# Establishing fin whale fibroblast culture conditions for toxicological studies

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# Abstract

Several marine mammal species have been hunted to near extinction by humans. As such, many of these species are vulnerable to population reductions due to changing climate, destruction of habitats, and environmental pollution. Environmental pollution is a huge problem and there is a lack of knowledge about how it impacts these vulnerable populations. Thus, toxicological studies of marine mammal species are important, also due to interspecies differences in reactions to toxicants. However, standard animal experiments are not options for most of these species. One alternative is to procure tissue and blood samples from individuals without killing them. These tissues can be used in several studies, e.g., determining toxicant concentrations in the individual or establishing cell cultures.

In this study, fibroblast cells from fin whales were used in toxicological studies. Firstly, better culture conditions were explored to improve cell growth. Secondly, the cells were exposed to benzo[a]pyrene and mono-(2-ethylhexyl) phthalate and the effects of expression of selected biomarker genes were tested. Lastly, the cells were exposed to a chemical cocktail in an attempt to reprogram the cells into mesenchymal stem cells (iMSCs).

The cells grew faster when bFGF was added to the medium while collagen coating had no detectable effect. No significant changes in gene expression levels of biomarkers were detected from cells exposed to the environmental toxicants. The MSC induction experiment resulted in cells of altered morphology that may suggest generation of MSC, but qPCR analysis of putative markers showed no significant expression changes.

# Abbreviations

| | |
|---|---|
| AHR | Aryl hydrocarbon receptor |
| AHRR | AHR repressor |
| ANOVA | Analysis of variance |
| APS | Ammonium persulphate |
| BaP | Benzo[a]pyrene |
| bFGF | Basic fibroblast growth factor |
| BSA | Bovine Serum Albumin |
| cDNA | Complementary DNA |
| CYP | Cytochrome P450 |
| DDT | Dichlorodiphenyltrichloroethane |
| DEHP | Di-(2-ethylhexyl) phthalate |
| DEX | Dexamethasone |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco's PBS |
| $EC_{50}$ | 50% of maximal effective concentration |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| ESC | Embryonic stem cells |
| FBS | Fetal Bovine Serum |
| FC | Fold change |
| HSP | Heat shock protein |
| IBMX | 3-isobutyl-1-methylxanthine |
| iMSC | Induced MSC |
| iPSC | Induced PSC |
| IWC | International Whaling Commision |
| LDH | Lactate Dehydrogenase |
| LIF | Leukemia inhibitory factor |
| MEHP | Mono-(2-ethylhexyl) phthalate |
| mRNA | Messenger RNA |
| MSC | Mesenchymal stem cell |
| MW | Molecular weight |
| NRT | No reverse transcriptase |
| OCT4 | Octamer-binding transcription factor 4 |
| OHC | Organohalogen contaminant |
| ORO | Oil red O |
| PAH | Polycyclic aromatic hydrocarbon |
| PBDE | Polybrominated diphenyl ether |
| PBS | Phosphate-Buffered Saline |
| PCB | Polychlorinated Biphenyl |
| PCR | Polymerase chain reaction |
| POP | Persistent organic pollutant |
| PPARG | Peroxisome proliferator-activated receptor gamma |
| PSC | Pluripotent stem cells |
| PVDF | Polyvinylidene fluoride |
| qPCR | Quantitative real time polymerase chain reaction |
| RNA | Ribonucleic acid |
| RT | Room temperature |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | SDS- polyacrylamide gel electrophoresis |
| SOX2 | Sex determining region Y-box 2 |
| TBS | Trisbuffered saline |

| | |
|---|---|
| TEMED | Tetramethylethylenediamine |
| TGF-β | Transforming growth factor-β |

# 1. Introduction

## 1.1 Background

Marine mammals consist of three living groups that spend most of — if not all of — their lives in the Ocean and bodies of water (Rice, 2008). The groups are cetaceans, pinnipeds, and sirenians. These are primarily oceanic; however, several members of each group spread into freshwater habitats. Additionally, several other species of mammals have become facultative or obligate members of the marine ecosystem even if their lives are more terrestrial, such as the polar bear (*Ursus maritimus*), the artic fox (*Vulpes lagopus*), and otters (family Mustelidae: subfamily Lutrinae).

Considering that bodies of water cover most of Earth's surface, their habitats are oftentimes extremely large, though, this is not always the case. Arctic species like the bowhead whale (*Balaena mysticetus*) and polar bears are restricted to arctic waters due to their ecological specialisation. On the other hand, the blue whale (*Balaenoptera musculus*) can be found in all oceans except some regional seas like the Mediterranean, Okhotsk, and Bering Sea (Cooke, 2018a). Unfortunately, this can make it difficult to study them. The ones with large habitats can be spread across it, making them sparse. Even the ones with a relatively small habitat can be difficult to find — especially the three groups that primarily lives in water as they can be spread across the entire volume of their habitat.

Whales (Cetaceans) are a diverse group of marine mammals (Ballance, 2017). They have a wide size range from less than one and a half meters long vaquitas (*Phocoena sinus*) to over 33 meters long blue whales (Cooke, 2018a; Rojas-Bracho *et al.*, 2018). As such, the group contains the largest animals in the world.

The great whales are a name given to the thirteen largest extant whales (McVay, 1966; Kenney, 2018). Most of these whales were greatly impacted by the commercial whaling and are now protected species (McVay, 1966; Gambell, 1977; Clapham and Baker, 2008).

## 1.2 Hunting and exploitation of marine mammals

Humans have a long history of hunting and exploiting marine mammals (Reeves, 2017). Every taxonomic group of marine mammals have been a target by human hunters. The rewards of felling them were high, giving the hunters substantial amounts of nutritious meat and fat, hides, ivory, sinews for sewing, and bones for making household implements or weapons. Though, some species were more attractive as prey. Hunters targeted species that gave them the rewards they were seeking, such as oil, meat, and baleen from the great whales; oil and pelts from pinnipeds; furs from otters and polar bears. However, sometimes they would target and kill the marine mammals that they came across while searching for specific species — e.g., capturing pinnipeds while searching for right whales mainly for their baleen (Tjernshaugen, 2018).

This hunting and overexploitation have decimated several marine mammal species, causing many of them to become endangered.

### 1.2.1 Traditional Whaling

Whaling has been a long-standing tradition for many different coastal communities, lasting over several hundreds of years (Ellis, 2017; Tjernshaugen, 2018). A lot of the earliest whaling history is lacking due to little or no records. We do have clear records that the Basques were a people of whale hunters. All records we have of them show their whaling activities and it is suspected that they were whaling long before records start. Most authors cite that they started around the year AD 1000. While that can be disputed, they did start the whaling industry by that time, being, as far as we know, the first people to hunt large whales in an organised and intentional manner. The industry took until the 12$^{th}$ century to fully develop. They established a pattern and methods for whaling that remained mostly unchanged until the onset of modern whaling in the late 19$^{th}$ century (Clapham and Baker, 2017; Ellis, 2017; Tjernshaugen, 2018).

Whalers pursued and fought whales with small boats powered by wind or the strength of the men rowing. At the start, the boats were dispatched from land when lockouts spotted whales with the help of towers along the shore. As navigation became better, they went further away from the shore and carried whaling equipment with sail boats. They still used the small boats, which they lowered from the bigger sailboat. These small boats were double ended which made it easier to manoeuvre despite the frenzy of the whale fights. The whalers rowed after the whales and attached the animals to the boats with hand-thrown harpoons. After fastening the whale to the boat, they could stab the being with lances. The fights often lasted for long periods of time and the whales could drag the boats long distances or potentially counterattack. Once they killed the whale, they initially flensed the carcass and packed the blubber into casks for home transport. The blubber was processed on shore. After tryworks, iron caldrons set in a brick furnace, evolved around the year 1750, the blubber processing happened on board of the ship. This expanded the hunting grounds for whales.

*Figure 1.1: Painting by Johannes Becx (fl. c1658-92) "A Fleet of Dutch Whalers Under Sail in the North." Oil on canvas from 1660s-70s Credit: New Bedford Whaling Museum*

However, not all whales were threatened by the traditional hunting methods (Clapham and Baker, 2017; Cooke, 2018b; Tjernshaugen, 2018). Some members of the rorquals, such as fin and blue whales, were basically impossible to hunt with traditional methods. This was due to several factors. The whales were streamlined and fast swimmers — fin whales are the fastest and can maintain speeds over 10 $ms^{-1}$ — which made it difficult to catch up to them with row boats. Another factor was that the whales had an immense strength that made them more dangerous for the men in the small boats. Additionally, these whales had a lower fat percentage which caused them to sink when they died. Thus, they would at least need a ship strong enough to support the dead weight of the whale if they wanted to harvest the whale bounty.

### 1.2.2 Modern Whaling

By the late 19$^{th}$ century, modern whaling was kicked into gear by a series of inventions (Clapham and Baker, 2017; Tjernshaugen, 2018). These inventions allowed the whalers to target and successfully capture whale species that, until this point, managed to avoid whaling. One of these inventions was the grenade harpoon invented by the Norwegian Sven Foyn. While this weapon became the industry standard, Foyn was not the first or only one who experimented with explosive harpoons — Thomas Welcome Roys created his own version independently of Foyn, though Roys inspired Foyn in his own work. Foyn's grenade harpoon consisted of a harpoon with a pointed grenade tip. They shot the harpoon from a bow-mounted cannon. The harpoon would pierce into the whale, fastening a line to it while the explosion from the grenade would kill or badly injure the whale. Thus, it made for an efficient weapon to kill and capture whales. Another advantage was that the whale was blown up with gasses from the explosions,

which helped keeping the whales afloat. As such, this weapon removed the danger of directly engaging with the whale while also keeping carcasses floating and accessible for harvest. Later on, whaling methods was further refined with the addition of a compressor that pumped air into whale carcasses immediately after death.

Another invention that was important for this development was the steam engine (Clapham and Baker, 2017; Tjernshaugen, 2018). The steam engine allowed the ships to reach the speeds of the fastest whales. No whale was able to outswim the whalers anymore. These inventions alone increased the catching rate of whales, though it was initially constrained by the need to use land stations for processing the carcass.

Factory ships further elevated whaling efficiency (Clapham and Baker, 2017; Tjernshaugen, 2018). Whaling ships dragged whale carcasses to these ships for processing. The first factory ships were dependent on protected harbours for their processing. However, in 1925 the first stern-slip factory ship created another increase in whaling efficiency. This ship was the first of many made and used. It allowed the whalers to hoist the whale carcasses onto the decks for flensing and processing. These ships could operate independently for months far out on the sea. They expanded the whaling range significantly and were astonishingly efficient at processing the carcasses supplied from an attendant fleet of catcher boats. Now there were no place for the whales to escape them.

This resulted in a collapse in many whale populations and the near extermination of the most heavily exploited species (McVay, 1966; Tjernshaugen, 2018). As an attempt to gain a sustainable whaling industry, the whaling nations developed a quota system in 1932, the "Blue Whale Unit" (bwu). Nations got a quota in bwu which determined the number of whales they could hunt. One bwu was equal to one blue whale, two fin whales, two and a half humpback whales, or six sei whales. When the International Whaling Commission (IWC) was established in 1946 to regulate whaling and overseeing whale stock research, they had difficulties in setting sustainable quotas for the different great whales. The bwu quota system remained in place until 1972 despite recommendations from IWC scientists to abolish it since 1963.

Nowadays, there are still some smaller scale whale catches in the world (Ellis, 2017; Kasuya, 2017; Tjernshaugen, 2018). Norway catches common minke whales (*Balaenoptera acutorostrata*) while Iceland may also catch fin whales. Inuits of Greenland still catch some fin whales, bowhead whales, and humpback whales for subsistence with permission from the IWC and following a set quota (Ellis, 2017; Tjernshaugen, 2018). On the other hand, Japan issued permits to catch whales for scientific and research purposes. However, it is debatable how useful this is for the research. The fact that the meat from the whales end up sold as food for human consumption causes more questions about the necessity of this scientific whaling.

While whaling now poses a small threat to whale populations, there are still other threats to the species (Cooke, 2018b; Panigada, Gauffier and Notarbartolo di Sciara, 2021). Climate change, ocean acidification, vessel collisions, entanglement in fishing gear, and environmental pollution are all potential threats to the large whales, including the fin whale. All of these can negatively impact the populations, and all contain factors of uncertainty. There is also the fact that it may be hard to separate these factors from each other: Climate change, ocean acidification, and pollution can affect the environment at several levels, making it hard to distinguish which factor caused what (Dietz *et al.*, 2019). On the other hand, people need to report in incidents to get an accurate measurement of how big of an impact vessel collisions and entanglement in fishing gear have on whale populations. Failure to report can have several reasons, one of which is because the incidents were undetected. As such, these factors may have a bigger impact than it currently seems.

## 1.3 The Fin whale

The fin whale (*Balaenoptera physalus*) is one of the ocean's giants, second only to the blue whale (Aguilar and García-Vernet, 2017). These large animals roam most of the world's oceans, though they mainly occur in offshore waters of the temperate and subpolar zones (Edwards *et al.*, 2015). This species used to have a significant number of individuals. However, the fin whale was one of the great whales that was hit hard by the whaling industry. Their abundance was decreased by over 70% during the commercial whaling era, from the late 19$^{th}$ century until ~1986

(Brownell and Yablokov, 2008; Cooke, 2018b). Even now, years later, the species is listed as vulnerable by the IUCN red list with approximately 100 000 mature individuals globally (Cooke, 2018b).

*Figure 1.2: Fin whale from above.*
*Credit: NOAA Northeast Fisheries Science Center/Peter Duley*

Fin whales are filter feeders that feeds on a wide variety of organisms (Aguilar and García-Vernet, 2017; Goksøyr and Routti, 2021). Their prey is often from the lower levels of the food chain.

While the fin whale population on a global scale is vulnerable, the population in the Mediterranean Sea is endangered (Cooke, 2018b; Panigada, Gauffier and Notarbartolo di Sciara, 2021). The Mediterranean Sea has heavy vessel traffic and has unusually high collision rates with fin whales with a mean annual fatal collision rate increased from 1 to 1.7 whales per year from the 1970s to the 1990s. Vessel disturbance and underwater noise are other factors that affect Mediterranean fin whales. These factors can interrupt and impact important behaviours, potentially leading them to be unable to perform natural behaviours required for their continued survival. Another threat is that the Mediterranean Sea has a limited water exchange and has several heavily populated and industrialised populations around it, which causes high levels of contamination by several environmental pollutants, including persistent organic pollutants (POPs) and microplastics.

These are all important facets when studying fin whale threats. However, looking at all of these factors is beyond the scope of this thesis. As such, this thesis will focus on one of them: Environmental pollution and its potential effects on fin whales, and how we can study them.

## 1.4 Environmental concerns

Environmental pollution is a rising concern and a massive global problem. Different species experience different levels of, and responses to, pollution. Their position in the food chain can affect their pollutant intake. Pollutants that bioaccumulate — e.g., POPs like PCB, DDT, and dioxins — are more present in top predators like the orca and polar bear while filter feeders like the fin whale bioaccumulate lower levels of the pollutants (Fossi *et al.*, 1992; Desforges *et al.*, 2018; Blévin *et al.*, 2020; Tartu *et al.*, 2020). Top predators gain higher levels of lipid contaminations due to biomagnification. For example, Blévin *et al.* (2020) found that female polar bears that depend on sea ice as a hunting

platform had a ΣPCB content of ~2183.5 ng/g lipid weight. Krahn *et al.* (2014) found a ΣPCB level of 1300 µg/g lipid weight in a female orca. On the other hand, Tartu *et al.* (2020) found that female blue whales had a ΣPCB of ~86 ng/g lipid weight, and that female fin whales had a ΣPCB of ~219 ng/g lipid weight. This reflects their trophic placement as the fin whale has a higher trophic level diet than the blue whale (Tartu *et al.*, 2020). These examples clearly illustrate that the potential effects — or at the least their level of exposure — of the environmental pollutants partially depends on their placement in the food chain.

The areas species inhabit can also affect contaminant levels due to differences in pollution levels, as Desforges *et al*. (2018) demonstrate for orca populations around the world, and Fossi *et al.* (2010) demonstrate between fin whales from the Mediterranean Sea and fin whales from the Gulf of California. They looked at several compounds and compared contaminant levels between populations and between the sexes. Male fin whales from the Mediterranean Sea had ~8000 ng/g dry weight (d.w.) content of ΣPCB and ~7000 ng/g d.w. content of ΣDDT. In comparison, male fin whales from the Gulf of California had ~1000 ng/g d.w. ΣPCB and ~3800 ng/g d.w. ΣDDT. An interesting thing to note is that females have lower levels of PCBs and DDTs. Mediterranean female fin whales had ~2000 ng/g d.w. ΣPCB and ~900 ng/g d.w. ΣDDT. Gulf of California female fin whales had ~900 ng/g d.w. ΣPCB and ~400 ng/g ΣDDT. This difference corresponds with another study by Aguilar and Borrel (1994) where they speculate that females potentially transfer their body burden of organochlorides to their foetuses through lipid transfer. They found that the body load of PCBs and DDTs decreased with age in females while it increased in males. Desforges, Ross, and Loseto (2012) investigated the transplacental transfer of PCBs and polybrominated diphenyl ethers (PBDEs) in beluga whales. They found that lighter congers transferred more readily, and the average transfer rate was 11.4% for ΣPCBs and 11.1% for ΣPBDEs. Wolkers, Lydersen, and Kovacs (2004) studied the lactational transfer of PCBs and pesticides from female harbour seals to their pups. They also found a lower concentration of ΣPCBs in the females compared to the males. Thus, sex and age of an individual likely influence accumulated contaminant levels.

Many marine mammals have reduced population, and some species experience further population reductions due to changing climate. Polar bears are affected by the reduced sea ice (Rode and Stirling, 2017). The ice-breeding seals have less ice area to bear their young (Bowen, 2017). Changes in ecosystems and food supplies can affect many different species — marine mammals and other animal groups (Hoegh-Guldberg *et al.*, 2007; Bowen, 2017; Marsh, 2017; Rode and Stirling, 2017; Dietz *et al.*, 2019). This means that adding stress from environmental contaminants can further stress and reduce animal populations. As such, it is important to study and estimate the threat-level of varying pollutants. Desforges *et al*. (2018) compiled data on blubber PCB concentrations within various orca populations and compared them to established concentration-response relationships for reproductive impairment and immunotoxicity-related disease mortality. Their models predicted that 10 of 19 orca population are at moderate or high risk of population-level effects.

As different species react differently to various threats, it is important to gain knowledge about these differences. This can give us a more accurate picture of how environmental toxicants may affect species. Normally, controlled lab experiments can give us this type of information. However, standard animal experiments are functionally impossible. Firstly, their low population and status as "protected species" make it unethical. It also causes limits for hunting them for research. Secondly, they are long-lived — some species of cetaceans can live well over 100 years — and have long generation times, which means that proper experiments would take years or even decades. Bowhead whale, for example, are assumed capable of living for over 200 years and has an estimated mean generation time of about 52 years (Cooke and Reeves, 2018). Thirdly, these animals are large and require massive areas to thrive. As previously stated, blue whales have a large habitat. They also perform diverse migratory patterns, some of which are long (Cooke, 2018a). Thus, it would be practically impossible to create a controlled environment for the experiment. Additionally, conservation efforts limit what types of experiments one can do. Therefore, scientists must develop experimental designs that are practical even with these restrictions.

One form of experimental designs is to procure tissue and blood samples from living individuals without killing the animal (Dietz *et al.*, 2019). Dietz *et al.* (2019) describes several correlation studies between molecular and physiological parameters, and in environmental contaminant levels measured in biopsies procured from animals. These samples can also be used as basis for cell cultures, on which different and relevant tests and analyses can be performed.

## 1.4.1 Toxicological studies in marine mammals

### *1.4.1.1 Finding non-lethal methods to study toxicological hazards in marine mammals*

When scientists first started to gain a scientific interest in the ecotoxicology of marine mammals several of these species were already threatened (Fossi *et al.*, 2000). Most toxicological studies in the earlier years used marine mammals killed by hunting, thus tacitly approving of this activity (Fossi and Marsili, 1997). As the studies were based on dead marine mammals, there was a limited number of available individuals — though this was more of a problem for the endangered species (Fossi and Marsili, 1997; Fossi *et al.*, 2000). The first biomarker studies were based on dead individuals, though this started to change in the early 1990s when non-destructive biomarkers were proposed as an alternative hazard assessment (Fossi *et al.*, 1992, 1997; Fossi, 1994).

Transcriptome studies can help with mapping gene expression that can function as biomarkers (Stahl *et al.*, 2012). Contaminants can cause changes within the transcriptome through activation of various receptors, such as the aryl hydrocarbon receptor (AHR). Thus, methods that register these transcriptional changes can function as biomarkers for pollutant exposure.

One proposed biomarker is the induction of CYP1A by evaluating the benzo[a]pyrene monooxygenase activity (BPMO), ethoxyresorufin O-deethylase activity (EROD), protein detection, or mRNA analysis. This biomarker can give information about exposure to some pollutants such as dioxin-like PCBs and dioxins, and it is a much-used biomarker in many vertebrates (Omiecinski, Redlich and Costa, 1990; Fossi, Casini and Marsili, 2007; Webb *et al.*, 2014). It is also relevant for marine mammals and has become a common biomarker in marine mammal toxicological studies (Watanabe *et al.*, 1989; Fossi and Marsili, 1997; Fossi *et al.*, 2003; Fossi, Casini and Marsili, 2006).

There are several advantages with using skin biopsies in toxicological studies, including using them as basis for measuring several biomarkers such as CYP1A induction or immunological responses (Fossi *et al.*, 2003; Marsili *et al.*, 2019). A second advantage lies in the fact that they can serve as basis for cell cultures. Also, they can tell us about the level of different contaminants in the individuals, which is helpful knowledge in toxicology. Knowing contaminant levels can tell us about the potential risk and stress the pollutants bring upon individuals. Another advantage is that skin biopsies does not require the death of an animal. It enables the ability to gather a large amount of samples without affecting populations by diminishing their numbers. The sampling method can be practically harmless to the sampled individuals.

### *1.4.1.2 Previous hazard studies in marine mammals*

Researchers have performed toxicological studies in several marine mammalian species. Dietz *et al.* (2015) evaluated the possible links between exposure to organohalogen contaminants (OHCs) and specific health effects in polar bears. They used Risk Quotient (RQ) calculations along with a cross-species analysis of critical body residues (CBRs) to estimate the potential risk and severity of polar bear OHC exposure. While this can give valuable information, it assumes comparable sensitivity and OHCs endocrine disruptive mode of action between the species used in the analysis.

Burkard *et al.* (2015) established the first successful humpback whale fibroblast cell lines (designated HuWa1 and HuWa2). Their intentions were to develop and assess an *in vitro* toxicity approach. They assessed the viability of human (HFb) and humpback fibroblast (HuWa1) cells exposed to varying concentrations of p,p,'-DDE. The difference in calculated $EC_{50}$ was significant, with HuWa1 having an $EC_{50}$ about six-fold higher than HFb. As these exposures only contained one compound compared to what the humpback whale encounters in nature, they also exposed the HuWa1 cells to a chemical extract obtained from the blubber of a stranded individual. To make the results comparable, the content of p,p,'-DDE was measured and used to express the concentrations of the chemical extract. The assessed $ED_{50}$ values were ~1880 times lower, which shows that the extract likely had chemical interactions between its components. However, the authors note that further toxicological studies are required to provide further insight to the cell line's responsiveness.

Jenssen *et al.* (1995) found that the relationship between ΣPCB and the retinol (vitamin A) content in corresponding plasma samples from grey seal pups had a significant negative correlation. They also found that there was a borderline significant negative correlation between ΣPCB and the ratio between total thyroxine and free thyroxine (TT4/FT4). While the biological significance is unknown, thyroxine depletion can have severe consequences.

Several studies also looked at metal exposure and potential risks of exposure. One of the metals studied is hexavalent chromium (Cr(VI)) and its cytotoxicity and genotoxicity in several marine mammals (Li Chen *et al.*, 2009, 2012; Wise *et al.*, 2010, 2011, 2015; Meaza *et al.*, 2020). Li Chen *et al.* (2009) directly compared the cytotoxicity and genotoxicity of Cr(VI) in right whale and human lung cells. Due to uncertainty of chromium exposure in right whales, they exposed cells to sodium chromate and lead chromate. They found that the North Atlantic right whales had a lower uptake of both chromate variants, leading to lower toxicity in right whales. After correcting for the different uptakes, they found that cytotoxicity in right whale cells exposed to lead chromate was higher compared to human cells. On the other hand, cytotoxicity in right whale cells exposed to sodium chromate was lower. Also, genotoxicity was lower in right whale cells exposed to both chromate variants. Wise *et al.* performed the same Cr(VI) cytotoxicity and genotoxicity experiment on Steller sea lion lung fibroblasts. They demonstrated that these fibroblasts are less sensitive to the cytotoxic effects than the human fibroblasts. However, they have the same sensitivity to genotoxic effects.

### 1.4.2 Toxicological studies in fin whale

Fin whales have also been the subject of toxicological studies. Many studies have investigated the relationship between organochlorine contaminants (OCs) and CYP1A activity (e.g., Fossi *et al.*, 1992, 2000, 2003, 2010; Marsili *et al.*, 1998). Fossi *et al.* (2003) compared the levels of OCs with endocrine disrupting (ED) capacity with CYP1A induction and found that CYP1A induction works as a valid early warning sign of OC exposure. They also found that fin whale samples from the Mediterranean Sea had a high percentage of the DDT metabolite op'DDT, which is a weak oestrogen and antiandrogen. This could affect reproduction of the whales. A comparison between the ΣDDT levels found in the fin whales and the ΣDDT levels found in bowhead whales from Barrow, Alaska shows that the fin whales have more than 10 times the amount of ΣDDT than the bowhead whales (5169 ng/g wet weight in fin whales vs. 410 ng/g wet weight in bowhead whales) (Hoekstra *et al.*, 2002; Fossi *et al.*, 2003). This comparison is interesting because two specimens of bowhead whales from Wainwright, Alaska showed pseudohermaphroditism, which could signify an impact of endocrine disrupting OCs (Tarpley *et al.*, 1995).

Wise *et al.* (2015) measured the levels of Cr(VI) in fin whales and measured genotoxic and cytotoxic effects using the same Cr(VI) cytotoxicity and genotoxicity experiment as Li Chen *et al.* (2009). They measured total Cr levels in skin biopsies from nine free-ranging fin whales, six males and three females. Cr levels ranged from 1,71 to 19,6 µg/g tissue wet weight with an average level of 10,07 µg/g. Lead chromate is both cytotoxic and genotoxic to fin whale cells in concentration-dependant manner. Sodium chromate is more cytotoxic than lead chromate, but has a similar genotoxic response, when adjusting for intracellular levels. Compared to right whales, fin whales are less susceptible to Cr(VI) induced cell death and chromosome damage.

Several studies look at the exposure and threat of phthalates, some using them as a tracer for microplastic intake (Fossi *et al.*, 2012, 2014, 2016; Routti *et al.*, 2021; Garcia-Garin *et al.*, 2022). Fossi *et al.* (2012) measured the concentration of the two phthalates di-(2-ethylhexyl) phthalate (DEHP) and its metabolite mono-(2-ethylhexyl) phthalate (MEHP) in surface water, neustonic and planktonic zooplankton, and fin whales from the Mediterranean Sea as a tracer for microplastic intake. Their data suggest that phthalates can serve this function and that Mediterranean fin whales risk ingesting great amounts of microplastics both directly from the water and indirectly from the plankton. This represents a problem as microplastics can adsorb, concentrate, and transport persistent organic pollutants (POPs) such as PCBs, DDTs, and PAHs, which increases their bioavailability (Rios, Moore and Jones, 2007; Fossi *et al.*, 2012; references therein).

Garcia-Garin *et al.* (2022) investigated phthalate concentrations in North Atlantic fin whale muscle over a thirty-year period (1986–2015). When they looked at the concentrations compared to biological variables (e.g., age, body length,

sex), they found no correlation, which can indicate a lack of bioaccumulation. They also found that the concentration of phthalates in fin whale muscle showed no significant temporal differences, which they considered unexpected. Though, they do note that their samples originate from outside of Iceland where the waters are likely less polluted by these contaminants.

## 1.5 Cell cultures

Cell cultures derived from tissue and blood samples create an experimental testing foundation (Freshney, 2016; Boroda, 2017; Boroda *et al.*, 2020). While they might not give a 100% accurate result, considering that they lack the biological complexity of living animals, they can give useful information about biological responses when used right. A huge advantage lies in the fact that it is possible to obtain cells from living animals — in fact, getting cell samples from living animals gives a higher chance of getting a bigger quantity of viable cells. Another advantage is that it is possible to get samples from the same individual over a time period, though that requires a way to identify individuals.

However, when working with cells, they need to be cultured in suitable conditions, as this will give better, more reproducible, and relevant results (Küppers-Munther *et al.*, 2004; Webb *et al.*, 2014; Lam *et al.*, 2020). As such, the cells must be cultured under conditions that fit the cell type and that support the studies they are used in.

Differing cell types have different needs and conditions they require to thrive. They can require different nutrients, hormones, gas levels, and various types of physiochemical environments. While some cells can grow floating in culture medium, most cell types require an anchoring substrate on which they can attach and grow (Freshney, 2016). In addition, type of species affects which conditions are optimal (e.g., insect vs. mammalian cell lines).

Marine mammals are elusive, and the populations of the great whales are reduced. Thus, finding and sampling the tissues of living or recently dead animals can be difficult (Boroda, 2017; Boroda *et al.*, 2020). It requires time and labour to find one animal, let alone several. However, the information and applications cell cultures can provide are worth the struggle of obtaining (Freshney, 2016; Lam *et al.*, 2020).

Another problem with marine mammal cell cultures is that they have been difficult to grow (Smith *et al.*, 1987; Wang *et al.*, 2011; Jin *et al.*, 2013; Burkard *et al.*, 2015). The cells often grow slowly and have an extremely limited number of passages before the cells change morphology and enter senescence. Thus, it can be difficult to perform significant experiments. Finding the optimal conditions could help. A second possibility could be immortalisation of cell lines as this can increase available cells for experiments (Lam *et al.*, 2020). However, this can irreversibly change important cell functions. As such, it needs to be confirmed that the cells display primary cell phenotypes that is critical for drawing biologically relevant conclusions.

### 1.5.1 Fibroblasts

Fibroblasts are the cells that makes the extracellular matrix (Thulabandu, Chen and Atit, 2018; desJardins-Park *et al.*, 2020). These cells are an important part of the stroma, and they play a critical role in several bodily functions. They are a part of several organs within the body (e.g., the heart, skin, intestine, and bladder) and contain a large degree of heterogeneity depending on several factors, which includes place and organ of origin (Driskell and Watt, 2015; desJardins-Park *et al.*, 2020; Plikus *et al.*, 2021).

While they contain high heterogeneity and diversity, they can still give valuable information in toxicological studies, especially considering that the heterogeneity is an important aspect of how these cells function (Driskell and Watt, 2015; Plikus *et al.*, 2021).

These cells are adherent and require a suitable surface and space to grow. Thus, cell cultures with fibroblasts are grown in cell culture plates or in cell culture flasks.

### 1.5.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent cells with potential to differentiate into several cell types (Caplan, 1991; Tocci and Donnamed, 2003; Wang *et al.*, 2014). With the right combination of growth factors and chemicals in cell cultures, these cells can differentiate into several mesodermal cell lineages such as cartilage, bone, fat, tendon, muscle, myocardium, and marrow stroma. Additionally, they also have the potential to transdifferentiate into tissues of different embryonic dermal origin. They also have low immunogenicity which allows allogenic usage (le Blanc *et al.*, 2003; Tocci and Donnamed, 2003; Wang *et al.*, 2014). These cells have a large expansive ability and are easy to manipulate which allows for a broad application of these cells in therapeutic studies.

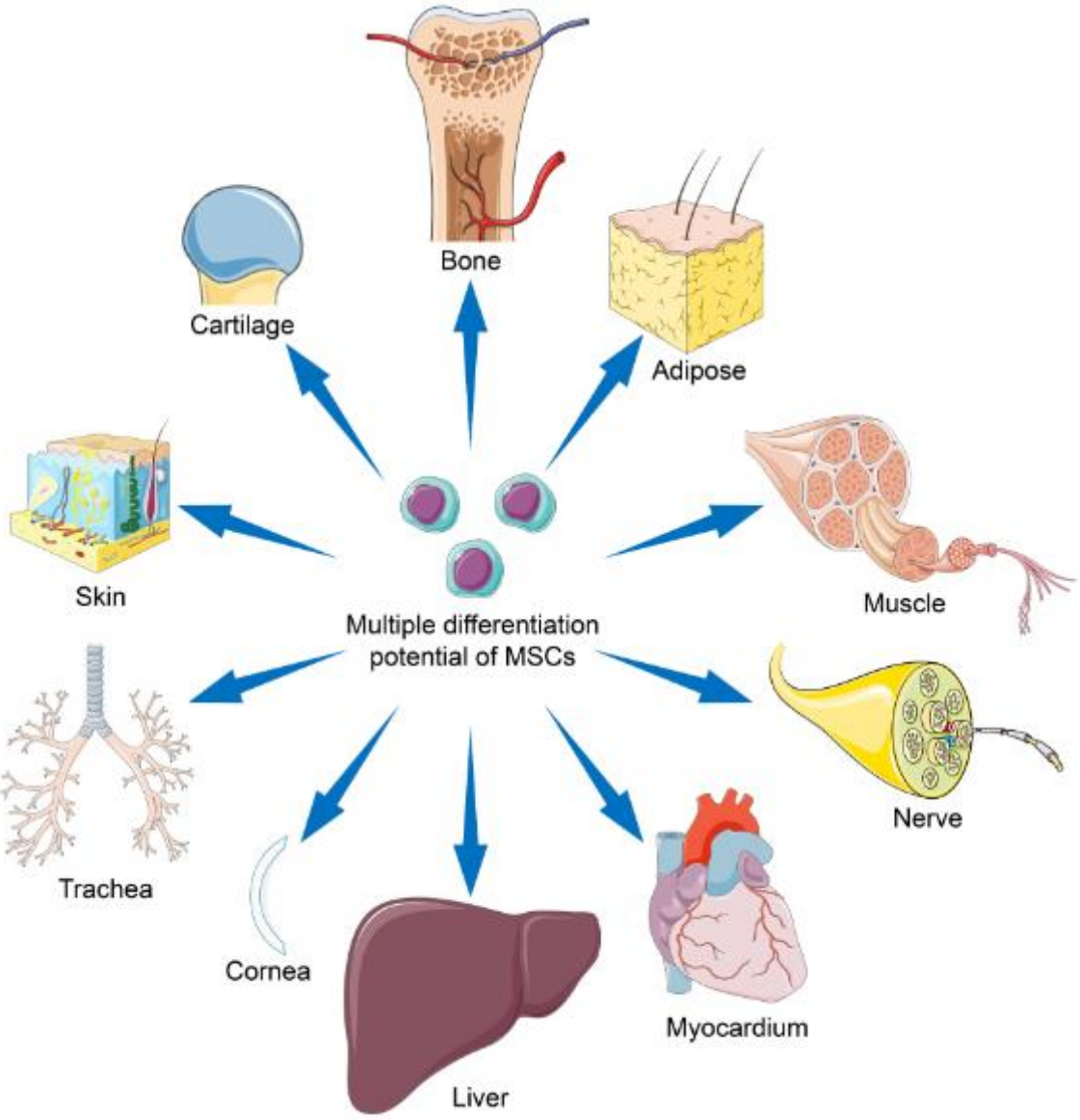


*Figure 1.3: Potential tissues originating from MSCs through their differentiation potential.*
*Figure source: Han et al. 2019*

However, the use of tissue derived MSCs contains many obstacles that limits their use (Dayem *et al.*, 2019; Han *et al.*, 2019). While MSCs can easily be isolated from various tissues and organs, the quality of the cells depends on several factors such as cell source, media composition, and cell passage. Cell morphology, DNA abnormalities, cell senescence, a decline in cell proliferation and differentiation capacity, and changes in cell plasticity reflect the quality (Rombouts and Ploemacher, 2003; Mimeault and Batra, 2009; Dayem *et al.*, 2019).

These cells have a lot of therapeutical potential, such as regrowing delicate tissue *in vivo* (Kimbrel *et al.*, 2014; Dayem *et al.*, 2019). However, *in vivo* experiments and clinical trials contain inconsistencies due to cell variations (Galipeau, 2013; Kimbrel *et al.*, 2014). Additionally, there is a high degree of heterogeneity of starting populations where different isolation protocols and culture methods can favour different selections of cell types and composition of subpopulations (Wagner and Ho, 2007; Kimbrel *et al.*, 2014).

### 1.5.3 Reprogramming cells to MSCs

As there can be several problems involving the use of tissue derived MSCs, scientists have been attempting to reprogram other cells into MSCs (Kimbrel *et al.*, 2014; Dayem *et al.*, 2019). One potential major source is pluripotent stem cells (PSCs). Embryonic stem cells (ESCs) were the first PSCs scientists attempted to induce into MSCs. Several methods exist ranging from using retroviruses to using three-dimensional spheroid cultures. However, there are ethical issues involved with using human ESCs as the source would be human embryos (Takahashi and Yamanaka, 2006). As such, scientists looked at other cell sources for MSC induction.

One potential source for MSCs is induced PSCs (iPSCs) (Dayem *et al.*, 2019). On their own, these cells bypass the ethical concerns associated with the use of ESCs, but they have their own challenges when it comes to clinical applications. Challenges with using iPSCs include immune rejection, teratoma formation, and epigenetic memory (Robinton and Daley, 2012; Dayem *et al.*, 2019). Inducing these cells into MSCs, creating iPSC-MSCs, could potentially circumvent these challenges. As such, several research groups have devised methods to produce iPSC-MSCs.

Another potential source is fibroblasts. Lai *et al.* (2017) developed a chemical cocktail consisting of six chemicals and three growth factors that consistently reprogrammed human dermal fibroblasts into MSCs. Their induced MSCs (iMCSs) had similar molecular structure to bone marrow MSCs and they expressed all the traditional MSC markers. One advantage with directly using fibroblast is that it circumvents the step of first creating iPSCs in a reliable manner. Thus, the MSC induction process can take less time.

As several marine mammals have reduced populations and are protected species, some forms of tissue and cells can be impossible to obtain for relevant studies such as toxicological studies (Lam *et al.*, 2020). One solution could be to reprogram obtainable cells (e.g., fibroblasts) into multi- or pluripotent cells. These reprogrammed cells can be used directly in for example exposure experiments, which can give information of how contaminants affect their differentiation into other cell types.

## 1.6 Aim of thesis

In this study, the overarching goal was to establish ways to use fin whale fibroblasts in toxicological studies. This was done by looking at three subgoals.

The first subgoal of this thesis was to optimise growth conditions for fin whale fibroblast cells from tissue biopsies. To determine cell growth, cells were seeded into plates, and observed every day through microscopy until they reached full confluence. The second subgoal was to detect cellular responses to various environmental toxicants and toxicant mixtures. Western blotting and quantitative polymerase chain reaction (qPCR) assay were used to determine cell responses to the environmental toxicants. The third, and final, subgoal was to reprogram the cells to mesenchymal stem cells (MSCs). The chemical induction cocktail used by Lai *et al.* was used to reprogram fibroblast cells into MSCs. qPCR assay and adipocyte differentiation were used to determine if the reprogramming was successful.

# 2. Materials

## 2.1 Chemicals and reagents

Table 2.1: Overview of used chemicals and supplier/product number

| **Chemical/Reagent** | **Supplier/Product number** |
|---|---|
| 2-log DNA Ladder | New England Biolabs/ N0469S |
| 3-isobutyl-1-methylxanthine (IBMX) | Sigma-Aldrich/410957 |
| Acrylamide/Bis-acrylamide, 40% solution | Sigma-Aldrich/A7802 |
| Agarose | Sigma-Aldrich/A9539 |
| AlbuMAX I | Gibco/11020021 |
| Ammonium Persulphate (APS) | Bio-Rad/ 1610700 |
| Anti-Actin | Sigma-Aldrich/A5060 |
| Antibacterial Antimycotic Solution (100x), Stabilized | Sigma-Aldrich/A5955 |
| Anti-CYP1A antibody | Abcam/ab124295 |
| Benzo[a]Pyrene | Sigma-Aldich/B1760 |
| Bovine Serum Albumin (BSA) | Sigma-Aldrich/A9647 |
| CHIR99021 (GSK3βi) | Sigma-Aldrich/SML1046 |
| Chloroform | Sigma-Aldrich/67-66-3 |
| Collagen, Type I solution from rat tail | Sigma-Aldrich/C3867 |
| Cytotoxicity Detection Kit (LDH) | Roche/ 11644793001 |
| Dexamethasone (DEX) | Sigma-Aldrich/46165 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich/D8418 |
| Dry milk | |
| Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (with phenol red, l-glutamine, HEPES) | Gibco/11330032 |
| Erythrosine B | Sigma-Aldrich/200964 |
| Ethanol absolute | VWR/ 20821.365 |
| Fetal Bovine Serum (FBS) | Sigma-Aldrich/F7524 |
| Formalin solution, neutral buffered, 10% | Sigma-Aldrich/HT501128 |
| Formamide | Sigma-Aldrich/F9037 |
| GelRed Nucleic Acid Stain, 10,000X in water | Biotium/41003 |
| GlutaMAX | Gibco/35050038 |
| Glycine | Sigma-Aldrich/G8898 |
| GlycoBlue™ Coprecipitant | Invitrogen/AM9515 |
| Gö6983 (PKCi) | Apollo Scientific/APOSBIFK0056 |
| Guanidine hydrochloride | Sigma-Aldrich/50933 |
| Hydrochloric acid (HCl) 37 % | Sigma-Aldrich/258148 |
| Insulin solution human | Sigma-Aldrich/19278 |
| iScript™ cDNA Synthesis Kit | Bio-Rad/170-8891 |
| Isopropanol | Kemetyl/603-117-00-0 |
| KnockOut™ DMEM | Gibco/10829018 |
| L-glutamine | Sigma-Aldrich/G7513 |
| LightCycler® 480 SYBR Green I Master mix | Roche/55499820 |
| MEM Non-Essential Amino Acids Solution (100X) | Gibco/11140050 |
| MesenPRO RS™ Medium | Gibco/10212293 |
| Methanol | Sigma-Aldrich/34860 |
| N2 supplement | Gibco/17502-048 |
| NaCl | |
| Oil Red O (ORO) | Sigma-Aldrich/O0625 |
| PageRuler | Thermo Scientific/26616 |
| PD0325901 (ERK1/2i) | Sigma-Aldrich/444968 |
| Phosphate buffered saline (PBS) | Sigma-Aldrich/P5493 |
| Phthalic acid mono-2-ethylhexyl ester | Sigma-Aldrich/CDS010608 |

| | |
|---|---|
| Phthalic acid mono-2-ethylhexyl ester | Sigma-Aldrich/CDS010608 |
| Pierce™ 600 nm Protein Assay Reagent | Thermo Scientific/22660 |
| Polyclonal Goat Anti-Rabbit Immunoglobulins/HRP | DakoCytomation/P0448 |
| Prestained SDS-PAGE standards | BIO-RAD/161-0318 |
| Primocin | InvivoGen/ant-pm-2 |
| Protease inhibitor Cocktail | Sigma-Aldrich/P8340 |
| QIAEX® II Gel Extraction Kit | Qiagen/20021 |
| Recombinant bFGF | Gibco/13256029 |
| Recombinant human LIF | Gibco/PHC9484 |
| Recombinant TGF-β1 | Gibco/PHG9214 |
| Rosiglitazone | Sigma-Aldrich/R2408 |
| Sample buffer 5X | |
| SB202190 (p38i) | Sigma-Aldrich/S7067 |
| Sodium Dodecyl Sulphate solution (20% in $H_2O$) | Sigma-Aldrich/05030 |
| Sodium Pyruvate | Sigma-Aldrich/S8636 |
| SP600125 (JNKi) | Apollo Scientific/APOSBIS1100 |
| StemPro™ Adipogenesis Differentiation Kit | Gibco/10154093 |
| SuperSignal™ West Pico PLUS Luminol/Enhancer Solution | Thermo Scientific/1863096 |
| SuperSignal™ West Pico PLUS Stable Peroxide Solution | Thermo Scientific/1863097 |
| TBE buffer | |
| TEMED | Thermo Scientific/17919 |
| TRI Reagent® | Sigma-Aldrich/T9424 |
| Trizma® base | Sigma-Aldrich/93352 |
| Trypsin-EDTA solution (0,25%) | Sigma-Aldrich/T4049 |
| TWEEN® 20 | Sigma-Aldrich/P5927 |
| Water, nuclease-free, molecular biology grade | VWR/4436912C |
| Y-27632 (ROCKi) | STEMCELL/TCIAY0018 |

## 2.2 Cell culture

### 2.2.1 Cell origin

A biopsy sample of an adult fin whale was taken 11th July 2019 at 13:00 with a biopsy arrow by crew from the Norwegian Polar Institute. The fin whale had the location of 78. 07549 N 13.489072 E, which is in Isfjorden, Svalbard, West of Barentsburg. A piece of ~1 cm containing the interface between the skin and blubber was cut with sterile equipment from the sample. This piece was used to establish the fin whale fibroblast cell culture. The cut tissue was shipped to the Environmental Toxicology lab at UiB (23 hours transport by air) while cooled (~4 °C) for processing.

### 2.2.2 Establishment of cell culture

The cell culture was established by Katharina Lühmann (2019).

The sample was processed according to the protocol by Burkard et al. (2015). Upon arrival, the sample was rinsed with 1x DPBS containing 100 µg/mL Primocin. While cutting, the sample lied on a folded layer of aluminium foil (folded to 4 layers, ethanol-sprayed and dried in sterile cabinet) in a petri dish. To avoid spreading fat droplets between sample pieces, the aluminium piece was exchanged for every cutting step.

The biopsy sample was roughly separated into epidermis, dermis, and blubber, which were separately cut into small pieces (max 3 $mm^2$) with sterile scalpel blades and tweezers. Then the pieces were washed trice with DPBS containing 100 µg/mL Primocin. ~10 pieces were transferred into a petri dish containing the wash solution. After 10

minutes, the pieces were transferred into a new petri dish with fresh wash solution for another 10 minutes before repeating the wash once more.

There were enough pieces to fill the wells of two 6-well plates with 5–10 pieces each with a sterile cover slip. The cover slip ensured contact to a surface for the fibroblast growth. 1 mL medium was added, and cells incubated at 37°C with 5% $CO_2$.

2 mL medium per well were added after ~12 hours. After, 50–75% of the medium was replaced every 2–3 days While the growth of the primary cells was checked under the microscope.

The primary cells were removed from the residual biopsy tissue after 17–24 days. First, the old medium was discarded, and the cells were washed twice with DPBS containing 100 µg/mL Primocin. Then 1 mL trypsin was added, and after incubating for 30 seconds at room temperature, the trypsin was removed, and the plate was incubated a further 5 minutes in the incubator (37°C and 5% $CO_2$). Cells were resuspended in 2 x 5 mL medium. The suspension was passed through 70 um sieve (Corning® cell strainer, Sigma) and seeded into petri dishes.

## 2.3 Culture media

Table 2.3: Standard fibroblast culture medium

| Components | Concentration | | |
|---|---|---|---|
| | Initial | Final | Unit |
| DMEM/F12 | 1 | 1 | X |
| FBS | 100 | 10 | % |
| L-glutamine | 200 | 1,5 | mM |
| MEM non-essential amino acids | 100 | 1 | X |
| Sodium Pyruvate | 100 | 1 | mM |
| Primocin | 50000 | 100 | µg/mL |

Table 2.4: MSC induction medium

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Knockout DMEM | 1 | X |
| AlbuMAX I | 10 | µg/mL |
| $N_2$ supplement | 1 | X |
| MEM non-essential amino acids | 1 | X |
| SB202190 | 10 | µM |
| SP600125 | 10 | µM |
| Gö6983 | 0,5 | µM |
| Y-27632 | 5 | µM |
| PD0325901 | 1 | µM |
| CHIR99021 | 3 | µM |
| Recombinant human LIF | 20 | ng/mL |
| Recombinant bFGF | 8 | ng/mL |
| Recombinant TGF-β1 | 1 | ng/mL |

Table 2.5: MSC expansion medium

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| MesenPRO RS™ Basal Medium | 1 | X |
| MesenPRO RS™ Growth Supplement | 1 | X |

| | | |
|---|---|---|
| GlutaMAX | 1 | X |
| Antibacterial Antimycotic Solution | 1 | X |

Table 2.6: Adipocyte induction medium version 1

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Standard fibroblast culture medium | 1 | X |
| IBMX | 500 | µM |
| DEX | 250 | ng |
| Rosiglitazone | 2 | µM |
| Insulin | 5 | µg/mL |

Table 2.7: Adipocyte induction medium version 2

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| StemPro® Adipogenesis Differentiation Basal Medium | 1 | X |
| StemPro® Adipogenesis Supplement | 1 | X |
| GlutaMAX | 1 | X |
| Antibacterial Antimycotic Solution | 1 | X |

## 2.4 Buffers and solutions

Table 2.2: Kit contents

| Kit | Contents | | |
|---|---|---|---|
| | Component | Volume | Unit |
| Cytotoxicity Detection Kit (LDH) | Catalyst (Diaphorase/NAD+ mixture) | 1 | mL |
| | Dye Solution (INT and sodium lactate) | 45 | mL |
| iScript™ cDNA Synthesis Kit | 5x reverse-transcription reaction mix | 400 | µL |
| | iScript reverse transcriptase | 100 | µL |
| | Nuclease-free water | 1.5 | mL |
| MesenPRO RS™ Medium | MesenPRO RS™ Basal Medium | 500 | mL |
| | MesenPRO RS™ Growth Supplement | 10 | mL |
| QIAEX® II Gel Extraction Kit | Buffer QX1 Solubilization buffer | 100 | mL |
| | QUIAX® II | 500 | µL |
| StemPro™ Adipogenesis Differentiation Kit | StemPro™ Adipocyte Differentiation Basal Medium | 100 | mL |
| | StemPro™ Adipogenesis Supplement | 10 | mL |

Table 2.8: 10X DNA loading buffer

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Glycerol | 3.9 | mL |
| SDS | 500 | µL |
| 0.5 M EDTA | 200 | µL |
| Bromophenol blue | 25 | mg |
| Xylene cyanol | 25 | mg |
| $H_2O$ | 5.4 | mL |
| Total volume | 10 | mL |

Table 2.9: Cytotoxicity detection working solution

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Catalyst (Diaphorase/$NAD^+$ mixture) | 50 | µL |
| Dye Solution (INT and sodium lactate) | 2.25 | mL |
| Total volume | 2.3 | mL |

### 2.4.1 SDS-PAGE solutions

Table 2.10: Resolving gel

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| 30% acrylamide/bis | 2.5 | mL |
| MilliQ | 4.835 | mL |
| 1.5 M Tris-HCl pH 8.8 | 2.5 | mL |
| 10% SDS | 100 | µL |
| 10% APS | 50 | µL |
| TEMED | 5 | µL |
| Total volume | 10 | mL |

Table 2.11: Stacking gel

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| 30% acrylamide/bis | 650 | µL |
| MilliQ | 2.995 | mL |
| 0.5 M Tris-HCl pH 6.8 | 1.25 | mL |
| 10% SDS | 50 | µL |
| 10% APS | 50 | µL |
| TEMED | 5 | µL |
| Total volume | 5 | mL |

### 2.4.2 Western blot solutions

Table 2.12: 5x sample buffer

| Components | Concentration |
|---|---|
| Tris HCl pH 6.8 | 250 mM |
| SDS | 10% |
| Glycerol | 30% |
| 2-β-mercaptoethanol | 5% |
| Bromophenolblue | 0.02% |

Table 2.13: Lysis buffer

| Components | Volume (µL) |
|---|---|
| 5 x Sample buffer | 900 |
| 10x PBS pH 7.4 | 450 |
| Protease inhibitor | 45 |
| MilliQ | 3 100 |
| Total volume | 4 495 |

Table 2.14: Transfer buffer

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Tris | 3.03 | g |
| Glycine | 14.4 | g |
| Methanol | 200 | mL |
| MilliQ | 800 | mL |
| Total volume | 1000 | mL |

Table 2.15: 10x TBS pH 7.5

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Tris | 48 | g |
| NaCl | 585 | g |
| MilliQ | 2000 | mL |
| Total volume | 2000 | mL |

Table 2.16: TBS-tween

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| 10x TBS | 50 | mL |
| Tween-20 | 0.25 | mL |
| MilliQ | 450 | mL |
| Total volume | 500 | mL |

Table 2.17: TBS-tween ver. 2

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| 1M Tris pH 7.5 | 10 | mL |
| 5M NaCl | 15 | mL |
| MilliQ | 475 | mL |
| Tween-20 | 0.5 | mL |
| Total volume | 500 | mL |

Table 2.18: 5% dry milk

| Components | Concentration | |
|---|---|---|
| | Final | Unit |
| Dry milk | 6.25 | g |
| TBS-tween | 125 | mL |

## 2.5 RNA extraction

Table 2.19: Quantitative polymerase chain reaction (qPCR) primers

| Oligo name | Sequence 5'–3' |
|---|---|
| Fw_actbf | GACAGGATGCAGAAGGAGATC |
| Fw_actbr | CACACGGAGTACTTGCGCTCA |
| FW_ahrrf1 | TACTGCGGTGAAGATGCAGAGT |
| FW_ahrrr1 | CGTGCAATTCTGGTTCACAGAG |
| FW_cyp1a1f1 | GCTTGAGAAACATTTAGAGCAGGC |
| FW_cyp1a1f2 | ACCAGGGCCTCTCCAATCTCTT |
| FW_cyp1a1r1 | ACCCCTGTGTCAGTATCCTGG |
| FW_cyp1a1r2 | AGATTGCTCAGCCTTGCATAAGC |
| FW_cyp1b1f1 | CAGTGGCTGCTCGTCCTCTTCA |
| FW_cyp1b1r1 | GTCCCTACCCACCACTTGATCC |
| FW_fabp4f1 | TGTAGGTACCTGGAAACTTGTCTCC |
| FW_fabp4r1 | ATCTTCATTTGTACTGATGGGCACA |
| FW_hsp70f1 | TGAGAATCAGCTGTTATGGCAGA |
| FW_hsp70r1 | TGCATTCTTAGCATCATTCCTCTC |

| | |
|---|---|
| FW_ppargf1 | GAGGGCGATCTTGACAGGAAAG |
| FW_ppargr1 | GGGGTGATGTGCTTGAACTTGA |
| nanog_f1 | GCAGAAATACCTCAGCCTCC |
| nanog_r1 | CAGTGTTGCTATTCCTCGGC |
| Oct4_f1 | GGATATACCCAGGCCGATGT |
| Oct4_r1 | CGTTGTTGTCAGCTTCCTCC |
| Sox2_f1 | CATGTCCTATTCGCAGCAGG |
| Sox2_r1 | CCTGGAGTGGGAAGAAGAGG |

## 2.6 Instruments

Table 2.20: Instruments used in analyses

| Category | Name | Manufacturer |
|---|---|---|
| Agarose gel and western blotting picture | ChemiDoc™ XRS+ System | Bio-Rad |
| Agarose gel visualisation | Chromato-vue TM-20 transilluminator | UVP, San Gabriel |
| Blotting | Mini PROTEAN® Tetra Cell | Bio-Rad |
| Cell counting | Bürker hemocytometer | Kebo Lab |
| Cell incubation | MCO-170AICUVL | Panasonic |
| | Hypoxia Incubator Chamber | Stemcell |
| Centrifugation | CT 15RE Himac | VWR |
| | Heraeus Multifuge X3R | Thermo Scientific |
| | Heraeus Pico 21 | Thermo Scientific |
| Electric power to electrophoresis | EPS 3501 XL Power Supply | Cytvia |
| Heat-block | Dry Block Heater 1 | IKA |
| Microscopy | DM IL inverted microscope | Leica |
| | Eclipse Ts2-FL | Nikon |
| | TMS-F No. 3 | Nikon |
| MilliQ $H_2O$ dispenser | MilliQ A10 advantage | Merck |
| PCR Thermo Cycler | CFX96™ RealTime PCR System C1000™ Thermal Cycler — qPCR | Bio-Rad |
| Platform shaker | HS 501 Digital | IKA-Werke |
| Spectrophotometry | Nanodrop One | Thermo Scientific |
| | EnSpire™ 2300 Multilabel Reader | PerkinElmer |
| Sterilized workspace for handling cells | CleanAir EuroFlowClass II biosafety cabinet | Baker |

# 3. Methods

## 3.1 Thawing protocol

Vials were removed from the liquid nitrogen storage and thawed by incubating them in a 37°C water bath for 1–2 minutes. The vial contents were transferred into centrifuge tubes containing 9 mL culture medium. Then the tube was centrifuged at 125 x g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 10 mL culture medium per vial. Cell suspension was transferred into 25 $cm^2$ culture flasks. TC 100 plates (10 mL per plate) or 6-well plates were used for culturing after the first thawing.

All cells were cultured at 37°C and ~5% $CO_2$.

## 3.2 Splitting protocol

Culture medium was removed, and the cells were washed twice with 5 mL PBS (or half the volume of culture medium). Then 1,5 mL 0,25% Trypsin EDTA (or 0,6 mL per TC35 dish/6-well plate) was added, and after

incubating for 45 seconds at room temperature, the trypsin was removed, and the plate was incubated a further 5 minutes in the incubator (37°C and 5% $CO_2$). The cells were resuspended in 10 mL medium (2 mL per TC35 dish) and distributed into receiving plates.

## 3.3 Freezing protocol

Followed the same protocol for splitting until resuspension in 10 mL. The resuspension was transferred into a centrifuge tube and centrifuged at 200 x g for 4 minutes. Supernatant was removed and cells were resuspended in 1–2 mL freezing medium (90% FBS + 10% DMSO) per centrifuge tube. The resuspension was transferred into cryovials, 1 mL per cryovial. Cryovials were placed into Mr. Frosty and stored in -80°C freezer for at least 24 hours. Lastly, cryovials were transferred into liquid nitrogen storage tank.

## 3.4 Cell growth determination

FIWA cells were seeded into TC35 standard plates, 50 000 cells per plate. Three plates with test condition(s) and three plates with control was prepared for every growth test. Plates were photographed through microscope every day until they reached full confluence. All cell culture pictures were obtained with a HDMI16AMDPX camera from DeltaPix mounted onto an Eclipse Ts2-FL unless specified otherwise.

### 3.4.1 Hypoxia chamber

Some cells were grown in a hypoxia chamber. The oxygen content of the air was presumed to be similar to *in vivo* oxygen levels (~5%) the cells would have experienced.

A Hypoxia Incubator Chamber (Stemcell) was used to create hypoxic conditions. The chamber was opened, assembled, and purged following the producer's protocol. Briefly on the exchange of gasses in (purging of) the chamber, both ports for the inlet and outlet tubing of the chamber were opened. After opening the tank valve control completely, the regulator valve control was opened to about 8–10 psi. The tank was connected to the hypoxia chamber with a flow meter between, which regulated the flow to 20 L/min. After 5 minutes, the tube was disconnected from the chamber and the tubing clamps were closed to seal the mixed gasses in the chamber. To stop the gas flow from the tube, the valve control was turned off, allowing the gas to purge from the regulator. Then the regulator valve was turned off. The chamber was put into the cell incubator for incubation.

The gas mixture used in this thesis was 5% $O_2$, 10% $CO_2$, and 85% $N_2$.

### 3.4.2 Fibroblastic growth factor (FGF)

When testing added FGF to the medium, two concentrations were tested. Three plates got medium containing 2 ng/mL FGF, three plates got medium containing 10 ng/mL FGF, and three plates were control with no added FGF.

### 3.4.3 Collagen coating

Three plates were coated with collagen before adding cells to test if it helps the cell growth. Three plates without collagen coating were controls.

Before coating the plates, a 0.01% collagen in water solution was prepared. First, the collagen was diluted 1:1000 in sterile milliQ to get a 0.1% solution. Then it was further diluted 1:10 to gain the 0.01% solution for coating of the plates. 1 mL 0.01% collagen solution was added to each TC35 plate, and the plates incubated in the incubator for ~4 hours. After incubation, excess fluid was removed, and the plates were left to dry overnight in a fridge. Plates were rinsed with PBS before use.

## 3.5 Exposure experiments

### 3.5.1 Exposure conditions

Cells were seeded into 6-well plates and exposed to two different compounds: BaP and MEHP. There were in total four sets of exposures for ~48 hours.

First exposure (Exposure 1) set was two 6-well plates. One had cells from hypoxic conditions, the other had atmospheric oxygen levels. Three wells of the two plates were exposed to 10 µM BaP and three was 0.1% DMSO controls. These were used in both western blot and qPCR. Only primers for CYP1A1 were used on these samples.

Second exposure (Exposure 2) set was intended for western blotting consisting of one 6-well plate with cells. Three wells were exposed to 5 µM BaP and three was 0.1% DMSO controls.

Third exposure (Exposure 3) set was four 6-well plates, each with cells in five of their wells, to perform a dose–response exposure. Five wells total were exposed to 5 µM BaP, five wells were exposed to 1 µM BaP, five wells were exposed to 0.2 µM, and the last five wells were 0.1% DMSO controls. These were only used for qPCR.

Last exposure (Exposure 4) set was two 6-well plates. One plate was used for BaP exposure, the other for MEHP exposure. Both plates had three different conditions with two replicates. The BaP plate had 10 µM BaP, 1 µM BaP, and DMSO control. The MEHP plate had 50 µM MEHP, 5 µM MEHP, and 0.1% DMSO control. These were only used for qPCR.

### 3.5.2 Cell viability test using Lactate Dehydrogenase (LDH) assay

Growth medium from wells with cells exposed to 5 µM BaP and DMSO controls was saved for the viability assay. LDH assay was performed according to manufacturer's protocols (Roche). Briefly, triplicates of 50 µL of each sample were pipetted into a clear 96-well plate along with three wells of DMEM/F12 medium as blank control. Then 50 µL of the spent medium from each well was mixed with 50 µL substrate solution from Roche LDH assay Kit and mixed. Then the plate was covered in aluminium foil and incubated for about 15 minutes at room temperature. After incubation, the plate was spun at 500 x g for about 3 minutes to remove bubbles. The absorbance at 490 and 650 nm was measured with an EnSpire plate reader.

## 3.6 Inducing Mesenchymal stem cells (iMSC)

The protocols described by Lai et al. (2017) were followed. Cells were treated with the chemical cocktail developed by Lai et al. (2017) to generate MSC-like cells. The cocktail contained six chemical inhibitors (SP600125, SB202190, Gö6983, Y-27632, PD0325901, and CHIR99021) and three growth factors (Transforming growth factor-β (TGF-β), Basic fibroblast growth factor (bFGF), and Leukemia inhibitory factor (LIF)). Cells were exposed to the cocktail for seven days with one medium change during the period.

This was done twice — once with 10 cm plates and once with 6-well plates. MSC expansion medium was not available after the first induction. These apparent iMSCs were cultured in standard culture medium without bFGF. The apparent iMSCs from the second experiment (see below) were cultured in MSC expansion medium

For the first reprogramming, several plates were harvested for qPCR during the seven-day induction period. On day 2 and 4, one plate from each condition was harvested. On day 7, three plates of reprogrammed and four control plates were harvested. Two plates with cells passaged once were harvested four days post reprogramming.

For the second reprogramming, eight wells from each condition were harvested for qPCR after the seven-day induction period.

Primers for specific stem cell markers (*OCT4*, *NANOG*, and *SOX2*) were used in qPCR. These three genes are a part of the pluripotency gene network.

### 3.6.1 Adipocyte induction

#### *3.6.1.1 Inducing conditions*

The induced cells were seeded in 6-well plates, 50 000 cells/well at first induction, 35 000 cells/well at second induction. After the cells reached around 60–70% confluence, the medium in three of the wells were changed to induction medium.

For the first induction experiment, adipocyte induction medium version 1 was used. Cells were induced for about 14 days.

For the second induction, adipocyte induction medium version 2 was used.

#### *3.6.2.2 Oil Red O staining*

Differentiation and culture medium were removed, and cells were washed with 1 mL PBS. 2 mL 10% formalin was added to each well and the plate was incubated for 1 hour at room temperature. After fixation, the cells were washed twice with 2 mL milliQ followed by 60% isopropanol. Cells were incubated for 5 minutes at room temperature before they were left to dry completely. 60% Oil Red O (ORO) working solution was prepared by mixing stock solution with milliQ, leaving it to incubate for 20 minutes, and filtered through 2 µm filter. 1 mL of the working solution was added to each well and incubated for 10 minutes. After ORO incubation, the cells were repeatedly washed with MilliQ until the water was no longer visibly pink.

The cells were viewed under a microscope.

## 3.7 Harvesting cells for analysis

### 3.7.1 RNA extraction

RNA extraction was performed using TRI reagent according to manufacturer's protocols (Sigma). Briefly, the first set of harvested cells (exposure 1) were initially suspended in 1 mL of exposure medium. After isolating cells with centrifugation, they were lysed in 1 mL TRI Reagent per Eppendorf with cells by repeated pipetting. The samples stood for 5 minutes at room temperature for dissociation of nucleoprotein complexes. 0.2 mL chloroform was added to each tube before the tubes were shaken vigorously for 15 seconds. Samples then stood for 15 minutes at room temperature before they were centrifuged at 12 000 x g for 15 minutes at 4 °C.

Every other set of harvested cells, cells were lysed directly on the plates by adding TRI Reagent, 1 mL per 6-well and 1.5 mL per 10 cm plate, after washing them twice with PBS. Cell solution was then transferred to Eppendorf tube and dissociated and centrifugated as above, 0.2 mL chloroform per 1 mL and 0.3 mL per 1.5 mL.

After centrifugation, the aqueous phase was transferred into a new Eppendorf tube (0.4 mL per 1 mL, 0.6 per 1.5 mL). RNA was precipitated by adding equal volume of isopropanol and centrifugation at 12 000 x g for 15 minutes at 4 °C. The RNA pellet was washed by adding 1 ml 75% ethanol, centrifugation at 75 000 x g for 5 minutes at 4 °C. The ethanol was removed, the pellet was dried at RT for 20 min and dissolved in nuclease free water by incubating at 60 °C for 20-30 min.

### 3.7.2 Protein extraction

Protein extraction was performed using TRI reagent according to manufacturer's protocols (Sigma). Proteins were extracted from the organic phase of TRI Reagent from cells harvested from exposure 1 for the first western blot. The remainder of the aqueous phase was removed, and 0.3 mL of 100% ethanol was added before mixing by inverting the tube. The mixture incubated for 3 minutes before getting centrifuged at 12 000 x g for 5 minutes at 4 °C. The phenol–ethanol mixture was transferred into a fresh tube for protein extraction.

1.5 mL isopropanol was added to the phenol–ethanol supernatant and incubated for 10 minutes before it was centrifuged at 12 000 x g for 10 minutes at 4℃. The supernatant was removed, and the pelleted proteins was resuspended in 2 mL of a wash solution of 0.3 M guanidine hydrochloride in 95% ethanol. After incubating for 20 minutes, the samples were centrifuged at 7500 x g for 5 minutes at 4 °C. The supernatant was then discarded, and the wash was repeated twice. After washing, the pellet was resuspended with a brief vortex in 2 mL ethanol. The mixture was incubated for 20 minutes before another centrifugation at 7500 x g for 5 minutes at 4 °C. The supernatant was removed, and the pellets were air dried for 10 minutes. After drying, the pellet was resuspended in 200 µL 1% SDS by pipetting up and down. To help with resuspension, the samples were incubated at 60 °C. Insoluble materials were removed by centrifuging the samples at 10 000 x g for 10 minutes at 4 °C. Supernatant was transferred into a fresh tube.

A second western blot was done with the same samples as the first.

The cells for the third western blot were harvested with the lysis buffer. Cells from exposure 2 were washed twice with 1 mL PBS before 650 µL lysis buffer was added. The plate was put on ice on a shaker for 5 minutes before the material was scraped loose with a cell scraper into an Eppendorf tube. The same scraper was used for the same conditions.

## 3.8 Protein analysis

### 3.8.1 Protein quantification

Protein quantification was done with Pierce 660 nm Protein Assay according to the manufacturer's protocols (ThermoFischer Scientific). Briefly, as the SDS concentration pf the protein samples were above 0.0125%, a 1:100 dilution in milliQ was prepared with part of the sample. A standard curve was prepared within the assay's working range by making a 1:2 serial dilution with BSA. 10 µL of the standard curve solutions, unknown samples, and appropriate blanks were added into the wells of a 96-well plate. 150 µL of the Protein Assay Reagent was added to each well, and the plate was placed on a plate shaker at medium speed for 1 minute for mixing. Then the plate incubated at room temperature for 5 minutes before having its absorbance at 660 nm read.

### 3.8.2 Western blotting protocol

#### *3.8.2.1 Preparation of sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

First the gel casting chamber was prepared. The glass plates were rinsed with $dH_2O$ and 70% ethanol. After drying the plates, the gel casting chamber was assembled. ~2 cm of $dH_2O$ was added and left between the plates to ensure no leakage.

Gel solutions were mixed in 15 mL falcon tubes, except APS and TEMED were left out. The water was removed from the casting chamber with Whatman paper, and the chamber was placed on a leveled surface. APS and TEMED was added to the resolving gel and mixed quickly by tilting the tube. A micropipettor was used to fill the appropriate amount of the gel into the casting chamber. Then $dH_2O$ was added on top to ensure an even surface. The gel was left to polymerise for about one hour. Polymerisation was checked by observing gel remains in the falcon tube.

After the resolving gel had polymerised, APS and TEMED was added to the stacking gel and mixed quickly by tilting the tube. The stacking gel was added to the casting chamber, filling the chamber, and the comb was placed in the stacking solution. Then the gel was left to polymerise for about 1 hour.

While waiting for the gel to polymerise, the samples were prepared. For the two western blots with the proteins isolated with TRI Reagent, 24 µL of the protein sample was mixed with 6 µL sample buffer consisting of β-mercaptoethanol and SDS. After mixing, the samples were heated for 5 minutes at 95℃ and carefully spun down. The samples for the last western blot were thawed and 100 µL was taken out for heating and adding to the SDS-PAGE.

The glass plates were removed from the casting chamber and assembled into the electrophoresis cell. Running buffer (0.1 x Tris/Glycine/SDS buffer) was added until it covered the wells before the comb was removed by pulling straight up. After making sure no air was trapped in the wells, the samples were applied into the wells along with one or two molecular standards. Then the cell was connected to a power source, which was turned on at 200 V. The power ran through the gel for about 45 minutes, until the bromphenol-blue front reached the bottom.

#### *3.8.2.2 Western blotting*

Pre-cut PVDF membranes (~9x6 cm) were equilibrated according to Millipore's protocol: First a 20 second bath in methanol, then a $dH_2O$ rinse, and lastly a bath in transfer buffer for 15–30 minutes. Filter papers were cut slightly larger than the membrane, two pieces on each side, which were saturated in transfer buffer along with fibre pads. The SDS-PAGE gel was equilibrated for 10 minutes in the transfer buffer.

After equilibrating the gel, the transfer unit was assembled and placed in the transfer cell. A cooling unit was placed next to the electrode unit and the tank was filled with transfer buffer. Then the mini gels were transferred at 100 V for 1 hour with a current of 350 mA.

To prepare the membrane for protein detection, it was transferred into a suitable box and was washed for 5 minutes in TBS-tween (Table 2.16 first two, Table 2.17 last time). Then the membrane was blocked in 100 mL 5% dry milk for 45 minutes, after which it got another two times 5 minutes TBS-tween wash.

Primary antibody was diluted in 15 mL 5% dry milk first with the dilution factor provided by the manufacturer. Wash solution was discarded and exchanged with the antibody–dry milk mixture. The membrane was incubated overnight on a shaker at 4°C, under shaking.

After the overnight incubation, the membrane was washed twice for 5 minutes in TBS-tween. HRP-conjugated secondary antibody was diluted in 15 mL 5% dry milk with the dilution factor provided by the manufacturer. The membrane was incubated in the secondary antibody dilution for 3 hours at room temperature, under shaking.

After incubation, the membrane was washed twice for 5 minutes in TBS-tween before another 5-minute wash in dH2O. Then the membrane was incubated in 1:1 mixure of SuperSignal chemiluminescent substrates solutions for 1 minute according to manufacturer's protocols (ThermoFischer Scientific) and imaged using the ChemiDoc™ XRS+ System (Bio-Rad).

## 3.9 RNA analysis

### 3.9.1 Quality control of RNA

The quality of the RNA was ensured through two steps: Spectrophotometric measurements and agarose gel electrophoresis.

#### *3.9.1.1 NanoDrop — Spectrophotometric Measurements*

A Nanodrop One spectrophotometer (A260nm/A260/280-ratio, Thermo Scientific) was used to measure the concentration and purity of the RNA samples. The sample's absorbance at 260 nm can be used to find the RNA concentration in the samples. Meanwhile, the ratio between the absorbance at 260/280 and the ratio between 230/260 nm can give insight to the RNA purity (Wilfinger, Mackey and Chomczynski, 2018). With an $A_{260/280}$ ratio between 1.8–2.0, the sample is considered as a pure RNA sample. However, it is important to note that pH and ionic strength of solutions used in these analyses can significantly affect the readout of the $A_{260/280}$ ratio. On the other hand, a low $A_{260/230}$ ratio indicates a phenol and protein contamination (Faraldi *et al.*, 2022).

*3.9.1.2 Agarose gel electrophoresis*

The integrity of the RNA can be established by performing an agarose gel electrophoresis (AGE). A 1% agarose gel was prepared in 1x TBE buffer with GelRed for visualisation of the samples. While the gel casted for about 60–90 minutes, the samples were prepared. First, 10x loading buffer was diluted 1:5 in formamide. Then 1–3 µL RNA sample was mixed with 9–7 µL diluted loading buffer before being incubated at 60°C for 5 minutes. The samples were loaded in the gel wells along with 2LOG DNA-Ladder to control gel integrity. The gel was covered in 1x TBE buffer while loading. After loading, the gel ran at 80V for about 1 hour. Gel images were analysed using Biorad Gel Doc XR Imaging System (Bio-Rad).

## 3.9.2 Quantitative polymerase chain reaction (qPCR) assay

*3.9.2.1 Primer design*

A series of primers were used in these analyses (Table 2.19). As there are currently no available transcriptome sequences for fin whales, the specific primers were designed from the genome sequence (NCBI GenBank assembly accession: GCA_008795845.1) by aligning genomic sequences of the genes with NCBI cDNA sequences of from a related organism (Killer whale, Orcinus orca) to identify exons and introns. The Primer3 software (https://primer3.ut.ee/) was used for designing primers.

*3.9.2.2 qPCR protocol*

First strand cDNA synthesis was performed using iScript cDNA synthesis kit following the manufacturer's protocols (Bio-Rad). Each RNA sample was prepared from 500 ng RNA in 10 µL water in a PCR strip. This mix (mix A) was then incubated at 70°C for 5 minutes to denature the RNA and then kept on ice. While denaturing the RNA, a mix of 4 µL 5x iScript Reaction Mix, 1 µL of iScript reverse transcriptase (RT), and 5 µL nuclease free water (mix B). Mix B was multiplied by the number of samples along with some extra. Pooled or one RNA sample was used to set up a no reverse transcriptase (NRT) control. 10 µL of mix B was added to mix A while the PCR strip was on ice. NRT control got a mix B where RT was exchanged with nuclease free water. After mixing by pipetting up and down, the samples were spun down and incubated in the PCR machine at the following temperatures:

25°C for 5 minutes
46°C for 30 minutes
95°C for 1 minute
Then kept at 4°C until further use

The cDNA was diluted 1:5 to create templates for qPCR. A Primer and Master mix was prepared: 5 µL of a 2 µM Primer mix (forward and reverse primers) was added to 10 µL 2x SYBR Green I Master mix. This mixture was multiplied by the number of samples along with some extra.

5 µL template was added to wells of a PCR plate, three replicas for each sample and NRT control. Then 15 µL of the Primer-Master mix was added and mixed by pipetting carefully up and down. A lid was fixed onto the plate before it was spun down at 500 x g for 2 minutes. Lastly, the plate was put in the qPCR machine and went through the following temperature profile

95 °C for 5 minutes
95 °C for 10 seconds
55 °C for 20 seconds
72 °C for 30 seconds
} x 40 cycles

Different experiments were prepared from varying RNA concentrations (250, 450, 500, or 750 ng).

### 3.9.4 Validation of primers

A standard curve with relative concentrations of xx was created to calculate the primer efficiencies. For actin and *CYP1A1* (F1R1 and F2R2) primers, the standard curve was prepared from a serial dilution of the first strand cDNA (from mixture of RNA from selected samples). After the synthesis, the cDNA was serial diluted 1:2, eight concentrations in total.

For *OCT4*, *AHRR*, *PPARG*, and *HSP70*, the standard curve was prepared using a 10-fold serial dilution gel-purified PCR fragments. The PCR products (from cDNA templates) using the respective primers were separated on a 2% agarose gel and purified using QIAX gel purification kit according to manufacturer's protocol (QIAGEN). The purified PCR products were diluted 1:100000. From this dilution, 10-fold serial dilutions were prepared for standard curves.

The primer pairs of *SOX2*, *NANOG*, *CYP1B1*, and fabp4 did not get standard curves since the amplifications from these primers were either too low/absent (*CYP1B1*) or their melting peaks showed the primers were not specific (*SOX2*, *NANOG*). Also, the PCR product from *OCT4*, *SOX2*, and *NANOG* were run through a 2% agarose gel.

*QIAEX II agarose gel extraction protocol*

The DNA bands were cut, with minimal excess, from the agarose gel using a clean, sharp scalpel, and visualising the bands with a UV transilluminator. The cut product was put into a 1.5 mL Eppendorf tube and weighted. 3 volumes of buffer QX1 was added to 1 volume of gel (300 µL buffer to 100 mg gel). 5 µL QIAEX II suspension was added and suspended into the solution. After, the mixture was incubated at 50°C for 10 minutes. During incubation, the mixture was vortexed every 2 minutes to keep QIAEX II in suspension. If the mixture had an orange or purple colour, 10 µL of 3M sodium acetate (pH 5.0) was added and the mixture incubated another 5 minutes.

After incubation, the sample was centrifuged for 30 seconds. The supernatant was removed, and the pellet was resuspended in 500 µL buffer QX1 by vortexing. Then the resuspension was centrifuged for another 30 seconds, the supernatant was removed, and the pellet was washed twice with 500 µL buffer PE by resuspending the pellet, centrifuging for 30 seconds, and removing the supernatant.

After washing, the pellet was air dried for 15 minutes before it was resuspended in 20 µL $H_2O$. The solution incubated for 5 minutes at room temperature to elute the DNA. Lastly, the mixture was centrifuged for 30 seconds, and the supernatant containing the DNA was transferred into a clean tube.

The purified DNA was diluted 1:10 000 before being serial diluted in a PCR strip.

### 3.9.5 Statistical analysis

The data from qPCR assay was analysed according to Schmittgen TD and Livak KJ (2008). In brief, the average Cq of each sample was calculated. Then ΔCq was calculated by subtracting the Cq of the housekeeping gene (Cq_actin) from the Cq of the target gene (e.g., AHRR). After, the average ΔCq of the control samples was calculated and subtracted from the ΔCq of every sample, finding the sample ΔΔCq. Then the fold change (FC, $2^{-\Delta\Delta Cq}$) was calculated and the average FC for control was found. The FC was normalised to the average FC for control and FC mean and standard deviation were found for each gene target group in the assay.

For the statistical comparison (at least triplicates), the FC values were log2 transformed before they were tested for normal distribution (Shapiro–Wilk test) and if there were any statistical outliers. The log2 transformed values were found to be normally distributed, and ANOVA and Dunnet's test or a t-test (for two groups) were performed. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA).

# 4. Results

## 4.1 Optimization of cell culture conditions

### 4.1.1 Initial observations — Hypoxia chamber and passage vitality

One major issue with the cell cultures were that they grew slowly. After passage 5, the growth slowed, and by passage 7 they barely grew and appeared senescent (Figure 4.1). No testing was done to confirm senescence, but their morphology resembled senescent cells.

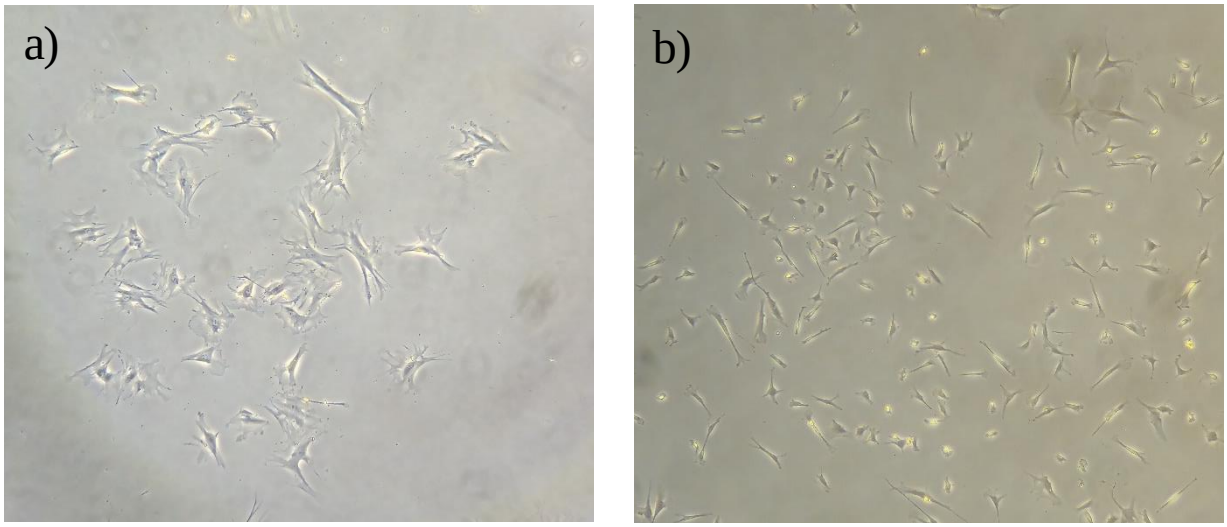


*Figure 4.1: Fin whale fibroblast cultures from a) passage 6 with abnormal morphology and b) passage 3 with normal morphology. Taken through microscope with the same zoom level. Pictures obtained with an HTC U11 phone camera.*

There were no direct comparisons made between standard oxygen levels and *in vivo* oxygen levels. This was due to the nature of the hypoxia chamber used. The chamber needed to be sealed and have the air inside changed into one with 5% oxygen content. Then it was sealed airtight and put into the incubator. Thus, several steps were required to ensure hypoxia for the cells. This made it more difficult to use it in direct cell growth comparisons. However, observations indicated that the cells lasted one passage longer before going senescent, which could mean that hypoxia may give slightly better growth conditions.

### 4.1.2 Effect of bFGF (FGF2)

Adding bFGF to the culture medium decreased the time it took for the cells to grow to full confluency by several days (Figure 4.2). There was little difference in the growth rate between 2 ng/mL and 10 ng/mL of bFGF added to the growth medium. On day 10, two plates with 10 ng/mL and one plate with 2 ng/mL were confluent. The next day, the remaining two plates with 2 ng/mL were confluent. The last 10 ng/mL plate was confluent on day 13. In comparison, the control plates were confluent on day 15. In summary, adding bFGF to cell culture medium gives better growth conditions for fin whale fibroblast cells.

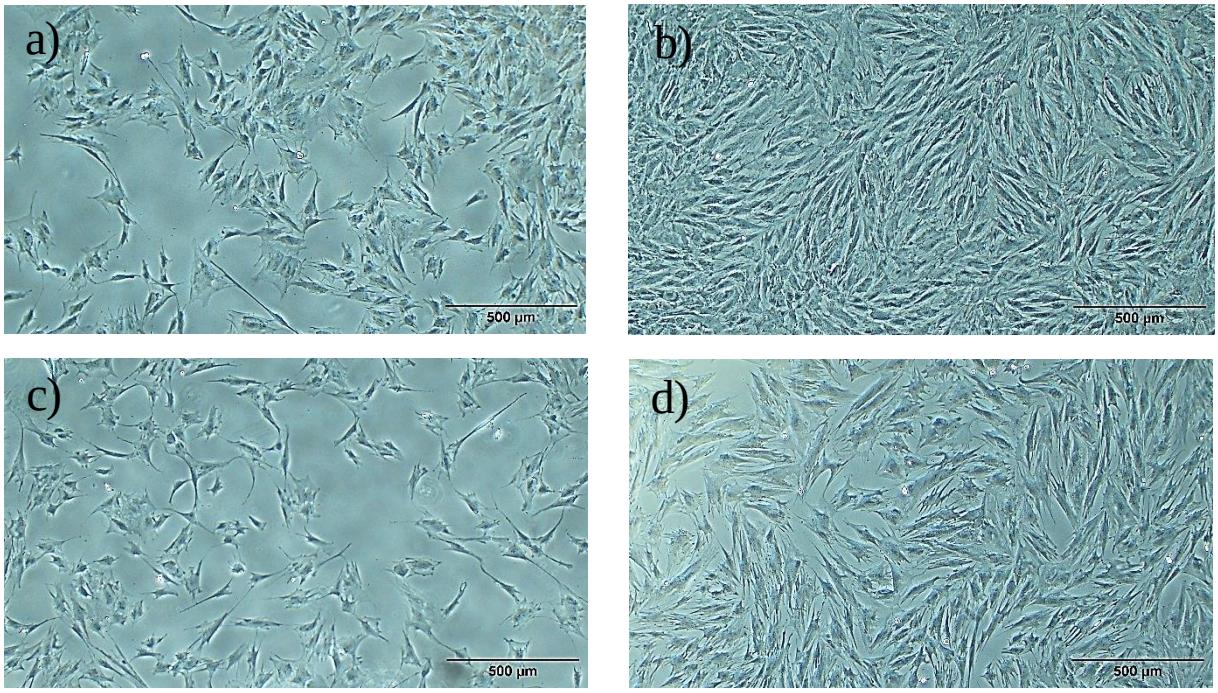


*Figure 4.2: Fin whale fibroblasts cultured with 10 ng/mL bFGF after a) five days and b) 10 days. Control cells without bFGF after c) five days and d) 10 days*

### 4.1.3 Effect of collagen coating

Unlike adding bFGF, collagen coating of the cell plates did not give any apparent difference in cell growth (Figure 4.3). Both the collagen coated plates and the control plates were fully confluent on day 15. As such, collagen coating on its own does apparently not increase the cells' growth rate.

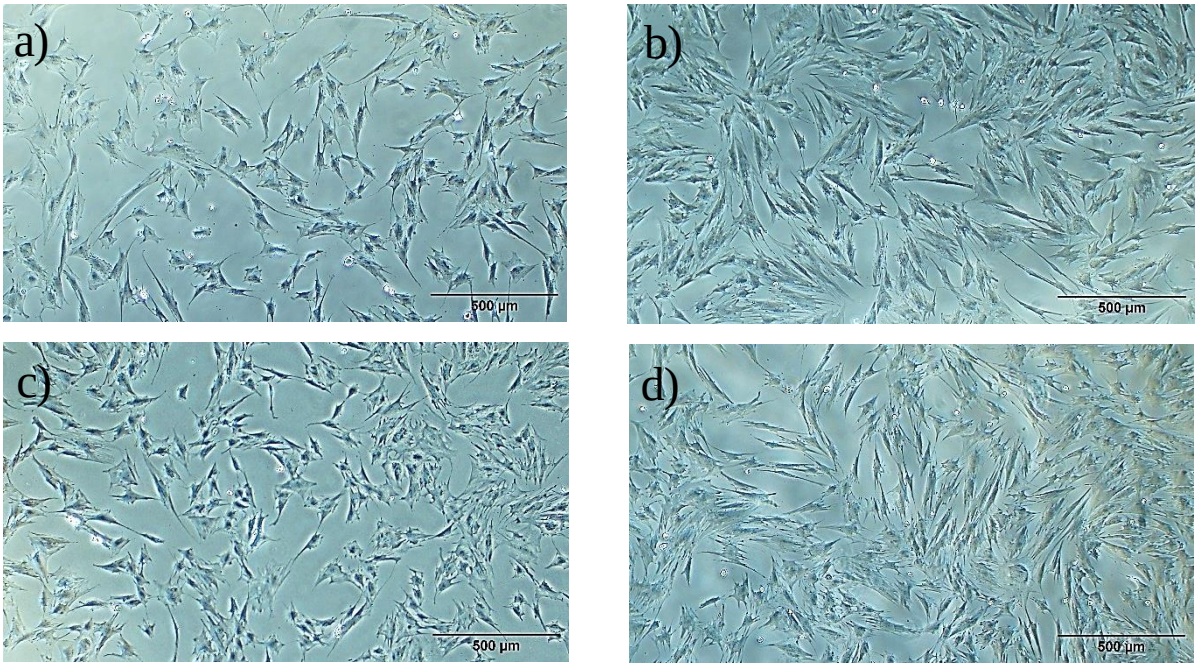


*Figure 4.3: Fin whale fibroblasts cultured with collagen coating after a) five days and b) 10 days. Control cells without collagen coating after c) five days and d) 10 days*

## 4.2 Exposure results

### 4.2.1 Western Blotting

The measured protein concentrations were low and unreliable (not shown). The loading controls of actin showed varying amounts within the different wells in the Western blots.
Western blotting was the first of two methods used to determine a stress response by checking the synthesis of CYP1A1. Expected molecular weight is 50–58 kDa.

Western blotting of Exposure 1 (se 3.5.1) did not show an increased synthesis of CYP1A1 (Figure 4.4). The exposure to 10 µM BaP did not cause the fibroblast cells to induce CYP1A1. On the other hand, Western blotting of Exposure 2 did not detect any CYP1A1 in the samples (Figure 4.5). Neither the samples exposed to 5 µM BaP nor the DMSO controls showed any signs of CYP1A1 in the western blot.

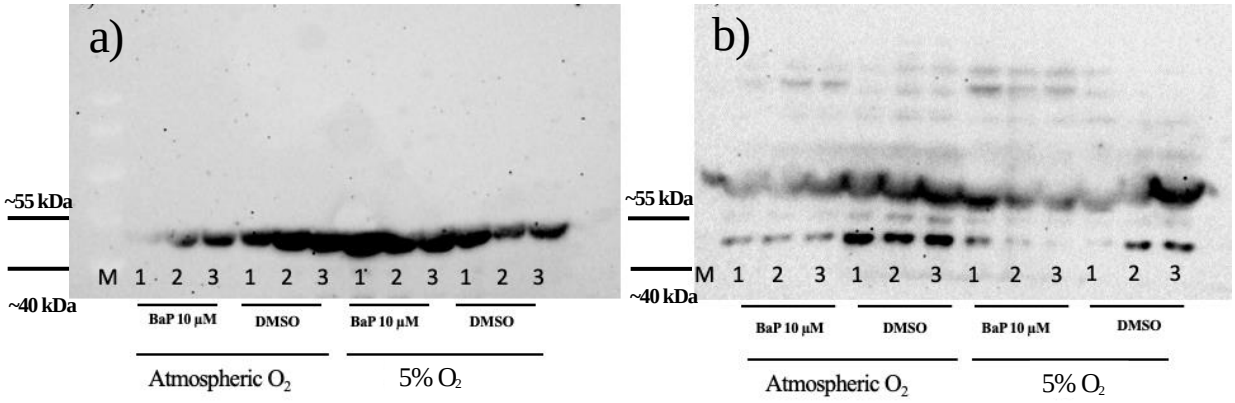


*Figure 4.4: Western blot of exposed and unexposed fin whale fibroblast cell samples from Exposure 1 probed with rabbit antibodies against a) CYP1A1 and b) actin.*
*Lanes are, from the left: molecular standard; BaP 10 µM 1, 2, 3, DMSO control 1, 2, 3 samples incubated in atmospheric $O_2$ levels; BaP 10 µM 1, 2, 3, DMSO control 1, 2, 3 samples incubated in 5% $O_2$ levels*

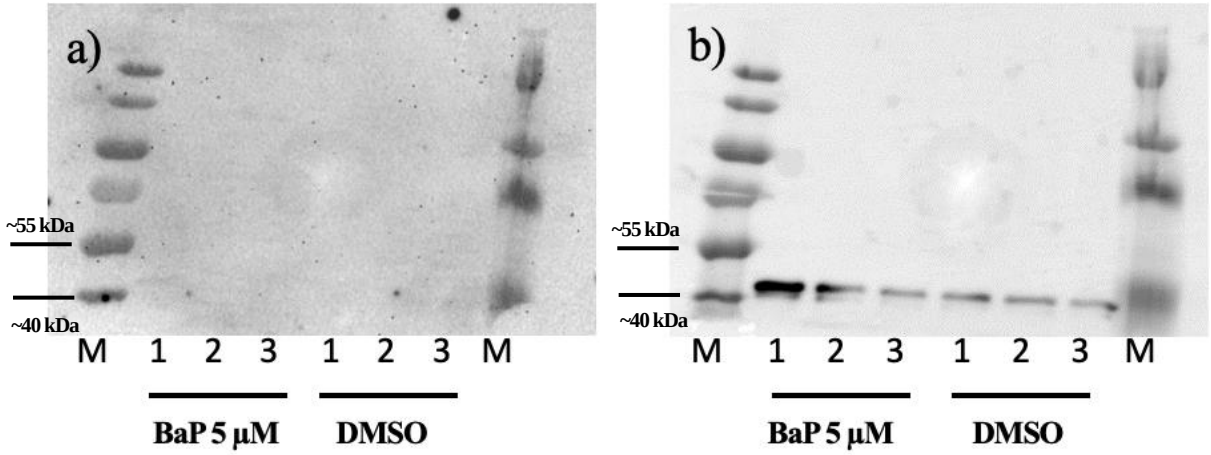


*Figure 4.5: Western blot of exposed and unexposed fin whale fibroblast cell samples from Exposure 2 probed with rabbit antibodies against a) CYP1A1 and b) actin. Lanes are, from the left: molecular standard, BaP 5 µM 1, 2, 3, DMSO control 1, 2, 3, molecular standard*

### 4.2.2 Viability assay

Cell viability was measured by using an LDH assay. Absorbance levels at 490 nm and 650 nm were measured and compared.

The cells exposed to 5 µM BaP have about the same absorbance level as the DMSO controls (Figure 4.6). A t-test showed no significant difference ($p = 0.94$) which indicate no difference in cell mortality. As such, exposure to 5 µM BaP did not affect cell viability.

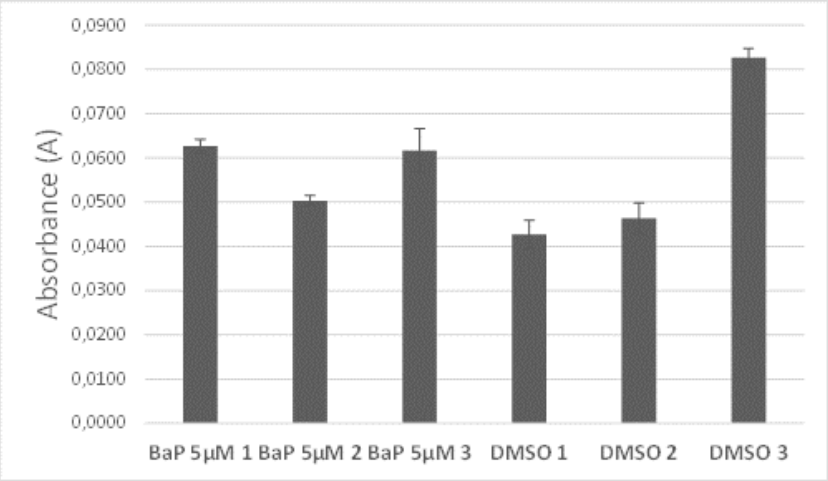


*Figure 4.6: Average absorbance level and standard deviation of the three exposure replicas and three DMSO controls*

## 4.2.3 qPCR output

### *4.2.3.1 Primer test*

The second method used to determine the stress response was qPCR with primer pairs relevant to the pathways affected by BaP and MEHP in the cells. As such, the various primer pairs were tested to validate the qPCR assay.

The tested *CYP1A1* primer pairs (F1+R1, F2+R2, F2+R1) and fabp4 primer pair (F1+R1) did not work with my fin whale fibroblast cDNA. They had melting peak graphs with several peaks, and thus were not amplifying the specific fragments. The standard curves of *CYP1A1* F1R1 and F2R2 also showed that the primer does not work properly. *CYP1B1* primer pair might be functional, but too low expression of the gene was detected in all the samples. The amplicon seems to have one melt peak when looking at the graph.

The remaining primer pairs all had amplification efficiencies within the desired range of 90–110%. Figure 4.7 shows the standard curve and efficiency of *AHRR*, *HSP70*, *OCT4*, and actin.

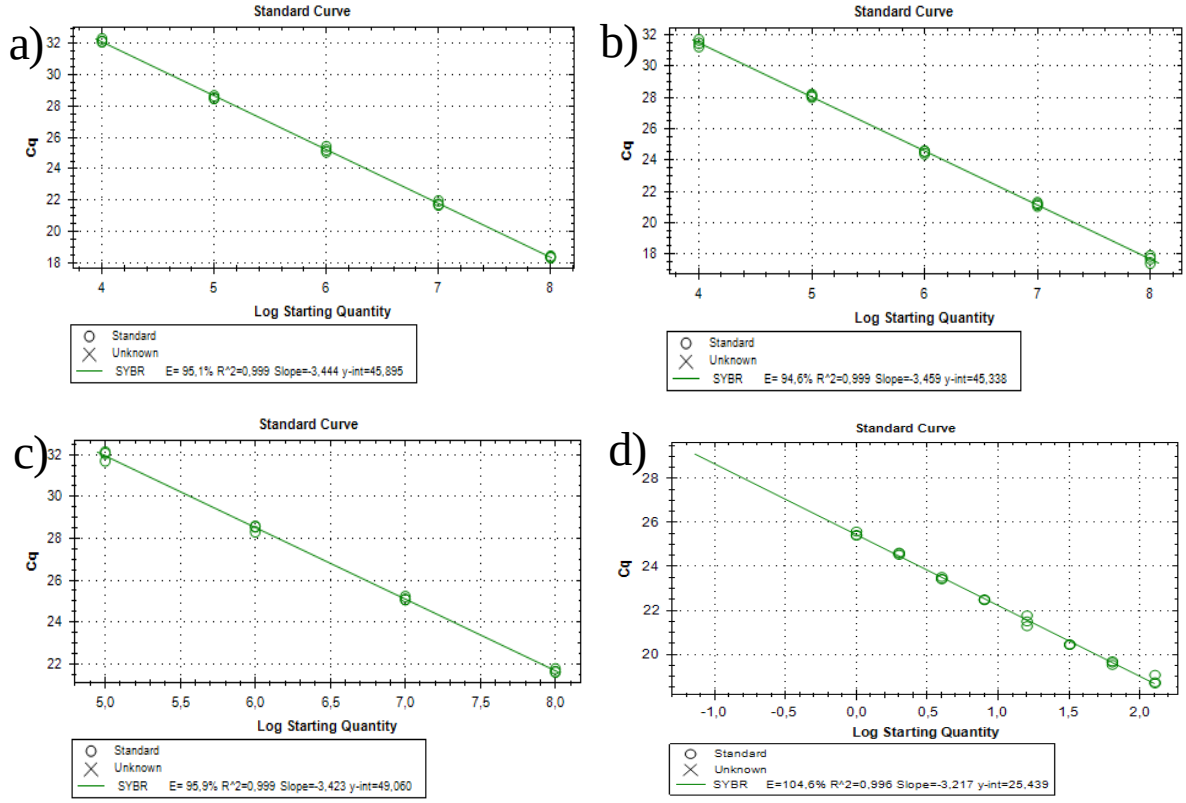


*Figure 4.7: Standard curve with efficiency for primers a) AHRR, b) HSP70, c) OCT4, and d) beta-actin on cDNA from fin whale fibroblasts*

*4.2.3.2 Effect of BaP exposure*

For Exposure 3, none of the qPCR FC values were found to be outliers, statistically. ANOVA tests showed an increase, but no statistically significant differences (between BaP treated samples and DMSO controls) in expression of both *AHRR* and *HSP70* (Figure 4.8). Thus, the effect of these concentrations of BaP on *AHRR* or *HSP70* is unclear.

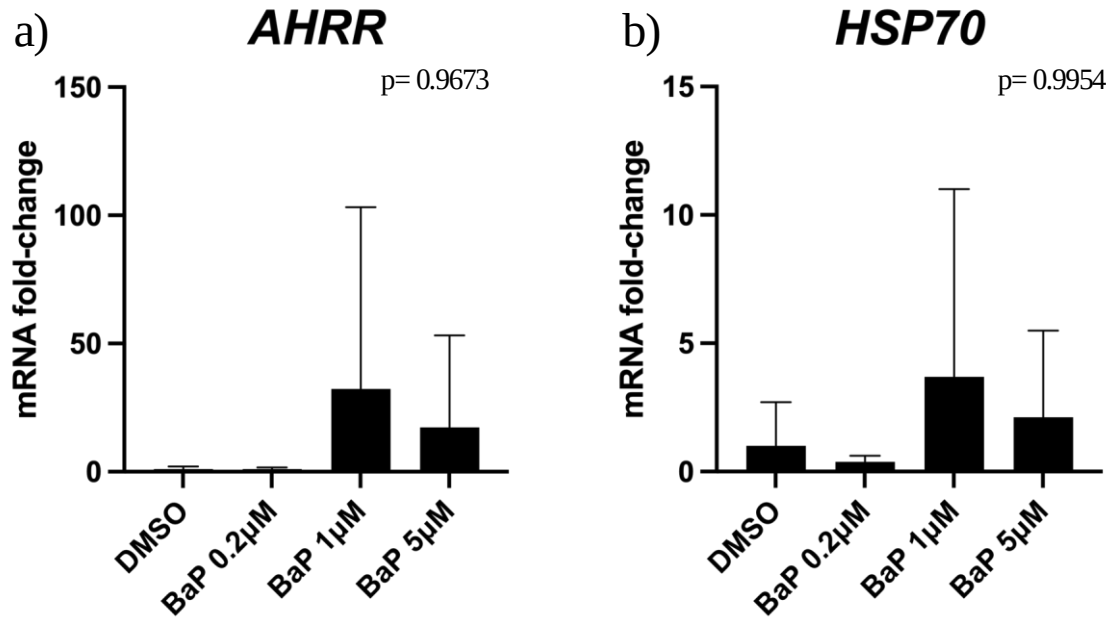


*Figure 4.8: Average fold-change in a) AHRR and b) HSP70 mRNA expression in fin whale fibroblasts after exposure to 0.2, 1, and 5 µM BaP with DMSO control (n=4 for 0.2 µM BaP and n=5 for the remaining ones)*

As results from Exposure 4 had only two replicas, no statistical test could be performed. Table A (*AHRR*) and B (*HSP70*) in Appendix show the calculated fold-change of BaP exposure. Again, no clear effect on these genes could be observed. On the other hand, the results from the qPCRs using *CYP1A1* and *CYP1B1* are not valid nor trustworthy due to non-functional primers (*CYP1A1*) or lack of expression (*CYP1B1*).

*4.2.3.3 Effect of MEHP exposure*

*PPARG* showed an apparent slight decrease in expression while *HSP70* showed an apparent massive decrease in expression (0.08 and 0.09 fold-change compared to DMSO control, Table C and D in Appendix). However, as there were only two replicas for each condition, no statistical testing could be done.

## 4.3 Reprogramming to MSCs

### 4.3.1 Morphology

Fin whale fibroblast cells were exposed to MSC induction medium as an attempt to induce the cells into iMSCs.

Cells exposed to reprogramming medium showed a visible change in morphology (Figure 4.9). This could indicate that something about the cells is altered compared to the fibroblasts.

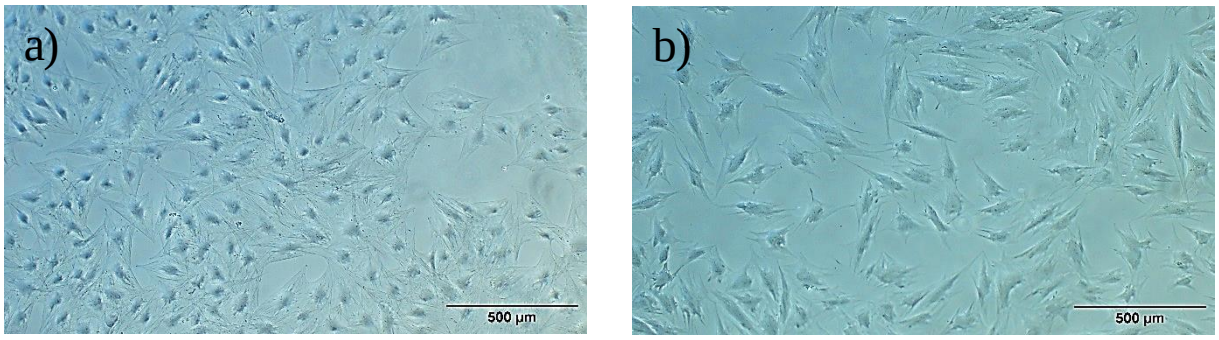


*Figure 4.9: a) Plate with fin whale fibroblast cells exposed to reprogramming medium after 5 days. b) Control cells cultivated with control medium after 5 days.*

### 4.3.2 qPCR output

RNA was extracted from fin whale fibroblast cells exposed to reprogramming medium for 7 days and control fibroblasts using TRI reagent. After assuring the RNA quality, cDNA was synthesised. The primer pairs of *OCT4*, *SOX2*, and *NANOG* were used as MSC markers as they are common markers for pluripotency and stem cells.

The first qPCR run gave no readable results, including the housekeeping actin gene.

The second qPCR run showed an apparent decrease in the expression of *OCT4*. However, this result is unreliable because there were only two replicas of the two conditions.

The qPCR FC values from the last qPCR showed a decreased expression of *OCT4* (Figure 4.10), but a t-test showed that the results were not significant (p = 0.8873).

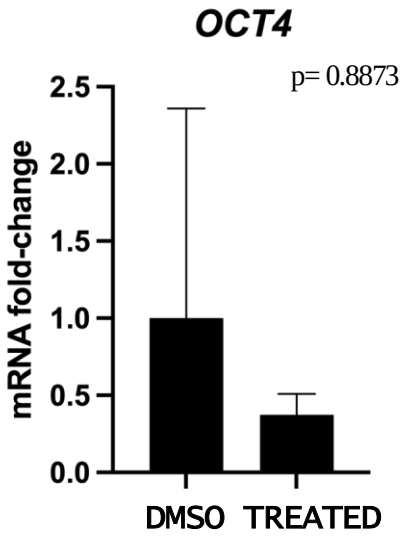


*Figure 4.10: Average fold-change in mRNA expression of OCT4 after 7 days exposure of fin whale fibroblasts to reprogramming medium compared to control cells (n=3 for iMSC (TREATED) and n=4 for DMSO control)*

The tested primer pairs of *SOX2* and *NANOG* did not work and there was no time to test other pairs. *SOX2* had a melting curve with several peaks, which indicates that the primer pair is unspecific. It also had several bands of background noise on the agarose gel. *NANOG* had a specific melting peak, but the agarose gel showed strong level of low MW background noise that may indicate primer dimers.

### 4.3.3 Adipocyte differentiation

Fin whale fibroblast cells exposed to MSC induction medium were exposed to adipocyte differentiation medium to test their differentiation ability. ORO staining was done after 15 days of differentiation.

The cells from the first adipocyte differentiation of apparent iMSCs showed a difference in morphology (Figure 4.11). The induced cells showed some signs of lipid droplets visible through microscope after 13 days of induction medium. However, ORO staining showed no signs of red droplets which would indicate the absence of lipid droplets.

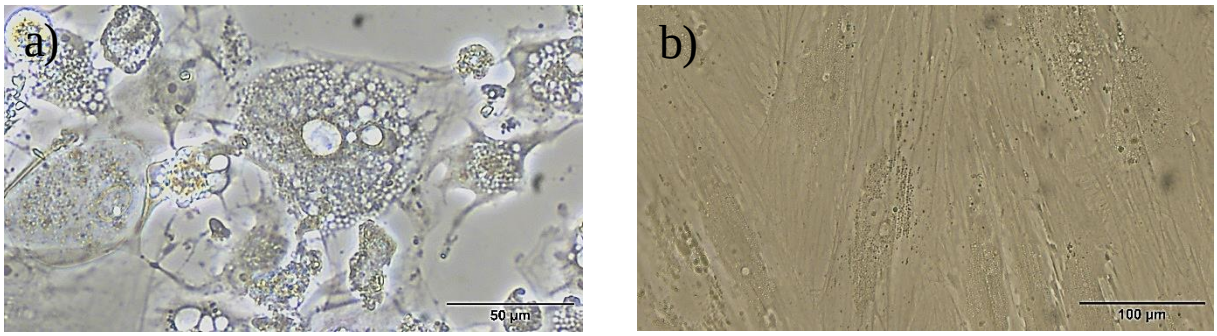


*Figure 4.11: Apparent iMSCs 13 days after a) differentiation into adipocytes and b) control with standard culture medium.*

The plates for the second adipocyte induction were contaminated by bacteria about mid-way through the differentiation period. Thus, the experiment was abandoned and there was no time to repeat it.

# 5. Discussion

Many reports have described the difficulty of working with marine mammal cell culture (e.g.,Smith *et al.*, 1987; Wang *et al.*, 2011)). This master's thesis project proved no different. Through my work, I struggled with slow growth, cell senescence, bacterial and mycoplasma contamination, among other things. Hence, I did not achieve all the goals I had set, but I did make some observations that may be useful for future work on these cells.

The main goal of this thesis was to establish ways to use fin whale fibroblasts in toxicological sturdies. To accomplish this, three subgoals were looked at. Firstly, a few growth conditions were tested to determine if these gave any improvements in cell growth. Secondly, the cells were exposed to two environmental toxicants, BaP and MEHP, and their responses were determined with western blotting and qPCR assay. Lastly, there was an attempt to reprogram fin whale fibroblasts into iMSCs.

## 5.1 Cell growth and culturing conditions

Cell culturing holds a lot of potential when it comes to toxicological studies in marine mammals despite the struggle of obtaining cell cultures from these elusive animals (Freshney, 2016; Lam *et al.*, 2020). Lam *et al.* (2020) summarises how cellular modelling with primary cells has been used in other non-traditional mammals with unique natural resistances. They illustrate how cell modelling was used to provide functional explanations of the mechanisms

lying behind different mammals' natural resistances to extreme conditions. It shows how valuable cell cultures can be in functional studies. However, to get reproducible and accurate results, the cell cultures need to be cultured in suitable conditions (Küppers-Munther *et al.*, 2004; Webb *et al.*, 2014; Lam *et al.*, 2020).

Adding bFGF to the cell culture medium increased the growth rate. The time until full confluency went down from 15 days to around 11 days. This means that it is beneficial to add bFGF to the fibroblast growth media. On the other hand, collagen coating showed no difference, also taking around 15 days to reach full confluency. Thus, there seems to be little point in coating the plates with collagen. However, Yashiki *et al.* (2001) show that fibroblast growth potential was enhanced by collagen coating, while Ivanova *et al.* (2009) showed that collagen fragments accelerated fibroblast adhesion. As such, there could be a potential in coating the plates with collagen even though this thesis did not see much difference. Also, there is the possibility that the method used to determine cell growth (visual inspection) was not sensitive enough to register a difference in growth between collagen coated and non-coated plates.

One thing to note is that the cells were not evenly distributed on the plate when starting this observation period. Every plate contained a part in the middle that started with ~90–100% confluence. The rest of the plates had a decently even distribution. This highly confluent part could potentially have affected the growth rate as the highly confluent parts could have depleted the medium of nutrients, build up toxic waste, and lowered the pH (Freshney, 2016). Thus, repeating the cell growth comparison between collagen coated and non-coated plates with a more sensitive detection method while making sure the cell distribution are entirely even would likely give a better assessment of its effect on fin whale fibroblast cells.

The fin whale fibroblast cells seemed to last about seven passages before appearing senescent and stopped growing with standard culture medium and atmospheric oxygen levels. Lower oxygen levels (5%) seemed to increase passage number to eight. Better suited growth conditions could potentially increase cell vitality and passage numbers. Higher cell vitality increases the ability to gain a higher cell number, which increases the possible experiments they can be used in. At the very least, better suited growth conditions give a higher cell growth as Burkard *et al.* (2015) shows with humpback whale cells. For example, the humpback whale cell numbers increased in a concentration-dependent manner from 5–20% FBS. They also grew best at 37°C. Fin whale cells could potentially show a similar pattern, which would certainly make experiments more accessible. Wise *et al*. (2015) cultured fin whale skin fibroblasts in DMEM/F12 medium with 15% cosmic calf serum, though they maintained the culture at 33°C. Both Burkard *et al.* (2015) and Jin *et al.* (2013) split the cells 1:3 which appears to give the cells a decent starting number. Jin *et al.* (2013) observed reduced proliferation in their humpback dolphin primary fibroblast cultures at passage 10 and senescence at passage 17. Burkard *et al.* (2015) reached passage 30 without observing slowed growth or senescence. Several people use DMEM/F12 medium, that was also used here, with supplements (Burkard *et al.*, 2015; Wise *et al.*, 2015; Meaza *et al.*, 2020).

## 5.2 Exposed cells showed no induction of AHR pathway or heat shock proteins

Both western blot and qPCR showed no induction of the AHR pathway genes in fin whale fibroblasts exposed to the AHR agonist BaP. Neither CYP1A1 (western blot) nor *AHRR* (qPCR) were activated despite the fact that both genes are inducible by AHR ligands (Karchner *et al.*, 2002). The cell viability assay showed that the cells survived the exposure to 5 µM BaP. Thus, one could argue that BaP should have induced *CYP1A1*. Part of the reason could potentially lie in the fact that fibroblasts have a lower sensitivity to AHR pathway-inducing compounds such as dioxin (Gradin *et al.*, 1999; references therein). Considering that BaP and dioxin trigger the same AHR pathway, this might explain the lack of induction of *CYP1A1* in the whale fibroblasts treated by BaP. Gradin *et al.* (1999) found that while dioxin have a low capacity to induce CYP1A1 in fibroblasts, adding the compound trichostatin A (TSA) along with dioxin resulted in increased induction of *CYP1A1* compared to dioxin and TSA separately. As such, it was an experiment to understand the mechanisms underlying the lower sensitivity of fibroblasts to AHR ligands. This could be interesting and informative to test with fin whale fibroblasts, determining if these fibroblasts do have a lower sensitivity to AHR ligands. On the other hand, several other studies have successfully induced *CYP1A1* in fin whale fibroblasts (Fossi et al., 2000, 2003, 2010; Fossi, Casini and Marsili, 2007). However, Fossi *et al*. (2010) found differences in responses from *in vitro* tests of fin whale biopsy slices from the Mediterranean Sea compared to fin

whales from the Gulf of California. Male individuals from the Gulf of California showed a dose–dependent induction of *CYP1A1*. Conversely, male individuals from the Mediterranean Sea showed no *CYP1A1* induction. They also measured contamination levels of various compounds, which included PCBs, DDTs, and PAHs. When they explored molecular biomarker responses, they found a statistically significant positive correlation between total PAHs and CYP1A1 induction. Thus, the lack of *CYP1A1* induction in the fin whale fibroblasts used in this thesis could potentially mean that the individual they originate from has endured a high toxicological load. Though, considering that Tartu *et al.* (2020) found a significantly lower pollutant level in fin whales from the same area (Svalbard), this seems highly unlikely. To demonstrate, Tartu *et al.* (2020) found a ΣPCB level of 219 ng/g lipid weight in female fin whales from Svalbard while Pinzone *et al.* (2015) found a ΣPCB level of 3776 ng/g lipid weight in the Mediterranean Sea. However, if the skin biopsy used in this thesis originate from a fin whale individual that was vagrant and visiting from a higher polluted area, there could potentially be some unknown toxicological load.

Heat-shock proteins (HSPs) are induced by a number of relevant physical and chemical agents and their purpose is mainly to protect the cells (Aït-Aïssa *et al.*, 2000; Rössner, Binková and Šrám, 2003). As these proteins have a large diversity of inducers, different toxicity mechanisms are thought to trigger these proteins. HSP70 is one of the proteins within this family and can thus be useful in toxicological studies. While *HSP70* does not represent a sensitive biomarker of toxicity compared to classical cell survival assays, it does give detailed information of the mechanisms behind the toxicity (Aït-Aïssa *et al.*, 2000). No induction of *HSP70* was detected in this thesis which could indicate that the cells were not stressed. However, another possibility is that BaP does not induce *HSP70* as Aït-Aïssa *et al.* (2000) show with transfected HeLa cells. They speculate that the lack of induction comes from an inefficient metabolic action of BaP in the cells, though some studies show the contrary (Rössner, Binková and Šrám, 2003; references therein). On the other hand, Grøsvik and Goksøyr (1996) found that BaP and PCBs induced HSP70 in primary cultures of salmon hepatocytes. Grøsvik and Goksøyr (1996) do note that the HSP70 response could be a secondary response to products of AHR mediated gene activation. If their speculation is correct, then the lack of *HSP70* induction in this experiment makes sense as the AHR pathway was not induced by BaP.

Cells were exposed to contaminants in pairs for Exposure 4 as it was an attempt to gain an overview dose–response assessment. There were limited cells to work with as time was running short and the cells grew slowly. Cells were exposed to MEHP as it is a compound that fin whales potentially encounter along with other phthalates (Garcia-Garin *et al.*, 2022). Studies indicate that phthalates can have several impacts, including acting as hormone sensitisers and nuclear receptors (Benjamin *et al.*, 2017; Baken *et al.*, 2019; Garcia-Garin *et al.*, 2022). Some of these compounds, including DEHP which metabolises to MEHP, appear to impair reproduction, metabolism, and development, and to cause neurological and carcinogenic effects. PPARG activation is highly likely to happen with phthalate exposure, which means it is a useful biomarker for such exposure (Baken *et al.*, 2019). Though, in this initial screening, the expression of *PPARG* seemed lower than the controls (Table C in appendix).

## 5.3 Exposure to MSC induction medium

The cells showed a visible difference in morphology after a seven-day exposure to MSC induction medium. This could indicate that they were reprogrammed into MSCs. However, the qPCR results showed a lower *OCT4* expression in the apparent iMSCs, though there were no statistical significance in this difference. Thus, these cells were likely not reprogrammed. Lai *et al.* (2017) successfully generated iMSCs from human dermal fibroblasts using the chemical cocktail used in this thesis. They tried out several chemical cocktails, with and without growth factors. While the six chemicals alone managed to convert the cells into iMSCs, including the three growth factors (TGF-β, LIF, and bFGF) appeared to give a better conversion rate. *OCT4* expression in their cells was upregulated compared to fibroblast controls, with similar expression as bone marrow MSCs. This shows that *OCT4* can potentially work as an indicator for MSC induction. Though, this marker is more relevant for iPSCs as they have a higher expression of *OCT4* than MSCs (Lai *et al.*, 2017; Dayem *et al.*, 2019). Thus, showing the presence of MSC markers and the differentiation potential of the cells would likely give better indication that cells were successfully induced into iMSCs (Wagner and Ho, 2007; Lin *et al.*, 2013; Lai *et al.*, 2017).

One thing to note about the induction experiment performed here is that no viability assay was performed on the cells exposed to the induction medium and the apparent iMSCs had a lower proliferation when expanded with MSC medium. When they grew in standard culture medium they seemed to revert into a fibroblastic morphology, which either suggests a redifferentiation or that the morphology change came from a stress response. However, MSCs are reported to have a fibroblast-like morphology, supporting the stress response angle (Rombouts and Ploemacher, 2003; Han *et al.*, 2019). On the other hand, they maintained the altered morphology when cultured in MSC expansion medium. This could indicate the apparent iMSCs redifferentiated into fibroblast, though it could be that this medium maintained the morphology from a stress response. The lower proliferation of the apparent iMSCs with the MSC expansion medium give some credit to the maintenance of a stress response angle, or it simply means they had a lower viability.

While microscopy showed signs of lipid droplets in the cells treated with adipocyte differentiation medium, the ORO staining gives the impression that the cells contained none. This could indicate that the cells did not differentiate into adipocytes. However, there is the possibility that something went wrong during the ORO staining which removed all lipid droplets from the cells. Considering that there was no red colouring from the ORO, even in the control cells, the possibility is present. If the cells did differentiate, this gives some indication that the cells were iMSCs. Although, due to sample loss, only two replicates were analysed. This analysis showed an apparent decrease in *OCT4* expression, so it probably had a similar result as the second induction.

## 5.4 Conclusions

In summary, throughout this study, the overarching goal was partly reached as some of the analyses showed potential uses in toxicological studies. The goals were only partly reached due to difficulties concerning the cell cultures. These cells grew slowly and lasted only to passage 7 before appearing senescent. They started to grow slower when they hit passage 6, also starting to show senescent morphology. Considering that the earliest passage available was passage 3, this made it difficult to gain enough cells for experiments. This, along with limited time, is why growth conditions were not that extensively tested and only two environmental toxicants were tested. The results of this thesis are: Firstly, adding bFGF to the medium gave a noticeable boost in growth rate. Secondly, no cellular response was detected from the exposures. Lastly, the cells showed an altered morphology after exposure to the MSC induction medium. However, *OCT4* expression showed no significant difference between induced and control cells. While this thesis was not that extensive, it does serve as a foundation for further work on these cells.

## 5.5 Future perspectives

One aspect that could be important in future work would be to find even better growth conditions for the cells. Here, hypoxic conditions were briefly looked at, while the effect of added bFGF and collagen coated plates were extensively checked. Further modifications of growth conditions and medium could potentially increase cell growth and viability. Things that could be relevant to assess include higher levels of FBS, lowering the oxidative stress (e.g., by adding a reducing agent), and combining conditions, checking for potentiating or synergistic interactions between the conditions. As stated, Burkard *et al.* (2015) found that humpback fibroblast grew better with 20% FBS. Additionally, several studies used 15%, or higher, serum supplementation (Mollenhauer *et al.*, 2009; Wang *et al.*, 2011; Jin *et al.*, 2013; Wise *et al.*, 2015; Yajing *et al.*, 2018; Meaza *et al.*, 2020). Thus, testing which FBS concentration give the best growth condition is certainly something to explore.

Both collagen and bFGF are a part of the extracellular matrix (ECM), which is the surrounding scaffolding for the cells throughout the body (Poole *et al.*, 2005; Ivanova, Kovaleva and Krivchenko, 2009; desJardins-Park *et al.*, 2020). Collagen is part of the insoluble factors of the ECM and helps with cell attachment. On the other hand, bFGF is one of the soluble factors that stimulates cell growth (Allouche' and Bikfalvi?', 1995). This thesis showed that collagen did not seem to affect cell growth while bFGF gave a significant boost. However, these two factors were tested separately. Considering that both collagen and bFGF are a part of the ECM, combining the factors could potentially

give the cells a better boost in growth, viability, or maybe both. Testing them in combination compared to on their own could be interesting. It could also be interesting to also include other ECM components.

Oxidative stress has a negative effect on several cell processes (Brownlee, 2001; Terada, 2006; Forbes, Coughlan and Cooper, 2008; Paravicini and Touyz, 2008; Lamers *et al.*, 2011). Thus, lowering the oxidative stress of the cells could possibly affect cell growth and viability. Research show that excessive glucose increases oxidative stress (Brownlee, 2001; Forbes, Coughlan and Cooper, 2008; Paravicini and Touyz, 2008). As such, using culture medium with a low glucose content could help the cells. Another possible solution is to add an antioxidant to the medium at a non-toxic, but working, concentration. Using the hypoxia chamber could lower the oxidative stress from higher oxygen levels (~21%) compared to the presumed *in vivo* oxygen concentration (~5%).

Future work should also set up more exposure experiments to toxicant and toxicant mixtures that are relevant for the fin whales. The experiments should include enough replicates so that a statistical analysis is relevant.

Further trials with the MSC induction method used here can be useful. Making sure that the cells are correctly identified is important. Performing immunohistochemistry with MSC markers and comparing the differentiation ability if apparent iMSCs with control fibroblasts might be better or more relevant identification methods. After successfully creating enough iMSCs, they can be used in toxicological screenings. Or the iMSCs can be differentiated into cells that are difficult or impossible to sample without killing the animal before being used in relevant experiments (Lam *et al.*, 2020). These cells have a lot of potential uses within toxicology.

Lastly, it might be beneficial to look into immortalisation of the cells (Lam *et al.*, 2020). Immortalised cells can divide indefinitely, which reduces the necessity to obtain new cell samples. Thus, it could significantly help when studying marine mammals. However, the cells might permanently change in a way that alters important functions and cell reactions. As such, it is important to test if the immortalised cells still carry the primary cell functions. If they do, they can give invaluable information in mechanistic investigations.

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# Appendix

## Fold change calculations from Exposure 4

Table A: Calculated *AHRR* response to BaP

| Sample | Cq_ahrr | Cq_actb | Normalised FC | Average FC | Standard deviation |
|---|---|---|---|---|---|
| DMSO 1 | 32.54 | 23.45 | 1.71 | | |
| DMSO 2 | 29.99 | 18.35 | 0.29 | 1.00 | 1.00 |
| BaP 10 µM 1 | 31.73 | 21.76 | 0.93 | | |
| BaP 10 µM 2 | 36.56 | 27.04 | 1.27 | 1.10 | 0.24 |
| BaP 1 µM 1 | N/A | 37.21 | N/A | | |
| BaP 1 µM 2 | 33.29 | 23.06 | 0.77 | N/A | N/A |

Table B: Calculated *HSP70* response to BaP

| Sample | Cq_hsp70 | Cq_actb | Normalised FC | Average FC | Standard deviation |
|---|---|---|---|---|---|
| DMSO 1 | 30.06 | 23.45 | 0.90 | | |
| DMSO 2 | 24.66 | 18.35 | 1.10 | 1.00 | 0.15 |
| BaP 10 µM 1 | 27.88 | 21.76 | 1.26 | | |
| BaP 10 µM 2 | 32.75 | 27.04 | 1.68 | 1.47 | 0.29 |
| BaP 1 µM 1 | 36.88 | 37.21 | 110.17 | | |
| BaP 1 µM 2 | 29.63 | 23.06 | 0.92 | 55.55 | 77.25 |

Table C: Calculated *PPARG* response to MEHP

| Sample | Cq_pparg | Cq_actb | Normalised FC | Average FC | Standard deviation |
|---|---|---|---|---|---|
| DMSO 1 | 36.59 | 24.72 | 0.78 | | |
| DMSO 2 | 36.27 | 25.05 | 1.22 | 1.00 | 0.32 |
| MEHP 50 µM 1 | 35.27 | 23.31 | 0.72 | | |
| MEHP 50 µM 2 | 30.52 | 18.55 | 0.72 | 0.72 | 0.003 |
| MEHP 5 µM 1 | 33.45 | 20.77 | 0.44 | | |
| MEHP 5 µM 2 | 33.47 | 21.51 | 0.73 | 0.58 | 0.20 |

Table D: Calculated *HSP70* response to MEHP

| Sample | Cq_hsp70 | Cq_actb | Normalised FC | Average FC | Standard deviation |
|---|---|---|---|---|---|
| DMSO 1 | 30.35 | 24.72 | 0.14 | | |
| DMSO 2 | 26.93 | 25.05 | 1.86 | 1.00 | 1.22 |
| MEHP 50 µM 1 | 29.63 | 23.31 | 0.09 | | |
| MEHP 50 µM 2 | 25.05 | 18.55 | 0.08 | 0.08 | 0.008 |
| MEHP 5 µM 1 | 27.24 | 20.77 | 0.08 | | |
| MEHP 5 µM 2 | 27.66 | 21.51 | 0.10 | 0.09 | 0.013 |