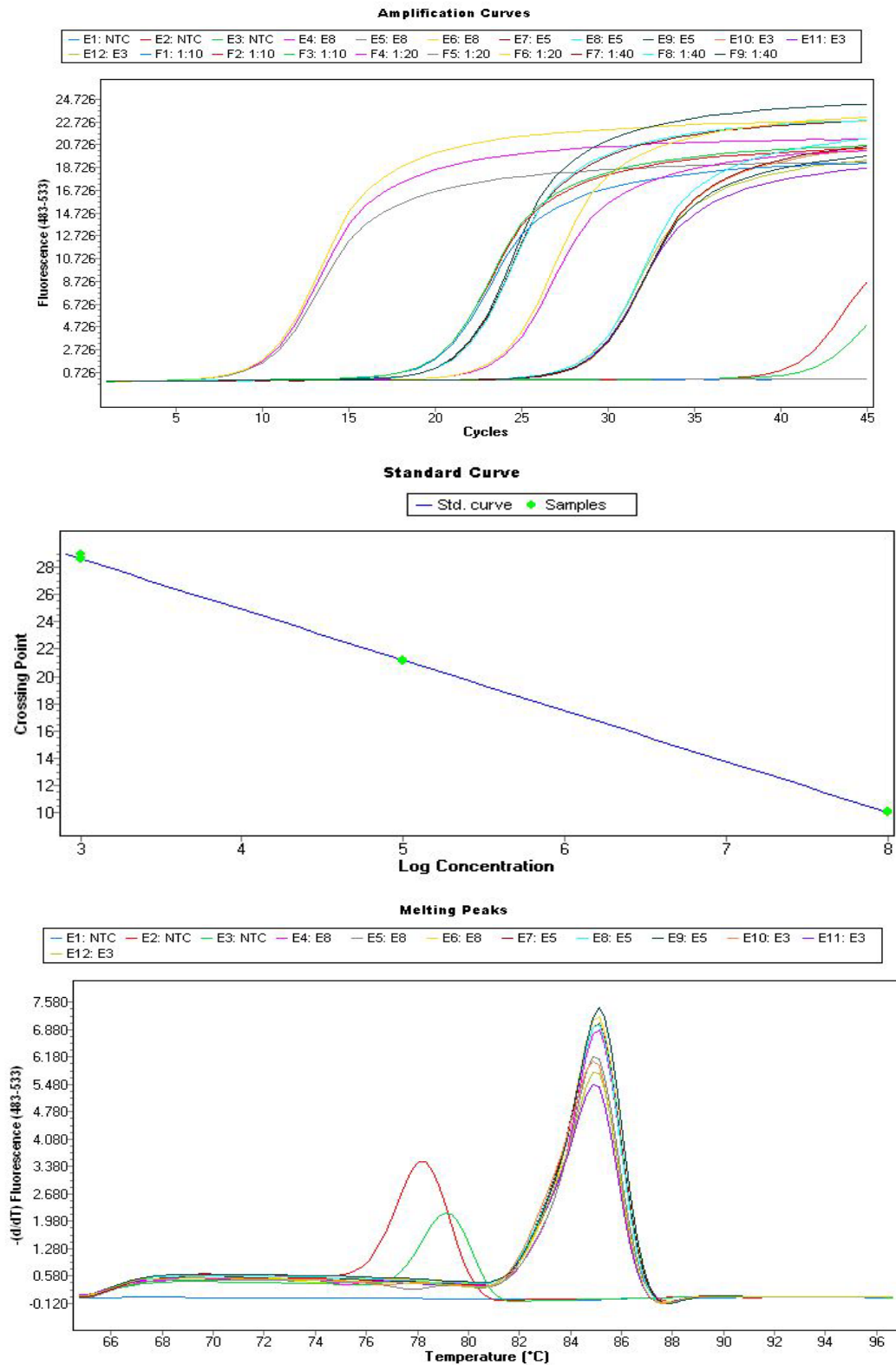


Figure C.2. Amplification, standard and melting curves of the pSC-A plasmid containing the pbPPIA gene fragment in a dilution series of  $10^7$ - $10^5$  molecules Amplification efficiency was 1.894.



**Figure C.3. Amplification, standard and melting curves of the pSC-A plasmid containing the pbPPAR $\gamma$  gene fragment in a dilution series of  $10^3$ ,  $10^5$  and  $10^8$  molecules. Amplification efficiency was 1.949.**



**Figure C.4.** Amplification, standard and melting curves of the pSC-A plasmid containing the pb*FABP4* gene fragment in a dilution series of  $10^3$ ,  $10^5$  and  $10^8$  molecules. Amplification efficiency was 1.857.

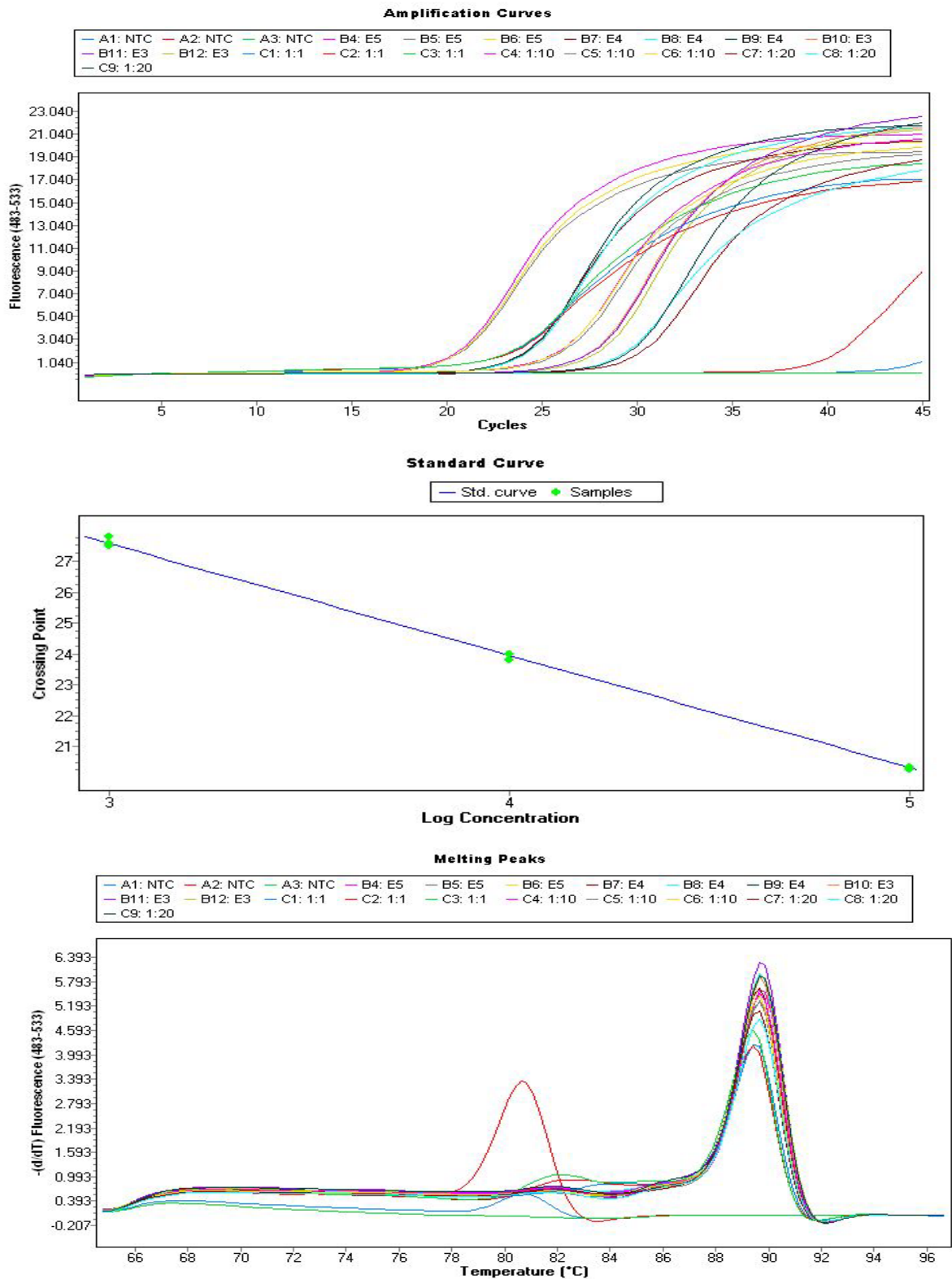


Figure C.5. Amplification, standard and melting curves of the pSC-A plasmid containing the pbLEP gene fragment in a dilution series of  $10^3$ ,  $10^5$  and  $10^8$  molecules. Amplification efficiency was 1.885.