Research Academy for Young Scientists

Analysis of Sarcomas Effect on the Proliferation of Fibroblasts in Vitro

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July 12, 2023

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Abstract

Cancer is a diverse disease and one of the leading causes of death in the world. Sarcoma is a relatively rare group of cancer with origins in the connective tissue. One aspect of cancer progression that has been put in the spotlight during later years is cancer associated fibroblasts (CAFs). There is little research regarding sarcomas correlation with CAFs and how primary fibroblasts may be activated by sarcoma cells and turned into CAFs. Therefore, the aim of this study is to analyse osteosarcomas effect on the rate of proliferation of primary fibroblasts, in vitro.

Flow cytometry was used to do a cell cycle analysis. The cell cycle analysis was executed in two separate experiments. The first compared one monoculture of fibroblasts with one indirect coculture containing fibroblasts and cell media from osteosarcoma. The second compared one monoculture of fibroblasts with one direct coculture with a 70:30 ratio of fibroblasts and osteosarcoma as well as one direct coculture containing a 50:50 ratio of fibroblasts and sarcoma.

The analysis showed a higher rate of proliferation in the direct cocultures compared to the other cultures. This may indicate that the osteosarcoma might increase the speed of proliferation of the fibroblasts in vitro. Further studies on the cell behavior of fibroblasts and osteosarcoma cells are sugested.

Acknowledgements

I would like to show my gratitude for my mentor, Dr. Wiem Chaabane Sivlér, for her unwavering support, providence of materials, aid and guidance through this project. This study would not have been able to be conducted without her.

Thank you to my co-worker and lab partner Imaan Muqbil, for the knowledge and help offered throughout this process.

Thanks to The Karolinska Institute for so graciously allowing us to conduct our research in their facilities.

A huge thanks to all of the amazing students at Rays–for Excellence for their relentless wit, motivation and energy, something which has greatly influenced not only this project but me personally. You have made this opportunity a once in a lifetime experience and I am forever grateful.

I wish to express a sincere thank you to the Rays 2023 counsellors, Julia Mårtensson, Linnea Jonröd, Max Eriksson and Viktor Sundström for their around-the-clock support, whether it was emotional or laboratory. Their fantastic lectures and helpful advice were invaluable during this process.

Credit is also due to Robert Khasan, Linnea Jonröd, Max Kenning and Johan Alm for their help during the writing process.

I would like to show my appreciation to Beijerstiftelsen and Bengt Lundqvists minne for making this summer research school possible.

Lastly, I would like to thank Rays – for Excellence for granting me this priceless opportunity.

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1 Introduction

Cancer is a large group of deceases and the second most common cause of death in the world. 9,6 million, or 1 in 6 deaths could be attributed to cancer worldwide [1]. Up to 50% of those deaths could have been avoided by taking preventative measures [1]. One relatively rare type of cancer is sarcoma with 400 adults and 35 children being diagnosed with sarcoma of the around 70 000 total cancer diagnoses in Sweden each year [2, 3].

Sarcoma is a collective name for over 70 different cancers and originates from the bodies supportive tissue, i.e. bone, fat tissue and blood vessels. The different types of sarcoma are often sorted into one of two categories: soft tissue sarcoma which originates in the soft tissue of the body or bone cancer.[4]

Osteosarcoma is the most common form of bone cancer with diagnosed patient often being in the pediatric demographic. [5]

The cancer micro environment greatly effects on cancer development. One group of cells with a large effect on the cancers environment is fibroblasts. Fibroblasts produce an extracellular matrix (ECM) which provides support for and aids the function of surrounding areas. [6]

The aim of this study is to understand the role of sarcoma cells in the activation of primary fibroblasts and, create a broader knowledge base for future development of cancer treatments.

1.1 Cell Cycle

The cell cycle consists of a series of phases that a cell undergoes to grow and divide. It is made up of four main phases: G1, S, G2, and M. In the G1 phase, the cell grows and prepares for DNA replication. The S phase involves DNA synthesis, where the genetic material is duplicated. During the G2 phase, the cell continues to grow and prepares for cell division. The M phase is the process of actual cell division. [7]

Two additional phases are firstly sub G1, sometimes called G0 phase, and secondly super G2. Cells in the sub G1 phase are defined by a having a lower DNA content compared to cells in other phases of the cell cycle. Whereas cells in the super G2 phase contain an

abnormally high amount of DNA. This means that cells in the sub G1 phase have lost DNA and cells in the super G2 phase have gained DNA by several cells being clumped together for example. [8]

1.2 The Mechanisms of Cancer

Cancer development is closely linked to the cell cycle and its regulatory mechanisms. Genetic mutations in key genes, such as oncogenes and tumor suppressor genes, disrupt the normal control of the cell cycle. Mutations can promote excessive cell division, due to oncogenes being activated, or inhibit cell death which is caused by tumor suppressor genes, leading to uncontrolled cell build up. Cancer cells often bypass cell cycle checkpoints, allowing damaged cells to continue dividing. Abnormalities in cell cycle regulators can also contribute to cancer progression. [9]

1.3 Fibroblasts

Fibroblasts are a type of connective tissue cell that plays a crucial role in the maintenance and repair of tissues. They are responsible for synthesizing and organizing the ECM. This refers to the network of molecules surrounding and supporting the cells within tissues. It provides structural integrity, mechanical support and biomedical cues for a verity of cellular functions. The ECM influences cell behavior, migration and proliferation. Fibroblasts secrete collagen, elastin, and other components of the ECM, contributing to tissue integrity and wound healing. Fibroblasts can be implicated in the development and progression of certain types of sarcomas. Fibroblasts in sarcomas can exhibit abnormal behavior, including increased proliferation and invasion into surrounding tissues. [10]

1.4 Tumour Micro Environment

The tumor microenvironment refers to the complex network of cells and non-cellular components present within and around a tumor. It consists of cancer cells, stromal cells (such as fibroblasts and immune cells), the extracellular matrix (ECM), blood vessels, and signaling molecules. [11]

In cancer, alterations in the ECM can contribute to tumor development, invasion, and metastasis. Understanding the ECM's role in osteosarcoma can lead to insights into tumor progression and the development of therapeutic strategies targeting the ECM.

The osteosarcomatic microenvironment plays a crucial role in tumor growth, invasion, metastasis, and response to treatment. Cancer cells interact with stromal cells and ECM components, which may promote tumor cell survival, proliferation, and migration. The immune cells present in the microenvironment can have both tumor-promoting and tumor-suppressing effects. Understanding the osteosarcomatic microenvironment is vital for developing effective therapeutic strategies that target not only cancer cells but also the supporting components within the tumor.

1.5 Growth Factors

In cancer, growth factors are proteins that play a significant role in promoting tumor development and progression. Cancer cells may overproduce signaling proteins leading to uncontrolled cell growth. Autocrine stimulation occurs when cancer cells facilitate growth factors that act on their own receptors, promoting self-stimulation of growth signals. Growth factors also contribute to angeogenesis.

Angeogenesis is when cancer cells release signaling molecules, such as vascular endothelial growth factor, that stimulate the growth of new blood vessels from existing ones. Inhibiting angiogenesis is an important therapeutic strategy in cancer treatment to starve tumors of their blood supply and impede their growth. [12]

Additionally, growth factors can enhance cancer cell invasion and metastasis by promoting cell motility and remodeling the extracellular matrix. Understanding the role of growth factors in cancer is important for developing targeted therapies to disrupt their signaling pathways and inhibit tumor progression. [13]

1.6 Flow Cytometry

Flow cytometry is used in biology and medicine to analyze the physical and chemical characteristics of cells or particles in a fluid suspension. It involves the measurement of

scattered light and fluorescence emitted by cells passing through a laser. By labeling cells with fluorescent markers specific to certain proteins or molecules, flow cytometry can provide detailed information about cell populations including cell size and intracellular components. It enables the identification, quantification, and sorting of different cell types enabaling study of immune cells, characterization of cancer cells, and investigation of various cellular processes in research and clinical settings. [14]

In this study, flow cytometry is used to study which phase in the cell cycle the cells currently are in. By first fixing the cells and then staining the DNA. The florescence is greater in the cells containing a larger amount of DNA, the cells in later stages of the cell cycle. The cultures with a higher percentage of cells in the later stages of the cell cycle have are considered to have a higher rate of proliferation in comparison with cultures with a lower percentage of cells in the later stages of the cell cycle.

2 Method

The use of flow cytometry is both precise and efficient if done correctly. This is an essential part of the experiment since that is when sarcoma has time to effect the proliferation of fibroblasts. Since several types of cells were used in some samples, the method needed to be able to distinguish which cells are the fibroblasts relevant for the study. The large variety of staining techniques make it possible to label the cells differently.

2.1 Materials

For materials, see section A.1 List of Materials in Appendix.

2.2 Patient Characteristics

The sarcoma cancer cells used in this study were osteosarcoma cells collected from a 14 year old male and is authenticated by European Collection of Authenticated Cell Cultures.

The fibroblasts used in this study were primary fibroblasts collected form healthy skin tissue.

2.3 Cultivating the Cells

Cultivating the cells was done in order to let the fibroblasts grow in different environments to see the potential effects on the cells proliferation.

2.3.1 Fibroblast Cell Suspension

Firstly, a microscope was used to make sure the solution containing the primary fibroblasts was confluent. All cell medium was removed from the flask using a micro pipett. The fibroblasts were washed with DPBS. 1 mL of trypsin was added and the flask was left to incubated for 5 min until all fibroblasts had detached from the flasks side. Thereafter, 9 mL of cell medium was added and the resulting solution was transferred to a falcon tube in order for it to be centrifuged for 5 min with 1100 RPM. All medium was removed from the falcon tube, leaving a pellet of cells behind. 3 mL of new cell medium was added and sample was mixed. Then, 1 mL was removed with a 1:3 ratio and placed aside for further cultivation.

2.3.2 Cell Count and Split

Thereafter, the remaining 2 mL of the solution where used for cell counting and cultivation. Essential steps for the rest of the study. 100 µL of the sample were extracted and placed into an Eppendorf-tube. 100 µL of reagent A were added and the resulting solution was mixed using vortex for 5 s. 100 µL of reagent B were added and the resulting solution was mixed using vortex for 5 s. The solution was sampled using a NucleusCassette which was placed into a NucleoCounter. The amount of cells per mL in the analysed solution was observed from the NucleoCounter. To make the cultivation solution 850 000 cells where needed. The volume containing this amount of cells was calculated based on the cell count and the resulting volume was put into flasks for cultivation. 10 mL of cell medium was added and the flask was rotated and microscoped to insure an even distribution of cells. This was repeated twice to create to cell cultivation which were placed in the incubator. The two additional cell cultivation's were to be used as the monoculture and indirect coculture.

2.3.3 Cultivation of Indirect Coculture

Microscoping of the fibroblasts was done to ensure healthy cells and a confluence of 40-50%. The sarcoma culture was microscoped to ensure healthy cells and a confluence of 60%. All cell medium from the sarcoma culture was collected and put into a syringe with a filter of 0.45 µm. The medium was filtered into a falcon tube. All cell medium was removed from one of the flasks containing fibroblasts and replaced with the medium from the sarcoma culture. This was left to incubate for 48 h together with the untouched fibroblast monoculture.

2.3.4 Cultivation of Direct Coculture

Three different cultures were prepared: The first contained 100% fibroblasts, the next contained 50% fibroblasts and 50% sarcoma cells, the last contained 70% fibroblasts and 30% sarcoma cells. First, cell suspension of the fibroblasts and sarcoma cells was executed, followed by cell count. The fibroblast were suspended in 2 mL of DPBS. 2 µL of CFSE was added and the solution was left to stain in the incubator for 20 min. An additional 2 mL of cell medium was added with 10% FBS to end the staining process and incubated for 5 min. This was to disable the CFSE from staining the osteosarcoma cells in later steps. Centrifuging of the solution was done and the supernatant was discarded. Resuspension of the fibroblasts was done in 4 mL of cell medium. According to the cell count, the volume of solution was calculated so that each coculture would contain 1 000 000 cells each with the above stated ratios. This was then added into three different flasks with 10 mL of cell medium. The flask was rotated and microscoped to insure an even distribution of cells. All cultures were then incubated for 72 h.

2.4 Flow Cytometry

Cell cycle analysis was executed in two experiments. The first compared one monoculture of fibroblasts with one indirect coculture containing fibroblasts and cell medium from osteosarcoma. The second compared one monoculture of fibroblasts with two direct co-cultures with different ratios of fibroblasts and osteosarcoma. One with a 50:50 split and

the other with a 70:30 split, 70%fibroblasts and 30% sarcoma.

2.4.1 Cell Fixation

Cell suspension was done and then each tube was washed with DPBS which was then removed after centrifugation. 150 µL of PFA was added and the cells were fixed in room temperature for 15 min. The sampels were then centrifuged at 3500 RPM for 5 min and the PFA was removed. Washing of the cells was then executed with PBS, making sure all PFA was removed since this could disturb the permeabilization.

2.4.2 Permeabilizing and Staining of the Cells

In order to stain the DNA in the fibroblasts, the cell membrane would have to be permabelized. Therefore, an antibody dilution buffer was prepared by adding BSA into DPBS to create a 0.5% buffer. After that, 150 µL of Triton X-100 was added to create a permebilizing buffer.

After fixing and washing of the cells, 1 µL of DAPI was added and the solution was transferred to the flow cytometry tubes which was left to stain the cells for 60 min before the flow cytometry analysis was performed.

2.4.3 Cell Cycle Analysis

The samples were placed in the NovoCyte for cell cycle analysis.

3 Results

The results of the flow cytometry analysis in the different experiments were as follows:

3.1 Indirect Coculture Analysis

As seen in Table 1 differences between the cell cycle analysis between the monoculture and the indirect coculture were minimal, however the distribution between the cell cycle phases were noticeable. The amount of cells was largest in G1, with S phase having noticeably

Table 1: Distribution in percent of cells from monoculture and indirect coculture in different phases of the cell cycle

Culture	G1	S	G2	Sub G1	Super G2
Monoculture	44.58 %	33.79 %	15.82 %	1.35 %	4.27 %
Indirect Coculture	48.52 %	31.48 %	15.28 %	0.9 %	3.83 %

less cells and even less in cells in G2 phase. Cells in sub G1 and super G2 were negligible due to their relatively low percentage and relevance to the study.

3.2 Direct Coculture Analysis

Table 2: Percentage of green cells each culture. It is only the green cells which are analysed in the cell cycle analysis

Monoculture	Direct Coculture 70:30	Direct Coculture 50:50
99.08 %	34.96 %	21.85 %

A substantial difference in the proportion of analysed cells in relation to the total amount of cells could be observed in Table 2. Almost all cells could be analysed in the monoculture compared to the 70:30 coculture with only about one third of the cells being analysed and even less with the 50:50 coculture.

Table 3: Distribution in percent of cells from monoculture and direct coculture in different phases of the cell cycle

Culture	G1	S	G2	Sub G1	Super G2
Monoculture	67.1 %	22.56 %	7.99 %	1.18 %	1.17 %
Direct Coculture 70:30	55.08 %	36.13 %	8.03 %	0.71 %	0.06 %
Direct Coculture 50:50	43.93 %	44.82 %	10.7 %	0.3 %	0.25 %

A difference in the amount of cells in could be observed both between the phases of the cell cycle as well as between the cultures. In the monoculture, a substantial difference in the amount of cells in the G1 phase compared to the S phase was noted. However, in

the 50:50 direct coculture, no difference was observed. The difference in the amount of cells in the 70:30 direct coculture in the G1 phase compared to the S phase was noted as smaller than for the monoculture but larger than the 50:50 direct coculture. The amount of cells in the G2, sub G1 and super G2 stayed consistent for the most part.

4 Discussion

Overall, the results appear to follow in line with previous studies and theory. According to the first experiment, the results from the two cultures were similar. This suggests that the fibroblasts' proliferation might not be so affected by the change of cell medium. The results from the second experiment show a variation of proliferation between the different cultures with the cells in the direct cocultures having a higher rate of proliferation. A comparison between the two experiments may indicate that cell contact is crucial of sarcomas effect on proliferation.

4.1 Indirect Coculture

Indirect coculture of the fibroblasts produced somewhat unexpected results, however they may be explained by the change of all media. The fibroblast produce their own growth factors which were removed with the medium when replaced with medium from the osteosarcoma. In addition to this, such an abrupt change in the cells extracellular environment could have resulted in stress placed on the cells which the cells might have been on their way to adapting to. Further research regarding this aspect might be needed. The experiment could be made more intricate if the ratio of fibroblast cell medium and osteosarcoma cell medium was optimized.

4.2 Direct Coculture

During analysis of the percentage of analysed cells, it was apparent that the sarcoma cells had a higher rate of proliferation compared to the fibroblasts because they constituted the majority of cells in the coculture even though the ratio was different in the beginning. This

corresponded well with previous research since osteosarcoma cells as well as cancer cells in general are commonly acknowledged to have a high rate of replication. Furthermore fibroblasts are often considered to have a low rate of proliferation and the final result was as expected.

The varying ratio of cells in the different phases of the cell cycle between the cultures indicate that the osteosarcoma cells had an impact on the proliferation of the fibroblasts. Since more cells were present in the later stages of the cell cycle in the direct cocultures compared to the monoculture the results hint at the osteosarcoma cells promote promote the proliferation of fibroblasts to some extent.

4.3 Further Studies

Further studies should consider optimizing the staining for the particular cell line to ensure the best results possible. It is also important to repeat the same protocol at least three times with different cell lines which produce similar results in order to confirm the significance. Next steps would be to analyse more cell behavior such as migration in vivo. Due to the speed at which fibroblast move through the body, the spread of sarcoma cells due to this would be a meaningful study.

4.4 Conclusion

The clear difference between the cultures show that there is a high probability that the fibroblasts rate of proliferation is affected by the sarcoma cells, however, more data is needed in order for this conclusion to be significant. Moreover, analysis of cell behavior such as cell migration in vivo would give a clearer picture on how fibroblasts and sarcoma cancer interacts which is information that could aid in the development of alternative treatments against osteosarcoma.

References

[1] “Cancer.” https://www.who.int/health-topics/cancer#tab=tab_1. [Online; accessed 4-July-2023].

[2] S. Schultz, “Sarkom.”

[3] J. Munter, D. Pettersson, and L. y. Holmberg, “Statistik om nyupptäckta cancerfall 2021,” *Socialstyrelsen*.

[4] Mayo Clinic Staff, “Sarcoma,” 2023. [Online; accessed 5-July-2023].

[5] S. Prater and B. McKeon, “Osteosarcoma,” *StatPearls*, May 2023. [Online; accessed 8-July-2023].

[6] M. V. Plikus, X. Wang, S. Sinha, E. Forte, S. M. Thompson, E. L. Herzog, R. R. Driskell, N. Rosenthal, J. Biernaskie, and V. Horsley, “Fibroblasts: Origins, definitions, and functions in health and disease,” *Cell*, vol. 184, no. 15, p. 3852–3872, 2021.

[7] “Cell cycle.” https://www.genome.gov/genetics-glossary/Cell-Cycle. [Online; accessed 4-July-2023].

[8] D. Plesca, S. Mazumder, and A. Almasan, “Dna damage response and apoptosis,” *Methods in Enzymology*, vol. 446, pp. 107–122, 2008.

[9] K. Collins, T. Jacks, and N. P. Pavletich, “The cell cycle and cancer,” *Proceedings of the National Academy of Sciences*, vol. 94, no. 7, p. 2776–2778, 1997.

[10] D. A. Mandal, “What are fibroblasts?,” Jun 2023.

[11] M. Ehnman, W. Chaabane, F. Haglund, and P. Tsagkozis, “The tumor microenvironment of pediatric sarcoma: Mesenchymal mechanisms regulating cell migration and metastasis,” *Current Oncology Reports*, vol. 21, no. 10, 2019.

[12] D. Hanahan and R. A. Weinberg, “The hallmarks of cancer,” *Oxford Textbook of Oncology*, p. 3–10, 2016.

[13] E. Witsch, M. Sela, and Y. Yarden, “Roles for growth factors in cancer progression,” *Physiology*, vol. 25, no. 2, p. 85–101, 2010.

[14] “Cell cycle analysis by flow cytometry.” https://www.thermofisher.com/se/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-cycle-assays-flow-cytometry.html. [Online; accessed 8-July-2023].

A Appendix

A collection of relevant information.

A.1 List of Materials

Dulbecco's phosphate-buffered saline (DPBS) - salt solution used to wash the cells, transport cells or dilute cells for counting.

Trypsin - enzyme used to remove the cells from the walls of the flask after cultivation of the cells.

Reagent A - acidic solution used to permeabilize the plasma membrane of the cells, allowing homogeneous staining of the nucleus.

Reagent B - weak alkaline solution used in combination with reagent A to raise the sample's pH value and avoid DNA degradation as well as improve the binding efficiency of DAPI.

NucleusCassette - cell sampling and staining device for viability and cell count applications with staining included.

NucleoCounter - automated cell counter.

Carboxyfluorescein succinimidyl ester (CFSE) - yellow dye.

Fetal bovine serum (FBS) - growth supplement for cells in vitro.

Paraformaldehyde (PFA) - used to fix the cells.

Bovine serum albumin (BSA) - protein to aid cell growth.

Triton X-100 - detergent used to lyse cells in order to enable the extraction of protein or organelles. It is also used to permeabalize the cells.

4',6-diamidino-2-phenylindole (DAPI) - blue dye used to stain the DNA.

A.2 Collection of Flow Cytometry Graphs

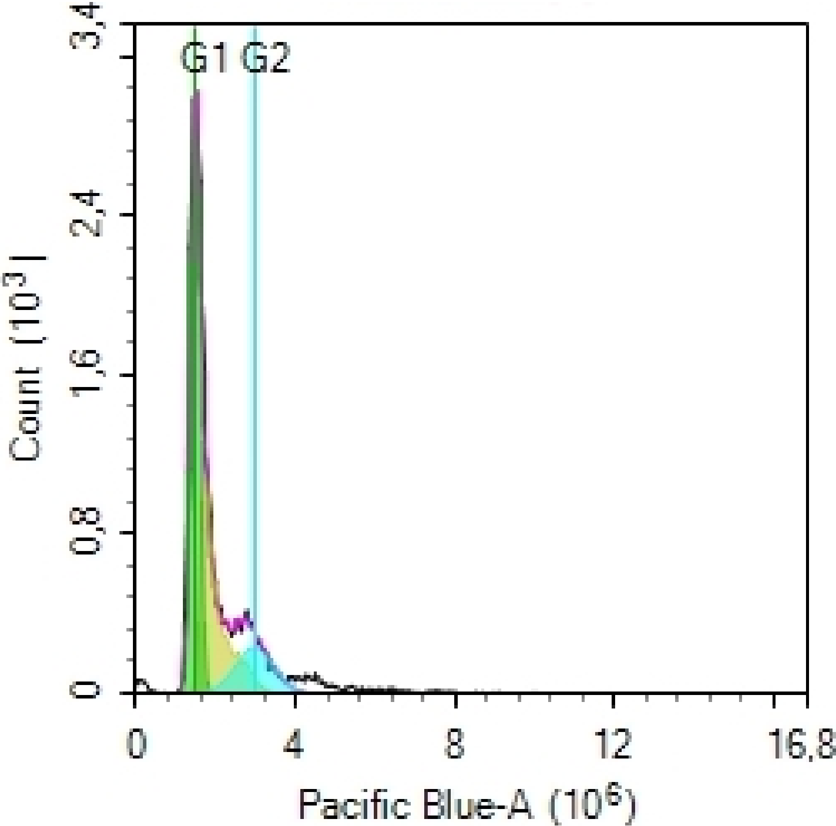

Figure 1: Cell cycle analysis of monoculture after 48 h using flow cytometry

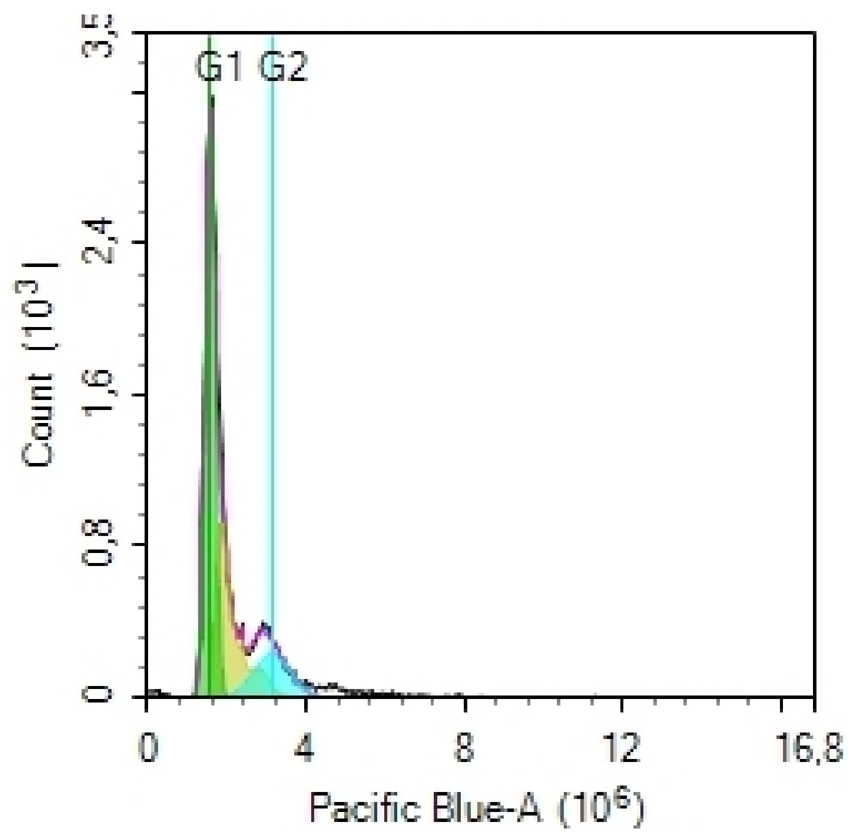

Figure 2: Cell cycle analysis of indirect coculture with 100% sarcoma cell media using flow cytometry

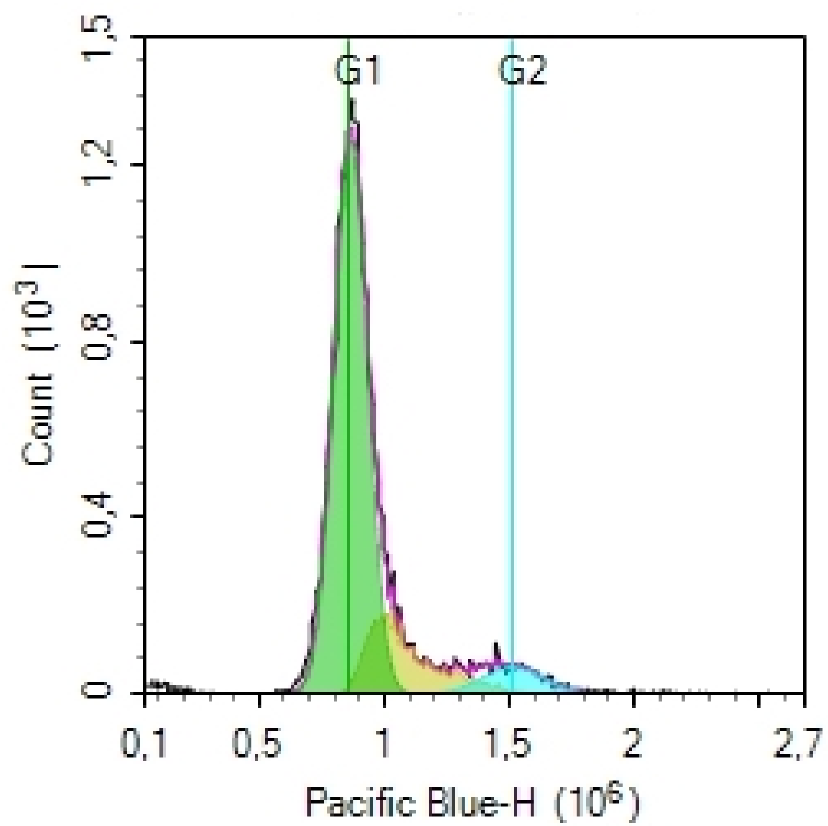

Figure 3: Cell cycle analysis of monoculture after 72 h using flow cytometry

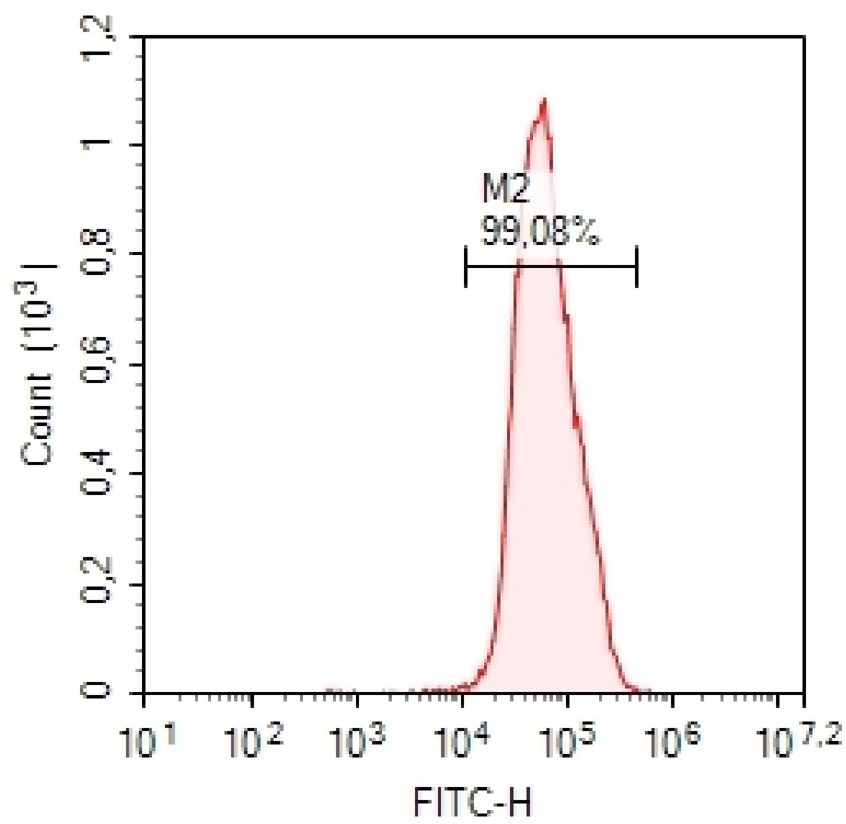

Figure 4: Analysis of dyed cells in monoculture after 72 h using flow cytometry

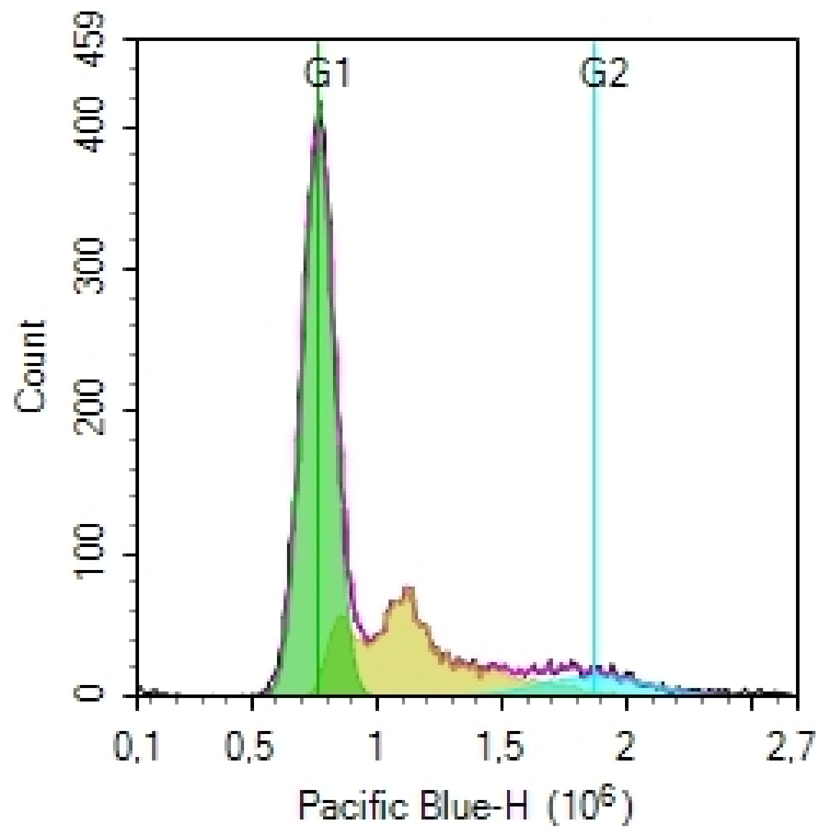

Figure 5: Cell cycle analysis of direct coculture with 70% fibroblasts and 30% sarcoma cells using flow cytometry

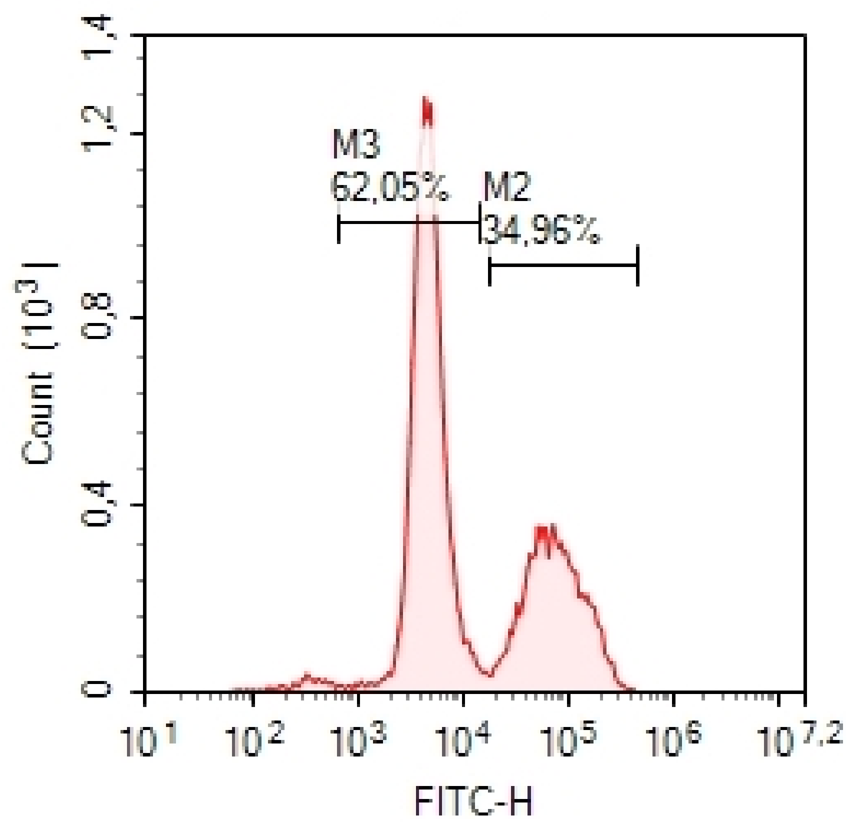

Figure 6: Analysis of dyed cells in direct coculture with 70% fibroblasts and 30% sarcoma cells using flow cytometry

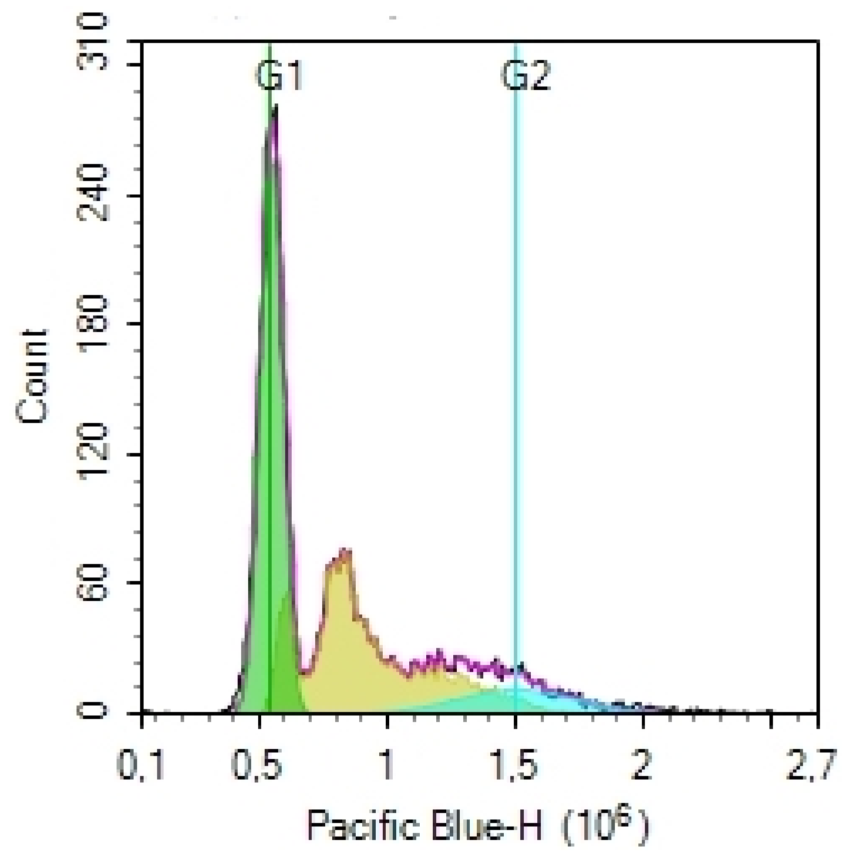

Figure 7: Cell cycle analysis of direct coculture with 50% fibroblasts and 50% sarcoma cells using flow cytometry

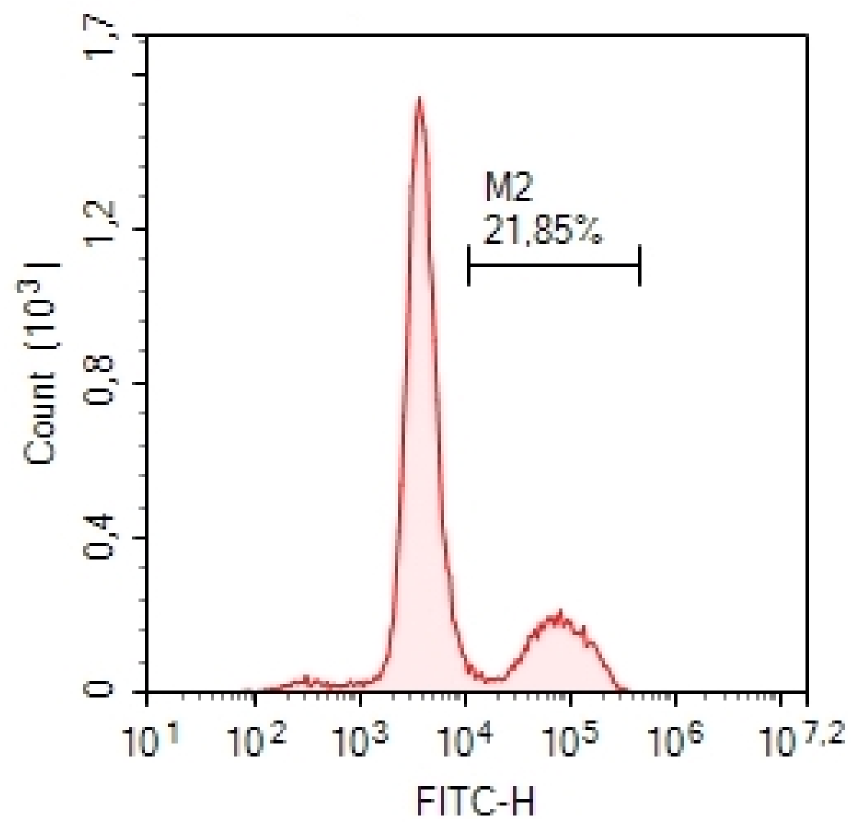

Figure 8: Analysis of dyed cells in direct coculture with 50% fibroblasts and 50% sarcoma cells using flow cytometry