Research Academy for Young Scientists

The Role of Osteosarcoma in the Activation of Fibroblasts

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Abstract

Sarcoma is a rare type of cancer derived from the connective tissue of the body. Due to the rareness of the disease, not much research has been conducted compared to several other cancer types. The role of fibroblasts in tumour development is well-known in several cancer types. However, their role in sarcoma remains unclear. This study aimed to investigate the influence of osteosarcoma on the activation of primary fibroblasts. By conducting direct, indirect cocultures and monocultures of primary fibroblasts and osteosarcoma, the cell cycle distribution was measured using flow cytometry enabling the study of the proliferation of fibroblasts. The results showed a higher proliferation in fibroblasts exposed to sarcoma, indicating that osteosarcoma can activate fibroblasts.

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1 Introduction

Cancer is one of the primary causes of death in humans causing millions of deceases every year [1]. By being a diverse type of disease with different origins, characteristics and development, it is difficult to find mutual therapeutic methods in order to cure cancer. Consequently, the understanding of cancer will be of importance in order to find new effective therapies towards the diverse types of cancers.

One aspect concerning cancer research is the tumour microenvironment in which cellular crosstalk between tumour and adjacent cells can induce tumorigenesis. Acquiring new knowledge about growth factors, cellular differentiation and other aspects regulating the alterations in these tissues are imperative. [2]

Sarcoma is a rare type of cancer in the connective tissue with predominance in the paediatric population [3]. Due to its infrequency, not much research has been conducted in this field. In order to find new efficient treatments, more research needs to be applied.

An aspect of sarcoma that has not yet been studied is the role of a certain specific type of cell in the connective tissue, named fibroblasts. Previous studies show that fibroblasts play a role in inducing proliferation and other tumourigenic changes in the tumour microenvironment of several cancer types, for instance in breast cancer [4]. However, their role in sarcoma remains unclear. Consequently, this study aims to better understand the role of fibroblasts in the tumour stroma of osteosarcoma by conducting cell cultures of these cell lines. Therefore, the study question is if osteosarcoma can influence the proliferation of fibroblasts.

1.1 The Cell Cycle

The cell cycle is strongly regulated by a diverse number of proteins and genes in order to maintain division when suitable. The cycle and development can be divided into five distinguishable parts: these are the G0-, G1-, S-, G2 and M-phase. G1-phase is the major phase of the cell cycle, in which the cell metabolism increases in order to prepare for cell division. The cell can also enter the G0-phase, which is an intermediate state where the cell metabolism is decreased. It is during the S- and M-phase in which division is prepared

and conducted in the cells. By replicating the DNA during the S-phase and eventually duplicate organelles, mitosis, the process in which a cell divides into two daughter cells, is executed. [5]

The decisive phase of which the cell commences to divide is in the phase transition between the G1- and S-phase. Through signal transduction and downstream gene regulations, the DNA replication can initiate. Some of these proteins are cyclin dependent kinases, that becomes activated or inhibited when bond to a cycline. These have an important role in the regulation of the signal transduction. One example of a cyclin-dependent kinases that when bond to cycline E or A becomes activated is cdk2. This is a S-phase promoting factor, which can phosphorylate DNA-polymerases. When DNA-polymerases are activated DNA-synthesis can be conducted in the cell and it consequently enters the S-phase. [6]

The above mentioned process is in turn regulated by a second level of growth factors and inhibitors. These are downstream regulated through gene expression and usually constitutes of transcription factors for the necessary kinases. The trancription factors that inhibits proteins activating DNA-replication are named suppressor genes, whilst the opposite are oncogenes.[6]

1.2 Mechanisms of Cancer

Mutations in genes regulating the cell cycle are one of the major causes of cancers. There are though several aspects resulting in proliferation in cancer cells and several hallmarks can be perceived. [7]

1.2.1 Mutations in Suppressor Genes and Oncogenes

Alterations in the gene expression of suppressor genes and oncogenes are the main reasons for malignant transformation. In normal cells proto oncogenes have a role in activating the signal transduction enabling cell division. Several of these genes are coding for growth factors or receptors for different growth factors. Through gene regulation, the expression of these genes are strongly regulated. In cancer cells, gene mutations can lead to overexpres-

sion of these genes. Mutations like translocations of chromosomes can alter the promotors for oncogenes, leading to an excessive transcription. In this way proto oncogenes becomes oncogenes. Consequently, this can cause malignant characteristics of the cells.

In contrast to oncogenes, suppressor genes are preventing cell division by for instance coding for proteins that inhibit the transition from G1- to S-phase. One example of a suppressor gene that tends to be inactivated in cancer cells is the p53 gene. p53 is a protein that can induce the activation of the CDK-inhibitor p21 which prevents the cell from entering the S-phase. p53 is usually activated after damage of DNA in order to prevent the proliferation of damaged cells. Therefore, suppressor genes have a role in preventing cancer development if activated. [5]

1.2.2 Evading Apoptosis

A way for cancer cells and other types of cells to obtain an unceasing proliferation is by increasing their life span. By evading apoptosis due to senescence and shrinking telomeres in the chromosomes as a result of DNA replication, the cancer cells can proceed proliferation. From growth factors or mutations the cells can sustain their telomeres during cell divisions. As a result, the cancer cells can divide more excessively. Previous studies have shown that the expression of the DNA polymerase synthezising telomere, telomerase, is on significant levels in contrast to nonimmortalized cells where the enzyme is absent. In this way the cancer cells are able to evade a state of declined proliferation called senescence. [7]

1.3 Sarcoma

Sarcoma is a diverse form of cancer that is derived from the connective tissue. The tumour development is usually initiated through mesenchymal stem cells in the connective tissue that starts to proliferate irregularly. Because of the origin, sarcoma can be distinguished into two different types: bone tissue and soft tissue sarcoma. The latter involves the development of a tumour in muscles, nervous tissue, blood vessels, adipocytes and cartilage. Bone tissue sarcoma on the other hand constitutes of for instance osteosarcoma. [8]

Regarding the origin of the tumour progression, there are several factors that can induce the cancer development of sarcoma. One of these include mutations in tumour specific genes altering the RB, p53 and other signal transduction pathways regulating the cell cycle. The increased synthesising of tyrosin-kinase growth factor receptors is another factor that increases the ligand contact with growth factors. This can in turn increase the cell proliferation leading to abnormal division and proliferation. [9]

Characteristic symptoms of the development of the tumour are bone pain, easily broken bones (mainly in osteosarcoma), weight loss and a hard lump that can be felt through the skin around the tumour [10].

1.4 Fibroblasts

Fibroblasts are a type of stromal cells with mesenchymal origin and that constitute the connective tissue in mammals. By producing and secreting fibrous proteins, for instance different types of collagen, fibroblasts play an important role in the production of the extracellular matrix (ECM). They also have a role in wound healing and fibrosis processes, in which damaged tissue becomes replaced by an excessive deposition of ECM. This process leads to the activation of fibroblasts into myofibroblasts. The activated phenotype receives larger endoplasmatic reticulum and a more prominent Golgi apparatus. Thus, the cells are able to produce ECM components in a higher extent, since the transportation of proteins becomes more effective. [2]

The activation of fibroblasts is regulated through several growth factors secreted from the injured cells. Some examples of these are Transforming Growth Factor-B (TGFB), Platelet-Derived Growth Factor (PDGF) and Fibroblast Growth Factor B (FGFB). These ligands can induce cell proliferation and activation of fibroblasts in different ways. For instance, TGFB induces both cell division and therefore proliferation of fibroblasts, as well as the signal transduction that can activate genes producing metalloproteinases (MMPs) [11] . These can degrade the ECM in the tissue and changing the composition [12].

Studies suggest that a similar activation of fibroblasts occurs in the tumour carcinoma, in which fibroblasts are becoming activated through paracrine signalling of growth

factors from the tumour [2] . A cellular crosstalk occurs through a reciprocal signalling, where fibroblasts in turn secret growth factors that induce tumorigenesis, angiogenesis and metastasis of cancer cells. Becuase of this, the activated fibroblast in the tumour stroma are usually named cancer associated fibroblasts (CAF). The tumour cells can secrete TGFB and PDGF which in turn can induce proliferation and activation of fibroblasts into myofibroblasts. The secretion of MMPs that degrade the ECM can enable the development of metastasis in the tumour by breakng physical barriers for the tumour. Furthermore, the activated fibroblasts can in turn produce other growth factors such as hepatocyte growth factor (HGF) that induces proliferation and increases the motility in the cancer cells. [4]

1.5 The Tumour Microenvironment

The tumour microenvironment contains a complex system of tissues and processes constituting paracrine communication and enabling exchange of nutrients and oxygen in the tumour. Immune cells, blood vessels, fibroblasts and ECM have an impact on the tumour progress and together they constitute the tumour stroma. [13]

1.5.1 Angiogenesis

In order for the tumour cells to obtain oxygen and nutrients for cell respiration and metabolism, the access to capillaries are needed. The development of capillaries in the cancer have an important role and is regulated by growth factors released from the cancer cells, like Vascular Endothelial Growth Factor (VEGF). The growth factor can be released by inflammatory cells and fibroblasts in the tumour microenvironment. The growth factor can activate endothelial cells in the tumour microenvironment, initiating the development of blood vessels. Since VEGF increases the permeability of the capillaries, plasma proteins such as fibrin can be released. A protein usually released during wound healing is fibrin, which therefore can activate fibroblasts and inflammatory cells to the tumour microenvironment, which in turn can activate the tumour. [2]

1.5.2 Metastasis

Metastasis is the process in which cells from the tumour migrate into blood vessels minting a development of new secondary tumours. The mechanisms of metastasis are complex, but they are strongly regulated by several cells types and the tumour microenvironment. The cells need to breach the basement membrane in order to migrate and thereafter find contact with endothelial cells to enter the blood stream. The alteration of ECM plays a role in the development and migration of metastais in the tumour microenvironment. [14]

1.6 Method Background

Using osteosarcoma and primary fibroblasts from human bone and skin the experimental design will be distributed through different cultures, containing untreated fibroblasts and fibroblasts with the nutrient solution, conditioned media, from sarcoma cancer.

1.6.1 Cell Counting

NucleoConter was used together with a dye containing propidium iodide which stains the DNA in order to count the number of cells. The reagent A100 is an inorganic acid that dissolves the cell membrane, leading to an increased permeability in the DNA for the dye [15]. In contrast to reagent A100, reagent B is an inorganic alkalic substance reducing the risk of DNA denaturation and neutralizing the cells [16] .

1.6.2 Coculture

By seeding primary fibroblasts together with conditioned media from osteosarcoma and together with osteosarcoma the cellular crosstalk between the sarcoma cancer and the fibroblasts can occur. These represent the indirect respectively direct cocultures. This makes it possible to study the activation of primary fibroblasts when exposed to growth factors secreted by the cancer cells in the conditioned media.

The cells that are used in the cultures are from ECACC (European Collection of Authenicated Cell Cultures). Osteosarcoma (MG-63) derives from paediatric bone and the primary fibroblasts (WS1), are from human skin.

The conditioned media contains of 85 vol% of medium (D-glucoe, L-glutamine and pyruvate), 10 vol% of serum fetal bovine and 5 vol% of antibiotics.

1.6.3 Flow Cytometry

Is a cellular analytic method that is frequently used during cell cultivations. The laser-based apparatus measures fluorescence dyed cells and can, for instance, be used for studying cell proliferation in cocultures. A dye, such as 4',6-diamidino-2-phenylindole (DAPI) or 5(6)-carboxyfluorescein diacetate N- hydroxysuccinimidyl ester (CFSE) can be used for measuring the DNA content of the cells. The molecule binds to adenine-thymine rich areas of the DNA and is fluorescent, enabling the laser-based analysis. A higher intensity of the dye implies a higher content of DNA. Since the cells in the G2 and M-phase obtain duplicated DNA, the fluorescence and therefore the DNA content is higher. The DNA replication during the S-phase results in a gradually increased DNA content. While cells in a non-dividing state, G1 and G0, have the same number of DNA. By displaying the results in a histogram, two peaks are expected, one with a simple set of DNA and one with a double set. Between these two peaks a prolonged intermediate phase will be perceived, corresponding the number of cells in the S-phase. By adding Triton-x100 to the antibody dilution buffert before applying flow cytometry, an increased permeabilization of the cell membrane can be acquired, since it becomes dissolved. This is of importance in order for the dye (DAPI) to bind to the DNA. Another part of the procedure is fixation of the cells using paraformaldehyde (PFA), which can stop cell metabolism and further division. This faciliates the study of the cells during one moment. [17]

1.7 Hypothesis

It is expected that primary fibroblasts will be activated in the indirect coculture with sarcoma. Consequently, a higher proliferation of fibroblast is expected where a larger share of the cells will be in the S-phase and the number of cells will have increased more significantly in contrast to the monocultures of fibroblasts.

2 Method

By applying indirect and direct cocultures of primary fibroblasts together with osteosarcoma and monocultures of primary fibroblasts, the activation of fibroblasts could be displayed. This was done through cell cycle analysis with flow cytometry and by counting the number of cells before and after two respectively three days of incubation.

2.1 Dissociation of Primary Fibroblasts

Firstly, a flask containing primary fibroblasts was investigated in a microscope to ensure the confluency of the cells. The cell medium was removed using a pipette. In order to enable dissociation of cells from the flask, Dulbecco's Phosphate Buffered Saline (DPBS) was added to the flask for washing the interior flask and then removed using a pipette. 1 mL of trypsine was pipetted in the flask and it was then put for incubation in 5 minutes. After 5 minutes, 9 mL of cell medium was added and the whole solution was then transferred to a falcon tube. The content of the falcon tube was centrifuged for 5 minutes and with a speed of 1100 RPM. After centrifuging, a cell pellet could be distinguished at the bottom of the tube, whilst the cell medium was at the top. The cell medium was subsequently removed from the tube. 3 mL of additional cell medium was added to the falcon tube. 1 mL was removed and put into the initial flask for further cultivation minting a proportion of 1:3 of fibroblasts and cell medium.

2.2 Cell Counting of Fibroblasts

Of the remaining cell medium solution 100 μL was added to an eppendorf tube. Then 100 μL of solution A100 was measured and added to the same eppendorf tube. The obtained solution was put in a vortex for rotation in 5 seconds. 100 μL of the B solution was added and the rotation was repeated. A Nucleus Cassate was used to absorb the mixed solution from the eppendorf tube and then put in a NucleoConter. The observed concentration (cell/mL) corresponded the number of cells in the eppendorf tube, which was diluted to one third.

2.2.1 Dilution of Cells

To prevent the risk of obtaining an overdensed flask, the fibroblast and cell medium solution in the falcon tube was pippetted to the total volume of 0.340 mL to each of the two flasks of a surface of 15 cm^2 in order to obtain a solution of $8.5 \cdot 10^5$ cells. After that 10 mL of cell medium was added to the two flasks and they were put in an incubator for 48 hours. See Appendix for the calculations of the numbers of cells. This was used as an initiation for further cultivation of these fibroblasts.

2.3 Indirect Coculture of Fibroblasts

The cell media from one of the flasks containing fibroblasts was used for the indirect culture. A flask containing osteosarcoma was observed in a microscope in order to study the confluency. The same procedure was conducted for the flasks of fibroblasts, which had been incubated for approximately 24 hours.

The flask containing osteosarcoma was filtered using a syringe and a syringe filter 25 mm/0.45 μm where 10 mL of the extraction was separated and added to a tube. The conditioned media from one of the flasks with fibroblasts was removed using a pipette and replaced by 10 mL of the conditioned media from osteosarcoma. In this way a medium transfer was conducted. The flask was then labeled and put in incubation for 48 hours.

After 48 hours the number of cells in the two flasks, monoculture and conditioned media from sarcoma, was counted using the same method as 2.2 Cell Counting of Fibroblasts.

2.4 Direct Coculture of Fibroblasts

When direct cocultures were applied three flasks were prepared. One containing a monoculture of fibroblasts, another one containing a coculture of sarcoma and fibroblasts with a cell ratio of 50% sarcoma and fibroblast and a third coculture with a ratio of 70% fibroblasts and 30% sarcoma. Prepared cultures of fibroblats and sarcoma were dissassociated as in 2.1 Dissociation of Primary Fibroblasts.

After centrifuging, the cell medium was removed from the falcon tube containing primary fibroblasts and 2 mL of DPBS was added and mixed with the cell pellet. 100 μL was put in an eppendorf tube used for counting the cells, see 2.2 Cell Counting of Fibroblasts. The remaining solution was stained with 2 μL of CFSE and the falcon was incubated for 20 minutes. After that 2 mL of cell medium was added and the falcon tube was incubated for 5 additional minutes.

The cell media from the second falcon tube containing sarcoma was removed after centrifuging and 4 mL cell media was added and mixed with the cell pellet. 100 μL was put in an eppendorf tube used for counting the sarcoma cells as in 2.2 Cell Counting of Fibroblasts.

The fibroblasts and the sarcoma cells were counted using NucleoConter. The corresponding volumes for the three different ratios were calculated, see Appendix for calculations. Then the volumes were added in the three flasks. The cells were then incubated for 72 hours before flow cytometry was applied.

2.5 Flow Cytometry Assay of Cell Cycle

Before conducting flow cytometry, an antibody dilution buffert was prepared. 2.5 g of bovine serum albumin (BSA) was measured and diluted with 50 mL of DPBS in a beaker, consequently a solution of 5% BSA was obtained. The solution was diluted to 0.5% BSA and this was done by adding 5 mL of the 5% BSA solution and adding 45 mL of DPBS. 50 μL Triton-x100 was measured and added to the antibody dilution buffert.

The flasks containing a monoculture of primary fibroblast and indirect coculture of fibroblasts were dissassociated using the same method as 2.1 Disassociation of Primary Fibroblasts. When all cell medium was removed after centrifuging, 5 mL of DPBS was added and mixed with the cell pellets. The solution was centrifuged and DPBS was removed. After that the cells were fixed with 150 μL of 4% paraformaldehyde (PFA). The falcon tubes were centrifuged at 3500 RPM and the PFA was removed. The solutions were in turn washed and mixed with 5 mL DPBS and then removed from the tube. 150 μL of the permiabilization solution, antibody dilution buffert, was added in order to stain the

tubes with 1 μL of DAPI each. These were then put into wells and analyzed through cell cycle analysis based flow cytometry using 405 nm wavelength.

The same procedure was repeated for the direct cocultures, with the difference that a gating was conducted were only the CFSE-marked cells were analyzed. Since the fibroblasts were initially stained with CFSE, these cells were of interest in the analysis.

2.6 Data Analysis

From the indirect cocultures the number of cells were measured before and after 48 hours of incubation. The number of cells in the indirect coculture was compared to the monoculture. Through flow cytometry the distribution of the cell cycle was measured. The differences in the cell cycle distribution between the monoculture and the indirect coculture was analyzed and compared. The same process was applied for the two ratios of direct coculture and the monoculture, with the exception that the number of cells was not counted because the direct cocultures contained two types of cells.

3 Results

The results from the indirect coculture and the monoculture as a reference are presented as a table showing the number of cells, a table and a diagram displaying the distribution of cell cycle are presented. For the direct coculture a table and diagram displaying the cell cycle distribution are presented.

3.1 Number of Cells

After having used NucleoConter for counting the cells initially and after two days of incubation there was a larger growth of the number of cells in the monoculture than in the indirect coculture with conditioned media, see Table 1

Table 1: The number of cells in the monoculture of fibroblasts and in the indirect coculture of fibroblast from the initial value (D0) and after 48 hours (D2).

Culture	D0	D2
Monoculture of fibroblasts	$8.50 \cdot 10^5$	$1.73 \cdot 10^6$
Indirect coculture of fibroblasts	$8.50 \cdot 10^5$	$1.33 \cdot 10^6$

3.2 Distribution of Cell Cycle

The distribution of the cell cycle shows that a higher share of the cells in the monoculture of fibroblasts were in the S- and G2-phase compared to the indirect cocultures of fibroblasts and sarcoma, see Table 2 and Figure 2.

Table 2: The distribution of the different phases of the cell cycle after 48 hours.

Culture	G1	S	G2
Monoculture of fibroblasts	44.58%	33.97%	15.82%
Indirect coculture of fibroblasts	48.52%	31.48%	15.28%

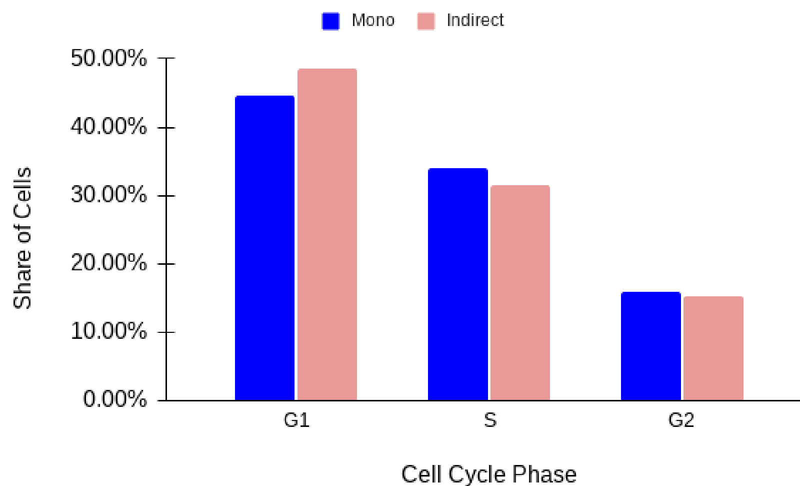

Figure 1: The share of cells in the different phases of the cell cycle in the indirect coculture and monoculture.

After having conducted direct cocultures the fibroblasts in the direct coculture with a ratio of 50/50 had the largest share of cells in the S and G2-phases compared to the two other cultures, with 44.82% of the cells in S-phase and 10.70% in G2. The monoculture with fibroblasts had the lowest share of cells in the S- and G2-phase, 17.14% respectively 6.70%. In this case the vast majority of the cells, 74.18% were in G1. The direct coculture with the ratio 70/30 had a higher share of cells in S and G2 than the monoculture, but

lower than the direct coculture with the ratio 50/50, with 31.48% in S-phase and 10.7% in G2-phase. See Table 3 and Figure 2 for all data.

Table 3: The distribution of the different phases of the cell cycle in the direct cocultures and a monoculture as reference after 72 hours.

Culture	G1	S	G2
Monoculture of fibroblasts	74.18%	17.14%	6.7%
Direct coculture of fibroblasts (50/50)	49.93%	44.82%	15.28%
Direct coculture of fibroblasts (70/30)	48.52%	31.48%	10.7%

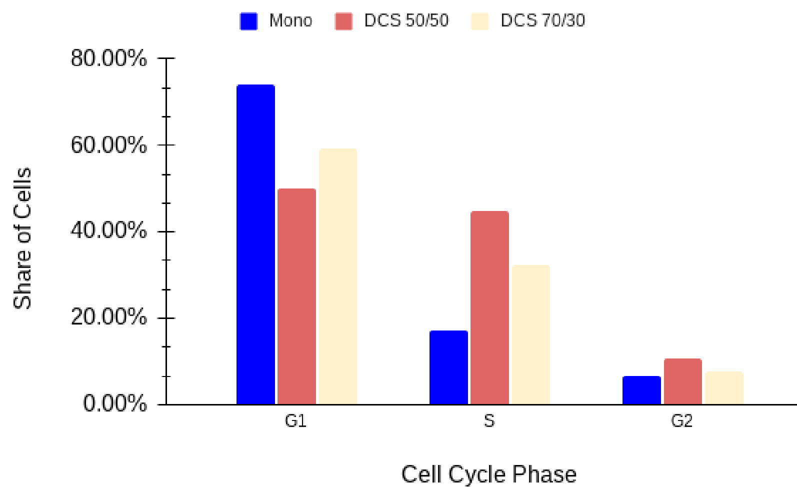

Figure 2: The share of cells in the different phases of the cell cycle in the direct-cocultures, DCS 50/50 and DCS 70/30 and the monocultures of fibroblasts.

3.3 Share of Apoptotic Cells

Through flow cytometry and cell cycle distribution, the share of fibroblasts that were apoptotic could be observed. In the indirect coculture the monoculture had the highest share of apoptotic cells whilst the indirect co-culture had the lowest, see Figure 3.

In the direct cocultures, the monoculture had the highest share of cells that were apoptotic, whilst the direct co-culture with the ratio 50/50 had the lowest share of apoptotic cells, see Figure 4.

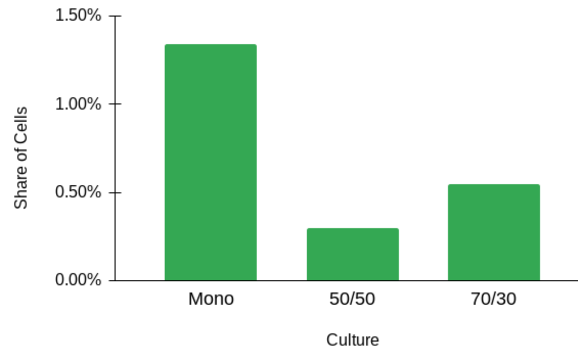

Figure 3: The share of apoptotic cells in the monoculture and indirect coculture.

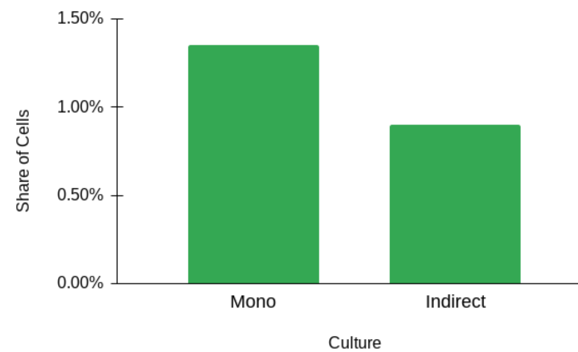

Figure 4: The share of apoptotic cells in the monoculture and direct cocultures.

4 Discussion

From the obtained results a significant change in the cell cycle distribution can be perceived from the direct cocultures compared to the monoculture. However, a significant difference in cell cycle distribution cannot be seen from the cells in the indirect coculture. Even though they were 500 thousand more cells in the monoculture compared to the indirect coculture, the small differences in the cell cycle distribution makes it difficult to draw conclusion. The lower rate of proliferation in the indirect coculture, which does not confirm the hypothesis in contrast to the results from the direct coculture, can have further explanations, see 4.2.2 The Use of Indirect Cocultures.

The direct coculture with the ratio of 50/50 had the highest proliferation of all three cultures, where over 40% of the cells were in the S-phase. The culture with the ratio 70/30 had a higher share of cells in the S-phase than the monoculture, but lower than the ratio of 50/50. This can indicate that the different samples correspond different phases of the tumour progression. When 50% of initial cells were osteosarcoma compared to 30% in the

70/30 culture, a later stage of the cancer can be depicted. Less number of cancer cells secrete less growth factors that can induce proliferation in the fibroblasts, which can then explain the obtained results.

4.1 Activation of Fibroblasts

According to the results from the direct cocultures with sarcoma and fibroblasts, a greater share of the cells was in S- and G2-phase compared to the monocultures that were not exposed to either growth factors or actual sarcoma cells. Apart from indicating a higher rate of proliferation of fibroblasts in direct contact with sarcoma, it can indicate the activation of primary fibroblasts by sarcoma. Of what previous research has suggested concerning the activation of fibroblasts in different types of cancers, this can indicate that osteosarcoma can provide fibroblasts with growth factors that induce proliferation and lead to the activation of cancer-associated fibroblasts (CAF). The significant increase of proliferation in which over 30% of the cells were in the S-phase in the direct cocultures compared to the monoculture, see Figure 2 and Table 3, shows that sarcoma has an influence on the proliferation of fibroblasts, even though no significant changes could be perceived from the indirect coculture, see 4.2 Method Discussion.

Since PDGF and TGFB are two growth factors secreted by other types of cancer and can induce proliferation in fibroblasts, it may be assumed that they can have a role in the cellular crosstalk between osteosarcoma and primary fibroblasts. The significant increase and decrease in the S-phase and G1-phase from the direct cocultures with the 50/50 ratio compared to the monoculture,see Figure 2 and Table 3, can in turn indicate a regulation of the cell cycle through the G1/S-restriction point, that is needed for the cell to enter DNA-replication and subsequently cell division. This regulation can be done through different signal transduction pathways but can be induced by ligands such as PDGF and TGFB that bind to tyrosine-kinase receptors of the fibroblasts. This can therefore lead to proliferation of the fibroblasts.

Another parameter studied through cell cycle analysis with flow cytometry is the share of apoptotic fibroblasts in each culture. The results indicated a lower share of apoptotic

cells in the cocultures, especially in the direct coculture with the 50/50 ratio, where only 0.30% of the cells were apoptotic. This can indicate that osteosarcoma can induce ligands evading apoptosis in fibroblasts. Since the mentioned growth factors such as TGFB has a role in the signal transduction pathway inducing proliferation, the same activated proto oncogenes can probably induce survival and evade proliferation.

Because the growth factors PDGF and TGFB are mediating phenotypical changings in the fibroblasts making them secret growth factors that in turn stimulate sarcoma to proliferate and develop metastases as well as altering the ECM enabling metastasis of the tumour, the increased proliferation can be associated with an activated cancerogenic phenotype. Nevertheless, the studying of the sarcoma cells proliferation is essential for confirming the cellular crosstalk and if the activated phenotype of fibroblasts is achieved, see 4.3 Further Studies.

4.2 Method Discussion

This experiment used two types of cultures; indirect cocultures and direct cocultures. The obtained results were different which can indicate a greater difference in the efficiency of the two methods. Another aspect that can have an influence on both tumour progression and the activation of fibroblasts is the conducting of in vitro experiments.

4.2.1 In Vitro Experiment

Conducting cell cultures and studying cellular crosstalk in the tumour microenvironment in vitro does not correspond to the complex tumour microenvironment. Since the microenvironment includes several types of cells such as fibroblasts, immune cells, endothelial cells, epithelial cells etc. only one factor can be examined. However, this method aimed to study the activation of fibroblasts by sarcoma, where mono- and cocultures can be seen as a beneficial method. But since the tumour becomes activated by several tumorigenic signals, which is partly induced by fibroblasts, partly by other types of cells, it becomes difficult to examine all factors affecting the growth of fibroblasts. Since there is a paracrine cellular crosstalk between the tumour and fibroblasts, the proliferation of the tumour has an

effect on the secretion of growth factors. Fibroblasts can for instance induce angiogenesis through VEGF by activating endothelial cells in the tumour microenvironment, leading to the development of capillaries sustaining the tumour with nutrients and oxygen. This can in turn enable the cancer cells to divide at a higher extent through an increased metabolism allowing the cells to enter the S-phase. The tumour progression leads to a higher secretion of growth factors that activate the fibroblasts.

The influence of angiogenesis is difficult to study in vitro, since it is dependent on a complex of reactions and cell differentiations occurring in the tumour microenvironment. But since it has an impact on the tumour development and in turn the proliferation and activation of fibroblasts, it is important to take that into consideration. If the rate of proliferation is studied over a longer period of time, this aspect will be of importance.

4.2.2 The Use of Indirect Cocultures

Using indirect cocultures can be one potential explanation why the proliferation rate of primary fibroblasts and therefore the activation of them were low. Since the media from fibroblasts only contains the nutrient solution from sarcoma together with secreted growth factors that may induce proliferation in fibroblasts, a low secretion of growth factors can be obtained. The concentration of TGFB and PDGF is therefore lower than if the cellular crosstalk occurred directly between the cells. The affinity to growth factors would increase if the sarcoma cells had a direct contact with fibroblasts, which could have been obtained using direct cocultures, instead of indirect.

Megan A. Cole et al suggests in a report about the role of ECM and fibroblasts in skin-ageing that the proliferation of fibroblasts are affected by the composition of the ECM [18]. Since the conditioned media containing fibrous proteins produced by fibroblasts were removed during the media transformation in the conditioned media of the fibroblasts, it can have been inhibiting the proliferation of fibroblasts even though additional growth factors from osteosarcoma were in the solution. When the adhesion to the ECM reduces it can lead to less cell division, even though the cells are exposed to growth factors from sarcoma.

The transferring of cell media has an impact on the cells and can cause stress during the exchange when an alteration in growth factors and ECM occurs [19]. This can therefore pose an explanation to the results from the indirect cocultures. Since there were not a significant difference in proliferation of the monoculture and indirect coculture of fibroblasts, the use of conditioned media in indirect cocultures can be concluded as less beneficial as direct cocultures.

4.2.3 The Rate of Proliferation

In order to study the rate of proliferation and growth over time, a prolonged culture process with gradual analysis of cell cycle distribution could have been applied to study the rate of proliferation and how it changes. Although, there are several limitations with this method concerning the time rate of which the experiment can be conducted. Primary fibroblasts, which have been used in the experiment, cannot divide for a long period of time with a high rate of proliferation before entering a phase of senescence, where the cell growth declines. However, the method that has been applied concerning direct coculture mirrors different concentrations of number of cells, which can depict two different levels of tumour growth. 50 percent of sarcoma was the initial share of cells in one of the direct cocultures, whilst only 30 percent of the cells were sarcoma. The latter can therefore include an earlier stage of the tumour development, where a lower rate of cell proliferation could be perceived.

4.3 Further Studies

In order to confirm the obtained results from the direct cocultures, the experiment needs to be repeated through new cultures of cells. In this case only one sample was made and more samples are needed to be tested in order to get statistically valid results. Thus, the significant results from the direct coculture can be confirmed.

An aspect that can be further investigated is the development of the cancer tumour in the experiment. Since this study was limited to the investigation of primary fibroblasts and their activation by sarcoma cancer, the influence on the tumour development

remains unclear. The paracrine cellular crosstalk between fibroblasts and sarcoma affects both types of cells. Since fibroblasts provide the cancer with growth factors, like HGF, when activated, it can increase the tumour progression through a higher proliferation. By applying flow cytometry on the tumour cells, the tumour progression concerning the proliferation of the sarcoma cells can be measured.

This study can indicate that growth factors such as TGFB and PDGF have a role in the activation of fibroblasts, but in order to confirm this, more experiments are needed. By investigating TGFB or PDGF receptors on the cell membrane of fibroblast, and studying their activation through staining, the activation of the receptors can be measured through flow cytometry. If an increased share of the studied PDGF receptors of fibroblasts is activated, then it will indicate that this growth factor has a role in the activation and proliferation of fibroblasts.

Not only the tumour progression through cell cycle analysis is of importance concerning the malignancy and invasiveness of the tumour. Another cell behaviour constituting metastasis has a role in the invasiveness. This could have been studied through different cell migration techniques in vitro, but it would not fully correspond to the tumour microenvironment. MMPs that are secreted from CAFs can degrade the ECM leading to an increased possibility for the tumour cells to metastasise by for instance breaking the physical barriers. Since the degradation of the basement membrane, that cannot be applied in vitro, of the tumour is essential for the invasive progression, it cannot be studied. Although this study would not correspond fully to the tumour microenvironment, it will give a view of the role of fibroblasts in the cell migration of the sarcoma and therefore indicate their role in the development of metastasis. By instead staining sarcoma cancer in direct cocultures with fibroblasts, a more nuanced view of the cellular crosstalk between sarcoma and fibroblasts can be perceived. A further study could therefore examine the role of fibroblasts in the tumour progression of sarcoma.

4.4 Conclusion

In conclusion, this study indicates that osteosarcoma can induce proliferation in primary fibroblasts. This can in turn imply that osteosarcoma can acivate fibroblasts into cancer-associated fibroblasts by providing it with essential growth factors. However, due to the lack of reliability it is not possible to draw that conclusion from the conducted experiment and further studies are therefore necessary.

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A Calculations of Cell Number

Indirect Co-Culture

Initial Values
Number from NucleoConter : $8.33 \cdot 10^5$ cells

Numbers of cells before dilution:
$8{,}33 \cdot 10^5 \cdot 3$ cells $= 2{,}5 \cdot 10^6$ cells

$2{,}5 \cdot 10^6$ cells $\Leftrightarrow$ 1 mL suspension

$8{,}5 \cdot 10^5$ cells $\Leftrightarrow$ 0,340 mL suspension

After 48 hours
Numbers from NucleoConter:
Indirect co-culture: $1{,}48 \cdot 10^5$ cells
Monoculture: $1{,}92 \cdot 10^5$ cells

Numbers of cells before dilution:

Indirect co-culture: $1{,}48 \cdot 10^5$ cells $\cdot\, 9 = 1{,}33 \cdot 10^6$ cells

Monoculture: $1{,}92 \cdot 10^5$ cells $\cdot\, 9 = 1{,}73 \cdot 10^6$ cells

Figure 5: Calculations for the monoculture and indirect cocultures

Numbers from NucleoConter:
Fibroblasts: $8{,}26 \cdot 10^5$ cells
Sarcoma: $1{,}11 \cdot 10^6$ cells

Numbers before dilutions

Fibroblasts: $8{,}26.. \cdot 10^5 \cdot 3$ cells $= 2{,}48 \cdot 10^6$

Sarcoma: $1{,}11.. \cdot 10^6 \cdot 3$ cells $= 3{,}34 \cdot 10^6$

Monoculture (100% fibroblasts)
$2{,}48 \cdot 10^6$ cells $\Leftrightarrow$ 1 mL suspension
$1 \cdot 10^6$ cells $\Leftrightarrow$ <u>0,404 mL suspension</u>

Direct co-culture (50% fibroblasts and sarcoma)

Volume of Fibroblasts (mL):

$1 \cdot 10^6 \Leftrightarrow$ 0,404 mL suspension
$0{,}5 \cdot 10^6 \Leftrightarrow$ <u>0,202 mL suspension</u>

Volume of Sarcoma (mL):

$3{,}34 \cdot 10^6 \Leftrightarrow$ 1 mL suspension
$0{,}5 \cdot 10^6 \Leftrightarrow$ 0,150 mL suspension

Figure 6: Calculations of the volumes in the direct cocultures.

Direct co-culture (30% sarcoma and 70% fibroblasts)

Volume of Fibroblasts:

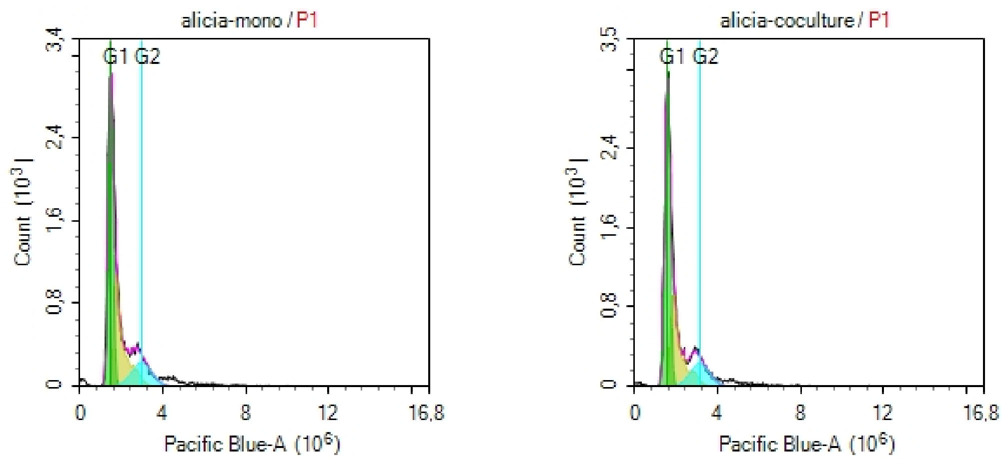
$1 \cdot 10^6$ cells ⇔ 0,404 mL suspension
$0,7 \cdot 10^6$ cells ⇔ 0,282 mL suspension

Volume of Sarcoma:

$3,34 \cdot 10^6$ cells ⇔ 1 mL suspension
$0,3 \cdot 10^6$ cells ⇔ 0,09 mL suspension

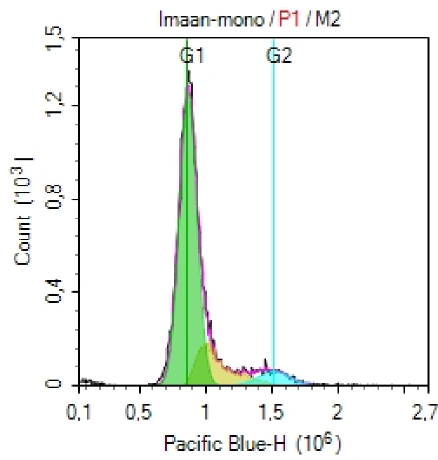
Figure 7: Continuation of calculations.

B Histograms from Flow Cytometry

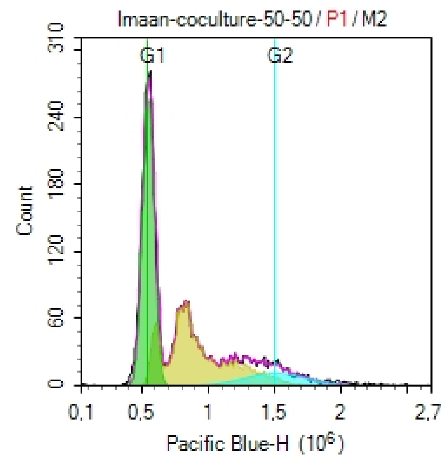


(a) Histogram from monoculture with fibroblasts

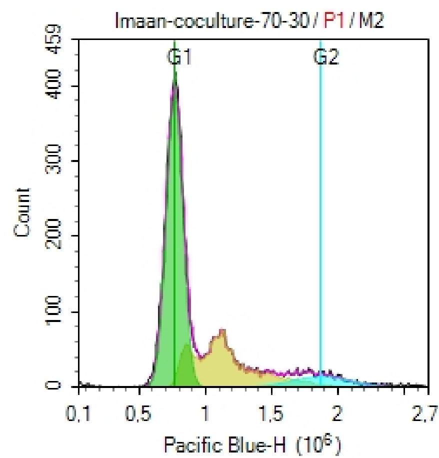
(b) Histogram from indirect coculture with fibroblasts.



(a) Histogram from monoculture with fibroblasts used as reference in direct co-cultures.



(b) Histogram from direct coculture with fibroblasts and the ratio 50/50.



(c) Histogram from direct coculture with fibroblasts and the ratio 70/30.

C List of Materials

Table 4: The chemicals and materials used during the experiment and their reference numbers.

Material	Reference Number
BSA	A3912-500G
CFSE	C34554
DAPI	D9542
DMEM	31885-023
DPBS	14190144
FBS	10500-064
Nucleo Casette	941-0001
PFA	158127
Reagent A100	910-003
Reagent B	910-0002
Syringe Filter	4614
Trypsin	9002-07-7
X-100 Triton	SLCD3084