Graduate thesis

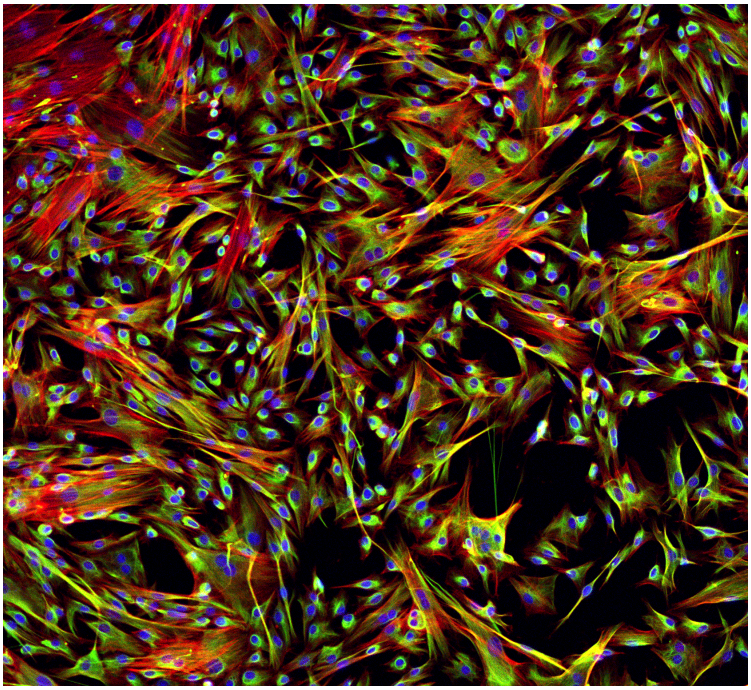
NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Sigrid Setsaas Hoven

Establishing robust 2D cultures of killer whale primary cells: an optimization study

Graduate thesis in Ocean Resources
Supervisor: Hanne Haslene-Hox
Co-supervisor: Anders Goksøyr, Heli Routti, Aman S. Chahal, Berit Løkensgard Strand

January 2023



Confocal microscopy image of killer whale fibroblasts. Photo: Aman S. Chahal.

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Abstract

Due to their trophic level and sources of prey, top predator Norwegian killer whales (*Orcinus orca*) are among the most contaminated marine species throughout the world. Accumulation of persistant organic pollutants (POPs) may have adverse effects on killer whale populations, by disrupting vital biochemical processes. Over the last decade, increased effort has been put into establishing *in vitro* model systems for marine mammals to assess the potential hazardous effects inflicted by environmental toxicants. The aim of this thesis was to establish a robust 2D cell culture with primary fibroblast cells derived from Norwegian killer whale skin tissue samples from free-ranging specimens by optimizing the cultivation conditions. Primary dermal cells exhibiting the typical spindle-shape of fibroblasts were isolated and identified as fibroblasts via primary vimentin antibody immunolabellering. Cell morphology was characterized with DAPI and phalloidin staining. The isolated killer whale fibroblasts were cultivated in various cultivation conditions, using Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 media with high or low concentration of glucose and varying selected supplementations. Different concentrations of fibroblast growth factor 2, fetal bovine serum or horse serum, non-essential amino acids, ascorbic acid and 2-Mercaptoethanol antioxidants, and gelatin or laminin surface coatings were tested to investigate the effect on cell growth and cell metabolism by the different parameters indiviually and in combinations. Cell proliferation and metabolism was measured by cell counting and metabolite analysis of glucose consumption and lactate accumulation. Common challenges associated with cetacean cell cultures were encountered, including limitation of cell sources, slow cell proliferation rates and senescence, which was confirmed in killer whale fibroblasts with the β-galactosidase senescence assay. Immunhistochemistry was successfully applied using primary Ki-67 antibody to identify proliferating fibroblast cells. FGF supplementation increased proliferation significantly at 1 ng/mL and an optimal concentration of 2 ng/mL. The optimized media composed of bFGF 2 ng/ml supplemented with 15 % FBS and 5 % horse serum and 2-Mercaptoethanol antioxidant, maintained a killer whale cell culture for 20 passages with a cell population doubling time of 40 hours without showing signs of reduced proliferation or senescence. The established cell culture was further used to assess cytotoxic effects of a cocktail which contained the most common POPs found in killer whale blubber after 48 hours exposure. A 25% loss in cell metabolism was detected in killer whale fibroblasts at the highest exposure concentration of 50X (202.1 µg/mL) the measured concentration in killer whale blubber with the CellTiter-Glo assay, while no cytotoxicity was detected with LDH release assay. Several POPs, including PCBs and *p,p'*-DDT, are known to bind to several receptors activating and changing gene expression and by so disrupting cellular mechanism. Further research is necessary to increase our knowledge about how killer whales are affected by environmental toxicants on genetic and biochemical level.

Sammendrag

Norske spekkhoggere (*Orcinus orca*) er som følge av deres trofiske nivå og føde blant de mest kontaminerte marine pattedyrene i verden. Akkumulering av persistente organiske forbindelser (POPs) kan føre til forstyrrelser av biokjemiske prosesser på cellulært nivå som kan ha alvorlige innvirkninger på populasjonene. I løpet av det siste tiåret er det lagt økt innsats i å etablere *in vitro* modellsystem for marine pattedyr med den hensikt å kunne studere hvilke konsenkvenser miljøgiftene kan ha på dyrene. Målet med denne masteroppgaven var å etablere en stabil 2D cellekultur med primære fibroblaster derivert fra vevsprøver tatt av norske fritt-levende spekkhogger gjennom å optimalisere kultiveringsforholdene. Primære dermale celler med fibroblast-typisk morfologi ble isolert og identifisert som fibroblaster via immunohistokjemi med primære vimentin antistoffer. Cellemorfologien ble også karakterisert med DAPI og Phalloidin farging av cellekjernen og intermediære aktinfilamenter. De isolerte spekkhogger fibroblastene ble dyrket i ulike kultiveringsforhold, med Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 medium med høy eller lav glukosekonsentrasjon and utvalgte supplementeringer. Vi testet ulike konsentrasjoner av fibroblast vekstfaktor 2, fetalt kalveserum eller hesteserum, ikke-essensielle aminosyrer, askorbinsyre og 2-mercaptoetanol antioksidanter og gelatin eller laminin overflate behandlinger for å undersøke effekten av de ulike parameterene alene og i kombinasjon på cellevekst og cellemetabolisme. Celleproliferasjon og metabolise ble målt kvantitativt ved celletellinger og metabolittanalyser av glukose konsum og laktatakkumulering. Vi støtte på utfordringer, som begrenset tilgang på vevsmateriale og celler, saktevoksende celler og lav replikativ kapasitet og tidlig irreversibel celledvale (senescence), som er vanlig med primære hvalcellekulturer. Senescente celler ble bekreftet med β-galaktosidase senescent analyse. Friske, prolifererende fibroblaster ble også idenfisert via immunohistokjemi med primære Ki-67 antistoffer. Tilsats av fibroblast vekstfaktor resulterte i betydelig økning i proliferasjon ved 1 ng/mL konsentrasjon og den optimale konsentrasjonen var 2 ng/mL. De beste parameterene for cellevekst ble kobinert og bestod av 2 ng/mL fibroblast vekstfaktor, 15 % fetalt kalveserum, 5 % hesteserum og 0.1 mM 2-mercaptoetanol. Med det optimaliserte mediet oppnådde vi 20 passasjer med primære fibroblaster fra spekkhoggere uten å se tegn til reduksjon i vekstrate. Populasjonsdoblingstiden ble beregnet til å være 40 timer. Den etablerte cellekulturen ble videre brukt til å måle cytotoksisk effekt av en miks med de 10 vanligste miljøgiftene som er målt i spekket hos norske spekkhoggere. Vi fant en dødelighet på 25 % for den høyeste eksponerte konsentrasjonen på 202.1 µg/mL, tilsvarene 50X den målte konsentrasjonen i spekket. Flere POPs, blant annet polyklorerte bifenyler og *p,p'*-DDT er kjent for å forstyrre cellulære mekanismer ved å binde seg til ulike reseptorer som aktivere og endrer genuttrykk i cellene. Videre forskning vil være nødvendig for å skaffe mer kunnskap om hvordan spekkhoggere påvirkes av miljøgifter på genetisk og biokjemisk nivå.

Acknowledgements

I would like to start by emphasizing how grateful I am towards Heli Routti, at the Norwegian Polar Institute, for giving me the opportunity to do this project. Ever since I began my bachelor's in marine biology, I wished to do my master's thesis on whales. Thus, when Heli assigned me to work with cell cultures from killer whales as part of a project on cellular responses to contaminant exposure in marine mammals from the Arctic at the Fram Centre, she made a small dream of mine come true. The project was a collaboration with the University of Bergen, were Anders Goksøyr became one of my supervisors. Both Anders and Heli have provided me with useful advices and help during my work which has been much appreciated!

Doing my thesis on killer whale cell cultures would not have been possible without Audun Rikardsen and Juni Bjørneset, at the University of Tromsø, and their work with sampling of killer whale skin biopsies. I know sampling is challenging and especially uncomfortable out on upon sea in Northern Norway during wintertime. I would also like to thank Juni once more for supplying me with isolated cells from her own project, otherwise my project would have ended far too early.

Further, a great appreciation is appointed to SINTEF Industry for taking a part in my project. SINTEF provided me a lab to work in and an extra supervisor. Hanne Haslene-Hox has been a major contributor to my project, by sharing her expertise in cell biology, assisting me in the lab and in the writing process and coming up with new ideas and always being available. For your time, engagement, enthusiasm and work with my thesis, I am sincerely grateful!

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Finally, I thank Mikael Harju at NILU for preparation of the toxmix that were used in the cytotoxicity assays and Demetri Spyropoulos from the Medical University of South Caroline for providing the SolnIX cryopreservation solution for this study. Demetri has a lot of experience with whale cell cultures which he kindly shared with us, and for that I would like to extend my biggest gratitude.

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List of Abbreviations

3Rs	Replacement, Reduction and Refinement
3T3	Mouse fibroblast cell line
AA	Ascorbic acid
ATP	Adenosine triphosphate
BME	2-Mercaptoethanol
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cell line
COS	Monkey kidney fibroblast cell line
CYP	Cytochrome P450 monooxygenase system
DDT	Dichlorodiphenyltrichloroethanes
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERA	Ecotoxicological risk assessment
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HuWa	Humpback whale
HS	Horse serum
KW	Killer whale
LDH	Lactate dehydrogenase
NEAA	Non-essential amino acids
PBDE	Polybrominated diphenyl ethers
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyls
PD	Petri dish
PDGE	Platelet-derived growth factor
PenStrep	Penicillin Streptomycin
POP	Persistent organic pollutant
p,p'-DDE	Dichlorodiphenyldichloroethylene
ROS	Reactive oxygen species
RT	Room temperature
SolIX	Special cryopreservation solution

1 Introduction

1.1 Killer whales – background

Killer whales (*Orcinus orca*) are the second largest toothed whale species. Unique subpopulations of the species, differing in morphology, foraging and social behaviour and genetics, are globally dispersed - inhabiting all the world's oceans (Forney et al., 2006). Since 1988, there has been an increased effort to understand more about the abundancy and biology of the killer whales in the North Atlantic, but knowledge is still lacking. Environmental changes due to global warming have changed their distribution patterns and feeding strategies, mainly by changing the prey and habitat availability (Jourdain et al., 2019). The rapid alterations in Arctic ecosystems possess large threats to killer whale populations in the North Atlantic and has resulted in higher occurrences of killer whales in Southeast Greenland and Northern Canada, thus increasing the conflict between them and human interests and the risk for native subsistence hunting (Higdon and Ferguson, 2009, Ferguson et al., 2010). The main threats to wild populations of killer whales are past and current unregulated hunting, depletion of prey populations, entanglement or other interactions with fisheries, acoustic disturbance, and chemical pollution. Among these, high levels of contaminants and prey limitations are of most concern in regards to population growth (Jourdain et al., 2019).

The main group of chemical compounds of concern for killer whales is persistent organic pollutants (POPs). This group is defined by the chemicals' ability to persist in the environment, be transported over long distances, bioaccumulate in organisms and bio-magnify through trophic levels and being toxic. In the marine environment, lipid-soluble POPs are absorbed by low trophic species and bioaccumulate in organisms' fat reservoirs, thus increasing in concentration as they move up the food chain (Fig 1). Polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs) and polybrominated diphenyl ethers (PBDEs) are some of the major chemical groups of POPs. They have been used as pesticides, organic dyes, plastics, electronics, and other manufactured products and released to the environment through wastewater, combustion and agricultural activities (Akhtar et al., 2021).

POPs are found to induce a variety of toxic effects on organisms through for example endocrine disruption, neurotoxicity and immunosuppression, which may lead to interference with reproductive and nervous system as well as developmental and behavioural mechanisms (Ross et al., 1996, Brouwer et al., 1989, Suzuki et al., 2020, Desforges et al., 2016). Some POPs, including several PCB congeners are metabolized into more or less toxic metabolites by cytochrome P450 monooxygenase system (CYP). Thus, the induction of CYP enzymatic activity is often used as a biomarker for contaminant exposure (Wolkers et al., 1998). Being a top predator, killer whales are particularly susceptible to accumulation of high concentrations of POPs through biomagnification which could have adverse health impacts on the populations.

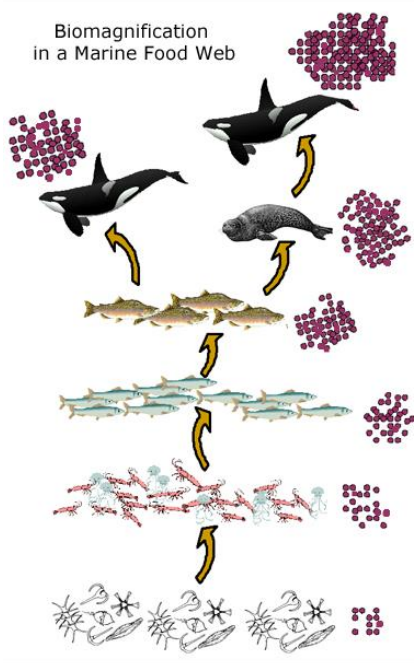


Figure 1: POPs are absorbed by phytoplankton at the bottom of the food chain. Moving up the chain, zooplankton feed on phytoplankton and accumulate the POPs from the phytoplankton. Further, the contaminant concentration increases as zooplankton are consumed by first, secondary and third consumers, such as herring, cod, and seals. Credit: https://ptmsc.org/programs/investigate/citizen-science/completed-projects/orca-project/contaminants-in-orcas

Outside of the Norwegian coast and throughout the Northeast Atlantic, one population of killer whales have developed a feeding strategy based on Norwegian spring-spawning herring (*Clupea harengus*). Since these killer whales are observed following the herring along their migration, this particular fish species is thought to be their main source of prey (Similä et al., 1996). The level of contaminants is significantly higher in herring compared to polar cod, and it is estimated that the killer whale diet is 10 to 15 times more polluted than the diet of beluga whales (Wolkers et al., 2007). As a result, Norwegian killer whales measure among the highest concentrations of PCBs and DDT in the Atlantic and Arctic waters (Wolkers et al., 2007).

Reproductive failure has been observed in killer whale populations inhabiting highly industrial regions around the Canary Island and Gibraltar (Jepson et al., 2016). Changes in gene expression linked to activation of the aryl hydrocarbon receptor, thyroid hormone α receptor and estrogen α receptor are also addressed in response to PCB exposure (Buckman et al., 2011). Further, *in vitro* exposure to POP cocktails derived from killer whale blubber induced immunotoxic effects on killer whale lymphocytes (Desforges et al., 2017).

The growing concern regarding chemical pollution in the marine environment has led to an increased effort to investigate and understand the ecotoxicological effects on marine mammals. The use of *in vitro* cell cultures has become important in ecotoxicological risk assessments (ERA), as it enables the study of induced cytotoxicity, genotoxicity and immunotoxicity by direct exposure to toxicants in accordance to the 3R's principle (replace, reduce and refine) in animal research (Sun et al., 2022). Cell cultures as a model system are most reliable when using cells derived from the species you want to study. Studying killer whales - enormous, free-living, fierce top predators – is naturally associated with some challenges, both practical and ethical. Most cetacean species are also protected by

laws due to risk of extinction. Therefore, establishing robust cell cultures from killer whales is crucial to gain knowledge about this species and improve the model system for cetacean ERAs (Yu et al., 2005).

1.2 Fibroblasts

Fibroblasts serve many critical functions in an organism and are useful instruments in numerous fields within cell biology (Buechler and Turley, 2018). They are relatively easily derived from dermal tissue and stored, which makes them suitable models for cell culture research. Also, harvesting skin biopsies is mildly invasive compared to deeper tissue sampling (Junker et al., 2010). Primary cell cultures with fibroblasts have been successfully established and used to measure cellular responses to POPs for several cetacean species, including humpback whales (*Megaptera novaeangliae*), pygmy killer whales (*Feresa attenuate*), Indo-Pacific finless porpoise (*Neophocaena phacaenoides*) and pantropic spotted dolphin (*Stenella attenuate*) (Rajput et al., 2018, Burkard et al., 2015, Yajing et al., 2018, Sun et al., 2022).

Fibroblasts are the most abundant cell type found in the connective tissue of animals. They are specialized in synthesizing and secreting precursors of the extracellular matrix (ECM), including collagens, elastin, fibronectin and proteoglycans. They also secrete different growth factors and cytokines and play an important role in development, immune responses, and wound healing. The biomechanical and biochemical properties of the ECM modulates diffusion dynamics and positional information important in signalling, proliferation, migration and differentiation of neighbouring cells. In response to tissue damage or during tissue development, fibroblasts are shown to increase proliferation, the secretion of ECM and reprogram into myofibroblasts (Plikus et al., 2021).

Fibroblasts can be identified by different markers, including several ECM encoding genes (e.g. *Col1a1*, *Col1a2*, *Fbln1*, *Fbln2*), fibroblast-specific protein 1 (FSP1), the intermedia filament proteins vimentin and cytokeratin and cell surface PDGF receptors. However, none of these markers are specific for fibroblasts, thus, morphological features should be included in the identification of fibroblasts (Kalluri, 2016, Muhl et al., 2020). Fibroblasts generally exhibit a spindle-shaped morphology with spikey extensions and an oval nucleus. In culture, they adhere to the plates creating dense monolayers (Fig 2) (Chang et al., 2014).

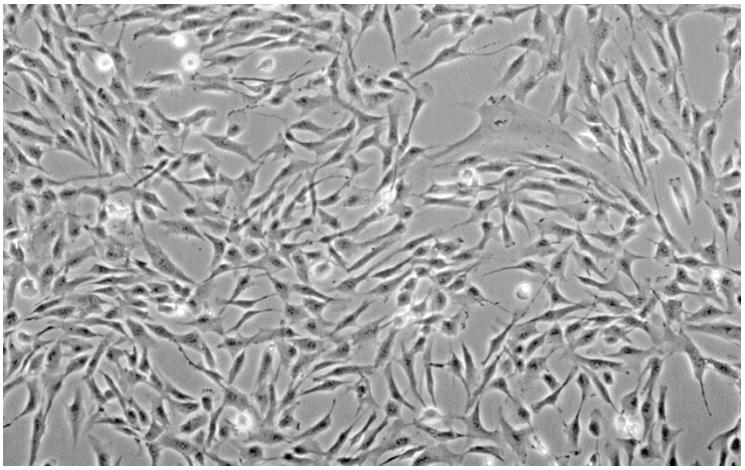


Figure 2. Spindle-shaped polygonal morphology exhibited by proliferating human dermal fibroblasts (Nilforoushzadeh et al., 2017).

1.3 Cell culture

Cultivation of cells *in vitro* has become one of the most important tools in cellular and molecular biology and is considered a valuable method to study cellular functions, mechanisms, and responses. Proliferation of cells outside of an organism has contributed to several fields of science including cancer research, drug development, genetics, stem cell biology and toxicology. In general, cells are removed from an organism and isolated into a primary culture where the cells proliferate. The cells are further sub-cultivated to retain the cells in an exponential growth phase, for efficient propagation of cells, that can subsequently be used for experiments. Normal cells have a limited lifespan and are destined to undergo apoptosis after a finite number of cell divisions as a defence mechanism against accumulation of cellular damages. The number of cell doublings that can be reached is highly dependent on the cell type, cells source and cultivation conditions (Masters, 2000). Cells can, however, be transformed into immortal cell lines with unlimited dividing capacity, either spontaneously or by chemical or biological (Fx SV40 Large T antigen) induction (Oyeleye et al., 2016).

The main advantages with cell cultures are that the physicochemical factors can be highly controlled during an experiment, which makes it possible to reproduce the results with clonal cells from the same batch. Cell lines including fibroblast, epithelial, endothelial, neural and organ-specific (parenchymal) cells have been successfully established from tissue derived from several animal species (Oyeleye et al., 2016). Some of the most commonly used cell lines are the mouse embryonic fibroblasts (3T3), human epithelial cells (HeLa), monkey kidney cells (COS) and the chinese hamster ovary cells (CHO) (Carter and Shieh, 2015).

2D culturing in Petri dishes, micro-well plates or culture flasks is the most common method to cultivate cells, as it generally yields high cell viability, is easy to use and has low costs. The main disadvantage with this method however, is restricting the cells to a hard substrate, glass or plastic, which inhibits natural growth and could impair or alter cell functions (Andersen et al., 2015). In their natural environment, living cells are organized in relation to other cells and embedded in an ECM, creating a complex three-dimensional (3D) structure of tissue and organs. The 3D structure facilitates and enables biochemical and physical signalling between cells, which is crucial for cellular functions, such as differentiation, migration, proliferation and apoptosis (Teti, 1992). When culturing cells *in vitro*, it is advantageous to create similar conditions as they would experience naturally to provide reliable results (Lee et al., 2008). Differences in gene expression and biological functions are shown in studies comparing 2D cell culturing and 3D cell culturing (Kenny et al., 2007). These findings illustrate how using 3D culturing methods can improve the biological relevance of cell responses obtained from *in vitro* experiments and how it can be of great importance in further drug development and cancer research, as well as toxicology studies.

A key challenge is to create a sterile cell culture when sourcing cells from live specimen in nature. Microbial contamination by bacteria, yeast or fungus often has serious consequences and can destroy whole batches of cell cultures. Bacteria and fungus generally grow faster than mammalian cells, causing depletion of media nutrients and change in pH making the culturing environment unfavourable for the cells. The source of contamination can be either through the media or other contaminated reagents, the air or work equipment in the laboratory, but most often it stems from dormant infections in the sampled specimen (Mathews et al., 1988). The most frequent contaminations in sampled tissue from marine

mammals are fungus (*Candida* spp.) and mycoplasmas (Marsili et al., 2000). Mycoplasma infections pose a great challenge, as they may inhibit cell metabolism, cell growth, alter nucleic acid synthesis, cause chromosomal aberrations, and alter the antigenicity of cell membranes and alter cell morphology (Barile, 1973). Other challenges include the influence of culturing conditions, such as growth media compositions, the finite lifespan of primary cells and oxygen conditions.

1.4 Cell metabolism

Like all living organisms, cells require various nutrients to live and proliferate and to produce biological molecules necessary for all other cellular functions. In *in vitro* cell culturing, growth media is used to provide essential nutrients for cell survival and growth. The growth media generally contains simple sugars, such as glucose or fructose, amino acids, inorganic salts, and vitamins (Arora, 2013). Most media also contain serum, such as fetal bovine serum, which provide important growth factors. Serum also provides vitamins, lipids, minerals, proteins, and hormones which stimulate cell growth. Additionally, antibiotics are often added to prevent bacterial and fungal contamination (Carter and Shieh, 2015).

The main energy source for animal cells is glucose. When oxygen is present in the cells, glucose is converted to pyruvate via glycolysis and oxidized through a series of events generally referred to as oxidative metabolism (Ronald Zielke et al., 1978). Glycolytic conversion rates might exceed the cells need and capability to turn over all the pyruvate, resulting in an inefficient coupling between glycolysis and the citric acid cycle. Pyruvate is then rather converted to lactate through aerobic glycolysis (Tsao et al., 2005). Glucose and lactate are commonly used as markers for interpreting *in vitro* cell metabolism and there is a positive correlation between glucose consumption and cell growth rates (Frame and Hu, 1991). However, excessive amounts of glucose and other nutrients, including glutamine, in the cultivation media may induce increased glucose/lactate turnover and cause accumulation of lactate and consequently acidification of the media (Hassell et al., 1991). Hassell et al. (1991) also demonstrated that high concentrations of lactate in the cell media (>20 mM) inhibits cell growth of mouse fibroblast and hybridoma cells and monkey kidney cells. It is found that using fructose or galactose as energy source resulted in lower consumption of sugar and less lactate accumulation compared to glucose but resulted to similar cell growth rates in CHO cultures (Wlaschin and Hu, 2007). Each molecule of metabolised glucose yields two molecules of lactate through the aerobic glycolysis. In fact, most of the glucose is actually converted to lactate in mammalian cell cultures (Hu et al., 1987). Thus, high level of glucose uptake does not necessarily reflect the rate of proliferation and w expect a positive correlation between consumed glucose and accumulated lactate in mammalian cell cultures (Jones and Bianchi, 2015).

Cytoplasmic lactate dehydrogenase (LDH) is responsible for the conversion of lactate to pyruvate and vice versa. When plasma membrane is damaged, through either lysis, necrosis, or apoptosis, LDH is released to the extracellular space. In cell cultures, accumulation of LDH in the cell culture medium indicates membrane leakage and is commonly used as a measure of cell damage and cell viability. Established LDH release assays are useful tools in e.g. toxicology, when determining cytotoxicity induced by different toxicants (Chan et al., 2013). The assays are commonly based on the LDH-catalyzed conversion of lactate to pyruvate, which produces NADH. NADH reduces tetrazolium, a yellow salt, into a red, water-soluble dye called formazan. The formation of

formazan is directly proportional to the amount of LDH in the media, and therefore a direct measure of amount of cell damage or cell death (Kumar et al., 2018).

1.5 Cell proliferation and senescence

The growth of cells and increase in cell number, termed cell proliferation, is essential in organismal development, growth, tissue restoration as well as tumour generations. The most important process in cell proliferation is the cell cycle, where the cells undergo a series of events that results in the replication of its content before it is divided into two identical daughter cells. Cell proliferation can be induced by extracellular hormones and growth factors that bind to cell surface receptors and activate intracellular signal transduction. Several growth factors known to regulate cell proliferation have been isolated and purified, among them platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factors (FGFs) (Schlessinger et al., 1983). The mitogenic effect of FGFs was demonstrated *in vitro* with 3T3 cells by increased cellular growth and proliferation (Gospodarowicz, 1974). A well-known cellular marker for proliferating cells is the monoclonal Ki-67 antibody. Ki-67 antigens, found in all vertebrates, interact with several other proteins, and are involved in regulation of the cell cycle. Expression of Ki-67 antigens is therefore associated with the state of the cell cycle, and elevated concentrations are detected within the nucleus during the active phases of the cycle (Gerdes et al., 1984).

Three mechanisms; differentiation, quiescence, and senescence, can delay or terminate the cell division cycle. Senescence occurs post-mitosis in response to different stressors, such as oxidative stress, telomere shortening, genotoxic stress and replicative stress, ultimately leading to oncogenic mutations and DNA damage responses (DDR). DDRs activates several pathways, involving p53 and retinoblastoma (RB) tumor suppressors, which further induces transcription of the Cdk inhibitors genes p21 and p16 (Chandler and Peters, 2013). Accumulation of p21 and p16 in the cell nucleus inhibits the cell from progressing through the initial phase of the cell cycle, causing the cell to enter a permanent cell cycle arrest (Kulaberoglu et al., 2021). Resting and senescent cells are shown to be negative for the Ki-67 antigen, which confirms its absence in non-proliferating cells (Gerdes et al., 1984).

Normal somatic cells, including fibroblasts, have finite life spans and generally go into a replicative senescent state prior to apoptosis. Senescence in *in vitro* cell cultures is highly depended on the cell source, in terms of age of the individual and telomere length e.g., and the culturing conditions. The limited life span of primary cell cultures poses a challenge, as it limits their time frame and number to perform relevant research studies. This issue of senescence can be solved by frequent sampling of new fresh tissue, which could be both practically and ethically challenging, or by increasing the cells lifespan through transfection-induced immortalization. Immortalized cell lines, however, often exhibit dissimilarities in gene expression and phenotypes compared to *in vivo* cells which could influence the research outcomes (Pan et al., 2009). For human fibroblasts derived from adult tissue, a replicative capacity of 14-29 doublings has been demonstrated *in vitro* (Nishio et al., 2001). Shortening of telomeres and accumulation of DNA damage through serial cell divisions are the main causes of growth arrest and cell death (Campisi et al., 2001).

The process of senescence impairs cellular structures and functions and is shown to cause changes in cell morphology (Fig 3). The changes involve flattening and enlargement of the cells, increased cell mass, increased production of reactive oxygen species (ROS) and

consequently increased accumulation of damaged cellular components mediated by ROS (Hwang et al., 2009) and increased expression of cell cycle inhibitors. Altered expression of cytoskeletal proteins during senescence is likely to affect cell structures, as well as migration and adhesion properties. Overproduction of vimentin intermedia filaments has been linked to changes in morphology observed in senescent human fibroblast (Nishio et al., 2001, Druelle et al., 2016).

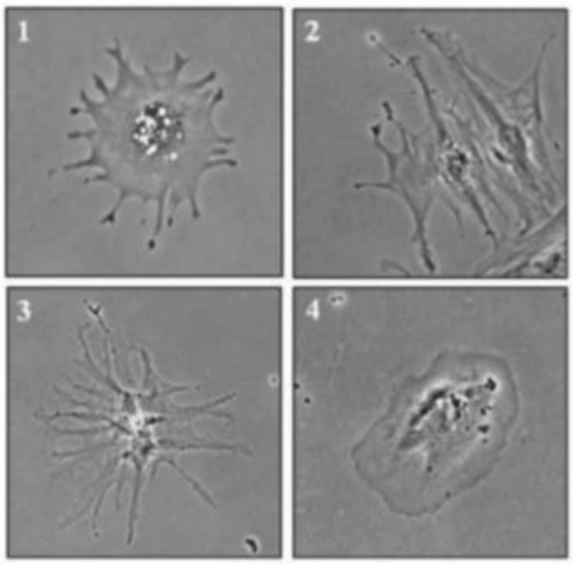


Figure 3. Morphological features of senescent human dermal fibroblast (HDF) cells, including irregular, enlarged and flattened shapes and increased number of protruding lamellipodia and filopodia (Cho et al., 2004).

1.6 Oxygen levels in cell cultures

Cell culturing aim to mimic the natural conditions to avoid unnecessary stress, thus creating a hypoxic/normoxic environment is advantageous. The standard atmospheric level of O_2 in an incubator is generally 18.6 % due to the water evaporation and addition of 5 % CO_2 (Maddalena et al., 2017). This level deviates from natural pericellular O_2 levels and is significantly higher than the physiological range of 1-5 % O_2 observed in different tissue (Habler and Messmer, 1997). Reduction of O_2 through cellular mechanisms produces ROS which can affect signalling pathways involved in metabolism and senescence among other cellular processes. Excessive levels of ROS causes oxidative stress in cells and possible oxidative damage of cellular structures such as nucleic acids, proteins and lipids (Maddalena et al., 2017).

Maddalena et al. (2017) studied the production of H_2O_2 in relation to oxygen levels during culturing. The results showed an increase in H_2O_2 production under standard cell culture conditions of 18% O_2 compared to the more physiologically relevant levels of 1-5 % (Maddalena et al., 2017). Higher levels of H_2O_2 induced by hyperoxia is shown to induce DNA damages and senescence in 3T3 and human fibroblasts (Parrinello et al., 2003), while lowering the O_2 could stimulate cell proliferation and increase their life span (Stone and Yang, 2006, Haque et al., 2013). This can be achieved by placing the culture plates in a hypoxic incubator chamber where the atmospheric O_2 concentration can be reduced and controlled.

1.7 Summary of previous *in vitro* studies involving cetaceans

I*n vitro* approaches of cetacean species have been increasingly applied during the last decade. Primary cell cultures and cell lines have been established for several cetacean species, listed in Table 1, most of which experienced challenges with early senescence and slow growth rates. Different cultivation media and culturing conditions have been tested, but optimisation specific to the species and cell types is still required.

Table 1. Summarized overview of a selection of previous established cell cultures and applications from several cetacean species. (P= passages, D= doublings, ND = not defined, FBS = fetal bovine serum, NEAA = non-essential amine acids, NaPyr = sodium pyruvate, PenStrep = penicillin streptomycin, LG = L-glutamine, EAA = essential amino acids, hEGF = human epidermal growth factor, BPE = bovine pituitary extract, CYP = cytochrome P450)

Species	Cell source/type	Passages/ doublings	Growth media/conditions	Application
Indo-Pacific Finless Porpoise (*Neophocaena phacaenoides)* (Ochiai et al., 2020)	Stranded individuals/ fibroblasts	8-10	DMEM/F12, 20 % FBS, 0.1 mM NEAA, 2 mM LG, 100 µg/mL PenStrep	Cytotoxicity and risk assessments to POPs and trace metals exposure
Pantropic spotted dolphin (*Stenella attenuate*) (Rajput et al., 2018)	Fibroblasts	9 (40 w/SV40 transfection)	37 ℃, DMEM/F12, 10 % FBS, 1.5 % LG, 0.4 % 200 µg/mL geneticin, 1 % EAA, 1 % NEAA, 100 µg/mL PenStrep	Cell viability and oxidative stress to PBDE exposure
Pygmy killer whale *(Feresa attenuata)* (Yajing et al., 2018)	Stranded individuals/dermal fibroblasts	Immortalized	DMEM/F12, 15 % FBS 0.1 mM NEAA, 2 mM LG, 100 µg/mL PenStrep	Karyotyping, SV40 T-antigen transfection, fibroblast identification w/vimentin
Humpback whale (*Megaptera novaeangliae*) (Burkard et al., 2015)	Skin biopsies/ Primary fibroblasts	30+ (P)	37 ℃, DMEM/F12, 10 % FBS, 0.1 mM NEAA, 1 mM NaPyr, 100 µg/mL PenStrep	Karyotyping, fibroblast identification w/vimentin, cytotoxicity by *p,p,'*-DDE exposure
Indo-Pacific Humpback dolphin (*Sousa chinensis*) (Jin et al., 2013)	Fibroblasts	10-17 / immortalized	DMEM/F12, 20 ng/mL hEGF, 15 % FBS, 15 mM HEPES, 1X NEAA, 3X EAA, 1X vitamins, 10 mM NaCl, 2 mM LG, 35 µg/mL BPE, 5 µg/mL Buffalo Insulin, 5 µg/mL transferrin, 100 µg/mL PenStrep, 5 µg/mL Amphotericin	Karyotyping, SV40 T-antigen transfection

Killer whale *(Orcinus orca)*(Marsili et al., 2012)	Skin biopsies of free-ranging individuals/ primary fibroblasts	ND	MEM Eagle earl's salts w/LG, 10 % FBS, 1 % MEM NEAA, 1 % PenStrep, 0.1 % Amphotericin	Genotoxic responses to contaminant exposure
North Atlantic right whale (*Eubalaena glacialis*) (Wise et al., 2008)	Tissue from dead individual kept in captivity/ lung and tests fibroblasts	ND	33℃, DMEM/F12, 15 % cosmic calf serum, 2 mM LG, 100 µg/mL Penstrep, 0.1 mM NaPyr	Cytotoxicity, and genotoxicity of hexavalent chromium
Fin whale (*Balaenoptera physalus*) (Fossi et al., 2006)	Skin and blubber biopsies/primary fibroblasts	ND	DMEM/F12, 10 % FBS, 1.5 mM LG, 1 mM NaPyr, 1 mM MEM NEAA, 100 µg/mL Primocin	Endocrine disruption (CYP induction) to contaminant exposure
Atlantic bottlenose dolphin (*Tursiops truncates*) (Yu et al., 2005)	Skin samples of free-ranging individuals/ epidermal cells	Immortalized	RPMI-1640 medium:Keratinocyte-SFM, bovine pituitary extract, hEGF, FBS 10 %, 50 µg/mL PenStrep, 20 µg/mL ciprofloxacin	Karyotype analysis, immortalization by SV40 T-antigen transfection

1.8 Aim of the study and experimental outline

Successfully establishing a killer whale fibroblast culture would enable us to study cellular mechanisms related to the species in detail. Ultimately, developing a robust culture of these cells will also allow us to understand how environmental toxicants impacts vital biochemical processes in marine mammals. Hence, this study is aimed at establishing a robust 2D primary cell culture with fibroblasts derived from killer whale skin biopsies by tailoring and optimizing the cultivation conditions. Subsequently an established cell culture of these cells was exposed to relevant POPs, where possible cytotoxic effects were evaluated. The specific objectives of this study were as follows:

1. Validate that cells isolated from biopsies are fibroblast in origin.
2. Determine the independent effects of FGF, glucose, serum, amino acids, and antioxidants concentration on KW fibroblast cell cultures.
3. Determine the effect of surface coatings on the establishment of KW cultures.
4. Determine if combining findings from media supplement studies (i.e FGF, serum, glucose, amino acids, antioxidants, and surface coatings) will improve cell growth of killer whale fibroblasts.
5. Assess possible cytotoxicity of POPs in killer whale primary cell cultures.

These specific objectives were studied predominantly using 12 and 96-well setups and employed a range of scientific methods as outlined in the illustration below (Fig 4). Details pertaining to the culture conditions are outlined in the materials and methods section.

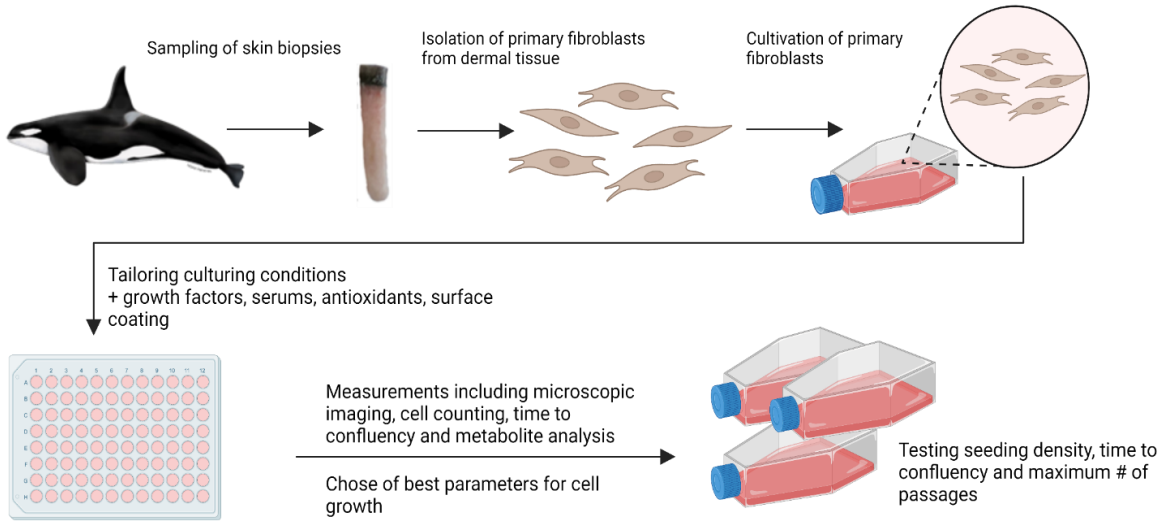


Figure 4. Experimental outline of study. Biopsies with dermal tissue was collected from free living killer whales outside of Reinfjorden in Northern Norway. Primary fibroblasts were isolated from cultivated tissue fragments derived different individuals of Norwegian killer whales. The isolated fibroblasts were further cultivated in different test conditions, including fibroblast growth factors, increased concentration of fetal bovine serum and horse serum, addition of 2-Mercaptoethanol and/or coating of the well surface with laminin or gelatin. The parameters that improved cellular growth and proliferation were combined to optimize the culturing conditions.

2 Methods and materials

List of reagents and products used in this study is attached in Appendix A.

2.1 Sampling of biopsies

Skin tissue were sampled from free-swimming killer whales in Kvænangen outside Reinfjorden in Northern Norway (Table 2). The sampling was performed by Audun Rikardsen (University of Tromsø) and his team. The biopsies were obtained by biopsy flotation darts (3.5 x 0.7 cm), which were shot approximately right below and slightly ahead of the dorsal fin of the individuals. The dart tips containing the biopsies were collected and the samples were immediately stored in 50 mL tubes containing a special cryopreservation solution ((SolnIX-SK (CGX), Cryogenix, LLC, South Carolina, US. (Baatz et al., 2014, Mancia et al., 2012)). The biopsies were further kept on ice and transported to SINTEF in Trondheim, where they were received and processed approximately 24 hours after sampling. Additionally, cryopreserved killer whale primary fibroblasts (KW-11-02) isolated by Juni Bjørneset (University of Tromsø) were received and used in this study (Bjørneset, 2022). The biopsy sampling details for KW-11-02 are included in Table 2.

Table 2. List of specimens that skin biopsies were obtained from with information about sampling date and location, and suggested age and sex as judged by the field specialists.

Species	ID	Date	Time	Location	Field age	Field sex
Killer whale	KW-1	02.12.21	07.30	Kvænangen, Reinfjord	Adult	Male
Killer whale	KW-2	02.12.21	08.00	Kvænangen, Reinfjord	Old	Female
Killer whale	KW-11-02	11.11.20	08.00	Skjervøy, Tromsø	Adult	Male

2.2 Processing of biopsies and isolation of primary cells

Processing of biopsies was done according to the protocol by Burkard et al. (2015). Two biopsies with killer whale skin tissue were received from Kvænangen and processed approximately 24h after sampling. Each biopsy was washed with distilled phosphate-buffered saline (dPBS) with 20% PenStrep and 2 % Geneticin for 10 minutes. The biopsies were then separated into epidermis, dermis and blubber with a scalpel (Fig 5). Separation of dermis and blubber was done on basis of visual inspection and firmness of the tissue – blubber has a more fat-like soft structure, while dermis is more dense and firm. The epidermis and blubber were discarded, while the dermis layer was used for further processing. The dermis layers were washed again with DPBS (20 % PenStrep, 2 % Geneticin) for 5 minutes before they were cut into ~2mm cross sections. The slices were washed again in DPBS (20 % PenStrep, 2 % Geneticin) for 5 minutes. Each slice of tissue was further processed individually and cut into smaller fragments ~1-2mm^3 before a final wash for 1-2 minutes. The tissue fragments were then transferred to 6-well plates, four-seven fragments in each well, and covered with sterile cover slip.

For cutting the biopsies and tissue fragments, sterile scalpels were used along with sterile tweezers. The cutting took place in a petri dish with sterile 4-folded layered aluminium.

The aluminium was changed for every new piece of tissue to avoid transferring of contaminants and fat droplets.

Figure 5. Processing of skin biopsy obtained from a killer whale. A) whole skin biopsy before processing. B) Separation of tissue into epidermis, dermis, and blubber. C) Cutting of dermis into small fragments. D) Cultivation of dermis fragments in well plate covered with coverslips and submerged in growth media.

The tissue fragments were submerged in 1 mL of same medium they were transported in, 1xSolnx and placed in an incubator at 37℃ and 5% CO_2.

When cells were observed to grow on the well bottom in confluent spots, fibroblasts were isolated from the respective wells containing the tissue fragments. The fragments were removed from the wells and the old media was discarded. The adhered cells were washed twice with PBS containing Primocin (100 ug/mL) and treated with TrypLE for 30 seconds at RT before the excessive TrypLE was removed. The cells were further incubated at 37℃ for 5 minutes. After incubation, the cells were resuspended in standard media (Control-H, Table 4) and the cell suspension was filtered through a 70 µm filter before seeding into 12-well plates for experimental tests. Tissue fragments that had not adhered sufficiently to the well bottom by this time were transferred to new wells.

2.3 Maintenance of cell culture

KW primary fibroblasts were cultivated in tissue-culture treated well plates (6 – 96-wells) and flasks (25, 75 and 175 cm^2) with standard growth media with modifications as listed in Section 2.4. 50-75 % of the media was change every 2-3 days (if not otherwise described). The cells were kept in an incubator at 37 ℃ and 5 % CO_2 and with ambient O_2.

The cells were passaged at 80-100 % confluence using either TrypLE or accutase as dissociation agent. For trypzinisation, the old media was removed the cells were washed with PBS. TrypLE was added to the wells and let sit for 30 second at RT. The TrypLE was further discarded, and the cells were incubated at 37 ℃ for 5 min before resuspension in fresh growth media. With accutase, the cells were incubated with an adequate volume of accutase at RT for 6 minutes with a couple of knocks given to the flask every other minute (or until fully detached), before resuspension in fresh growth media.

The cell suspension was either split in ratio or according to cell density, specified when relevant.

Isolated and cultivated primary fibroblasts were cryopreserved in freezing media. The cell suspensions were centrifuged at 200 G for 4 minutes and the supernatant was removed. The cell pellet was then resuspended in freezing media and transferred to 1 mL cryovials and frozen in cell freezing containers (Corning CoolCell LX Cell Freezing Container, CLS432002) at – 80 ℃ for minimum 24 hours before they were transferred to liquid nitrogen (N_2) tank (-197 ℃).

Cryopreserved vials (1 mL) with primary fibroblasts were thawed from a N_2 tank by transferring the vials directly from N_2 to a water bath holding 37 ℃. The vial was stirred carefully in the water for 2 minutes till the cell suspension was completely thawed. The thawed cells suspension was the quickly pipetted into a tube with fresh growth media which was either seeded directly in culture plates/flasks or centrifuged at 200 G for 4 minutes before resuspending the cell pellet in fresh growth media prior to seeding.

Different microscopes were used for imaging and qualitative measurements of the killer whale cells, including phase contrast microscope, confocal laser scanning microscopes and EVOS microscope. Microscopic images were processed with ImageJ software. The cells were counted with Trypan Blue stain (0.4 %) and Countess 3 Automated Cell Counter (AMQAX2000) in the beginning of this study and later replaced with Norma-XS cell counter (IPrasense) with Horus Software.

2.4 Growth media preparation

Several growth media were prepared for the different optimization experiments performed in this study, listed in Table 3, Table 4 and Table 5.

Table 3. Composition of media used with KW-1. Varying parameters across the different media are highlighted in yellow.

Reagent	**Medium**				
	Control-H	**bFGF1-H**	**Low glucose**	**AA**	**NEAA**
DMEM/F12	-	-	-	-	
Glucose	3.15 g/L	3.15 g/L	1 g/L	1/3.15 g/L	3.15 g/L
FBS	10 %	10 %	10 %	10 %	10 %
L-Glutamine	4 mM	4 mM	4 mM	4 mM	4 mM
Sodium pyruvate	1 mM	1 mM	1 mM	1 mM	1 mM
HEPES	1 %	1 %	1 %	1 %	1 %
Primocin	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL
bFGF		1 ng/mL			
AA				50 µg/mL	
NEAA					1 mM

Table 4. Composition of the different media used in metabolite analysis with KW-11-02. Varying parameters across the different media are highlighted in yellow.

Reagent	Medium						
	Control	**bFGF1**	**bFGF2**	**bFGF4**	**BME**	**FBS20**	**HS**
DMEM/F12	-	-	-	-	-	-	-
Glucose	1 g/L	1 g/L	1 g/L	1 g/L	1 g/L	1 g/L	1 g/L
FBS	10 %	10 %	10 %	10 %	10 %	20 %	5 %
GlutaMAX	4 mM	4 mM	4 mM	4 mM	4 mM	4 mM	4 mM
Sodium pyruvate	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM	1 mM
HEPES	1 %	1 %	1 %	1 %	1 %	1 %	1 %
Primocin	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL	100 µg/mL
bFGF		1 ng/mL	2 ng/mL	4 ng/mL			
HS							5 %
BME					0.1 mM		

Table 5. Composition of media combining optimized parameters. Varying parameters across the different media are highlighted in yellow.

Reagent	Medium		
	Mix1	**Mix 2**	**Mix 3**
DMEM/F12	-	-	-
Glucose	1 g/L	1 g/L	1 g/L
FBS	20 %	5 %	15 %
GlutaMax	4 mM	4 mM	4 mM
Sodium pyruvate	1 mM	1 mM	1 mM
HEPES	1 %	1 %	1 %
Primocin	100 µg/mL	100 µg/mL	100 µg/mL
bFGF	1 ng/mL	1 ng/mL	2 ng/mL
HS		5 %	5 %
BME	0.1 mM	0.1 mM	0.1 mM

Low glucose medium was prepared by using DMEM/F12 without glucose (Biowest, L0091) and adding glucose to a concentration of 1 g/L.

A fibroblast growth factor 2 (bFGF) stock solution of 5 µg/mL was used for preparation of bFGF containing media.

BME was added directly to the cells at given concentration (0.1 mM) with every media change.

For cryopreservation, a freezing media composed of FBS (90 %) and DMSO (10 %) was prepared.

2.5 Coating of well plates

Laminin is a glycoprotein secreted by fibroblasts and one of the main components of the ECM scaffolds where it facilitates and support adhesion and proliferation of cells. Gelatin is a derivate of collagen, another main component of the ECM also synthesized by fibroblasts. Different experiments in this optimization study involved coating of well plates with either gelatin (0.1 %) or laminin (2 µg/cm^2) to test if any of the substrates could support and increase growth of KW fibroblasts by creating a more natural environment. The gelatin solution was added to the wells for 30 minutes and removed, directly followed by seeding of cells. A laminin stock solution (1 mg/mL) was diluted in PBS to a working solution of 10

µg/mL which was added in adequate volumes to the wells. The laminin-coated wells were incubated for 2 hours at RT before the laminin working solution was removed. Laminin-coated plates were covered with parafilm and stored at 4℃ prior to cell seeding.

2.6 Growth determination with KW isolated primary cells

Table 6. Overview of primary fibroblasts obtained from different specimens used in different experiments for optimization of the culturing conditions. Experiments were carried out on cells at different passage numbers and with varying well plate types. Technical replicates included for each test condition in the different experiments are listed. Cells used in in Mycoplasma detection test was cultivated in a Petri dish (PD), while the assay was performed in a 96-well plate

Individual	Passage	Experiment/media tested	Plate	Replicates	Cryopreserved
KW-1	1,2	bFGF1/Laminin coating	12-well	2	No
	1,2	bFGF1/Laminin coating	12-well	2	No
	3	Mycoplasma test	6cm PD/ 96-well	1	No
	3	High/Low Glc +/- AA	12-well	3	Yes
	3	NEAA	12-well	6	No
	4	bFGF 1, 2, 4 ng/mL	12-well	3	No
	5	High/Low Glc +/- AA	12-well	3	No
	5	DAPI/Phalloidin staining	12-well	3	No
	5	Senescence assay	96-well	6	No
KW-11-02	3	bFGF1-4, FBS20, HS, BME, laminin/gelatin coating	96-well	3	Yes
	4-6	bFGF1-4, FBS20, HS, Mix1, Mix2	96-well	3-6	Yes
	6 →	Mix1	T25 flask	1	Yes
	5 →	Mix3	T25 flask	1	Yes, 2x
	6	Staining DAPI/phalloidin,	96-well	5	Yes, 2x
	6	Immunolabelling vimentin, Ki-67	96-well	5	Yes, 2x
	6	Senescence assay	96-well	4	Yes, 2x
	9	POPs exposure, cytotoxicity assays (LDH, CellTiter-Glo)		6	Yes, 2x

2.6.1 Fibroblast Growth Factor 2 and Laminin-Coating

The effect of fibroblast growth factor 2 (bFGF, 1 ng/mL) with and without laminin-coating on cell growth was tested on the isolated KW primary cells (P1) and compared to a control group with standard conditions.

KW isolated cells were seeded in 12-well plates at a density of 20,000 cells/mL and 10,000 cells/mL, 2 mL per well with control-H media. Each condition included two technical replicates. For the replicates where bFGF was going to be tested, all media was changed 24 hours after seeding and replaced with bFGF-H media (Table 3).

2.6.2 bFGF concentrations

The effect of three different concentrations of bFGF (Table 4) on cell growth was tested on KW-1 passage 4 and KW-11-02 passage 3 to 6 fibroblasts and compared to the control media. The test concentrations were 1 ng/mL, 2 ng/mL and 4 ng/mL. KW-1 were seeded in a 12-well plate including three replicates per concentration, while KW-11-02 were seeded in 96-well plates and included three to six replicates per concentration.

2.6.3 Low/High Glucose and Ascorbic Acid

The effect on cell growth by low (L, 1 g/L) and high glucose (H, 3.15 g/L) concentrations with and without ascorbic acid (AA, 50 ug/mL) was tested on KW-1 passage 5 and passage 3. The cells were seeded in 12-well plates, including three replicates for each of the following conditions: L/-AA, H/-AA, L/+AA and H/+AA.

2.6.4 Non-Essential Amino Acids

The effect of MEM non-essential amino acids (NEAA) on cell growth was tested on KW-1 passage 4 cells. The cells were suspended in control media and seeded 45,000 cells/well in a 12-well plate. Since they had been previously treated with bFGF-H, both control media and NEAA media contained bFGF-H (1 ng/mL) to limit the parameter changes. After 24 hours, all the media was removed and replaced with respective test media to ensure that the cells were only exposed to their respective test condition.

2.6.5 bFGF, FBS, HS, BME, Gelatin and Laminin coating

Cryopreserved primary fibroblasts from KW-11-02, isolated by Juni Bjørneset at University of Tromsø, was seeded 100 μL of cell suspension per well in a 96-well plate. Some of the wells had been coated with laminin and gelatin (0.1 %) prior to cell seeding. After 24 hours, all the media was removed from each well and replaced with different test conditions. The experiment included three replicates for each treatment; bFGF 1 ng/mL, bFGF 2 ng/mL, bFGF 4 ng/mL, FBS 20 %, Horse serum (HS) 5 %, 2-Mercaptoethanol (BME, 0.1 mM), gelatin coating (Gel), Gel + bFGF 1 ng/mL, Gel + BME, Gel + FBS 20 %, Gel + HS 5 %, laminin coating (Lam), Lam + bFGF 1 ng/mL and Lam + BME and control group with standard medium. BME was freshly added with every media change.

The media in which the cells had been cultivated was analysed for content of metabolites, including glucose, lactate, LDH, ammonia (NH_3) and glutamine. The metabolic analysis was performed with Cedex Bio Analyzer. 50 μL supernatant from each well was transferred to Eppendorf tubes, combining the three replicates for each treatment into one replicate to ensure enough volume. The supernatant was stored at 4 ℃ overnight before the analysis.

Data obtained for Gln and NH_3 were excluded from the results, as the media were supplemented with GlutaMAX and not L-Glutamine.

2.6.6 Combined growth conditions

To test if the parameters that yield increased growth separately in previous experiments would increase cell growth when combined, KW-11-02 passage 4 cells were exposed to seven different treatments, including bFGF1, bFGF2, bFGF4, FBS20 and HS and either combination media Mix 1 (bFGF 1 ng/mL, FBS 20 %, BME 0.1 mM) or Mix 2 (bFGF 1 ng/mL, HS 5 %, BME 0.1 mM) (Table 4 and 5). At confluence, the cells were dissociated and counted. According to the cell density, the cells were split with different ratio and seeded with approximately similar density into a 96-well plate and cultivated further with the same treatments to investigate the effect on KW cell growth.

2.6.7 Maximum number of passages of KW-11-02 primary cell culture

KW-11-02 passage 6 was used to test how many passages could be achieved, using the optimized media, Mix1 (bFGF, 1 ng/mL), (FBS, 20 %) and (BME, 0.1 mM). Three different seeding ratios, 1:4, 1:5 and 1:10, respectively were also tested. Figure 5 illustrates the procedures carried out from cryopreserved KW-11-02 passage 5 fibroblasts were thawed and further upscaled to perform exposure studies. Excessive cells suspension was cryopreserved for future studies.

From passage 9, split ratio was replaced with seeding density. KW-passage 10 was further seeded at 45,000 cells/mL, equal to a 1:4 split ratio. Three different seeding densities were tested later on: 20,000, 40,000 and 60,000 cells/mL, with passage 10 to 17, to see what seeding density was most optimal in terms of splitting frequency and cell growth. Cells were counted and cell viability was measured for each passaging and used to construct a growth curve with the obtained passages.

The experiment was repeated to test if it could be reproduced and improved with increased concentration of FGF and HS using cryopreserved KW-11-02 passage 4 cells. The cells were cultivated with Mix3 media composed of 2 ng/mL bFGF, 15 % FBS and 5 % HS and 0.1 mM BME. At 80-90 % confluence, the cells were split and seeded 40,000 cells/mL in new culture flasks (culture treated, vented). In the beginning of the experiment, the cells were seeded in 25cm^2, 75cm^2 and 175cm^2 flasks for upscaling to perform toxicological experiments and store cell material for future studies. From P10, only T25 flasks were used (Fig 6).

For passage 5 to 13, the splitting protocol included accutase as dissociation reagent as previously described, while TrypLE was used from passage 14.

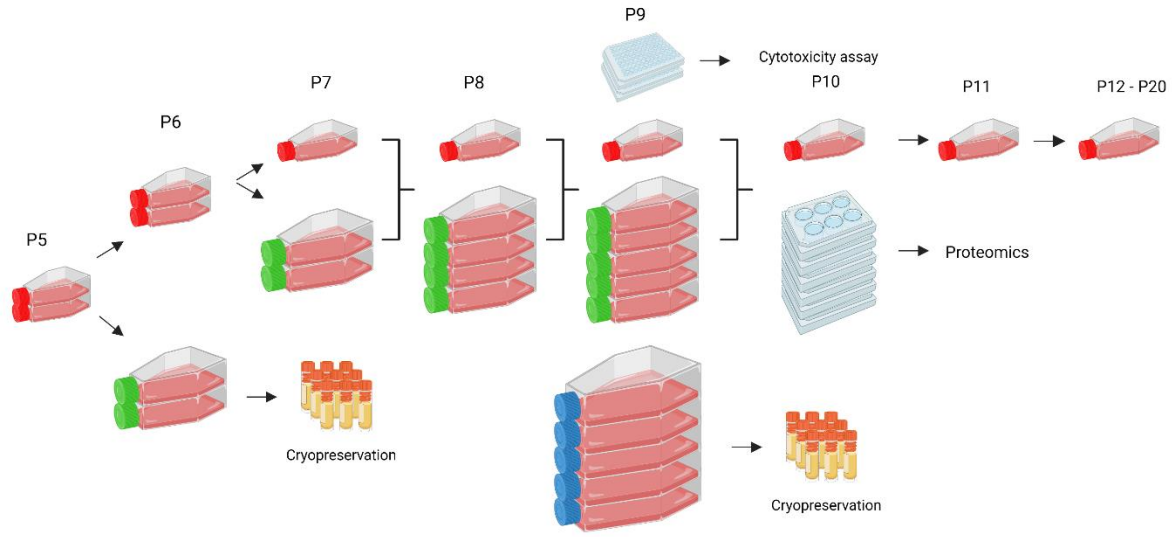


Figure 6. Flow chart of KW-11-02 culturing from thawing and seeding at passage 5 until passage 20. The cells were cultivated in tissue culture treated T25 flasks (red cap), T75 flasks (green cap) and T175 flasks (blue cap). Excessive cell suspension was cryopreserved at P6 and P9. P9 was also seeded in 96-well plates and exposed to a POPs mixture for cytotoxicity studies. P10 seeded in 12-well plates was exposed to the same POPs mixture and the cell pellet was collected and stored for possible further proteomics studies. From P11, the cells were cultivated and passaged until replicative senescence.

2.7 Mycoplasma Detection Assay

KW-1 primary cells at passage 3 were tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit. The cells were cultivated for three weeks in a 60 mm Petri Dish using the standard medium without antibiotics or antimycotics. The assay was performed according to the manufacturer's instructions.

Positive and negative controls were reconstituted. To produce a positive control, 1 mL of MycoAlert Assay Buffer was added to the vial containing lyophilized MycoAlert Assay Control. The same MycoAlert Assay Buffer served as negative control. The controls were incubated at RT for 15 minutes.

The media, which the cells had been cultivated in, was transferred to a 15 mL tube and spun down at 200G for 5 minutes. The supernatant was used as test sample for Mycoplasma contamination in the cells.

Three replicates were made for each testing condition: the positive and negative controls, and the cell supernatant test. First, 100 µL lyophilized MycoAlert Reagent was added to all the respective wells of 96-well plate (flat/white bottom). Then, 100 µL of the controls or supernatant sample was added to the wells. The plate was incubated for 5 minutes for the reagents to interact with the samples. Emitted luminescence was measured with a Plate Reader (read A). At last, 100 µL lyophilized MycoAlert Substrate was added to each well and the plate was incubated for another 10 minutes. Emitted luminescence was measured again (read B). The ratio between read A and read B was used to determine whether Mycoplasma was present or not in the cell media in relation to the negative and positive controls.

2.8 Fluorescent Imaging and Immunohistochemistry

KW-1 passage 5 and KW-11-02 passage 6 fibroblast cells were fixed and permeabilized with 4 % formaldehyde and 0.1 % Triton X100 for 30 minutes at RT. Further, the cells were incubated with a DAPI 1:1000 (blue) and Phalloidin 1:400 (red) staining solution for 1 hour. DAPI was used to identify cells by staining the nuclei, while Phalloidin was used to stain the actin filaments to determine cell morphology. The staining solution was removed, and the cells were washed three times with PBS before a final addition of PBS to each well to prevent the cells from drying out.

Immunolabelling with vimentin antibody (primary antibody conjugated with Alexa Fluor 488 FITC) was used for specific cell type identification, and Ki-67 antibody (primary antibody conjugated with Alexa Fluor 488 FITC) was used as a marker for proliferating cells. Both vimentin and Ki-67 antibodies were diluted 1:200 in PBS:BSA 1%. Before adding the antibodies, the fixed cells were incubated with 5 % BSA blocking solution at RT for 1 hour. Then, 100 µL of antibody solution was added to separate wells for vimentin and Ki-67 and stored overnight at 4℃. Next day, the antibody solution was removed and a mix with DAPI 1:1000 and Phalloidin 1:400 was added to each. The DAPI/Phalloidin staining solution was let sit for 40 minutes at RT before removal. After removing the staining solution, each well was washed with PBS one time. Finally, 200 µL PBS was added to each well and the plate was sealed and covered with foil and stored at 4 ℃ before imaging.

All confocal microscopy images, excluding senescence, were taken by Aman S. Chahal.

2.9 Senescence Assay

A senescence assay was performed using CellEvent™ Senescence Green Detection Kit. The assay enables detection of senescent cells, using the increased activity of β-galactosidase (β-Gal) as a biomarker by adding a specific β-Gal substrate. The enzyme is situated in the lysosomes and cleaves the fluorescent substrate, and the product remains within the cells due to covalent bonds to intracellular proteins where it emits a fluorescent signal between 490/514 nm.

The senescence assay was applied to KW-1 fibroblasts passage 5 that had been seeded at a density of 33,000 into 12 wells on a 96-well plate (black, clear bottom). Six out of 12 wells were coated with gelatin (0.1 %). The cells were allowed to attach to the plate and sit for four days with one media shift in between, before performing the assay.

The media was removed from the wells and the cells were washed with PBS. 100 µL of fixation solution of 2 % formaldehyde in PBS was added to each well and incubated at RT covered in aluminium foil for 10 minutes. In the meantime, the working solution was prepared by diluting the CellEvent™ Senescence Green Probe 1000x in pre-warmed CellEvent™ Senescence Buffer. The fixation solution was removed, and the cells were washed with PBS + BSA 1 % before adding 100 µL of working solution to each well. The cells were covered with foil and incubated for 2 hours at 37 ℃ without CO_2. Before imaging, the working solution was removed and the cells were washed with PBS three times and at last 100 uL PBS was added to each well and the cells were screened with (confocal microscope with FITC filter, 500 nm).

The assay was performed with proliferating KW-11-02 passage 6 fibroblasts additionally, to see if they differed in β-Gal expression compared to the KW-1 fibroblasts. The cells were seeded 20,000 cells/mL in four wells on a 96-well plate and cultivated for three days before they were fixed and stained as described above.

2.10 Cytotoxicity of POPs mixture

Cytotoxicity induced by a specific mixture of POPs was measured on fibroblasts from KW-11-02 at passage 9 cultivated with Mix3 growth media (Table 5). The POP mixture dissolved in DMSO was prepared by the Norwegian Air Research Institute. The mixture reflected the composition and the concentrations of the ten most abundant POPs measured in blubber of killer whales from Northern Norway (Blevin et al., unpublished, attached in Appendix B). The DMSO stock represents the 20,000-fold concentrated level of POPs measured in Norwegian killer whales. For POPs toxicity assays, the stock was diluted 1:400, resulting in a DMSO concentration of 0.25 % and 50 times higher than the median concentrations measured in killer whale samples (50X). A 1:2 dilution series was prepared and the cells were exposed to the nine following concentrations of the POP mixture: 50X, 25X, 12,5X, 6,25X, 3,125X, 1,562X, 0,781X, 0,39X, 0,195X. A DMSO control with 0.25 % DMSO and a non-exposed control group with only growth media was included per plate. Each concentration and control group included six technical replicates. KW-11-02 passage 9 had been seeded 6000 cells/well in two 96-well plates and left to attach to the well surface for 24 hours. Prior to the exposure, all the old media was removed from the wells and replaced with 50 uL fresh media. 50 uL of the prepared POP mixture/DMSO solution was added to each well. The cells were exposed for 48 hours before measuring induced cytotoxicity with both CellTiter-Glo assay and Lactate Dehydrogenase (LDH) release assay.

Table 7. Composition of POP toxicant mixture and the respective concentrations used in exposure studies. The killer whale POP toxmix is based on the 10 most abundant POPs found in Norwegian killer whale blubber measured as molar concentration (uM). The concentration of each compound in undiluted POP toxmix was 20,000-fold the concentration measured in the killer whale blubber. KW-11-02 passage 9 fibroblasts were exposed to nine different concentrations of the toxmix, ranging from 0.195X to 50X the concentration measured of the different compound in Norwegian killer whales (Blévin et al., 2021 [Unpublished]).

Selected compounds in POPs-mix	POPs in KW blubber 1x (uM)	Concentration of POPs in DMSO stock (µg/mL)	Exposure conc. X (µg/mL)
***p-p'*-DDE**	4.477	28477	0.195-50 (0.3-71.2)
PCB153	1.741	12568	0.195-50 (0.1-31.4)
PCB138	1.310	9453	0.195-50 (0.1-23.6)
tNC	0.990	8797	0.195-50 (0.1-22.0)
PCB180	0.637	5037	0.195-50 (0-12.6)
PCB101	0.519	3386	0.195-50 (0-8.5)
PCB52	0.519	3029	0.195-50 (0-7.6)
PCB99	0.483	3155	0.195-50 (0-7.9)
PCB187	0.451	3569	0.195-50 (0-8.9)
PCB118	0.441	2882	0.195-50 (0-7.2)
Total		**80853**	**0.195-50 (0.8-202.1)**

2.10.1 Celltiter-Glo assay

The CellTiter-Glo Luminescent Cell Viability Assay (Fig 7) measures the number of viable cells in a culture. To perform the assay, 100 µL of CellTiter-Glo 2.0 (Promega, G9243) reagent was added to each well of one 96-well plate. The plate was shaken for 2 minutes to induce cell lysis. The cells were incubated with the reagent for 10 minutes at RT before the luminescence was measured using a SpectraMax iD5 Reader with a preconfigured CellTiter-Glo protocol in SoftMax® Pro Software. The obtained data was analyzed, and the cell viability was measured relative to the emitted luminescence.

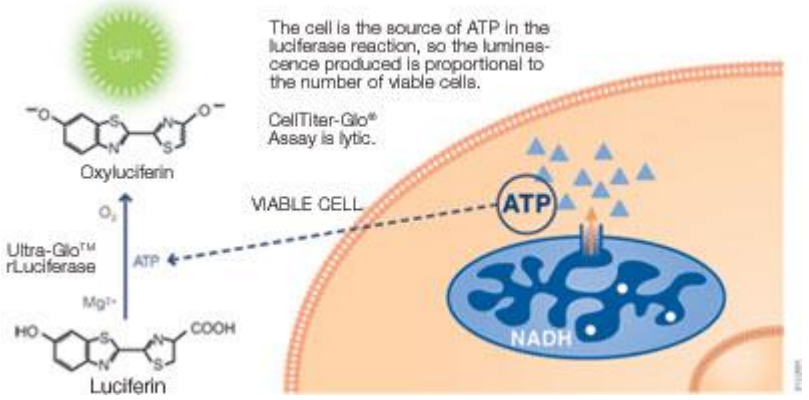


Figure 7. CellTiter-Glo 2.0 reagent contains the enzyme Luciferase which catalyses the oxidation of Luciferin to Oxyluciferin in the presence of Mg2+ and ATP produced by living cells. Oxyluciferin emits light proportionally to the number of viable cells, thus the cell viability is a relative measurement of

the measured luminescence. Credit: https://no.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-luminescent-cell-viability-assay/?catNum=G7570

2.10.2 Lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase (LDH) release assay was performed by using LDH cytotoxicity assay kit (Abcam, Ab65391). First, Triton X100 (0.1 %) was added to 6 wells with cells to induce cell death for a dead control group. The cells were incubated with Triton X100 for 10 minutes at 37 ℃. Then, 50 μL media from the wells were transferred to a half area 96-well plate before 50 μL of already prepared LDH solution was added to each well. The plate was shaken at 1000 RPM for 30 seconds and incubated at RT for 10 minutes. Colorimetric measurement (at 490 nm) was performed with SoftMaxPro and the data was analyzed and used to determine the cytotoxic effects of the POPs mixture.

2.11 Data processing and evaluation

Imaging analyses were done by qualitative evaluation of confocal images. Cell growth was evaluated semi-quantitative by visual inspection of cultures and quantitative measured of cell densities and metabolites. Due to low number of parallels and limited culture volumes single measurements were performed at the majority of sampling points. The number of replicates is reported where quantitative results are presented. Thus, statistical analysis was not relevant to apply for the results presented in this thesis.

3 Results

3.1 Isolation of primary cells

Biopsies sampled from two individuals of killer whales were used to isolate primary fibroblasts (KW-1 and KW-2). The dermal tissue fragments were cultivated as described in Methods 2.4. Out of the two sampled biopsies, primary fibroblasts were successfully isolated from KW-1. Mycoplasma negative cultures of KW-1 was ensured by the MycoAlert detection assay.

3.1.1 Killer whale individual 1 (KW-1)

New primary fibroblasts derived from the skin sample were first observed 13 days post processing along the edges of the tissue fragments showing good adherence to the well. These fragments were cut closest to the epidermis of the biopsy. Morphologically, the cells grew adherent in the well and exhibited typical fibroblastic morphology with spindle-shaped, polygonal appearance (Fig 8, 9, 10).

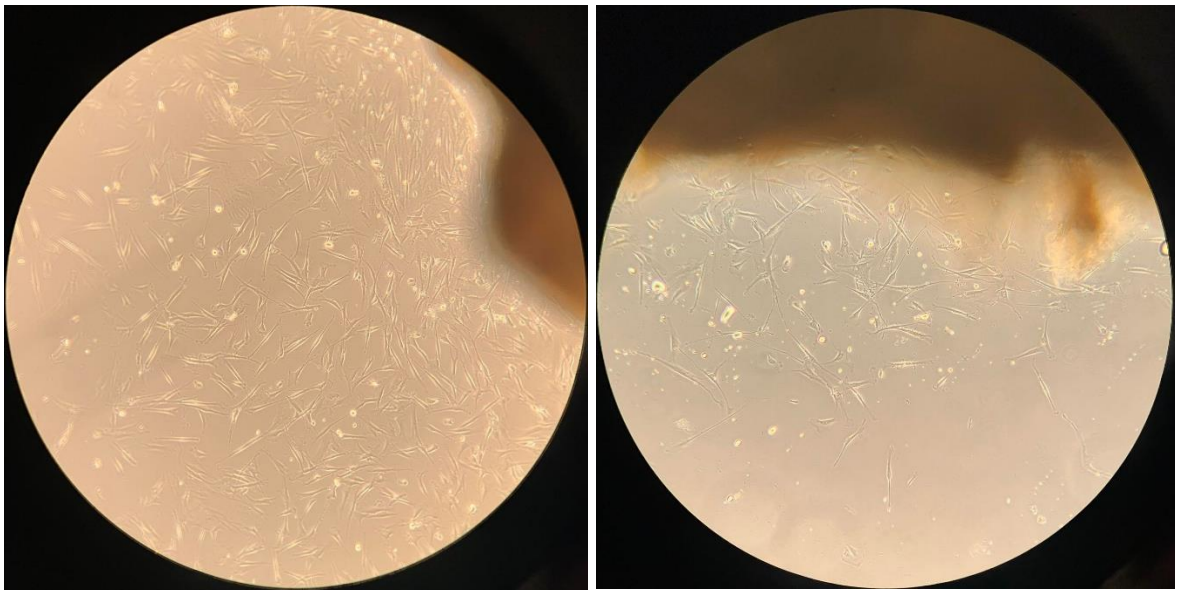


Figure 8. New primary cells derived from KW-1 skin samples photographed through phase contrast microscope. Magnification 4X.

At day 18 post processing, primary fibroblasts were isolated from both wells (8 mL), yielding 20,000 cells/mL and 10,000 cells/mL, respectively, that were used in further experiments.

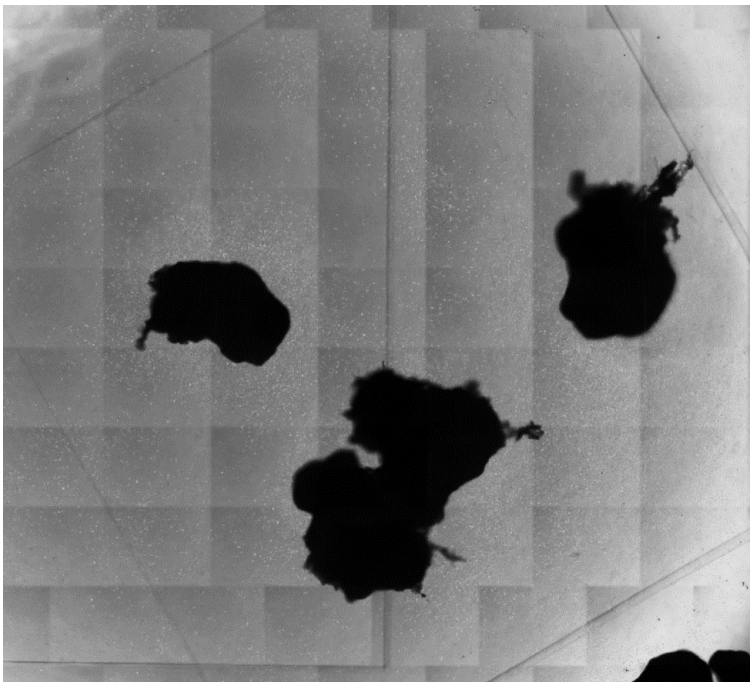


Figure 9. Tissue fragments from KW-1 seen as large black patches. The fragments were cultivated in 6-well plates and covered with coverslips. New primary fibroblasts observed around the fragments and dispersed around the well. Image taken at day 18 post processing. Magnification 4x.

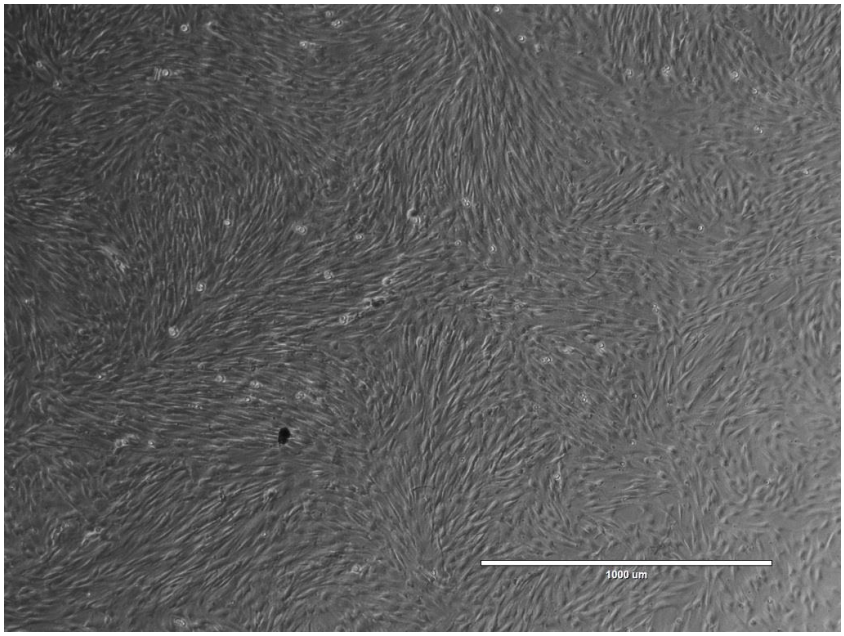


Figure 10. Close up image of confluent KW-1 primary fibroblasts derived from the tissue fragments. Scale bar 1000 µm.

Remaining tissue fragments were cultivated for a total of 29 days before isolation. The isolated primary cells derived from KW-1 were cryopreserved at 1.2 x 10^6 cells/mL (1 mL) at passage 2.

3.1.2 Killer whale individual 2 (KW-2)

The biopsy from KW-2 was smaller compared to KW-1 and yield three wells with tissue fragments. At day 13, one of the wells had become heavily contaminated with fungus. Seven days after processing, new primary cells were observed growing at the edges of the

tissue fragments. However, the cells stopped proliferating and the cell density did not increase after another nine days in culture. Due to this, isolation of primary cells from KW-2 was unsuccessful and the tissue fragments and the cells were discarded.

3.2 Effects of Fibroblast Growth Factor 2, Laminin Coating and Seeding Density on Cell Growth

Primary fibroblasts originating from KW-1 passage 1 were plated at two different cell densities (20,000 and 10,000 cells/mL). The cells were seeded in normal tissue-coated or laminin-coated plates and grown in media with or without fibroblast growth factor 2 (bFGF1, 1 ng/mL) added. The cells plated at 20,000 cells/mL and treated with bFGF1-H, reached confluence after six days. All cells from the different treatments were dissociated and counted simultaneously (Fig 11). bFGF1-H treated cells plated on laminin-coated plates had grown the most with 102,000 ± ~20,400 cells/mL. The second highest growth was observed for bFGF1-H treated cells without laminin-coating with an average number of 93,800 ± ~ 5800 cells/mL. Laminin-coating of the plate alone also increased cell growth with 70,300 ± 5800 cells/mL compared to the control with 44,000 ± 8800 cells/mL.

The cells seeded 10,000 cells/mL took 17 days before the first wells showed patches of confluence. Here, the bFGF1-H treated cells had the highest growth with 79,000 ± 32 000 cells/mL, compared to bFGF1-H + laminin-coating with 55,000 ± 2600 and laminin-coating alone with 20,000 ± 8800. The average density of the cells in the control group seeded at 10,000 cells/mL was 8,800 ± ~3000 cells/mL after 17 days, which illustrates that the cells did not thrive well when seeded at a lower density.

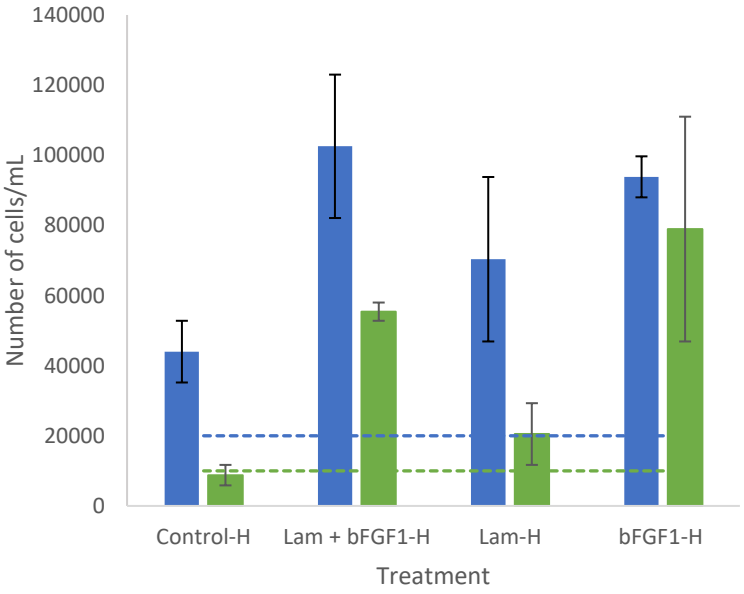


Figure 11. Number of KW-1 fibroblast cells counted after treatment with and without laminin-coating of well surface and/or fibroblast growth factor 2 (1 ng/mL). The measures are shown as average based on two replicates with standard deviations of the mean. The cells were seeded either 20,000 cells/mL (blue bars) or 10,000 cells/mL (green bars). The dotted lines represent the two seeding densities. The cells were counted when the first wells reach confluence. (H = high glucose concentration)

3.3 Effects of Non-Essential Amino Acids

KW-1 fibroblasts of passage 3 were used in the experiment to test if supplementation with MEM Non-Essential Amino Acids (NEAA) would affect cell growth. After seeding, observation through phase contrast microscope revealed that a lot of cells did not manage to adhere to the well plate and were floating in the media. There was also an issue with aggregation of the cells during the passaging, which can be seen in the microscopic images (Fig 12). No proliferation could be observed within the first days in cells that did adhere and survive, neither for the control nor the NEAA-treated cells. Thus, images were taken of the cultures at a later time point to compare with earlier images. After 16 days in culture, there were still no visual increase in cell number for either condition. The cell cultures were kept going for 28 days, and by that time it seemed like there were less cells left in the wells than initially, and that the cells were dying rather than growing. A few days later, the cells were trypsinized and reseeded without dilution with their respective treatments. Very few cells managed to attach to the well surface then, and most of the cells died.

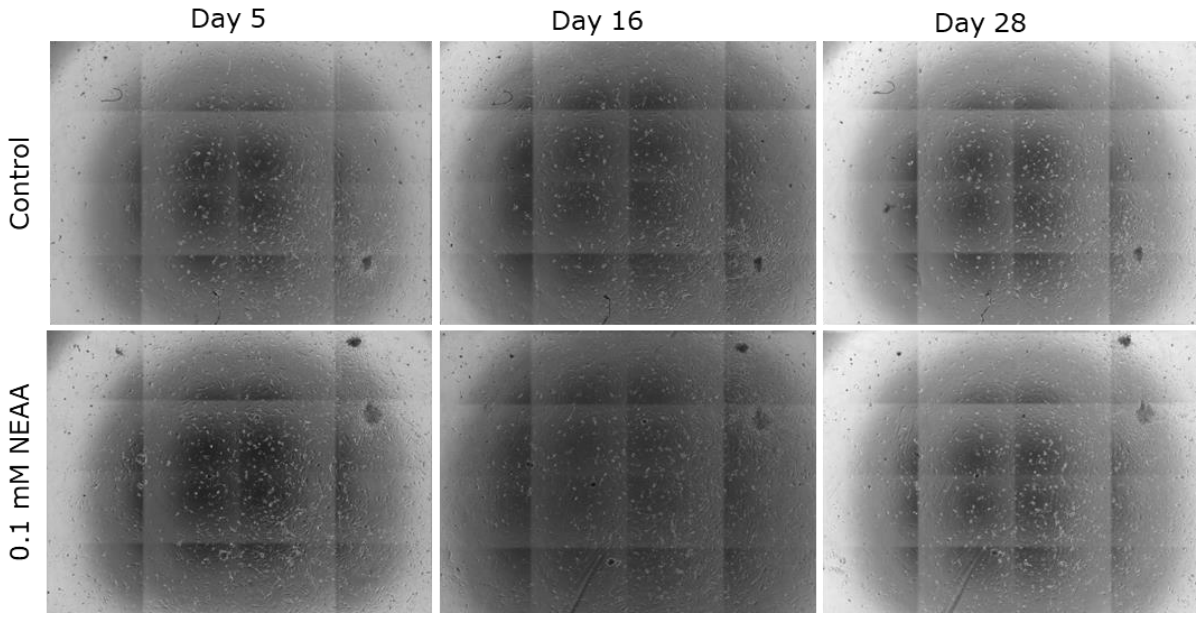


Figure 12. Microscopic images of KW-1 passage 3 fibroblasts taken at day 5, 16 and 28 post seeding. Control group treated with standard media (n = 3) and KW-1 treated with 0.1 mM non-essential amino acids (n = 6, passage 3) in 12-well plates. 50-75 % of the media was changed every 2-3 days and bFGF (1 ng/mL) were added in both test media.

3.4 Effects of different bFGF concentrations on cell growth

KW-1 passage 1 fibroblast showed significantly improved growth when supplemented with bFGF at 1 ng/mL (Section 3.2). Thus, an experiment with increasing concentrations of bFGF (1, 2 and 4 ng/mL) was performed with KW-1 at passage 4. From the microscopic images it seemed like cells treated with bFGF at 2 ng/mL and 4 ng/mL had increased growth compared to control and bFGF 1 ng/mL after 12 days (Fig 13). However, the cells grew strangely and did not exhibit very fibroblastic features in terms of morphology and monolayer sheet-forming. For all test conditions, the cells grew unpredictably and unconditionally to each other, and did not create typical patterns for confluent fibroblasts,

as can be seen from the images of KW-1 passage 0 before isolation (Fig 15). After 21 days in culture, none of the treatments reached confluence and it looked like the cells grew only in size, rather than in number, which also complicated the confluence determination.

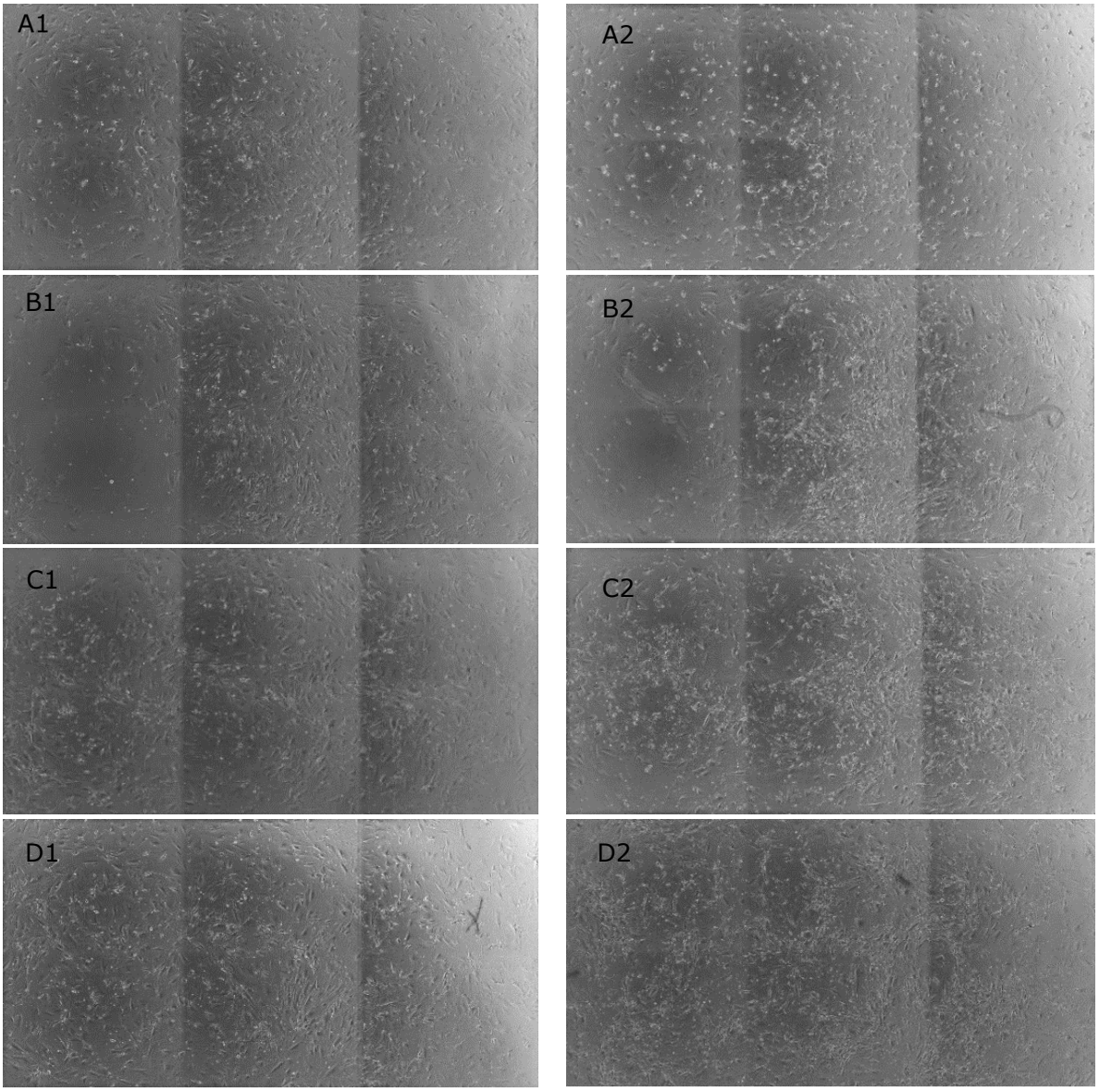


Figure 13. Microscopic images of KW-1 passage 4 fibroblast cells. Images numbered as 1 were taken 12 days post seeding while images numbered as 2 were taken 32 days post seeding. The test conditions were either A) control group with standard media, B) KW-1 fibroblasts treated with bFGF (1 ng/mL), C) KW-1 fibroblasts treated with bFGF (2 ng/mL) or D) KW-1 fibroblasts treated with bFGF (4 ng/mL). The cells were seeded in 12-well plates with 50-75 % change of media every 2-3 days. Each condition included three replicates, where one replicate is shown here.

3.5 Effects of Glucose Concentration and Ascorbic Acid on Cell Growth

The effect of low (1 g/L) and high (3.15 g/L) concentration of glucose with and without AA (50 µg/mL) on cell growth was tested on KW-1 passage 5. When KW-1 passage 4 were passaged and seeded for this experiment, very few cells were able to attach to the plate and most of them died during and after the passaging. By visual inspection of the well plate, it was observed that the media turned more yellow in the wells that contained high concentration of glucose, which indicates that the pH was reduced faster in high glucose condition. The cells that survived and managed to attach to the well plates did not proliferate and rather increased in size than numbers. The cells were stained with DAPI and Phalloidin to detect the actin filaments and nuclei and demonstrate the large cell surface area (Fig 14).

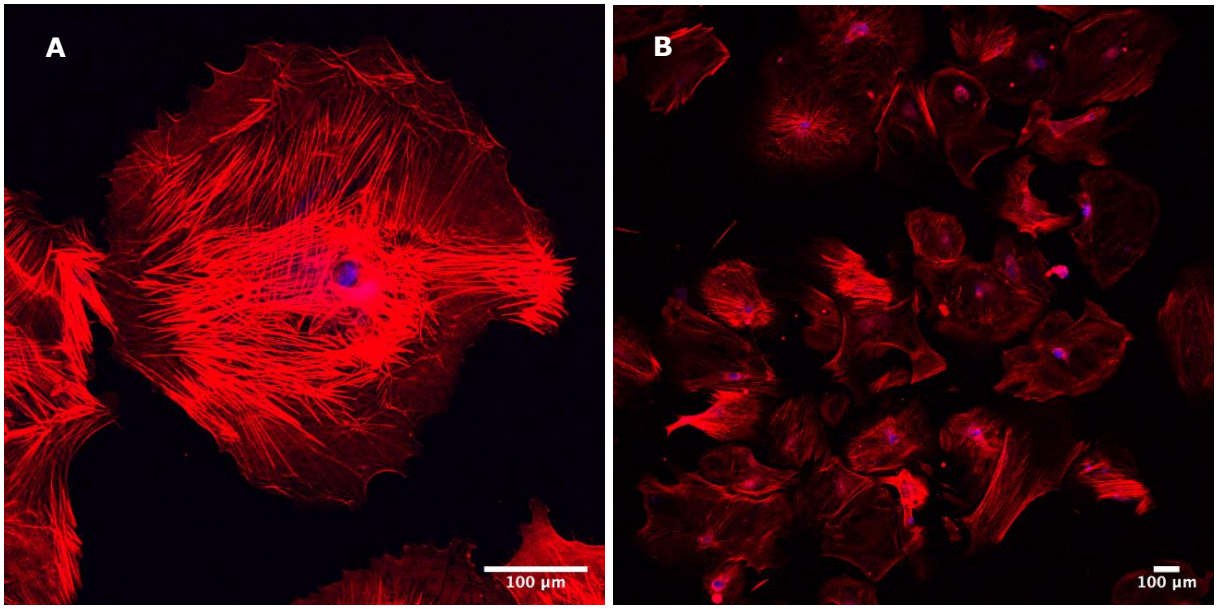


Figure 14. Confocal images of KW-1 passage 5 fibroblasts. A) Close-up view of KW fibroblast. B) Overview of KW fibroblasts. The cells were fixed with formaldehyde 4 % and permeabilized with 0.1 % Triton X100 for 30 minutes at RT. The nuclei were stained with DAPI (1:1000 PBS, blue) and actin filaments stained with Phalloidin (1:400 PBS, red). Scale bar, 100 µm.

The effect of low/high glucose and AA could not be determined with KW-1 passage 5 as their ability to proliferate was severely reduced. The experiment was therefore repeated with KW-1 passage 3 that had been cryopreserved once. Similar changes in morphology, including enlargement as well as disrupted structural behaviour, was observed. Before isolation of the primary cells, microscopic images were taken of KW-1 P0 fibroblasts at high confluence. Compared to the microscopic image of KW-1 P3, the KW-1 P5 fibroblasts portrayed a significant increase in cell size and change in structural morphology (Fig 15B). Senescence was later confirmed with β-Galactosidase senescent assay.

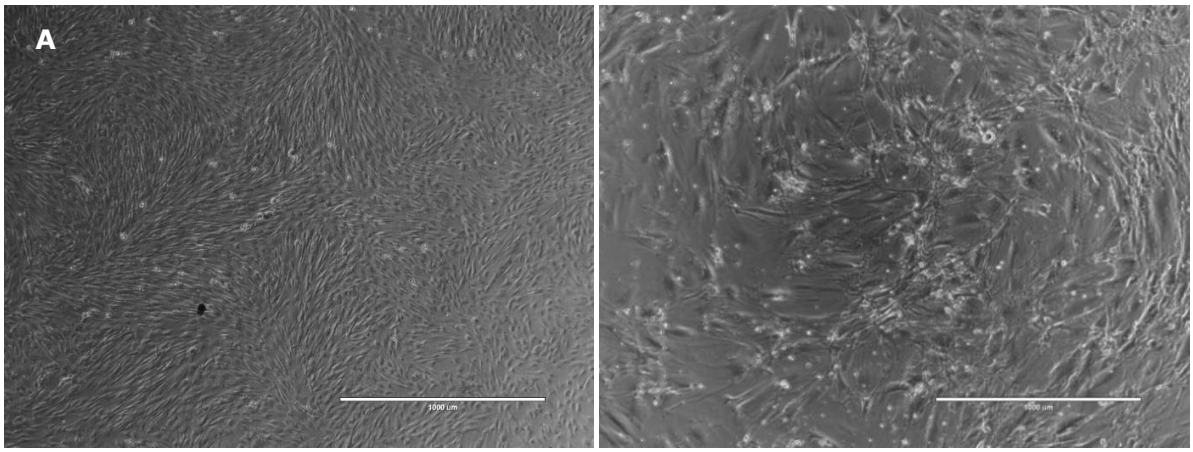


Figure 15. A) Image of confluent KW-1 fibroblasts at passage 0, before isolation. B) Image of KW-1 at passage 3 after 16 days in culture after cryopreservation and thawing. KW-1 passage 3 was part of the low/high glucose ± AA trial and had been previously cultivated with high glucose, without AA. Scale bar, 1000 µm.

3.6 Senescence Determination

As the morphological features observed for KW-1 were indicative of replicative senescence, a senescence assay based on the enzymatic activity of β-Galactosidase was conducted with KW-1 at passage 5 which exhibited similar features in both proliferation and morphology. The cells were cultured with combination media, Mix3, (See Section 2.4, Table 5). Microscopic observation of the cells showed poor growth and lack of proliferation after seeding. There were a lot of dead cells floating in the media and many cells had shrunken into round, dark dots still attached to the well. The round, shrunken cells, assumingly in the initial phase of apoptosis, exhibited the strongest enzymatic activity (Fig 16A). Staining also revealed great cytoplasmic expansions that were not visible in phase contrast microscope prior to fixation and staining. The high expression of β-gal detected in the senescence assay confirmed that KW-1 were in a replicative senescent state. Proliferating KW-11-02 passage 6 fibroblasts were stained similarly as control and expressed visibly less β-gal activity (Fig 16B) in addition to being smaller. However, a few of the KW-11-02 cells had grown bigger and expressed higher levels of β-gal compared to the other, which indicates that they had become senescent as well.

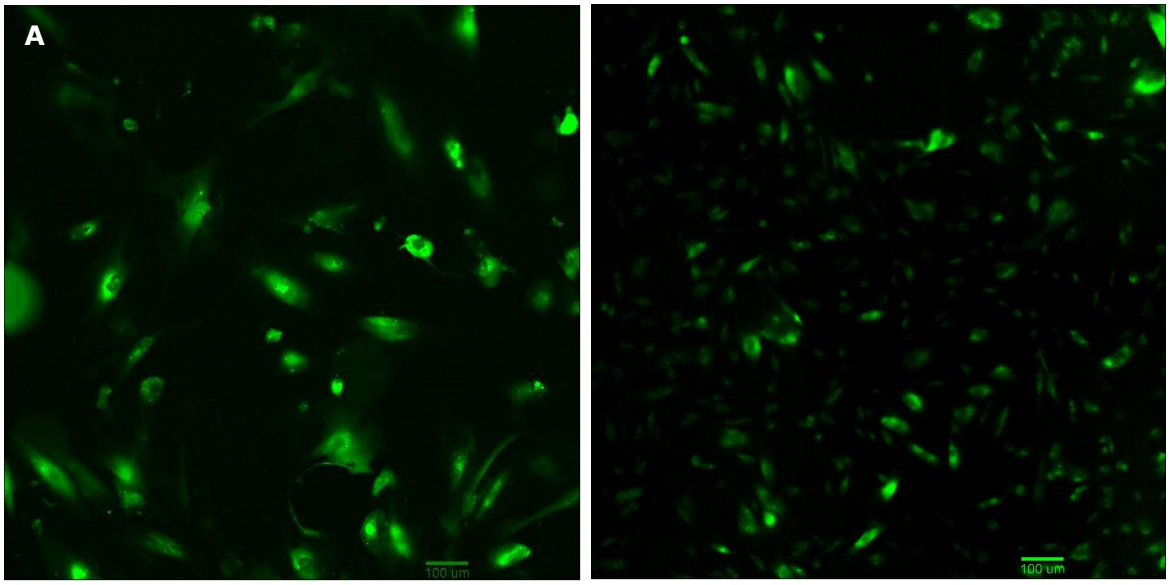


Figure 16. Confocal images of A) KW-1 passage 5 fibroblasts. B) KW-11-02 passage 6 fibroblasts. The cells were cultivated for four days after seeding with 50 % media change every other day. The cells were fixed with 2 % formaldehyde for 10 minutes at RT and stained with CellEvent™ Senescence Green Probe, a fluorescent substrate for β-Gal before performing the imaging. Scale bare, 100 µm.

3.7 Effect of bFGF, FBS, HS, BME, Gelatin and Laminin treatment on cell growth

Analyses of glucose, lactate and LDH were used to determine the effect of various media and coating on metabolic activity in KW-11-02 passage 3 fibroblasts. Different concentrations of fibroblast growth factor 2 (bFGF), fetal bovine serum (FBS), horse serum (HS) and 2-Mercaptoethanol (BME) were applied with or without well surface coating with

gelatin or laminin (Fig 16). For reference, the initial concentrations of the metabolites were measured in fresh cultivation media, for both the control media, FBS20 media and HS media.

In the first measurement after six days in culture, glucose consumption by the control group was measured at 2.2 mM. Treatment with bFGF 1, 2 and 4 ng/mL and FBS 20 % with and without surface coating had the highest glucose consumption with small variations. Horse serum plus laminin coating also yield similar increasement in glucose consumption compared to the control. The lowest glucose consumption was measured in cells treated with the control media and in cells only supplemented with BME and/or surface coatings. Gelatin-coating and laminin-coating seemed to slightly increase the metabolic activity of the cells compared to the control after six days. However, after 13 days, neither surface coating increased the glucose consumption compared to the control group.

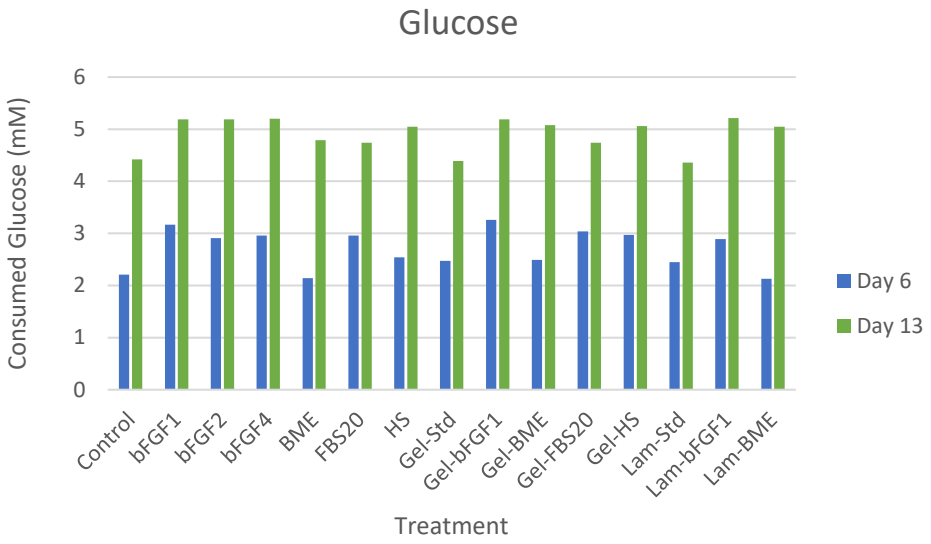
Treatment of killer whale cells by BME with/without coating increased glucose consumption by 8-15 % only after 13 days. No difference in glucose consumption was seen in the treated vs. control cells after 6 days (Fig17).

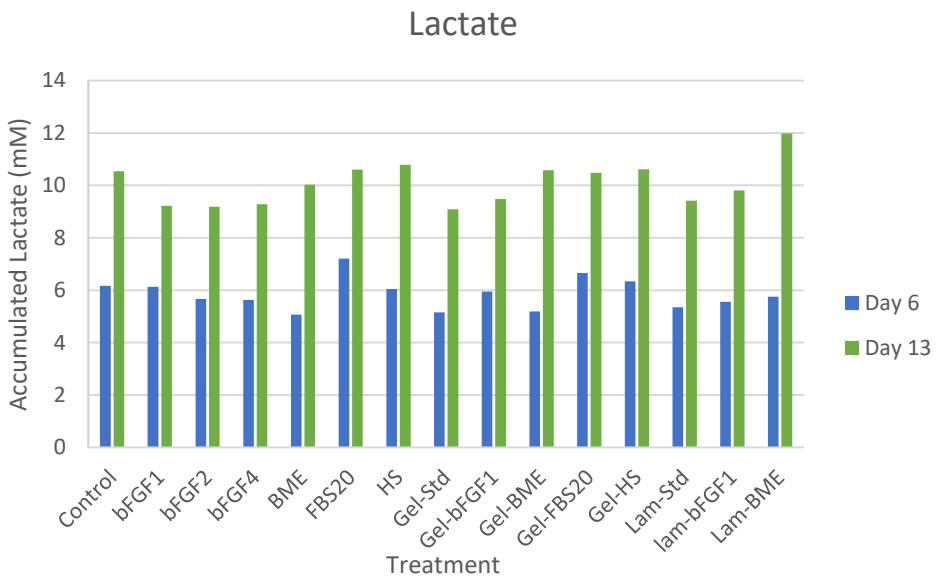
At day 13 post seeding, several wells withvdifferent treatments had reached confluence and were fully covered with cells. That included all bFGF treatments, as well as FBS 20 % and horse serum ± surface coatings. The cells treated with 1, 2 and 4 ng/mL bFGF regardless of surface coatings had consumed all the available glucose in the media. That was also the case for the horse serum and FBS 20 % treatments. None of the wells treated with only BME or the control media reached confluence within 13 days. These treatments also had glucose left in the media by the time of measurement. Nevertheless, both surface coatings with additional BME had increased glucose consumption compared to BME alone and the control group, and they had almost metabolized all the available glucose (Fig 17).

The condition which increased glucose consumption the most was bFGF, which was also the condition that accumulated the least lactate in general. Surface coatings yield similar accumulation of lactate, but also showed less glucose metabolism. The control group had the lowest glucose consumption, but also the highest accumulation of lactate (Fig 17).

The concentration of LDH, which was used as an indicator of poor cell health or death, was greatly increased for all conditions over one week, from day six to 13. LDH accumulation naturally increases with increasing cell numbers, and as mentioned, all wells except for the control group, BME with and without surface coating treatments, and surface coating treatments alone, were completely confluent by day 13. Although increasing the cell numbers the most, bFGF treatments had accumulated the lowest concentrations of LDH in the first measurement. The control group had the highest concentration of LDH six days post seeding, and also relatively high concentration at day 13 considering these wells were the least confluent at this time (Fig 17).

Noteworthy was the relatively low accumulation of LDH in cells treated with standard media surface coatings, both gelatin (Gel-std) and laminin (Lam-std) compared to the control group treated with only standard media. Horse serum treatment caused slightly elevated levels of LDH compared to Gel-std and Lam-std, but simultaneously increased the cell growth.


Glucose
Consumed Glucose (mM)
0
1
2
3
4
5
6
Control
bFGF1
bFGF2
bFGF4
BME
FBS20
HS
Gel-Std
Gel-bFGF1
Gel-BME
Gel-FBS20
Gel-HS
Lam-Std
Lam-bFGF1
Lam-BME
Treatment
Day 6
Day 13


Lactate
Accumulated Lactate (mM)
0
2
4
6
8
10
12
14
Control
bFGF1
bFGF2
bFGF4
BME
FBS20
HS
Gel-Std
Gel-bFGF1
Gel-BME
Gel-FBS20
Gel-HS
Lam-Std
lam-bFGF1
Lam-BME
Treatment
Day 6
Day 13

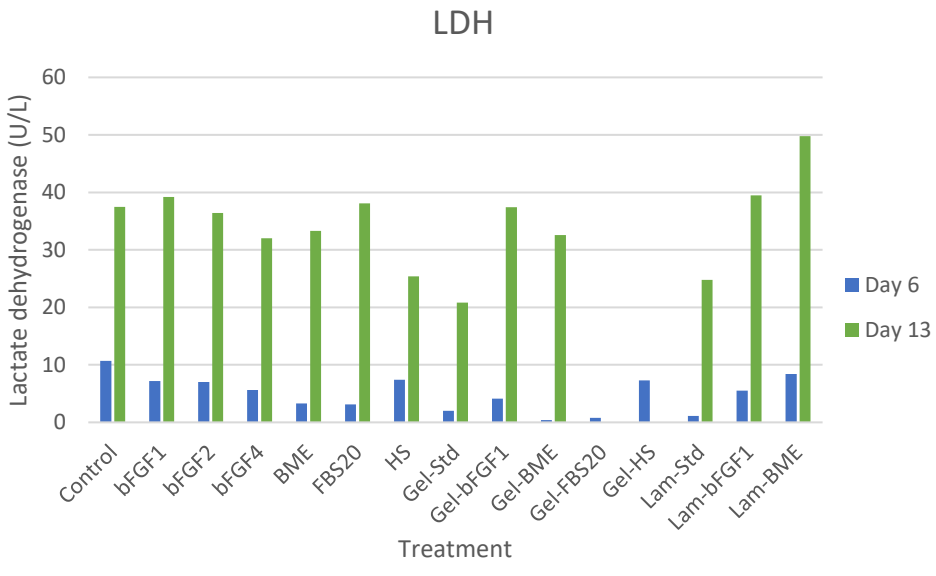

LDH
Lactate dehydrogenase (U/L)
0
10
20
30
40
50
60
Control
bFGF1
bFGF2
bFGF4
BME
FBS20
HS
Gel-Std
Gel-bFGF1
Gel-BME
Gel-FBS20
Gel-HS
Lam-Std
Lam-bFGF1
Lam-BME
Treatment
Day 6
Day 13

Figure 17. Consumption of glucose and accumulation of lactate and LDH for KW-11-02 passage 3 fibroblasts cultivated under different conditions, including 1, 2 and 4 ng/mL fibroblast growth factor 2 (bFGF1, bFGF2 and bFGF4, respectively), BME (0.1 mM), 20% fetal bovine serum (FBS20), 5% horse serum (HS) with or without, gelatin (Gel) and laminin (Lam) surface coating. Each bar represents the measurement done on one pooled sample (from three replicates) for each treatment at day six (blue) and day 13 (green) post seeding. A) Glucose consumption by KW-11-02 fibroblasts. Control levels are given by the dotted lines. Given values were calculated by subtracting the measured concentration of glucose in cultivation media from the wells, from the initial concentration of glucose in the fresh growth media. B) Accumulated lactate (mmol/L) by KW-11-02 fibroblasts. Values calculated by subtracting the measured concentration of lactate in the fresh growth media from the concentrations measured in the media taken from the cultivation wells. C) Accumulated LDH (U/L) measured in media for KW-11-02 fibroblasts for the different culturing conditions. LDH was not measured at day 13 for Gel-FBS20 and Gel-HS.

3.8 Optimization of Cultivation Media

As the metabolite analysis indicated that media supplemented with bFGF 1 ng/mL, 20 % FBS, 5 % horse serum and BME increased the metabolic activity, these parameters were combined in two different media, Mix1 and Mix2 (Table 5), to optimize the growth conditions. Mix1 and Mix2 were compared to bFGF at concentrations 1, 2 and 4 ng/mL (bFGF1, bFGF2 and bFGF4 respectively), FBS 20 % and horse serum alone (Fig 18). Overall, bFGF2 yield the highest growth with a total of 275,000 cells/mL ± 5,000 followed by Mix1 with an average cell density of 225,000 ± 15,000 cells/mL.

Cells in conditions with either 20 % FBS or horse serum grew poorly compared to the other treatments and were therefore excluded after the first passage of this trial. FBS20 also gave the lowest viability with 60 %, and the second lowest viability was observed for Mix1 which also consisted of 20 % FBS with 76.5 % (Appendix 2). Both optimized media increased growth compared to bFGF at 1 ng/mL alone, which indicate that supplementing the media with extra serum, either increased concentration of FBS or by replacing half the FBS with horse serum was more optimal compared to only bFGF at 1 ng/mL. Further it seems obvious from these results that bFGF is the most efficient growth factor, as bFGF at 2 ng/mL stimulated significantly more cell growth than bFGF at 1 ng/mL and Mix1 which combined both bFGF 1 ng/mL and extra serum.

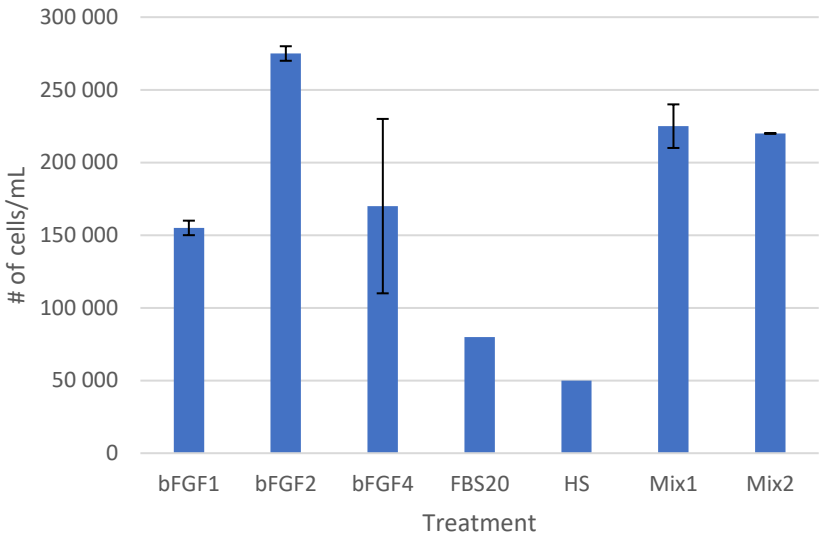


Figure 18. Number of KW-11-02 fibroblasts cells counted (cells/mL) as an indicator of cell proliferation induced by different cultivation conditions. The different media treatments were standard media supplemented with either 1, 2 and 4 ng/mL fibroblast growth factor 2 (bFGF1, bFGF2 and bFGF4, respectively), fetal bovine serum 20 % (FBS20) or horse serum 5 % (HS) and the optimized composite media Mix1 (bFGF 1 ng/mL, 20 % FBS, 0.1 mM BME) and Mix2 (bFGF 1 ng/mL, 5 % HS and 0.1 mM BME). The cells were cultivated until the first wells were confluent with 50 % media change every other day. At confluence, the cells were counted and split with ratio according to the cell densities. The experiment was repeated over two passages (P4-P5); hence each bar represents the mean number of cells counted for each passage. Standard deviations are given by the error bars. FBS20 and HS lack standard deviations as they were only counted in the first passage.

3.9 Maximum Cell Culture Passages

The optimized media with 1 ng/mL bFGF, 20 % FBS and 0.1 mM BME (Mix1) was used further to cultivate KW-11-02 for as many passages that could be achieved. KW-11-02 fibroblasts were thawed at passage 3 and reached a total of 18 passages over a period of 15 weeks before the cells stopped proliferating. Time until confluence and counted cell density for passage 9 to 18 is given in Figure 19.

Passage 10 was seeded at 53,000 cells/mL density and took three days to reach confluence. The seeding density was reduced to 20,000 for the next passage, and the cells became confluent in one week. When seeded at 60,000 cells/mL the first time, KW-11-02 P12 took three days to reach confluence, while P13 took four days. A sudden drop in cell viability was observed after P12, from approximately 75 % to 50-60 % for the next passages. This happened at the same time as new cultivation media was mixed, and the sodium pyruvate concentration was accidently increased from 1 mM to 2 mM.

It seemed more optimal to split the cells once per week, in terms of disturbance and stress related to dissociation of the cells and collecting of cells for other experiments. Hence, the seeding density was reduced to 40,000 cells/mL for P14-P18. The growth rate decreased gradually until P16, and after P16, a rapid decline was observed. By 18 passages, the cells struggled to attach to the culture plate and stopped proliferating.

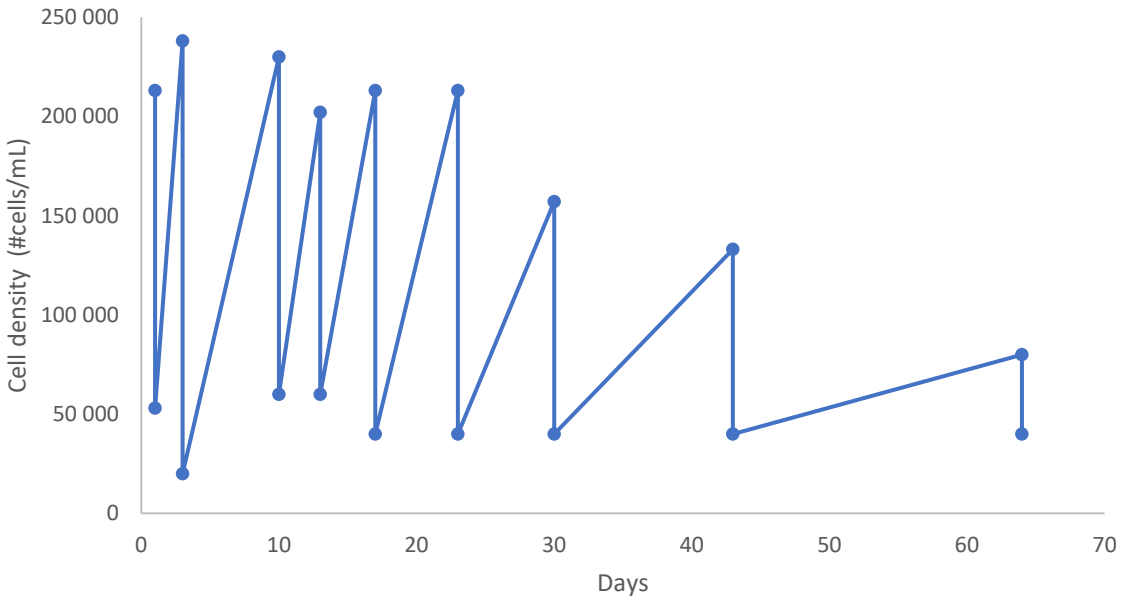


Figure 19. Constructed growth curve based on cell number (cells/mL) from cultivation of KW-11-02 fibroblasts from P9-P18 over a 9-week period with Mix1 growth media supplemented with fibroblast growth factor 2 (bFGF, 1 ng/mL), fetal bovine serum (FBS, 20 %) and 2-Mercaptoethanol (BME, 0.1 mM). Until passage 9, the cells were split according to ratio and not cell density, hence they are not included. The cells were cultivated in T25 tissue-culture treated flasks with 50 % media change every other day. At 90-100 % confluence (top bullets), the cells were split and seeded with seeding densities 20,000-60,000 cells/mL (bottom bullets).

The experiment was repeated to see if the results were reproducible, and the same number of passages could be achieved with KW-11-02 cells cryopreserved at passage 4. This time, the cultivation media was composed of 2 ng/mL bFGF, 15 % FBS, 5 % horse serum and 0.1 mM BME. The cells grew 80-90 % confluent within two to four days for all passages and the population doubling time was calculated to approximately 40 hours. By passage 20, no significant decrease in growth rate was observed, nor did the cell morphology change considerably. The high cell density measured for P8 was due to the cell suspension from one 25cm^2 flask and four 75cm^2 flasks being combined, which yield a different surface:volume ratio (Fig 20).

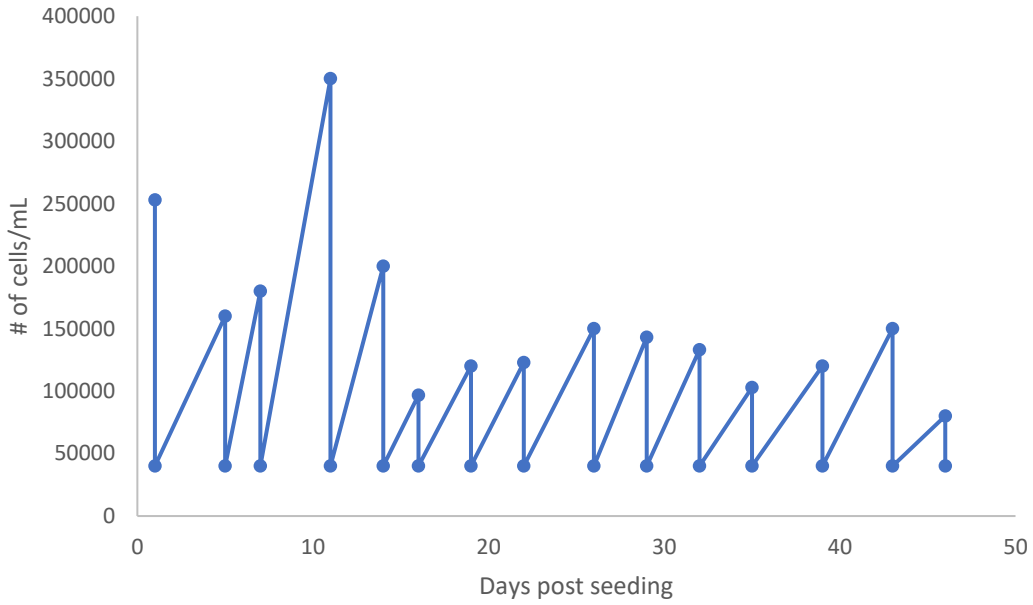


Figure 20. Constructed growth curve based on cell number (cells/mL) from cultivation of KW-11-02 fibroblasts from P4-P20 over seven weeks with Mix3 growth media. The cells were seeded at 40,000 cells/mL (bottom bullets) and passaged at 80-90 % confluence (top bullets) The growth media was supplemented with fibroblast factor 2 (bFGF, 2 ng/mL), fetal bovine serum (FBS, 15 %) horse serum (HS, 5 %) and 2-Mercaptoethanol (BME 0.1 mM). The cells were cultivated in T25 tissue-culture treated flasks with 50 % media change every other day.

3.10 Cell type identification and Ki-67 expression

To determine if the isolated KW primary cells were indeed fibroblasts, they were labelled with a vimentin antibody which is normally highly expressed by this cell type. Vimentin labelling revealed clear indication that the isolated cells were fibroblasts, both in terms of morphology and vimentin expression (Fig 21).

Ki-67 is a well-established marker for proliferating cells and was used to determine if the KW-11-02 cells were proliferating. Labelling with Ki-67 demonstrated that many of the cells were in a proliferating state. However, the number of DAPI stained nuclei exceeds the number of Ki-67 expressing cells, which indicates that there was non-proliferating, potentially senescent cells present in the culture also (Fig 21).

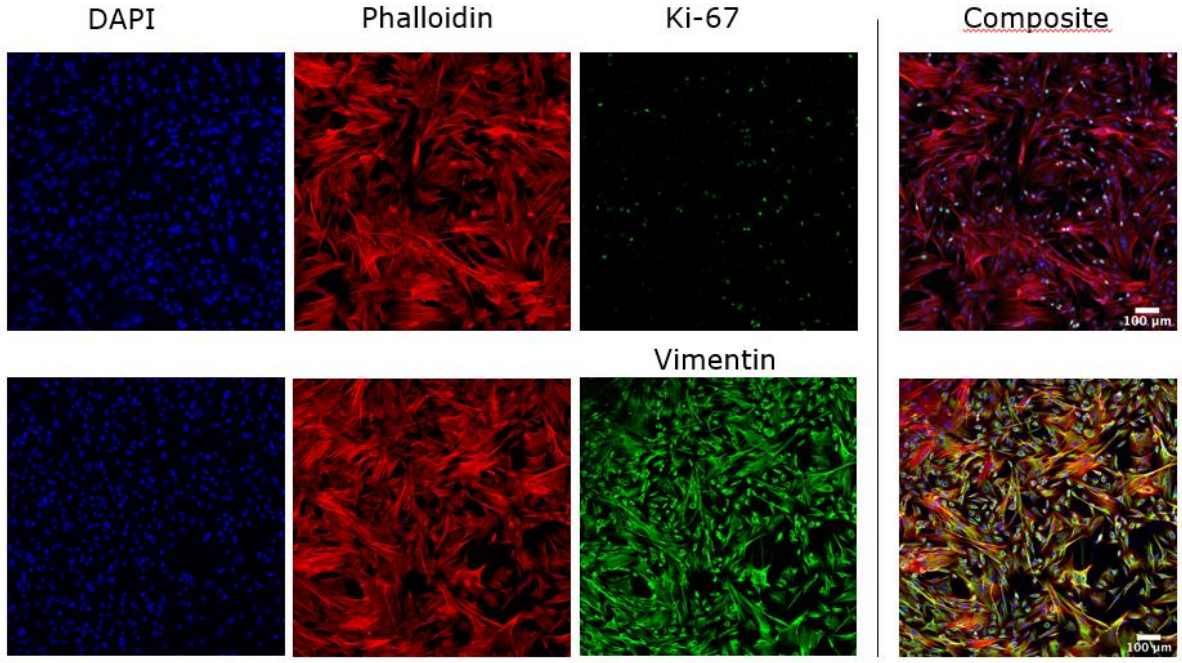


Figure 21. Confocal images of KW-11-02 passage 6 fibroblasts. The cells were fixed and permeabilized with 4 % formaldehyde and 0.1 % Triton X100 solution. After fixation, the cells were incubated with a BSA 5 % blocking solution 1 hour before addition of vimentin primary antibody conjugated with Alexa Fluor 488 FITC (green, 1:200 PBS:BSA 1 %) and Ki-67 primary antibody conjugated with Alexa Fluor 488 FITC (green, 1:200 PBS:BSA 1 %). The cells were incubated with the antibodies for 24 hours at 4 ℃ before removal and further stating with DAPI (blue, 1:1000 PBS) and Phalloidin (red, 1:400 PBS) mastermix. DAPI/Phalloidin was added to each well and incubated for 40 min at RT. Upper row show KW-11-02 nuclei stained with DAPI, actin filaments stained with Phalloidin and expression of Ki-67. Lower row show KW-11-02 nuclei and actin filaments stained with DAPI and Phalloidin and the expression of vimentin intermediate filament labelled with vimentin antibodies. Scale bar, 100 µm.

3.11 Cytotoxicity of POP mixture

Cytotoxicity of the POP mixture that reflect pollutants present in killer whale blubber was applied at different concentrations on KW-11-02 passage 9 fibroblasts and measured by CellTiter-Glo assay (Fig 22) and lactate dehydrogenase (LDH) release assay (Fig 23).

3.11.1 CellTiter-Glo assay

KW-11-02 passage 9 was exposed to different concentrations of relevant POP mixture to measure induced cytotoxic effects. Cell viability was used as an indirect measure of cell death caused by the toxicants. The highest applied concentration of 201.1 µg/mL (50X) induced a clear cytotoxic effect of 25 % ± 2.5. Further it was observed that 101.1 µg/mL (25X) reduced the cell viability with approximately 10 % ± 3.5 %. For lower concentrations of POP, no cytotoxicity was observed. Hence, a concentration-dependent loss in cell viability for KW-11-02 fibroblasts exposed to the POP mixture was demonstrated with an estimated threshold 64 µg/mL. The DMSO control (0.25 %) did not influence the cell viability.

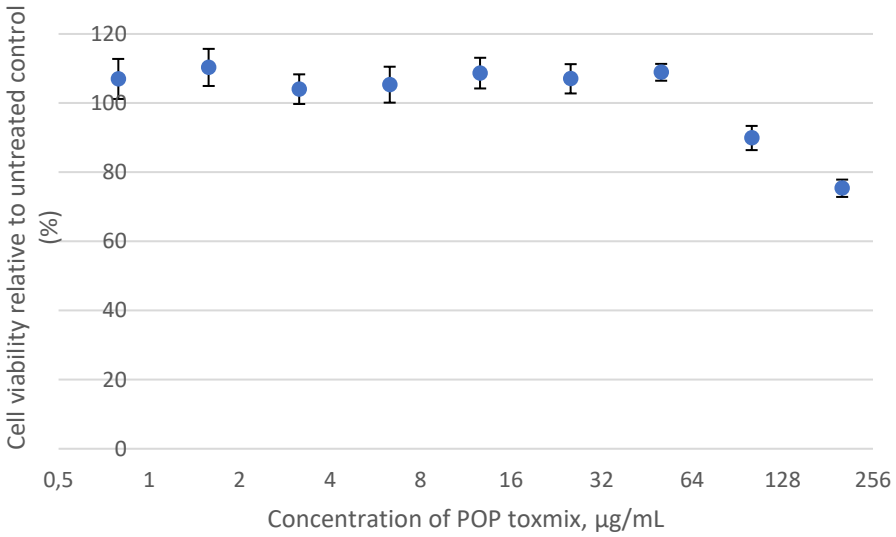


Figure 22. Measured cell viability (%) relative to untreated control group. KW-11-02 passage 9 fibroblasts was seeded 6000 cells/well in 96-well plates and left to attach for 24 hours before exposure to nine different concentrations of POPs mixture: 202.1, 101.1, 50.5, 25.3, 12.6, 6.3, 3.2, 1.6, 0.8 µg/mL and 0.25 % DMSO. The cells were exposed for 48 hours before cytotoxicity analysis with CellTiter-Glo. Each bar represents the average measure of six technical replicates and the error bars indicate the standard deviation of the mean.

3.11.2 LDH release assay

The LDH release assay measured no clear indications of cytotoxic effects by the POP mixture on KW-11-02 fibroblasts. Relative to the Triton X100 (0.1 %) dead control, LDH leakage caused by the highest applied POPs concentration of 202.1 µg/mL was 0 ± 1.5 %. The non-treated control group measured the most LDH leakage with 5.2 ± 2.2 %.

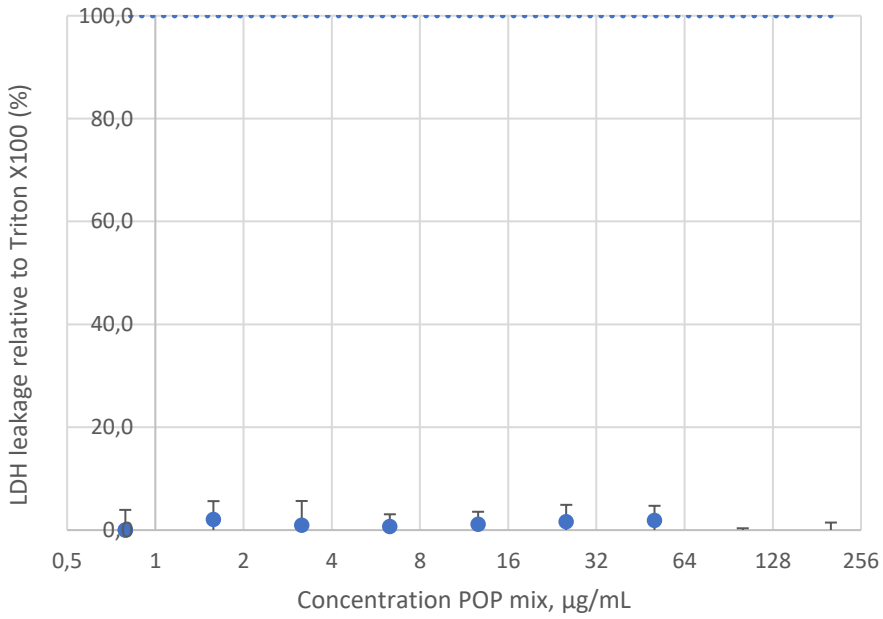


Figure 23. Measured LDH leakage by LDH release assay as an indicator of cell damages in response to a POP mixture. The values are given in % relative to Triton X100 dead control (dotted). KW-11-02 passage 9 cells were exposed to concentrations of POPs ranging from 0.8-202.1 µg/mL and 0.25 % DMSO for 48 hours before analysis. Each measure is given as the mean of six technical replicates with error bars representing the standard deviation of the mean.

4 Discussion

This thesis focused on establishing a robust cell culture of primary fibroblasts derived from Norwegian killer whales by optimizing the cultivation media and culturing conditions. Numerous studies have previously reported that cultivation of cetacean cells is accompanied with great challenges due to slow growth and senescence (Table 1) and this study revealed no exceptions.

4.1 Killer whale cell sources

The primary cells used in this study were derived from skin biopsies sampled from three different individuals of free-ranging Norwegian killer whales. Two of the biopsies were processed as part of this study, while the third had been processed a year earlier in another study. Out of the two processed biopsies, primary fibroblasts were successfully isolated from KW-1 at day 18, which is compatible with the successful isolation of primary fibroblasts from humpback whales between day 17-24 post processing (Burkard et al., 2015). Based on visual observations of the size of the animal and shape and size of their dorsal fin, two individuals were classified as large adult males, named KW-1 and KW-11-02, while the third animal most likely was an old female or possible a young male in which the sex cannot be determined without genetic testing (KW-2). The biopsies were processed immediately at receival in the lab, approximately 24 hours after sampling and cultivated until new cells grew out from the tissue fragments. Primary fibroblasts were first observed around the edges of KW-1 tissue fragments 13 days post processing. The new cells proliferated from those fragments which had managed to attach well to the plates, while no cells grew in the wells were the fragments were floating around. This demonstrates the importance of cell attachment and the benefit of using cover slips to facilitate the attachment for derivation of primary cells from tissue cultures (Mathews et al., 1988).

Literature shows that primary cultures from whales are prone to contamination from microbes present on the skin of the animals (Marsili et al., 2000). Tissues and cells are therefore treated with antifungals and antibiotics to reduce the risk of infection of the cultures and all cell cultures were tested for presence of Mycoplasma, another common contaminant. It was observed infection in cultures from one individual, likely fungal due to its white cotton-wool appearance during the phase when were cells grew out from tissue fragments. The infected cells were discarded, and no infections or mycoplasma was detected at later stages. The pre-treatment and isolation procedure thus enabled us to take out sterile cell cultures from the primary tissue samples.

4.2 Cell type identification

Identification of whale fibroblasts with vimentin antibodies has been previously performed with cells derived from humpback whales, pygmy killer whales and beluga whales (Burkard et al., 2015, Yajing et al., 2018, Boroda et al., 2020). Vimentin antibody was therefore chosen for immunolabelling in this study to identify KW-11-02 cells as fibroblast. In addition to having the typical polygonal spindle-shape of fibroblasts, the high immunoreactivity to vimentin exhibited by KW-11-02 cells confirmed that they were indeed fibroblasts. Vimentin is not unique for fibroblasts, thus if the killer whale cells were to be further identified, using Collagen type I (Col I) antibody could have been an option, as this

marker induced strong immunoreactivity in HuWa fibroblasts. Col IV and cytokeratin are other markers for fibroblasts, but the immunoreactivity induced by these antibodies were either weak or lacking in HuWa fibroblasts (Burkard et al., 2015).

4.3 Senescence and longevity of killer whale primary cells

Most KW-1 showed decreased proliferating rated already at passage 3 and by passage 5, no cells could be propagated further. Similar observations were demonstrated by Bjørneset (2022) working with KW primary fibroblasts which entered total growth arrest at passage 5-7. Microscopic imaging demonstrated great enlargement and flattening of the KW-1 cells with nearly 4x expansion of the cell dimension between senescent KW-1 (Fig 14) and proliferating KW-11-02 fibroblasts (Fig 21), identical to the morphological features shown for senescent fibroblasts by Cho et al. (2004) (Fig 3). Previous studies with cetacean primary cell cultures have reported reduced growth rates and senescence occurring at passage 6-9 for bowhead whale fibroblasts (Smith et al., 1987), 9 for pantropic spotted dolphin fibroblasts (Rajput et al., 2018), 8-10 for finless porpoise fibroblasts (Jin et al., 2013) and 10-17 for humpback dolphin fibroblasts (Jin et al., 2013). While some cetacean cell lines have been immortalized by SV40 T-antigen transfection, Burkard et al. reached 30 passages without signs of senescence or reduced proliferation with HuWa primary cells and became the first to establish a spontaneous cell line with fibroblasts from humpback whales (Burkard et al., 2015).

The replicative capacity of cells derived from animal KW-1 and KW-2 were severely poorer than of KW-11-02. KW-11-02 was cultured until passage 20 without exhibiting any clear decrease in proliferation rates or changes in morphology related to senescence. The medium used, Mix3, differed from that of KW-1 cultures, but that alone should not be sufficient to support the improved lifespan of KW-11-02. The inherent replicative capacity of KW-11-02 compared to KW-1 was significantly better, which suggest that individual differences may influence the establishment of KW primary cell cultures. Negative correlation between donor age and replicative capacity and *in vitro* lifespan of cells is well documented (Schneider and Mitsui, 1976, Balin et al., 2002), thus the poor growth potential of KW-2 may support the classification of the animal as an old female rather than a young male.

When testing the lifespan of KW-11-02 with Mix1 media in the first trial, the cells reached passage 16 before exhibiting signs of senescence and the cells could not be propagated beyond 18 passages. In the second trial, bFGF was increased from 1 ng/mL to 2 ng/mL (Mix3) which resulted in faster growth. Thus, the cells could be split more frequently resulting in an overall less time in culture to achieve same and higher passage numbers compared to the first trial. Also, in the first trial, KW-11-02 were grown too confluent before passaging (up to 100 %), which probably induced apoptosis and caused cell lysis and leakage of nucleic acids which made the cells aggregate during dissociation. The aggregation made both cell counting and dispersal of the cells during seeding difficult. The culturing procedure was therefore modified, and the cells were grown only 80-90 % confluent in the second trial, which eliminated those issues while also improving the growth of KW-11-02.

4.4 Effect of selected cultivation parameters on cell growth

4.4.1 Fibroblast growth factor 2

Fibroblast growth factor 2 is known to stimulate cell growth and proliferation of mouse and human fibroblasts (Gospodarowicz, 1974, Makino et al., 2010). The significant increase in KW-1 proliferation induced by bFGF demonstrates its mitogenic effect on KW primary cells and the benefit of including bFGF to the growth media in KW cell cultures. However, after 3 passages, the effect was strongly reduced for KW-1. Microscopic observation of the cells showed a great increase in cell size and cytoplasmic expansions and protrusions, and the cells were confirmed senescent. It is reported that cells near or at replicative senescence do not respond well to mitogenic signals (Cristofalo and Pignolo, 1993). The effect of bFGF was shown most clearly in proliferative cells (KW-11-02), but the increase in cell growth was not concentration-dependent. The optimal mitogenic effect in human dermal fibroblasts is induced by 1 ng/mL bFGF (Imaizumi et al., 1996). KW-11-02, however, experienced a significant increase in proliferation with 2 ng/mL bFGF compared to 1 ng/mL, but further decreased growth with 4 ng/mL. These findings could indicate that a plateau was reached between 2 ng/mL and 4 ng/mL bFGF and that 2 ng/mL is the optimal concentration for KW primary fibroblast cultures.

4.4.2 Glucose concentration

The first observation in the high/low glucose concentration experiment was that the high glucose (3.15 g/L) caused the pH of the media to decline faster. The experiment was conducted on KW-1 passage 5 and passage 3. For either passage, no difference in cell growth was observed between the glucose conditions. Higher glucose/lactate turnover caused by excessive glucose likely lead to the pH decline. Further, in contrast to most other cell types (lymphocytes e.g), fibroblasts exhibit increased metabolic activity when they are in a quiescent state confirming that high glucose consumption is not necessarily equal to high proliferating rates (Jones and Bianchi, 2015).

Low glucose (2mM) increased proliferation of skeletal muscle cells under adherent culture conditions (Furuichi et al., 2021). Experiments with KW-11-02 suggested that cell growth was not limited by glucose as the cells reached passage 20 in low concentration of glucose (1g/L). High glucose might in turn be excessive for KW fibroblasts, as it may induce oxidative stress and higher lactate formation and, reduce the pH in the media (Hassell et al., 1991). This may in turn influence cell growth and migration as reported for mouse embryonic fibroblasts (Lamers et al., 2011). The metabolite analysis also showed that bFGF-treated cells (with the highest proliferation rates) consumed more glucose but accumulated less lactate compared to the control cells when they were depleted in glucose which indicates that glucose metabolism was more efficient in these cells. Depletion of glucose only occurred when the cells were cultured at 100 % confluency over days, hence feeding them more glucose was not considered necessary to improve growth.

4.4.3 Serum supplementations

Serum is commonly used in cell cultures to provide essential hormones and growth factors. Previous studies have reported the need for extra serum in cetacean cell cultures (Mollenhauer et al., 2009), and humpback whale (HuWa) cells showed an concentration-dependent increase in proliferation with FBS concentrations ranging from 5 % to 20 % (Burkard et al., 2015). Enriching the media with 20 % FBS rather than 10 % in the standard media also resulted in increased killer whale cell growth and glucose metabolism. Although FBS is the most used serum, some cells benefit from replacing FBS with horse serum. For

example, it is reported enhanced functionality of equine dendritic cells when they were differentiated in the presence of horse serum rather than FBS (Ziegler et al., 2016). A study with chick embryo cells also reported increased proliferation and maintenance of morphology with horse serum compared to FBS (Verger, 1979). To our knowledge, horse serum had not yet been tested with whale cell cultures. Our study found that that replacing 5 % of the FBS in the standard media with 5 % horse serum increased glucose consumption and proliferation compared to the control. However, following experiments with combined conditions showed slightly more cell growth by 20 % FBS. Noteworthy, 20 % FBS also contained twice the total serum concentration compared to the horse serum treatment. Horse serum should therefore have been tested at the same concentration as FBS to conclude further which serum had the best effect on cell growth and health. But, a study with mouse neural cells reported that 20 % horse serum improved cell attachment but decreased proliferation, whereas 5 – 10 % horse serum stimulated higher proliferation (Fedoroff and Hall, 1979). Thus, killer whale cells might not necessarily benefit from increased horse serum concentrations. In our composite media, Mix3, the total serum concentration was 20 %, with 15 % FBS and 5 % horse serum. The cells cultured with this media reached the highest passage number and had the highest proliferation rates, while maintaining the typical fibroblast morphology which suggest that combining horse serum and FBS could be optimal for killer whale cells.

4.4.4 Antioxidants

Antioxidants, such as Ascorbic acid (AA) are important cofactors in numerous cellular functions and support a variety of processes including differentiation, proliferation and ECM synthesis (Nowak and Schnellmann, 1996) , and they also protect the cells from oxidative damage by scavenging ROS (Poljsak et al., 2013). Several reports have proved AA to stimulate increased collagen synthesis and proliferation rates in human skin fibroblasts (Phillips et al., 1994, Rowe et al., 1977, HATA et al., 1988). But, as it turns out, AA is highly unstable in culture media incubated at 37 ℃ and as much as 88-98 % of it was reported gone after 24 hours (Peterkofsky, 1972). The rapid degradation severely limits the availability of AA to the cells (Chepda et al., 2001), and unless daily supplemented to the culture media, its effect on growth could be difficult to determine. Adding AA daily instead of every 2-3 days might have been advantages for KW growth, by avoiding the frequent depletion of AA in the media. AA has shown promising results with other cells and that could as well be the case for KW fibroblasts. But, due to challenges with senescence of the KW cells and degradation of AA in the media, further studies should be performed with proliferative cells and daily supplementation of AA in order to conclude whether it could be positive for KW growth or not.

2-Mercaptoethanol (BME) is a thiol antioxidant that supports growth and proliferation of several cell types, including lymphocytes, leukocytes, murine spleen cells and osteoprogenitors derived from bone marrow fibroblasts (INUI et al., 1997). Here, no difference in glucose consumption was measured after six days, but a slight increase compared to the control was measured by BME, gelatin + BME and laminin + BME at day 13. BME also reduced the accumulation of LDH compared to the control which indicates less cell damage in this treatment. Addition of BME to bovine embryo cultures subject to oxidative stress due to the hypoxic environment of a standard incubator (~ 20 % O_2) almost improved development to the rates of normoxic culturing (5 % O_2) (Takahashi et al., 2002). All this considered could suggest some beneficial properties of BME in long-term KW cell cultures.

4.4.5 Well plate surface coating

Both gelatin and laminin are part of the ECM secreted by fibroblasts. Fibroblasts are also adherent-dependent, requiring physical anchoring to its environment in order to survive and grow. Thus, the idea that coating of the well plates with either ECM protein would support KW fibroblast health growth was not unlikely. Laminin-coating proved to increase growth of KW-1 fibroblasts, possibly by facilitating attachment to the plates (Couchman et al., 1983) and reducing the stress associated with cell contact dependency (Balin et al., 2002).

KW-11-02 cells treated with bFGF 1 ng/mL in combination with either laminin or gelatin showed no increase in cell growth compared to bFGF 1 ng/mL separately, and no difference in glucose consumption was measured among these treatments. Further, lactate and LDH accumulations were fairly similar for all the three conditions. Thus, the effect of surface coating on KW-11-02 cell growth was considered negligible.

4.4.6 Seeding density

Seeding density also had a significant influence on the growth of KW primary cells. An inverse relationship between proliferative life span and seeding density (10,000 cells/cm^2) has been demonstrated for e.g., fetal skin fibroblasts (Balin et al., 2002). KW-1 seeded approximately 2700 cells/cm^2, exhibited reduced growth over 17 days in culture with standard conditions, and illustrates how KW fibroblasts do not thrive when seeded at densities <3000 cells/cm^2 also proposed for KW fibroblasts by Bjørneset (2022). These findings are comparable with those of Boroda et al. on beluga whale fibroblasts which ceased to proliferate when seeded at 2000 cells/cm^2 or lower densities (Boroda et al., 2020).

4.4.7 Amino acids

The standard media used in this study was initially based on the reported growth media used with fibroblast cell cultures obtained from fin whales (Fossi et al., 2006) with modifications. MEM non-essential amino acids (NEAA) was excluded from the media after Burkard et at. reported a reduction in HuWa growth rates by NEAA supplementation (Burkard et al., 2015). Their results contradicted other studies which state that cetacean cells require amino acid enriched cultivation media to support cell growth (Nielsen et al., 1989, Fossi et al., 2006, Marsili et al., 2012, Jin et al., 2013). KW-1 passage 3 was used to test the effect of NEAA in this study, but no change in cell growth could be observed. Neither the control group nor the NEAA test group seemed to proliferate after seeding. The lack of proliferation and ability to attach and survive after next passaging, indicated that the cells were in a senescent state already, which made it impossible to determine whether supplementation with NEAA could improve growth or not. Nevertheless, no decrease in growth, compared to the control, was observed for NEAA either. Because the replicative capacity of the cells was comprised from the start, the actual effect of NEAA cannot be concluded.

4.5 Glucose/lactate metabolism

Measuring cell metabolism through metabolite analysis was attempted to estimate growth without having to dissociate and harvest the cells, as that inflicts mechanical and chemical stress to the cells. Glucose is converted to lactate through anaerobic and aerobic glycolysis. The glucose-lactate turnover yields two molecules of lactate per molecule of glucose, thus

it is expected to see a positive correlation between consumed glucose and accumulated lactate in the cell media if the glucose is not completely oxidized by the mitochondria (Hu et al., 1987). The condition which increased glucose metabolism the most was bFGF. Also, bFGF treated cells accumulated the lowest concentrations of lactate, which indicates that glucose oxidized more efficiently by these cells. The control group had the lowest glucose consumption, but also the highest accumulation of lactate. Thus, glucose was not efficiently oxidized by the cells, and rather converted to lactate caused by the excessive concentration of glucose/available energy sources (Chen et al., 2017). Accumulation of high amounts of lactate (> 20- 50 mM) has adverse effect on culture growth, productivity, and viability of mammalian cells (Mulukutla et al., 2010), but measured concentrations in killer whale cells were considerably lower than allegedly toxic concentrations.

LDH is released to the extracellular space when the cell membrane is damaged due to apoptosis or cell necrosis. Accumulation of LDH in the cell growth media was measured and used as an indicator of poor cell health or death (Chan et al., 2013). The control group demonstrated elevated accumulation of LDH without increasing cell numbers, which indicates that the standard media without bFGF was not optimal for culturing of killer whale fibroblasts. Coating the well surface with gelatin or laminin reduced the accumulation of LDH but did not increase the cell growth noticeably, which indicated that surface coatings might be beneficial for survival of killer whale fibroblasts but not necessarily growth. However, treating the cells with bFGF in addition to gelatin or laminin coating revealed no reduction in LDH accumulation compared to bFGF treatment alone. This suggests that improved cell survival by surface coatings might be limited to otherwise poor growth conditions. The increased LDH accumulation for bFGF and 20 % FBS treatments compared to the control measured at day 13 was probably due to higher cell densities and possible induced apoptosis and cell lysis causing release of cell debris and LDH to the media as these cells had been confluent for several days. Nevertheless, LDH concentrations were overall relatively low and cell death was not an issue at this point.

LDH is present in serums used in the growth media (Hiebl et al., 2017), which means that LDH is added to the media with every media changed between the measurements as well, which may influence the measured LDH concentrations. 20 % FBS media contained more LDH initially, which caused a higher addition of LDH with every media shift compared to the other treatments. The opposite was the case for horse serum, which contained less LDH and therefore less LDH was added with every media shift. Similar issues occurred for glucose, which is added, and lactate which is partially removed from the media with every media shift. To avoid this confounding factor, metabolites should have been measured with every media shift and several replicates should have been included to calculate statistical significances. That would have enabled cumulative mass-balance calculations which could have given further insight into nutritional requirements and growth properties of the cell cultures. Such measurements on cell growth obtained without the need for harvesting of the cells would be highly advantageous in slow-growing sensitive cell cultures, such as cetacean primary cell cultures.

4.6 Cytotoxicity of POPs mixture

Cytotoxicity assay was conducted on KW-11-02 passage 9 cells. As the cells from this individual proliferated for at least 16 passages, it was unlikely that senescence impacted the cell viability. Humpback whale cells demonstrated a concentration-dependent loss in cell viability in response to *p,p'*-DDE exposure (Burkard et al., 2015) measured with a

membrane integrity-based fluorescent assay. They found the lowest effect concentration (LOEC) to be 1500 µg/L with an estimated EC_{50} at 1693 µg/L when *p,p'*-DDE was applied alone. HuWa cells were also exposed to a POP cocktail, like the case in this study, which induced EC_{50} at 0.9 µg/L which is ~ 1880 times decrease in EC_{50} compared to EC_{50} of *p,p'*-DDE alone. The toxmix used in this study contained relevant POPs found in blubber of Norwegian killer whales and assessing the effect of these in combination is therefore highly relevant, as this is what the animals are exposed to in real life. The enhanced toxicity of POP cocktails, due to additive and synergistic effects when different chemicals are combined, has been demonstrated with the HuWa cells and other studies (Kortenkamp et al., 2009, Desforges et al., 2017, Burkard et al., 2015) Desforges et al. performed an *in vitro* study with a contaminant cocktail derived from killer whale blubber on immune T-cells and found a rapid decline in proliferation and a cytotoxic threshold at 8 µg/mL (Desforges et al., 2017). It was found that KW fibroblasts exhibited a threshold for cytotoxicity around 64 µg/mL. Different cocktail composition and cell type might explain the higher sensitivity to contaminant exposure reported by Desforges et al. compared to our study and may also suggest that fibroblasts are less sensitive to POPs compared to immune T-cells.

Cytotoxicity was also assessed indirectly by LDH leakage, with the same experimental set up (similar POPs conc., exposure time, KW-11-02 batch and passage number). No induced cytotoxicity could be measured for any of the tested POP concentrations. Nevertheless, several POPs are known to induce other adverse effects in cells than direct cytotoxicity, including changes in gene expression, DNA damages and chromosome aberration which can have severe long-term impacts on cellular as well as individual level without killing the cells immediately. This study performed a short-term cytotoxicity assay, but the cytotoxic effects of long-term exposure are yet to assess. Another possible explanation why cytotoxicity was not detected by the LDH release assay could be that the cell plasma membrane was not damaged, thus no LDH was released to the media. Further, the cytotoxicity detected with CellTiter-Glo does not necessarily translate into number of dead cells. The assay measures amount of ATP produced by viable cells, thus the reduction in detectable fluorescent emitted by the 50X and 25X exposed groups could be due to reduced cell metabolism and/or inhibition of proliferating induced by the contaminants rather than cell death.

5 Conclusion

This study has demonstrated that fibroblast growth factor 2 is a highly potent inducer of proliferation in killer whale primary fibroblasts. Further it was shown that 2 ng/mL is the optimal concentration for killer whale fibroblast growth, and that a plateau is reached between 2 and 4 ng/mL bFGF. Additionally, enriching the media with 20 % serum (20 % FBS or perhaps even better 15 % FBS and 5 % horse serum improved growth of KW fibroblasts. A composite media with 2 ng/mL bFGF, 20 % FBS and 0.1 mM BME could sustain a killer whale primary cell culture for 20 passages without exhibiting clear signs of senescence or decline in proliferating rates. Nonetheless, cytotoxic effects were induced by POP mixture concentration >101.1 µg/mL and NOEC at 50.5 µg/mL. Overall, in this study, I was able to produce a great number of cells for *in vitro* research while highlighting parameters critical to the success of culturing killer whale fibroblast cells. Finally, while culture parameters are known to vary among species, it was also demonstrated that proliferating capacity and lifespan of the cells varies greatly among the different cell donors.

The main challenges encountered in this study mainly pertain to the limited availability of cells, which gave little material to work with. Additionally, poor growth and senescence made evaluating the different conditions difficult and the lack of quantitative methods to measure growth that does not require harvesting of the cells. In terms of future perspectives, the contaminant levels in killer whales are concerning and further *in vitro* research is necessary to assess the potential hazardous effects of POPs on e.g., contaminant metabolism, gene expression, immune responses, and endocrine disruption. This study had provided an optimized method for culturing killer whale fibroblasts up to at least 20 passages with a population doubling time 40 hours, which could ensure enough cell material to perform RNA sequencing and proteomics analysis to better understand how pollutants modulate cellular processes on gene and protein levels. It may also open doors for establishing cell cultures for other marine mammals to study toxic responses and cell physiology, and also facilitate further attempts to differentiate fibroblasts into other cell types, such as mesenchymal stem cells and adipocytes. Unfortunately, due to limited time and budget allocated to this study, proteomic analysis with KW fibroblasts could not be performed. However, cell pellets of untreated KW cells and cells exposed to different concentration of the POP toxmix were collected and frozen, which can be analyzed in the future. Since contaminants generally accumulate in the adipose tissue, exposure studies with adipocytes could be of higher relevance than fibroblasts. A next step could be to try to induce differentiation of KW fibroblasts into mesenchymal stem cells and adipocytes. Finally, once robust 2D cultures of killer whale cells are established, efforts should be directed towards validating 3D cultures using hydrogels that would better mimic *in vivo* conditions of the killer whale.

References

Akhtar, A. B. T., Naseem, S., Yasar, A. & Naseem, Z. 2021. Persistent organic pollutants (POPs): sources, types, impacts, and their remediation. *Environmental Pollution and Remediation.* Springer.

Andersen, T., Auk-Emblem, P. & Dornish, M. 2015. 3D cell culture in alginate hydrogels. *Microarrays,* 4**,** 133-161.

Arora, M. 2013. Cell culture media: a review. *Mater methods,* 3**,** 24.

Balin, A. K., Fisher, A. J., Anzelone, M., Leong, I. & Allen, R. 2002. Effects of establishing cell cultures and cell culture conditions on the proliferative life span of human fibroblasts isolated from different tissues and donors of different ages. *Experimental cell research,* 274**,** 275-287.

Bjørneset, J. 2022. *Establishment of killer whale cell cultures and their responses to pollutant exposure.* Unpublished master's thesis, University of Tromsø

Blévin, P., Eulaers, I., Aaes, J., Andvik, A., Biuw, M., Borgå, K., Bytingsvik, J., Fisk, A., Haug, T., Herzke, D., Lyche, J., Lydersen, C., Kovacs, K., Rikardsen, A. & Routti, H. 2021. Wide range of POPs contamination in marine mammals from the Norwegian Arctic.

Boroda, A. V., Kipryushina, Y. O., Golochvastova, R. V., Shevchenko, O. G., Shulgina, M. A., Efimova, K. V., Katin, I. O. & Maiorova, M. A. 2020. Isolation, characterization, and ecotoxicological application of marine mammal skin fibroblast cultures. *In Vitro Cellular & Developmental Biology-Animal,* 56**,** 744-759.

Brouwer, A., Reijnders, P. & Koeman, J. 1989. Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (Phoca vitulina). *Aquatic toxicology,* 15**,** 99-105.

Buckman, A. H., Veldhoen, N., Ellis, G., Ford, J. K., Helbing, C. C. & Ross, P. S. 2011. PCB-associated changes in mRNA expression in killer whales (Orcinus orca) from the NE Pacific Ocean. *Environmental Science & Technology,* 45**,** 10194-10202.

Buechler, M. B. & Turley, S. J. A short field guide to fibroblast function in immunity. Seminars in immunology, 2018. Elsevier, 48-58.

Burkard, M., Whitworth, D., Schirmer, K. & Nash, S. B. 2015. Establishment of the first humpback whale fibroblast cell lines and their application in chemical risk assessment. *Aquatic toxicology,* 167**,** 240-247.

Baatz, J. E., Newton, D. A., Riemer, E. C., Denlinger, C. E., Jones, E. E., Drake, R. R. & Spyropoulos, D. D. 2014. Cryopreservation of viable human lung tissue for versatile post-thaw analyses and culture. *In vivo,* 28**,** 411-423.

Campisi, J., Kim, S.-H., Lim, C.-S. & Rubio, M. 2001. Cellular senescence, cancer and aging: the telomere connection. *Experimental gerontology,* 36**,** 1619-1637.

Carter, M. & Shieh, J. C. 2015. *Guide to research techniques in neuroscience*, Academic Press.

Chan, F. K.-M., Moriwaki, K. & De Rosa, M. J. 2013. Detection of necrosis by release of lactate dehydrogenase activity. *Immune homeostasis.* Springer.

Chandler, H. & Peters, G. 2013. Stressing the cell cycle in senescence and aging. *Current opinion in cell biology,* 25**,** 765-771.

Chang, Y., Li, H. & Guo, Z. 2014. Mesenchymal stem cell-like properties in fibroblasts. *Cellular Physiology and Biochemistry,* 34**,** 703-714.

Chen, Y., Fry, B. C. & Layton, A. T. 2017. Modeling glucose metabolism and lactate production in the kidney. *Mathematical biosciences,* 289**,** 116-129.

Chepda, T., Cadau, M., Girin, P., Frey, J. & Chamson, A. 2001. Monitoring of ascorbate at a constant rate in cell culture: effect on cell growth. *In Vitro Cellular & Developmental Biology-Animal,* 37**,** 26-30.

Cho, K. A., Ryu, S. J., Oh, Y. S., Park, J. H., Lee, J. W., Kim, H.-P., Kim, K. T., Jang, I. S. & Park, S. C. 2004. Morphological adjustment of senescent cells by modulating caveolin-1 status. *Journal of Biological Chemistry,* 279**,** 42270-42278.
Couchman, J. R., Höök, M., Rees, D. A. & Timpl, R. 1983. Adhesion, growth, and matrix production by fibroblasts on laminin substrates. *The Journal of cell biology,* 96**,** 177-183.
Cristofalo, V. J. & Pignolo, R. J. 1993. Replicative senescence of human fibroblast-like cells in culture. *Physiological reviews,* 73**,** 617-638.
Desforges, J.-P., Levin, M., Jasperse, L., De Guise, S., Eulaers, I., Letcher, R. J., Acquarone, M., Nordøy, E., Folkow, L. P. & Hammer Jensen, T. 2017. Effects of polar bear and killer whale derived contaminant cocktails on marine mammal immunity. *Environmental Science & Technology,* 51**,** 11431-11439.
Desforges, J.-P. W., Sonne, C., Levin, M., Siebert, U., De Guise, S. & Dietz, R. 2016. Immunotoxic effects of environmental pollutants in marine mammals. *Environment international,* 86**,** 126-139.
Druelle, C., Drullion, C., Deslé, J., Martin, N., Saas, L., Cormenier, J., Malaquin, N., Huot, L., Slomianny, C. & Bouali, F. 2016. ATF6α regulates morphological changes associated with senescence in human fibroblasts. *Oncotarget,* 7**,** 67699.
Fedoroff, S. & Hall, C. 1979. Effect of horse serum on neural cell differentiation in tissue culture. *In vitro,* 15**,** 641-648.
Ferguson, S., Higdon, J. & Chmelnitsky, E. 2010. The rise of killer whales as a major Arctic predator. *A little less Arctic.* Springer.
Forney, K. A., Wade, P. R. & Estes, J. 2006. Worldwide distribution and abundance of killer whales. *Whales, whaling and ocean ecosystems,* 145**,** 162.
Fossi, M. C., Marsili, L., Casini, S. & Bucalossi, D. 2006. Development of new-tools to investigate toxicological hazard due to endocrine disruptor organochlorines and emerging contaminants in Mediterranean cetaceans. *Marine environmental research,* 62**,** S200-S204.
Frame, K. K. & Hu, W. S. 1991. Kinetic study of hybridoma cell growth in continuous culture: II. Behavior of producers and comparison to nonproducers. *Biotechnology and bioengineering,* 38**,** 1020-1028.
Furuichi, Y., Kawabata, Y., Aoki, M., Mita, Y., Fujii, N. L. & Manabe, Y. 2021. Excess glucose impedes the proliferation of skeletal muscle satellite cells under adherent culture conditions. *Frontiers in cell and developmental biology,* 9**,** 640399.
Gerdes, J., Lemke, H., Baisch, H., Wacker, H.-H., Schwab, U. & Stein, H. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *The journal of immunology,* 133**,** 1710-1715.
Gospodarowicz, D. 1974. Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. *Nature,* 249**,** 123-127.
Habler, O. P. & Messmer, K. F. 1997. The physiology of oxygen transport. *Transfusion science,* 18**,** 425-435.
Haque, N., Rahman, M. T., Abu Kasim, N. H. & Alabsi, A. M. 2013. Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *The Scientific World Journal,* 2013.
Hassell, T., Gleave, S. & Butler, M. 1991. Growth inhibition in animal cell culture. *Applied biochemistry and biotechnology,* 30**,** 29-41.
Hata, R. I., Sunada, H., Arai, K., Sato, T., Ninomiya, Y., Nagai, Y. & Senoo, H. 1988. Regulation of collagen metabolism and cell growth by epidermal growth factor and ascorbate in cultured human skin fibroblasts. *European Journal of Biochemistry,* 173**,** 261-267.
Hiebl, B., Peters, S., Gemeinhardt, O., Niehues, S. M. & Jung, F. 2017. Impact of serum in cell culture media on in vitro lactate dehydrogenase (LDH) release determination. *Journal of Cellular Biotechnology,* 3**,** 9-13.
Higdon, J. W. & Ferguson, S. H. 2009. Loss of Arctic sea ice causing punctuated change in sightings of killer whales (Orcinus orca) over the past century. *Ecological Applications,* 19**,** 1365-1375.

Hu, W., Dodge, T., Frame, K. & Himes, V. 1987. Effect of glucose on the cultivation of mammalian cells. *Developments in biological standardization,* 66**,** 279-290.
Hwang, E. S., Yoon, G. & Kang, H. T. 2009. A comparative analysis of the cell biology of senescence and aging. *Cellular and Molecular Life Sciences,* 66**,** 2503-2524.
Imaizumi, T., Jean-Louis, F., Dubertret, M.-L., Bailly, C., Cicurel, L., Petchot-Bacqué, J.-P. & Dubertret, L. 1996. Effect of human basic fibroblast growth factor on fibroblast proliferation, cell volume, collagen lattice contraction: in comparison with acidic type. *Journal of dermatological science,* 11**,** 134-141.
Inui, K., Oreffo, R. O. & T Triffitt, J. 1997. Effects of beta mercaptoethanol on the proliferation and differentiation of human osteoprogenitor cells. *Cell biology international,* 21**,** 419-425.
Jepson, P. D., Deaville, R., Barber, J. L., Aguilar, À., Borrell, A., Murphy, S., Barry, J., Brownlow, A., Barnett, J. & Berrow, S. 2016. PCB pollution continues to impact populations of orcas and other dolphins in European waters. *Scientific reports,* 6**,** 1-17.
Jin, W., Jia, K., Yang, L., Chen, J., Wu, Y. & Yi, M. 2013. Derivation and characterization of cell cultures from the skin of the Indo-Pacific humpback dolphin Sousa chinensisDerivation and characterization of cell cultures from the skin of the Indo-Pacific humpback dolphin Sousa chinensis. *In Vitro Cellular & Developmental Biology-Animal,* 49**,** 449-457.
Jones, W. & Bianchi, K. 2015. Aerobic glycolysis: beyond proliferation. *Frontiers in immunology,* 6**,** 227.
Jourdain, E., Ugarte, F., Víkingsson, G. A., Samarra, F. I., Ferguson, S. H., Lawson, J., Vongraven, D. & Desportes, G. 2019. North Atlantic killer whale Orcinus orca populations: a review of current knowledge and threats to conservation. *Mammal Review,* 49**,** 384-400.
Junker, J. P., Sommar, P., Skog, M., Johnson, H. & Kratz, G. 2010. Adipogenic, chondrogenic and osteogenic differentiation of clonally derived human dermal fibroblasts. *Cells Tissues Organs,* 191**,** 105-118.
Kalluri, R. 2016. The biology and function of fibroblasts in cancer. *Nature Reviews Cancer,* 16**,** 582-598.
Kenny, P. A., Lee, G. Y., Myers, C. A., Neve, R. M., Semeiks, J. R., Spellman, P. T., Lorenz, K., Lee, E. H., Barcellos-Hoff, M. H. & Petersen, O. W. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Molecular oncology,* 1**,** 84-96.
Kortenkamp, A., Backhaus, T. & Faust, M. 2009. State of the art report on mixture toxicity. *Contract,* 70307**,** 1-391.
Kulaberoglu, Y., Hergovich, A. & Gómez, V. 2021. The role of p53/p21/p16 in DNA damage signaling and DNA repair. *Genome Stability.* Elsevier.
Kumar, P., Nagarajan, A. & Uchil, P. D. 2018. Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harbor Protocols,* 2018**,** pdb. prot095497.
Lamers, M. L., Almeida, M. E., Vicente-Manzanares, M., Horwitz, A. F. & Santos, M. F. 2011. High glucose-mediated oxidative stress impairs cell migration. *PloS one,* 6**,** e22865.
Lee, J., Cuddihy, M. J. & Kotov, N. A. 2008. Three-dimensional cell culture matrices: state of the art. *Tissue Engineering Part B: Reviews,* 14**,** 61-86.
Maddalena, L. A., Selim, S. M., Fonseca, J., Messner, H., Mcgowan, S. & Stuart, J. A. 2017. Hydrogen peroxide production is affected by oxygen levels in mammalian cell culture. *Biochemical and biophysical research communications,* 493**,** 246-251.
Makino, T., Jinnin, M., Muchemwa, F., Fukushima, S., Kogushi-Nishi, H., Moriya, C., Igata, T., Fujisawa, A., Johno, T. & Ihn, H. 2010. Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways. *British Journal of Dermatology,* 162**,** 717-723.
Mancia, A., Spyropoulos, D. D., Mcfee, W. E., Newton, D. A. & Baatz, J. E. 2012. Cryopreservation and in vitro culture of primary cell types from lung tissue of a stranded pygmy sperm whale (Kogia breviceps). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 155**,** 136-142.

Marsili, L., Fossi, M., Neri, G., Casini, S., Gardi, C., Palmeri, S., Tarquini, E. & Panigada, S. 2000. Skin biopsies for cell cultures from Mediterranean free-ranging cetaceans. *Marine environmental research,* 50**,** 523-526.
Marsili, L., Maltese, S., Coppola, D., Caliani, I., Carletti, L., Giannetti, M., Campani, T., Baini, M., Panti, C. & Casini, S. 2012. Test tube cetaceans": from the evaluation of susceptibility to the study of genotoxic effects of different environmental contaminants using cetacean fibroblast cell cultures. *New approaches to the study of marine mammals***,** 49-76.
Masters, J. 2000. *Animal cell culture: a practical approach*, OUP Oxford.
Mathews, E. A., Keller, S. & Weiner, D. B. 1988. A method to collect and process skin biopsies for cell culture from free-ranging gray whales (Eschrichtius robustus). *Marine Mammal Science,* 4**,** 1-12.
Mollenhauer, M. A., Carter, B. J., Peden-Adams, M. M., Bossart, G. D. & Fair, P. A. 2009. Gene expression changes in bottlenose dolphin, Tursiops truncatus, skin cells following exposure to methylmercury (MeHg) or perfluorooctane sulfonate (PFOS). *Aquatic Toxicology,* 91**,** 10-18.
Muhl, L., Genové, G., Leptidis, S., Liu, J., He, L., Mocci, G., Sun, Y., Gustafsson, S., Buyandelger, B. & Chivukula, I. V. 2020. Single-cell analysis uncovers fibroblast heterogeneity and criteria for fibroblast and mural cell identification and discrimination. *Nature communications,* 11**,** 1-18.
Nielsen, O., Kelly, R. K., Lillie, W. R., Clayton, J. W., Fujioka, R. S. & Yoneyama, B. S. 1989. Some Properties of a Finite Cell Line from Beluga Whale (Delphinapterus leucas). *Canadian Journal of Fisheries and Aquatic Sciences,* 46**,** 1472-1477.
Nilforoushzadeh, M. A., Ashtiani, H. R. A., Jaffary, F., Jahangiri, F., Nikkhah, N., Mahmoudbeyk, M., Fard, M., Ansari, Z. & Zare, S. 2017. Dermal fibroblast cells: biology and function in skin regeneration. *Journal of Skin and Stem Cell,* 4.
Nishio, K., Inoue, A., Qiao, S., Kondo, H. & Mimura, A. 2001. Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts. *Histochemistry and cell biology,* 116**,** 321-327.
Nowak, G. & Schnellmann, R. G. 1996. L-ascorbic acid regulates growth and metabolism of renal cells: improvements in cell culture. *American Journal of Physiology-Cell Physiology,* 271**,** C2072-C2080.
Ochiai, M., Kurihara, N., Hirano, M., Nakata, A. & Iwata, H. 2020. In vitro cytotoxicity and risk assessments of environmental pollutants using fibroblasts of a stranded finless porpoise (Neophocaena asiaeorientalis). *Environmental Science & Technology,* 54**,** 6832-6841.
Oyeleye, O., Ola, S. & Omitogun, O. 2016. Basics of animal cell culture: Foundation for modern science. *Biotechnology and Molecular Biology Reviews,* 11**,** 6-16.
Pan, C., Kumar, C., Bohl, S., Klingmueller, U. & Mann, M. 2009. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. *Molecular & Cellular Proteomics,* 8**,** 443-450.
Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S. & Campisi, J. 2003. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nature cell biology,* 5**,** 741-747.
Peterkofsky, B. 1972. The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Archives of biochemistry and biophysics,* 152**,** 318-328.
Phillips, C. L., Combs, S. B. & Pinnell, S. R. 1994. Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts. *Journal of Investigative Dermatology,* 103**,** 228-232.
Plikus, M. V., Wang, X., Sinha, S., Forte, E., Thompson, S. M., Herzog, E. L., Driskell, R. R., Rosenthal, N., Biernaskie, J. & Horsley, V. 2021. Fibroblasts: Origins, definitions, and functions in health and disease. *Cell,* 184**,** 3852-3872.
Poljsak, B., Šuput, D. & Milisav, I. 2013. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxidative medicine and cellular longevity,* 2013.

Rajput, I. R., Xiao, Z., Yajing, S., Yaqoob, S., Sanganyado, E., Ying, H., Fei, Y. & Liu, W. 2018. Establishment of pantropic spotted dolphin (Stenella attenuata) fibroblast cell line and potential influence of polybrominated diphenyl ethers (PBDEs) on cytokines response. *Aquatic Toxicology,* 203**,** 1-9.
Ronald Zielke, H., Ozand, P. T., Tyson Tildon, J., Sevdalian, D. A. & Cornblath, M. 1978. Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. *Journal of cellular physiology,* 95**,** 41-48.
Ross, P., De Swart, R., Addison, R., Van Loveren, H., Vos, J. & Osterhaus, A. 1996. Contaminant-induced immunotoxicity in harbour seals: wildlife at risk? *Toxicology,* 112**,** 157-169.
Rowe, D. W., Starman, B. J., Fujimoto, W. Y. & Williams, R. H. 1977. Differences in growth response to hydrocortisone and ascorbic acid by human diploid fibroblasts. *In vitro,* 13**,** 824-830.
Schlessinger, J., Schreiber, A. B., Levi, A., Lax, I., Libermann, T. & Yarden, Y. 1983. Regulation of cell proliferation by epidermal growth factor. *Critical Reviews in Biochemistry,* 14**,** 93-111.
Schneider, E. L. & Mitsui, Y. 1976. The relationship between in vitro cellular aging and in vivo human age. *Proceedings of the National Academy of Sciences,* 73**,** 3584-3588.
Similä, T., Holst, J. C. & Christensen, I. 1996. Occurrence and diet of killer whales in northern Norway: seasonal patterns relative to the distribution and abundance of Norwegian spring-spawning herring. *Canadian Journal of Fisheries and Aquatic Sciences,* 53**,** 769-779.
Stone, J. R. & Yang, S. 2006. Hydrogen peroxide: a signaling messenger. *Antioxidants & redox signaling,* 8**,** 243-270.
Sun, Y., Zeng, Y., Rajput, I. R., Sanganyado, E., Zheng, R., Xie, H., Li, C., Tian, Z., Huang, Y. & Yang, L. 2022. Interspecies differences in mammalian susceptibility to legacy POPs and trace metals using skin fibroblast cells. *Environmental Pollution,* 315**,** 120358.
Suzuki, T., Hidaka, T., Kumagai, Y. & Yamamoto, M. 2020. Environmental pollutants and the immune response. *Nature Immunology,* 21**,** 1486-1495.
Takahashi, M., Nagai, T., Okamura, N., Takahashi, H. & Okano, A. 2002. Promoting effect of β-mercaptoethanol on in vitro development under oxidative stress and cystine uptake of bovine embryos. *Biology of reproduction,* 66**,** 562-567.
Teti, A. 1992. Regulation of cellular functions by extracellular matrix. *Journal of the American Society of Nephrology,* 2**,** S83.
Tsao, Y.-S., Cardoso, A., Condon, R., Voloch, M., Lio, P., Lagos, J., Kearns, B. & Liu, Z. 2005. Monitoring Chinese hamster ovary cell culture by the analysis of glucose and lactate metabolism. *Journal of biotechnology,* 118**,** 316-327.
Verger, C. 1979. Proliferation and morphology of chick embryo cells cultured in the presence of horse serum and hemoglobin. *In vitro,* 15**,** 587-592.
Wise, J. P., Wise, S. S., Kraus, S., Shaffiey, F., Grau, M., Chen, T. L., Perkins, C., Thompson, W. D., Zheng, T. & Zhang, Y. 2008. Hexavalent chromium is cytotoxic and genotoxic to the North Atlantic right whale (Eubalaena glacialis) lung and testes fibroblasts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis,* 650**,** 30-38.
Wlaschin, K. F. & Hu, W.-S. 2007. Engineering cell metabolism for high-density cell culture via manipulation of sugar transport. *Journal of biotechnology,* 131**,** 168-176.
Wolkers, H., Corkeron, P. J., Van Parijs, S. M., Similä, T. & Van Bavel, B. 2007. Accumulation and transfer of contaminants in killer whales (Orcinus orca) from Norway: indications for contaminant metabolism. *Environmental Toxicology and Chemistry: An International Journal,* 26**,** 1582-1590.
Wolkers, J., Burkow, I., Lydersen, C., Dahle, S., Monshouwer, M. & Witkamp, R. 1998. Congener specific PCB and polychlorinated camphene (toxaphene) levels in Svalbard ringed seals (Phoca hispida) in relation to sex, age, condition and cytochrome P450 enzyme activity. *Science of the Total Environment,* 216**,** 1-11.

Yajing, S., Rajput, I. R., Ying, H., Fei, Y., Sanganyado, E., Ping, L., Jingzhen, W. & Wenhua, L. 2018. Establishment and characterization of pygmy killer whale (Feresa attenuata) dermal fibroblast cell line. *PLoS One,* 13**,** e0195128.

Yu, J., Kindy, M. S., Ellis, B. C., Baatz, J. E., Peden-Adams, M., Ellingham, T. J., Wolff, D. J., Fair, P. A. & Gattoni-Celli, S. 2005. Establishment of epidermal cell lines derived from the skin of the Atlantic bottlenose dolphin (Tursiops truncatus). *The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology: An Official Publication of the American Association of Anatomists,* 287**,** 1246-1255.

Ziegler, A., Everett, H., Hamza, E., Garbani, M., Gerber, V., Marti, E. & Steinbach, F. 2016. Equine dendritic cells generated with horse serum have enhanced functionality in comparison to dendritic cells generated with fetal bovine serum. *BMC veterinary research,* 12**,** 1-11.

Appendix A

Table A1. List of chemicals and reagents with respective suppliers and product number used in this study.

Chemical/Reagent	Supplier	Product number
2-Mercaptoethanol 1000X 55 mM in DPBS	Thermo Fisher	21985023
Accutase solution	Sigma-Aldrich	A6964
Alexa Fluor 568 Phalloidin	Thermo Fisher	A12380
Bovine Serum Albumin solution	Sigma-Aldrich	A8577
CellEvent Senescence assay kit	Thermo Fisher	C10850
CellTiter-Glo 2.0	Promega	G9243
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher	D1306
D-Glucose anhydrous	Quality Biological	QBIC20181
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2650-100ML
Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (w/o L-Glutamine, w/o HEPES, w/o glucose)	Biowest	L0091-500
Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (with Phenol red)	Thermo Fisher	21331020
EmbryoMax 0.1% Gelatin Solution	Sigma-Aldrich	ES-006-B
Ethanol absolute	VWR	20824.365
Fetal bovine serum	Sigma-Aldrich	F7524
Formaldehyde 4 % Formalin 10 % Buffered	R&L Slaughter	9713.1000
Geneticin	Thermo Fisher	15710-049
GlutaMAX-I 100X	Thermo Fisher	35050-061
HEPES	Thermo Fisher	15630-056
Horse serum	Thermo Fisher	16050-122
Human Heat Stable bFGF Recombinant Protein	Thermo Fisher	PHG0367
Ki-67 Monoclonal Antibody (SolA15), Alexa Fluor 488	Thermo Fisher	53-5698-82
Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane	Sigma-Aldrich	L2020-1MG
L-Ascorbic Acid	Sigma-Aldrich	102262126
LDH assay kit (cytotoxicity)	Abcam	Ab65391
L-Glutamine	Sigma-Aldrich	G7513-100ML
MEM Non-Essential Amino Acids	Thermo Fisher	11140-050
MycoAlert detection kit	Thermo Fisher	LT07-118
Oxoid Phosphate Buffered Saline Tablets	Thermo Fisher	BR0014G
Penicillin Streptomycin	Thermo Fisher	15140-122
POPs toxicant mixture		
Primocin	Fisher Scientific	NC9141851
Sodium Pyruvate	Thermo Fisher	11360-070
SolnIX-SK	Cryogenix, LLC, South Carolina, US.	
Triton X-100 solution	Sigma-Aldrich	102521699
Trypan Blue	Thermo Fisher	T10282
TrypLE Express Enzyme (1X), phenol red	Thermo Fisher	12605010
Vimentin	Thermo Fisher	11-9897-82

Appendix B

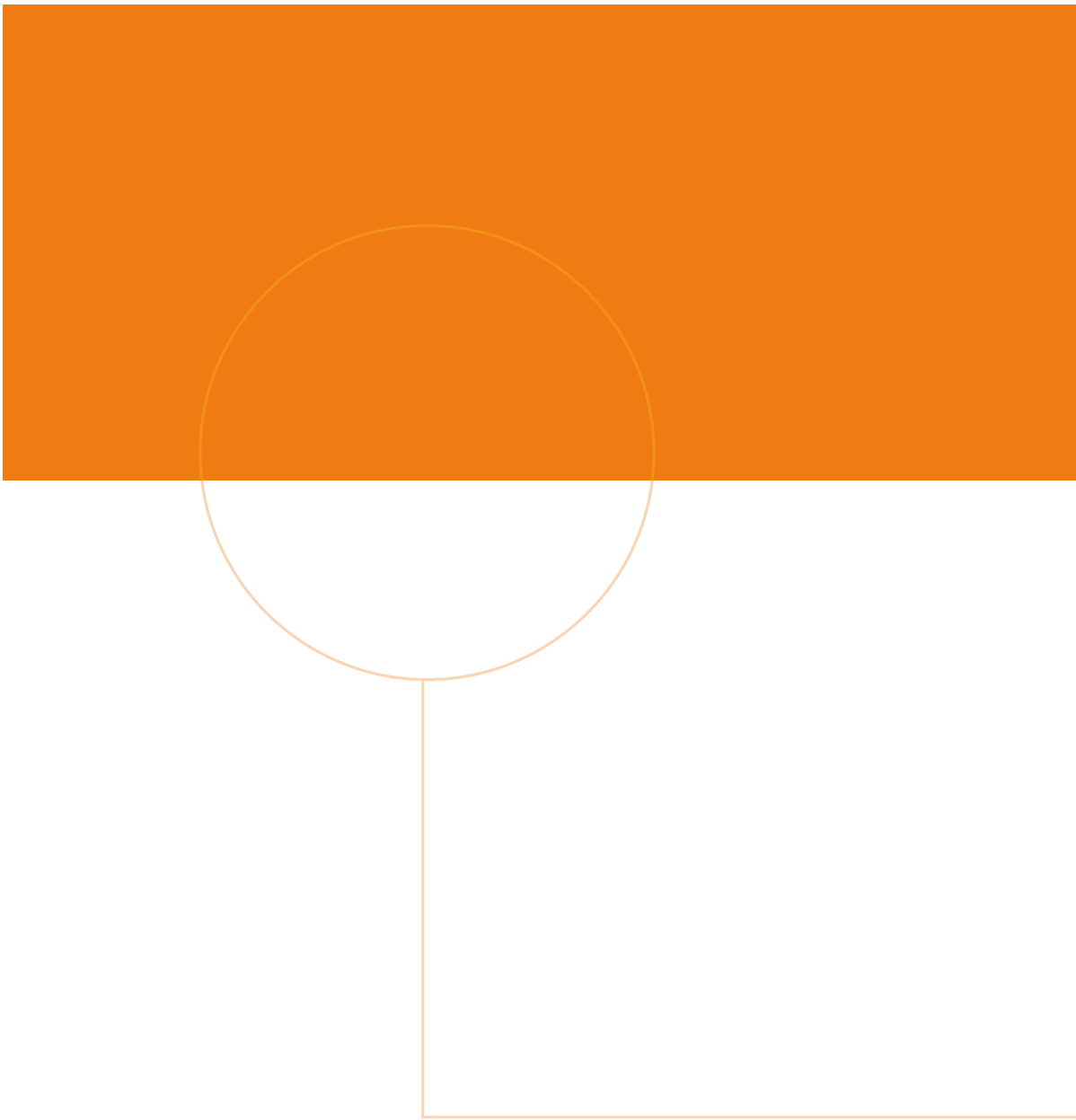
*Table B1. List of the 10 most common persistent organic pollutants found in blubber of Norwegian killer whales measured in molar concentration (uM). The compounds are solved in DMSO (µg/mL). The concentration in DMSO in concentrated POP-mix is 20,000-fold the concentration measured in lipid wet weight (ww). DMSO constitutes 0.25 % when concentration factor is 50X (*Blevin et al., unpublished).

Compounds	CAS	1x uM	1x ng/g ww	Conc. in DMSO in concentrated POP-mix (µg/mL)
***p,p'*-DDE**	72-55-9	4.477	1423.9	28477
PCB153	35065-27-1	1.741	628.4	12568
PCB138	35065-28-2	1.310	472.6	9453
tNC	39765-80-5	0.990	439.9	8797
PCB180	35065-29-3	0.637	251.9	5037
PCB101	37680-73-2	0.519	169.3	3386
PCB52	35693-99-3	0.519	151.4	3029
PCB99	38380-01-7	0.483	157.8	3155
PCB187	52663-68-0	0.451	178.5	3569
PCB118		0.441	144.1	2882
	Max conc. factor X			50
	when DMSO % in the well is			0.25

Appendix C

Table C1. Number of KW-11-02 cells counted of passage 4 and passage 5 and respective cell viabilities obtained by different cultivation conditions.

	P4	P5			
Treatment	**15.jun**	**20.jun**	**Mean**	**SD**	**Viability**
bFGF1	1,6E+05	1,5E+05	1,55E+05	5000	77,55
bFGF2	2,7E+05	2,8E+05	2,75E+05	5000	90
bFGF4	2,3E+05	1,1E+05	1,70E+05	60000	86,4
FBS20	8,0E+04		8,00E+04		60
HS	5,0E+04		5,00E+04		78
Mix1	2,4E+05	2,1E+05	2,25E+05	15000	76,5
Mix2	2,2E+05	2,2E+05	2,20E+05	0	87,55



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