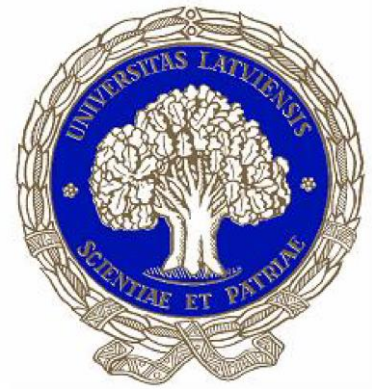

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Faculty of Biology

ZANE SIMSONE

Doctoral Thesis

ANTICANCER DRUG-TOLERANT CELL–MICROCELL CHARACTERIZATION ACCORDING TO STEM CELL LIKE CELL MARKER EXPRESSION

Promotion to the degree of Doctor of Biology

Subfield of Human and Animal Physiology

Supervisor: Tālivaldis Freivalds, Dr. biol.

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Supervisor: lead researcher, Dr. Biol. Tālivaldis Freivalds

Reviewers:

1) *Ph.D., associate professor Zbigņevs Marcinkevičs (University of Latvia);*
2) *Habilitate doctor of medicine, professor Valērija Groma (Riga Stradiņš University);*
3) *Ph.D. Hava K. Avraham (Northeastern University, Center for Drug Discovery and Departments of Pharmaceutical, USA).*

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Chairman of the Doctoral Committee _____/ *Ģederts Ieviņš/*
(paraksts)

Secretary of the Doctoral Committee _____/ *Vita Rovīte/*
(paraksts)

ABSTRACT

Cancer cells are highly heterogeneous and polymorphic within a tumor (Malihi et al. 2018; De Francesco, Sotgia, and Lisanti 2018). The heterogeneity of cancer cells is described at both the genetic and phenotypical level (Rueff and Rodrigues 2016; Gay, Baker and Graham 2016). Cancer resistance and stem cell-like properties, such as proliferation and self-renewal, have been linked to heterogenetic cells (Rueff and Rodrigues 2016; Jin Jin, and Kim 2017; Galofré et al. 2020). Therapeutic resistance in cancer resistance is frequently associated with chemoresistance, which interferes with proliferation (Islam, Gopalan, and Lam 2018; Salmina et al. 2017).

Different cells express various markers to identify cell populations, called biomarkers. Biomarkers are molecules, including proteins, nucleic acids, antibodies, and peptides, among others, and have the potential to identify diseases, cancer, and/or normal cells in patients. To date, studies have shown that cancer stem cells have DNA repair mechanisms, avoiding the effects of medication. Experiments with cell cultures and treating these cultures with chemotherapy have revealed the presence of small cells, with a small amount of cytoplasm that can be intensively stained with azure eosin, called microcells. Microcells developed during sporosis from a damaged tumor macrocell. After anticancer therapy in tumor cells, a defective macrocell may produce one or more microcells.

This study aims to induce microcells using anticancer chemotherapeutic agents and following markers already associated with cancer regeneration, metabolic activity, and resistance to develop criteria for the morphological characterization.

Paclitaxel-treated cancer cells show stronger expression of stem-associated ALDH2, SOX2, and Nanog markers than untreated cells. The proliferation of nuclear antigens in cells, the synthesis of DNA and RNA in microcells indicate cell self-defense, and renewal which promotes resistance to applied therapy. Microcell renewal was observed during the time-lapse experiment, and GFP expression was observed in the microcells. Additionally, the results of the nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) coenzyme activity assay showed that the microcells were still alive after anticancer treatment. Together, these results show that cancer microcells are resilient, capable of withstand the stress brought on by anticancer medications, and susceptible to the intake of chemical components from the environment, such as the incorporation of NRU and GFP into DNA.

KOPSAVILKUMS

Ļaundabīgie audzēji ir viens no biežākajiem mirstības iemesliem un ieņem otro vietu tūlīt aiz sirds un asinsrites sistēmas slimībām ne tikai Latvijā, bet visā pasaulē. Latvijā katru gadu tiek reģistrēti vairāki tūkstoši jaunu onkoloģisko saslimšanu. Turklāt vēl joprojām plaši tiek pielietota ķīmijterapija un staru terapija. Ir pētījumi, kas parāda, vēža šūnu polimorfismu un neviendabīgumu audzējā (Malihi et al. 2018; De Francesco, Sotgia, and Lisanti 2018). Vēža šūnu neviendabīgums tiek saistīts ar vēža rezistenci un cilmes šūnām līdzīgo īpašību veidošanos, piemēram, proliferācija un pašatjaunošanās, kā arī heteroģenētiskās īpašības (Rueff and Rodrigues 2016; Jin, Jin, and Kim 2017; Galofré et al. 2020). Vēža šūnu populācija, kurai piemīt pašatjaunošanās spējas un tā var būt rezistenta vai toleranta pret pielietoto pretvēža terapiju, skaita ziņā ir neliela (Yu et al. 2012; Mamun et al. 2018).

Šīs šūnas var raksturot, izmantojot dažādus marķierus – biomarķierus. Biomarķieri ir molekulas, tādas kā proteīni, nukleīnskābes, antivielas un peptīdi. Izmantojot šos marķierus, var diagnosticēt un identificēt dažādas saslimšanas, tajā skaitā vēzi. Pētījumi par nukleīnskābēm liecina, ka vēža cilmes šūnām līdzīgām šūnām piemīt spēja labot DNS, kas palīdz izvairīties no medikamentu iedarbības. Veicot pētījumus šūnu kultūrās, tās apstrādājot ar ķīmijpreparātiem, ir novērotas maza izmēra šūnas - mikrošūnas ar nelielu citoplazmu un intensīvi krāsotu kodolu. (Buiķis, Harju, and Freivalds 1999). Mikrošūnu attīstība tiek saistīta ar sporozes mehānismu, kad no bojātas audzēja makrošūnas terapijas ietekmē veidojas viena vai vairākas mikrošūnas.

Šī pētījuma mērķis inducēt mikrošūnas, izmantojot pretvēža ķīmijterapijas līdzekļus un noteikt mikrošūnu ekspresētos marķierus, kuri ir saistīti ar vēža reģenerāciju, vielmaiņas aktivitāti un rezistenci, lai izstrādātu mikrošūnu morfoloģiskās raksturošanas kritērijus.

Ar PTX apstrādātās melanomas šūnu kultūras šūnas (Sk-Mel-28) ekspresē ALDH2, SOX2 un Nanog antigēnus izteiktāk nekā kontroles paraugos. PCNA, DNS un RNS sintēze liecina par šūnu pašaizsardzību un pašatjaunošanos. GFP ekspresija un NADPH koenzīma aktivitāte norāda uz šūnu dzīvotspēju un metabolisko aktivitāti. Kopumā pētījuma rezultāti parāda, vēža mikrošūnu izturību pret pielietoto ķīmijterapiju un radīto šūnas stresu, kā arī mikrošūnām piemīt endocitotiskas īpašības ar spējām uzņemt vielas no ekstracelulārās vides, piemēram, neitrāli sarkano krāsvielu un GFP ieslēgšanās DNS.

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ABBREVIATIONS

4/21 cell line – Dzungaria hamster fibroblastoma cell line

ALDH – aldehyde dehydrogenase

ANS – 8-anilinonaphthalene-1-sulfonic acid

Anti-PCNA – anti-proliferating cell nuclear antigen

ATTC – American Type Culture Collection

BRAF, BRAF - Serine/threonine-protein kinase B-Raf, gene

BRCA1/2, BRCA1/2 - Tumor suppressor breast cancer protein 1 and 2, genes

CAD - caspase-activated DNAse

Casp-2 – Cysteine aspartate-specific protease -2, Caspase-2

Casp-3 – Cysteine aspartate-specific protease -3, Caspase-3

Casp-6 – Cysteine aspartate-specific protease -6, Caspase-6

CD24 – the cluster of differentiation 24

CD44 – the cluster of differentiation 44

CPCs – Cancer progenitor cells

CSCs - Cancer stem cells

DMEM - Dulbecco's modified Eagle's medium

DNA – desoxyribonucleic acid

DOX - doxorubicin, a type of chemotherapeutic drug against different types of cancer

DTP cells - drug-tolerant persister cells

EdU - 5-ethynyl-2'-deoxyuridine, a thymidine analog that is an alternative to BrdU

EtBr – ethidium bromide

FBS – fetal bovine serum

FFPE samples – formalin-fixed paraffin-embedded samples

GFP – green fluorescent protein

HCl – hydrochloric acid

HeLa - Human cervical carcinoma

Hs-68 – human skin fibroblasts

ICC staining – immunocytochemical staining

ICIs - immune checkpoint inhibitors

IHC staining – immunohistochemical staining

IOD – integrated optical density, is obtained by deducting the background pixel value of

a certain field of interest from the value of a single pixel

ITH – Intra-tumor heterogeneity

MHC II - major histocompatibility complex

NADPH - β-Nicotinamide adenine dinucleotide phosphate reduced sodium salt hydrate

Nanog – homeobox transcription factor Nanog

NLS - Nuclear localization signal is a short stretch of amino acids that mediates the transport of nuclear proteins into the nucleus.

NRU – neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) uptake

NTB – nitro blue tetrazolium

Oct3/4 - Octamer binding transcription factor 3/4, also known as Oct4 / 4 and POU5F1, is a transcription factor from the POU homeodomain family expressed in embryonic stem cells and germ cells

OD – optical density, the absorption of light by a sample proportional to the density of cells in the sample

PCNA – proliferating cell nuclear antigen

PBS – phosphate buffer saline

PhC – phase contrast

PI – propidium iodide

PI3/AKT - phosphoinositide-3-kinase–protein kinase B/Akt

PTEN - phosphatase and tensin homolog deleted on chromosome ten

PTX – Paclitaxel, a type of chemotherapeutic drug against different types of cancer, could stop cancer cell dividing and proliferation

RNA – ribonucleic acid

SCs – Stem cells

Sk-Mel-28 – Melanoma cell line

SOX2 – SRY-box transcription factor 2

TEM – transmission electron microscopy

Thio-TEPA – triethylenethiophosphoramide, is a trifunctional alkylating agent with a broad spectrum of antitumor activity.

TRIS – tris(hydroxymethyl)aminomethane

TSG101 – tumor susceptibility gene 101

INTRODUCTION

Cancer is the second most common cause of death after cardiovascular diseases. Billions of new cancer cases are registered worldwide. There are estimated over 4 million new cancer cases in Europe in 2020 (Dyba et al. 2021). In addition, nearly two million cancer deaths were indicated in 2020 in Europe (Dyba et al. 2021; OECD/European Union 2022, 6011:98). Despite advanced cancer diagnostics and treatment abilities, the available therapies are not effective enough, and there are hundreds of cases of cancer mortality per month (OECD/European Union 2022; Siegel, Miller, and Jemal 2019). Furthermore, this may indicate the continuous change of cancer cells and their ability to self-renew. It is known that cancer is heterogeneous, consists of multiple types of cells (Gay, Baker, and Graham 2016; Kim et al. 2018). Cancer heterogeneity is the basis of tumor resistance, and this resistance is most often associated with cancer stem cells (Dalerba, Cho, and Clarke 2007; Vallette et al. 2019). Cancer cell resistance could be inherited when tumor cells at the beginning are resistant to applied therapy (Bray et al. 2018; Vallette et al. 2019). Additionally, resistance could be acquired resistance when initially tumor cells respond to anticancer therapy, but during cell proliferation formed therapy or drug-tolerant cells with gene mutations or changes in protein expression (Vallette et al. 2019; Rueff and Rodrigues 2016). Furthermore, cancer cells and their properties are studied for therapy before anticancer drug therapy or cancer immunotherapy, and based on this information, the effect of therapy on tumors is modeled and its effectiveness evaluated (Walker et al. 2017; De Francesco, Sotgia, and Lisanti 2018). However, it is just as important to look at cancer cells after therapy to see how they change over time. Some authors have observed that under the influence of stress, for example, under the influence of triethylenethiophosphoramide, the formation of so-called "microcells" is induced. Microcells are formed from a mother cell or macrocell. Microcells have been described as round or oval in shape, small amount of cytoplasm, and homogeneous, intensively staining nuclei (Freivalds, Buikis, and Harju 1996; Buiķis, Harju, and Freivalds 1999; Buiķis et al. 2002) cells. Microcells show an increased capacity for endocytosis, which indicates their increased metabolic activity and viability (Bema et al. 2008). Other authors have been observed by other authors, such as Bonghan microcells and Raju cells. Bongham microcells are small-sized cells that flow through the Bongham canal and have the pluripotent ability to divide and differentiate in the same way as adult stem cells (Baik et al. 2009; Baik, Dobberstein, and Soh 2007). Raju cells, on the other hand, arise from a

polyploid cell that has formed due to DNA damage and dies as a result of mitotic catastrophe. However, some polyploid cells undergo neosis characterized by karyokinesis (Sundaram et al. 2004). Raju cells have a long mitotic life span. The path of formation of spores and microcells is similar, that is, they are formed from a dying macrocell/polyploid cell (Freivalds, Buikis, and Harju 1996; Sundaram et al. 2004). Therefore, it would be important to identify and study the resistant cancer cell population under the influence of therapy. The focus of this study is based on microcells that have been described as a natural tumor component, but interestingly their relative numbers increase after cancer therapy has been initiated. The hypothesis explains the process of microcell development by sporosis, which is a process of microcell formation from a damaged tumor macrocell.

The novelty of the promotion work: This study is based on microcell research during anti-cancer therapy. Most of the tumor cells are going to die. Otherwise, smaller parts of tumor cells survive by repairing DNA breaks or removing substances from the cell through a P-glycoprotein pump. Cancer stem cells are considered to be cells that have survived anticancer therapy. In turn, microcells develop as a result of anti-cancer therapy from damaged cancer cells. Microcells are viable, small-sized – up to 2 to 5μm tiny, metabolic active with endocytic ability that indicates the cell`s ability to be resistant.

The objective of this study is to use this information to improve cancer treatment therapy. Delaying the process of resistant cells, thus the formation of new clones could significantly improve the effectiveness of classical anticancer therapy.

This study aims to induce microcells using anticancer chemotherapeutic agents and following markers already associated with cancer regeneration, metabolic activity, and resistance to develop criteria for the morphological characterization.

The main **objectives** were as follows:

1. To initiate the formation of microcells and characterize the metabolic activity of microcell viability *in vitro*;

2. Characterize microcells as a possible prognostic factor for the effectiveness of anticancer therapy *in vitro*;

3. To evaluate differences in the integrated optical density of breast cancer cells in histological samples using the Feulgen method;

4. Determine the intensity of EdU synthesis in microcells after exposure to the chemical preparation *in vitro.*

1. LITERATURE REVIEW

1.1. Cancer Biology and Development

Cells that undergo uncontrolled cell division and transformation contribute to the development of cancer. Cancer cells have modified signal pathways and metabolism (Upadhyay 2021; Katzman et al. 2012). Cancer is heterogeneous and consists of polymorphic cell populations in various developmental stages in the same cancer, making it difficult to identify the cancer's origin (Malihi et al. 2018; De Francesco, Sotgia and Lisanti 2018; Rueff and Rodrigues 2016). Cancer is defined as a chronic disease related to uncontrolled cell growth disregarding the normal cell cycle (Murthy and Mathew 2004; Hejmadi 2010). The accumulation of mutations promotes the selectivity of growing cancer cells. These genetic changes can alert to glucose consumption in cells, indicating cancer as a metabolic disease (Gyamfi, Kim, and Choi 2022; Seyfried et al. 2014).

In cancer, a cell subpopulation with high genetic heterogeneity creates an opportunity for the development of a resistant cell population. Most of the time, this is explained by genetic resistance through natural selection. However, genetic mutations occur at random in single cells, suggesting the development of cancer (Islam, Gopalan, and Lam 2018; Fiorillo, Sotgia, and Lisanti 2019). Drug resistance has also been shown not to be associated with genetic alterations, which refer to functional adaptations (Inde and Dixon 2017; Rueff and Rodrigues 2016). DTP (drug-tolerant persister) cancer cells have been found in colorectal cancer cells, basal-like breast cancer cells, and non-Hodgkin’s lymphoma cells (Sharma et al. 2011; Risom et al. 2018; Klener and Klanova 2020). DTP cells reduce growth and alter metabolism, facilitating greater tolerance to antidrug. The population of DTP cells is small, and cells arise with a low incidence of about 0.3% to 5% (Sharma et al. 2011; Bell and Gilan 2020; Rehman et al. 2021).

There are two main hypotheses for cancer development. The first cancer develops from cancer stem cells, which are a rare cancer subpopulation associated with malignancy, prognosis of therapy, and avoidance of it (De Francesco, Sotgia, and Lisanti 2018; Debeb et al. 2010). The second - characteristic changes during the development of the cancer cell population with the emergence of cell subpopulations of altered and even aggressive subpopulations of cancer cells (De Francesco, Sotgia, and Lisanti 2018; Greaves and Maley 2012).

1.1.1. Cancer Cell Clonality

One of the hallmarks of cancer is clonality, the formation of tumors from single cells or many that proliferate abnormally (Cooper and Hausman 2000; Nowell 1976; Cheng et al. 2013). However, the clonal origin of cancer does not indicate that the primary cancer cell or cancer stem cell causes a tumor to rise making evolutional neoplasia (Nowell 1976; Nicoś and Krawczyk 2022). As well it does not mean cancer-origin cells have initially acquired all of the characteristics of a cancer at all (Nicoś and Krawczyk 2022; Muzza et al. 2021; Cooper and Hausman 2000).

Cancer clonality can be explained in four ways: linear, branching, neutral, and punctuated, as it is indicated in Figure 1.

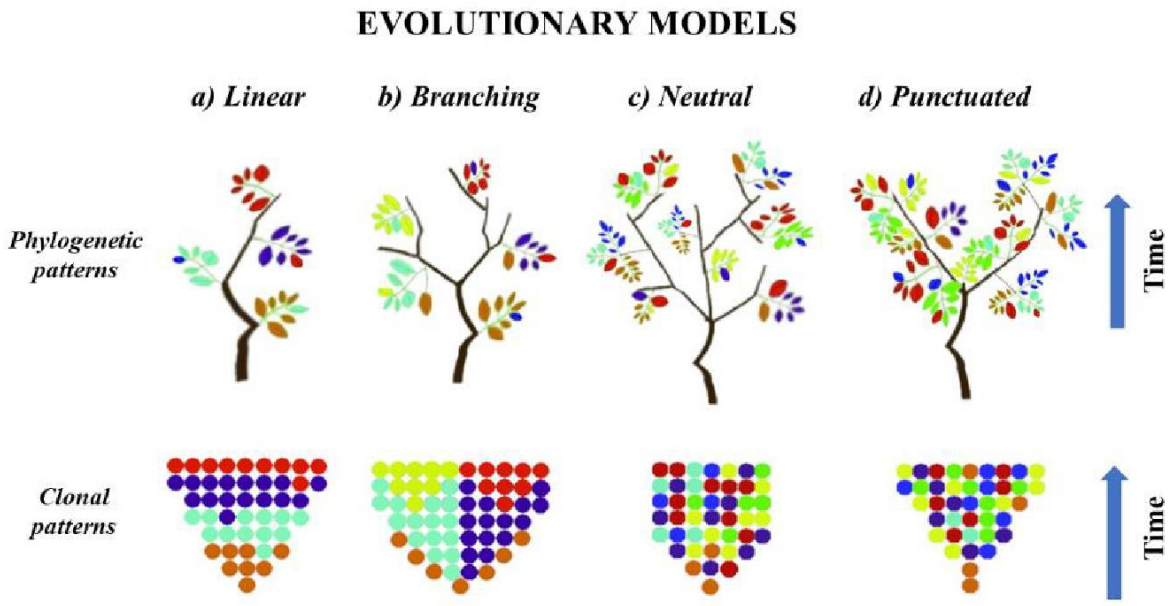


Figure 1. Clonal proliferation of cancer cells explained by evolutionary models. Dots and leaves of branches indicate genetically different cancer cell clones. (A) Linear model represents selective spindling away based on the dominant genotype. (B) The branching model represents multiple clone selection simultaneously. (C) Neutral model represents a lack of selective spindling, but shows adventitious accumulation of the genetic variation due to time. (D) Punctuated model represents a lack of selective spindling, but shows the appearance of the genetically diverse cancer type at the early stage of the tumorigeneses, without further subclones selection (adapted after Nicoś and Krawczyk 2022).

The cancer cell clone that consists of favorable genomic background rises and becomes dominant selectively spindled out weaker clones, in the linear clonal model (Greaves and Maley

2012; Davis, Gao, and Navin 2017). Cancer mass forms from the tumor dominant clone that expands in the early stage of tumorigenes by subclonal diversity in the branching, neutral and punctuated clonal models. Multiple subclonal lineages develop parallelly origin tumor environment cells and exist independently within the primary tumor creating wide intra-tumor heterogeneity (ITH), in the branching model (Nicoś and Krawczyk 2022; Davis, Gao, and Navin 2017; Mahalingam 2018).

Multiple subclonal lineages develop parallel origin tumor environment cells and exist independently within the primary tumor creating wide intratumor heterogeneity, in the branching model. Furthermore, in the branching model, clonal cells could form an aggressive subclone that leads heterogenous cancer profile (Reiter et al. 2018; Nicoś and Krawczyk 2022; McGranahan and Swanton 2017).

Whereas, punctuates and neutral models perform a random accumulation of changes resulting in ITH (Nicoś and Krawczyk 2022; McGranahan and Swanton 2017). Meanwhile, the progression of the cancer occurs by tumorigenesis at the beginning, in these two models (Reiter et al. 2018; Nicoś and Krawczyk 2022). The main difference between the neutral and punctuated models is that genomic changes pass in a short time in the earliest stages of the tumorigenesis in the punctuated model. However, in the neutral model, genomic dispersion is underway fast in the late stages (Davis, Gao, and Navin 2017; Shlush and Hershkovitz 2015; Dang et al. 2020).

All of these clonal patterns have different clinical challenges. Linear and punctuated evolution patterns represent the whole tumor by a single biopsy. The linear evolution model has low IHT, while the punctuated model has early generated IHT with rare subclone formation (Nicoś and Krawczyk 2022; Kim et al. 2018). Branching and neutral evolutional models show a vast IHT and there is a need for spatial and temporal biopsy (Kim et al. 2018).

Analyzing the literature on the origin models of cancers, it can be seen that all evolutionary models have a single ancestor cell. This cell is called a stem cell, more specifically, a cancer stem cell or stem cell-like cancer cell (Gay, Baker, and Graham 2016).

1.2. Cancer Progenitor Cells

Progenitor cells, known as stem cells, can proliferate longer than nonstem cells, as well as having the properties to create multiple cell types in the whole organism. These abilities indicate that progenitor cells are not differentiated (Ju et al. 2022; Lozach 2020; Gaillochet and Lohmann 2015). Nevertheless, cancer progenitor cells have proliferation potential. Normal

stem cells are normally in rest or inactive quiescent state (in G1/G0) and are preserved from cell damage or mutation (Ju et al. 2022).

Cancer progenitor cells (CPCs) have characteristics as stem-cell-like cells and are considered to be a main cause of tumor heterogeneity because they are capable of producing the full repertoire of cancer cell types (Jin, Jin, and Kim 2017; De Francesco, Sotgia, and Lisanti 2018; Piña-Sánchez et al. 2021). In addition, CPCs can form tumors; in contrast, non-tumorigenic cancer cells cannot form. CPCs can differentiate into multiple cell types such as persistent tumor or metastatic cells, as well as causing tumor relapse (Walcher et al. 2020). The environment and/ or specific stimuli can encourage the rise of new stem cells as cells that self-maintain the ability to dedifferentiate and return to primitive states of the development (Aponte and Caicedo 2017; Jin Jin, and Kim 2017). There are multiple theories and hypotheses of cancer stem cells (CSCs) origin (Figure 2). The hypothesis most described and analyzed is that CSCs form from progenitor cells, stem cells or differentiated cells present in adult tissue both somatic and germ cells (Bell and Gilan 2020; Karakaş, Cevatemre, and Ulukaya 2014; Aponte and Caicedo 2017; Pribluda, De La Cruz, and Jackson 2015).

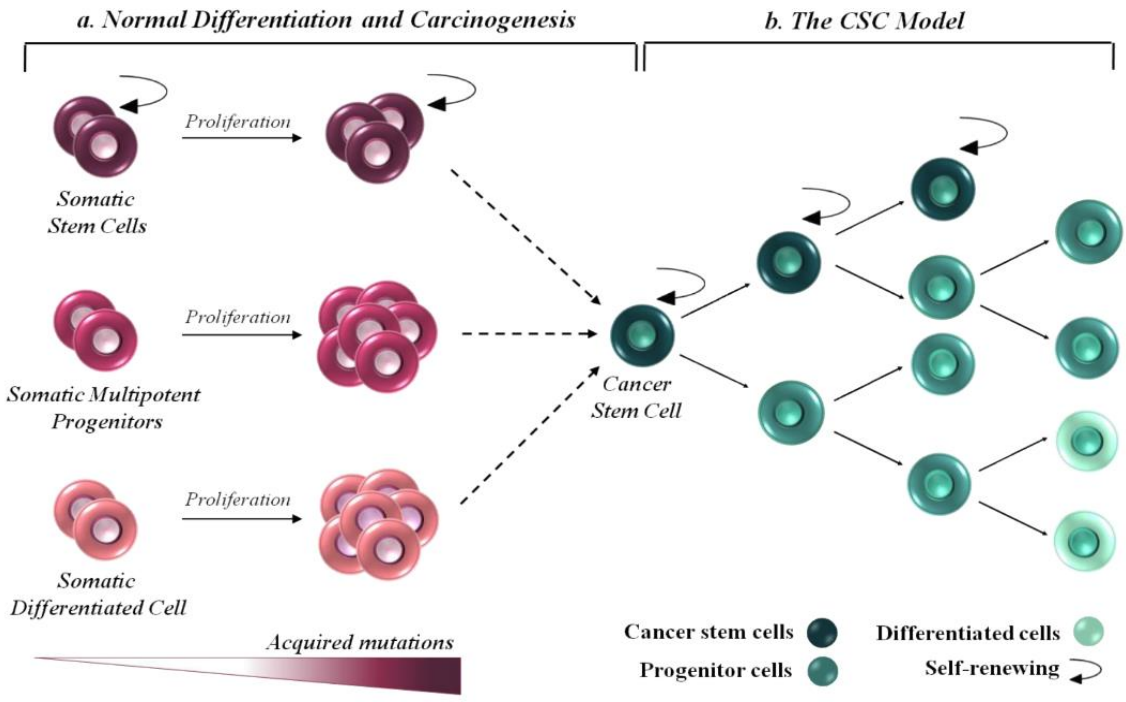


Figure 2. Cancer stem cell hypothetical origin models. (**A**) Normal somatic stem cells, somatic multipotent progenitors, and somatic adult cell differentiation and mutation accumulation during the proliferation process result in cancer stem cells (CSCs). (**B**) Cancer stem cells develop from different cells in various ways. Cancer stem cells can self-renew and proliferate as progenitor cells (Karakaş, Cevatemre, and Ulukaya 2014).

Cancer stem cells could originate from mutated normal stem cells during self-renewal ways. The other opinion is that CSC originations from differentiated cells acquire stem cell-like properties after several mutations. These functions include the self-renewal property and progenitor generation through asymmetrical division to create more committed progenitor cells or differentiated cells (Dalerba, Cho, and Clarke 2007; Karakaş, Cevatemre, and Ulukaya 2014). Cell differentiation promotes multiple gene mutation formation resulting in cell genomic instability or oncogene-induced plasticity (Walcher et al. 2020; Hotfilder et al. 2018; Karakaş, Cevatemre, and Ulukaya 2014). The heterogeneity as well as hierarchy development results from asymmetric division of CSCs (Karakaş, Cevatemre and Ulukaya 2014). Genetic instability may contribute to the development of drug-resistant cancer stem cells. However, drug resistance can be non-genetic but phenotypically tolerant to applied anti-cancer drugs (Risom et al. 2018; Marine, Dawson, and Dawson 2020; Inde and Dixon 2017).

1.3. Cancer cell drug resistance

Chemotherapy and radiation therapy are the most widely used anticancer treatments worldwide in most cases. Furthermore, during this anticancer treatment, a resistant cell population with different subpopulations is developed (Mirzaei et al. 2022; Schaefer and Lengerke 2020).

Cancer drug resistance is the avoidance by cancer cells of anti-cancer treatment, mainly chemotherapy and radiotherapy (Kohsaka et al. 2019; Chang 2016). Drug resistance is mostly associated with genetic changes in the cancer cell, which creates resistant cell clones. One of the characteristics of cancer resistance with drug resistance to the tumor and consistent before therapy starts – is intrinsic resistance. The tumor is reduced, but does not disappear and stops reacting to the treatment and starts regrowing (Wang, Zhang, and Chen 2019; Bai et al. 2020). The second character is outer or extrinsic resistance, which develops into the tumor cells reacting to the treatment. This kind of reaction creates gene mutation or protein expression changes that allow it to be resistant (Wang, Zhang, and Chen 2019; Bai et al. 2020). Thus, several different cell lines from one tumor. However, there are also drug-tolerant cancer cells called drug-tolerant persister cells. These cells develop in drug-free media, in cell cultivation *in vitro*, after treatment. DTP cells recover proliferation without acquired mutational resistance, and the new cell population is just as sensitive as the parental population (Recasens and Munoz 2019; Risom et al. 2018).

1.3.1. Genetic cancer resistance

Genetic changes could occur due to cancer cell proliferation as well as under the influence of external factors such as anticancer treatment. Therefore, cancer resistance is assigned by acquired and inherited genetic changes. The resistance cancer subclone population could adapt rapidly to applied therapy and the cancer intratumor environment (Rueff and Rodrigues 2016). The inherited mutations are only a small proportion, 5 – 10%, of many cancers resulting in a high risk of developing specific cancers (Jefford, Wu, and Irminger-Finger 2006; Hodgson 2008). Tumor suppressor genes, oncogenes, genes encoding proteins involved in DNA repair, and cell cycle control may be involved in causing relapse of cancer and angiogenic process.

At initial treatment cancer cells respond to therapy, immunotherapy is effective, but could relapse or progress after a period of applied treatment, called acquired cancer genetic resistance (Bai et al. 2020). Increased resistance of patients to the use of immune checkpoint inhibitors (ICIs) has appeared, especially in melanoma patients (Bai et al. 2020).

Common genetic changes associated with a mutation in BRAF, BRAF, BRCA1/2, BRCA1/2 in melanoma, breast, ovarian and prostate cancer (Castro and Eeles 2012; Thang et al. 2015; Tiago et al. 2014; Boussios et al. 2022). BRCA1/BRCA2 is the breast cancer suppressor gene of the breast cancer protein 1 and 2 genes, which are associated with up to 67% higher risk of breast cancer in life (O'Donnell, Axilbund and Euhus 2018; Meijers-Heijboer et al. 2001). BRAF encodes the proto-oncogene of B-Raf and serine/threonine kinases. BRAF gene mutations are the most frequently identified mutation in melanoma, as well as colorectal cancer, thyroid carcinoma, non-small cell lung carcinoma, non-Hodgkin lymphoma, hairy cell leukemia, and adenocarcinoma of the lung (Śmiech et al. 2020; Ko et al. 2019; Thang et al. 2015; "BRAF B-Raf Proto-Oncogene, Serine/Threonine Kinase [Homo Sapiens (Human)]" 2023).

1.3.2. Non-genetic Cancer Resistance and Drug Tolerant Persister Cells

The non-genetic and epigenetic changes in the tumor cell are changes in the activity of genes that appear independently from DNA sequencing variety (Bell and Gilan 2020; Marine, Dawson, and Dawson 2020). It is defined that the cell condition accidental changes could promote non-genetic cell-cell variability. These changes are essential in maintaining a single-

cell population as cancer. One of those types of variation is in the biological signal of information pathways according to intracellular cascades (Marine, Dawson, and Dawson 2020).

Genetic mutations occur at random in single cells, suggesting the development of cancer (Islam, Gopalan, and Lam 2018; Fiorillo, Sotgia, and Lisanti 2019). Drug resistance has also been shown to not be associated with genetic alterations, referring to functional adaptations (Rueff and Rodrigues 2016; Inde and Dixon 2017). Drug-tolerant persister cells (DTP) are characterized as non-genetic anticancer drug resistant cells, especially against chemotherapy (Rehman et al. 2021; Risom et al. 2018). DTP cancer cells have been found in colorectal cancer cells, basal-like breast cancer cells, and non-Hodgkin lymphoma cells. DTP cells reduce growth and alter metabolism, facilitating greater tolerance (Sharma et al. 2011; Risom et al. 2018; Klener and Klanova 2020). The DTP cell population is small, and cells arise at a low incidence of about 0.3% to 5%. Resistance to DTP cells may be reversible after treatment if proliferation is low (Bell and Gilan 2020; Rehman et al. 2021). However, occasionally, DTP cells have rare mutations that occur in a dormant state and these cells can proliferate in the presence of anticancer agents (Recasens and Munoz 2019).

1.4. Cancer stem cell markers

Each cell in an organism has its multiple characteristics, and these cells can be identified by their markers. Cell markers are variable and different, such as cluster differentiation number (CD), cell surface markers, and markers to identify SCs, CSCs, and metabolic active cells (Brier and Jayanti 2007; Walcher et al. 2020). Markers that indicate stemness are SOX2 and Nanog. SOX2 is involved in the maintenance of stem cells during embryogenesis. It has a major role in the cell regeneration process, reprograming, and homeostasis, and promotes proliferation and cell survival (Mamun et al. 2018). CD24 and CD44 are used as breast cancer stem cell markers (Piña-Sánchez et al. 2021; Islam, Gopalan, and Lam 2018; Freivalds et al. 2012).

1.4.1. Cluster of Differentiation Number 24

Cluster of differentiation 24 (CD24) is a glycoprotein that is typically found on a cell membrane surface (Even-Zohar et al. 2022; Altevogt et al. 2021). An important marker of cell proliferation and clonal expansion in the immune system is CD24 (Altevogt et al. 2021; Tan et al. 2016). CD24 is known to act as a costimulator of T cells during homeostasis and proliferation, while in B cells it is functionally involved in cell activation, differentiation, and

maturation (Tan et al. 2016; Mensah et al. 2018; Malihi et al. 2018). Also, there are findings that CD24 in lymphoid tumor cell lines and various types of cancer (Altevogt et al. 2021; Even-Zohar et al. 2022; Dong et al. 2019).

CD24 expression is detected in cancers and is related to tumor aggressiveness, cell invasion, and stem cell cancer (Mensah et al. 2018; Dong et al. 2019). The expression of the cell membrane marker CD24 is found in human granulosa tumor cell cytoplasm and cell nuclei of human granulosa tumor cells (Dong et al. 2019). CD24 is a glycosyl-phosphatidyl-inositol (GPI) anchored glycoprotein that localized at the plasma membrane in normal cells. However, in cancer cells, CD24 can accumulate in the cytoplasm as a GPI anchor in the endoplasmic reticulum (ER), deposit in lipid droplets, and could be a late endosomal compartment to release extracellular vesicles (EVs) (Altevogt et al. 2021; Dong et al. 2019).

High expression of CD24 in multiple cancers such as the breast, prostate, ovary, pancreas, and bladder were shown to correlate with metastasis and poor prognosis (Even-Zohar et al. 2022). CD24 can cause cell migration into blood flow associated with P-selectin or palates to promote metastasis (Sihombing et al. 2022). CD24 is widely used as a CSC marker, especially in breast cancer in combination with CD44, SOX2, and ALDH (Ortiz-Montero et al. 2018; Allison et al. 2017; Sihombing et al. 2022; Islam, Gopalan, and Lam 2018).

1.4.2. Cluster of Differentiation Number 44

The biological marker CD44 is a protein, a cell surface adhesion molecule, involved in the interaction of cells and cell-matrix (Debeb et al. 2010; Z. Yu et al. 2012). CD44 is a transmembrane protein and its cytoplasmic tail is important in signal transmission to interact with cytoskeletal elements such as actin and spectrin to activate cell migration and adhesion (Yaghobi et al. 2021). The CD44 antigen is expressed in various cell types including B lymphocytes, hepatocytes, endothelial cells, and mesenchymal cells (Novak et al. 2020; Malihi et al. 2018). CD44 activates B lymphocytes and their migration to the target site, a biomarker that also demonstrated high clonogenic capacity *in vitro* and tumorigenicity *in vivo* in human ovarian cancer cell lines (Zhou et al. 2014; Xu et al. 2020).

Based on the physiological function it indicates involvement in tumor metastasizing (Mesrati et al. 2021; Xu et al. 2020). Furthermore, CD44 has discovered that it positively modulates the nuclear factor of erythroid-2-like 2 (the main regulator of antioxidant genes) in DOX-resistant breast cancer cells (Xu et al. 2020; Yaghobi et al. 2021). Although the connection between CD44 and metastasis is known, only CD44 alone cannot cause metastasis

(Gargini et al. 2015; Yong Yang et al. 2017). Furthermore, CD44 in combination with CD24, CD29, CD90, and CD133 has been used as CSCs identificatory to isolate tumor cancer cells in different tumors (Yu et al. 2012). Furthermore, high expression of CD44 predicts poor overall survival, while ALDH-1 coexpression is associated with very negative overall survival in patients with endometrial cancer (Xu et al. 2020; Chen et al. 2018). As a CSC marker, CD44 is the main factor for epithelial-mesenchymal variation, tumor development, and cell cytoskeletal modification. CD44 is involved in multidrug resistance regulation (MDR) and can cause cancer cell phenotypes that are chemically resistant because it affects the efflux of intracellular cytotoxic drugs. It is important to target CD44 markers to provide wide opportunities for successful anticancer chemotreatment (Yaghobi et al. 2021; Mesrati et al. 2021).

1.4.3. SOX2

SOX is the SRY homology box family protein (Schaefer and Lengerke 2020; Novak et al. 2020; Shuchen Zhang 2014). SOX2 expression is observed early in embryonic development (Novak et al. 2020; Shizhen Zhang, Xiong, and Sun 2020). SOX proteins are determined mainly as DNA binding elements and are called high mobility groups (HMGs) associated with mature transcription regulators (i.e. SEX determining factors Y and SRY) so that SOX/Sox proteins are functionally DNA binding elements. It plays an important role in various stages of embryonic development and preservation of independent embryonic stem cells (EMCs) (Schaefer and Lengerke 2020; Zamzam et al. 2021). Several researchers have observed a connection between SOX2 expression and various clinical aggression as the well as development of the cancer resistance including lung, breast, and prostate cancers (Zamzam et al.,2021).

Furthermore, SOX2 has found a strong oncogene-characterized aggressive phenotype in gastric cancer and nonsmall cell lung carcinoma (Schaefer and Lengerke 2020). In addition, *Helicobacter pylori* infections affect SOX2 expression of SOX2 in a gastric cancer. SOX2 was described as a reactivator of PTEN (phosphatase and tensin homologs deleted on chromosome 10) and thus inhibited cell cycle progression by PI3/AKT (phosphoinositide-3-kinase–protein kinase B/Akt) pathways and anti-apoptosis effects (Schaefer and Lengerke 2020; Carbognin et al. 2019; Hemmings and Restuccia 2012).

1.4.4. ALDH

There are various members (ALDH1, ALDH1A3, ALDH2, ALDH3A1) of the ALDH family of proteins, and they are the enzymes that oxidize alcohol (Debeb et al. 2010; Vassalli 2019; Shortall et al. 2021). Furthermore, members of the ALDH family have cell-protective biological significance through aldehyde detoxification of the aldehydes and cell proliferation, differentiation, and survival (Rebollido-Rios et al. 2020). ALDH2 expression is related to high metabolic activity in a cell through the cytosol or mitochondria, distinguished by their electrophoretic motilities (Raha et al. 2014; Allison et al. 2017). ALDH expression indicates the ability of cells to resist treatment and cytotoxic drugs, such as hydro-peroxy-cyclophosphamide (4-HC) and doxorubicin (Yan and Wu 2018; Vassalli 2019). The detoxifying enzyme ALDH is responsible for intracellular oxidation of ALDH; thus acting as a mediator of self-defense and resistance to the alkylating agent and is used in anticancer therapy (Yan and Wu 2018; Raha et al. 2014). A positive ALDH is significantly related to a poor prognosis of mammalian gland and lung cancers. ALDH and its isoform ALDH-1 are being used as functional markers to identify high tumorigeneses and metastatic potential, as to well as detect resistance in various epithelial tumors (Fiorillo, Sotgia and Lisanti 2019; Moharil et al. 2017).

1.4.5. PCNA

The expression of proliferation cell nuclear antigen (PCNA) is mainly is related to cell replication and is involved in DNA repair systems; otherwise, in control cells, PCNA expression is observed in the nuclei and cytoplasm (Zhang et al. 2021; Peng et al. 2018; Golubnitschaja et al. 2020; Simsone et al. 2020). It plays a significant role in cell cycle regulation, especially with high expression during the G1 and S phases (Ye et al. 2020). PCNA expression in cancer cells is more likely as a result of post-translation modification, and it is related to malignant breast cells, melanoma, mesotheliomas, and bladder cancer (Peng et al. 2018; Malkas et al. 2006; Matsushita et al. 2011; Melaiu et al. 2012; Golubnitschaja et al. 2020; Simsone et al. 2020). PCNA is possible to use as a marker of cellular proliferation in both normal and cancer tissues and cells (Woods et al. 1991).

PCNA expression is mostly observed in cancer cell nuclei, not in normal cells (Ye et al., 2020; Malkas et al. 2006). In some studies, PCNA expression was observed in the cancer cell cytoplasm not in normal cells and it is related to cell stress regulation (Malkas et al. 2006; Wang

et al. 2018; Røst et al. 2020). The strong expression in cancer cell nuclei is significantly associated with malignant tumors, and the high expression has been related to pathological grade, TNM (tumor, node, and metastasis) stage, and the number of metastases (Ye et al. 2020).

1.4.6. Oct3/4

Octamer binding transcription factor 3/4 (Oct3/4), also known as Oct4 and POU5F1, is a transcription factor of the POU homeodomain family expressed in embryonic stem cells and germ cells. Oct3/4 belongs to a family of octamer-binding proteins that specifically bind to the ATTTGCAT regions of transcriptional control genes (Zhou et al. 2014; Yoo et al. 2013; Walcher et al. 2020; Mao et al. 2021). The presence of Oct3/4 is crucial during embryogenesis in the formation of pluripotent stem cells. Oct3/4 is responsible for germ cell expression in tumors and their metastases that show pluripotency characteristics, including seminoma, dysgerminoma–ovarian tumor, germinoma–germ cell tumor, and embryonal carcinoma (Cheng et al. 2013). The expression in embryonic stem cells, mouse embryonal carcinoma, and primordial germ cells was demonstrated in several independent studies (Zhou et al. 2014; Cheng et al. 2013; Yoo et al. 2013).

The researcher Mao and their colleagues (Mao et al. 2021) noted a pronounced increase in Oct3/4 expression of cancer bladder tissues compared to healthy tissues without infection. At the same time, the suppression of Oct3/4 is compatible with the ability to reduce the proliferation, migration and invasion grade of bladder cancer cells *in vitro* and prevent tumor growth *in vivo* (Mao et al. 2021; Yu Yang et al. 2019). This suggests that Oct3/4 may act as an oncogene in some cancers. The up-regulation and expression level of Oct3/4 is associated with cervical, colon, and breast cancers, testicular germ cell tumors, as well as drug-resistant and undifferentiated tumor-inducet cells that develop (Baek, Choi, and Pei 2020; Mao et al. 2021; Pierpont et al. 2017).

1.5. Apoptosis Markers - Caspases

Caspases (cysteine aspartate-specific proteases) are aspartate-specific cysteines that depend on the protease family members with the main function of promoting participation in programmed cell death, or apoptosis (Julien and Wells 2017; Lamkanfi et al. 2007). Human caspases are involved in other biological functions that regulate neither apoptosis, as well as inflammation, dendrite trimming, and cell differentiation functions on noncell death (Julien and

Wells 2017; Zheng et al. 2020; Lamkanfi et al. 2007). Human caspases have been divided into several main groups based on their function and sequence similarity (Julien and Wells 2017; Zheng et al. 2020). Caspases can be inflammatory caspases -1, -4, -5 and -11 and apoptotic caspases -3, -6, -7, -8, -9, -10 (Balderstone et al. 2019; Zheng et al. 2020; Julien and Wells 2017). Apoptotic caspases can be classified based on their functionality as initiator caspases (caspases -2, -8, -9 and -10) and effector or executioner caspases (caspase-3, -6, and -7) (Zheng et al. 2020; Kamiya et al. 2010; Julien and Wells 2017).

1.5.1. Caspase-2

Caspase-2 (Casp-2) functions as an initiator caspase of the caspase family of proteases, which responds by stimulating the initiation of the apoptotic cascade (Ando et al. 2017; Brown-Suedel and Bouchier-Hayes 2020). Casp-2 can be localized endogenously and nuclearly. The endogenous Casp-2 exists throughout the cell, but is especially come across the Golgi apparatus, mitochondria, endoplasmic reticulum (ER), cytoplasm, and nucleus (Susin et al. 1999; Mancini et al. 2000; Vigneswara and Ahmed 2020; Kopeina and Zhivotovsky 2021In turn, abnormal bilateral nuclear imports of pro-Casp-2 through the importation are regulated by two nuclear location signals (NLSs) located in the pro-domain, indicating that the nuclear location of Casp-2 may be essential to its activation prior to cell death (Mancini et al. 2000; Vigneswara and Ahmed 2020). The study explained that Casp-2 was maintained in the nucleus until the end of apoptosis, but Casp-2's ability to transfer the nucleus could not be correlated with its ability to mediate cell death (Paroni et al. 2002; Vigneswara and Ahmed 2020; Kopeina and Zhivotovsky 2021). Mostly, Casp-2 is located in the nucleus and can eventually penetrate into the nucleus substrate, especially in response to the signal of genotoxic stress (Ando et al. 2017; Sidi et al. 2008; Brown-Suedel and Bouchier-Hayes 2020; Vigneswara and Ahmed 2020).

Casp-2 is involved in the development regulation of hematopoietic stem cells (HSCs) and progenitor cell differentiation. Additionally, as the lack of Casp-2 in animals increases short-term HSCs and multipotent progenitors increase, causing abnormal differentiation (Lim, Dorstyn, and Kumar, 2021). Casp-2 is shown to retain multiple cellular functions to prevent genomic instability and tumor progression of tumor (Fava et al. 2012; O'Byrne and Richard 2017; Boice et al. 2021).

1.5.2. Caspase-3

Caspase-3 (Casp-3) is involved in the execution of apoptosis and plays a role in the evaluation of tumor regression (Toumia et al. 2020). Casp-3 is described as a prototypical apoptotic executioner that spilled many other functionally critical proteins that lead to cell apoptosis after activation of initiator caspases (Zhou et al. 2018). Casp-3 responds to carcinogenesis promotion after cellular exposure to chemicals and radiation (Zhou et al. 2018; Yosefzon et al. 2018). Thus, anticancer researchers use Casp-3 activation as a surrogate marker for evaluating anti-cancer therapy (Bareiss et al. 2013; Zhou et al. 2018).

Casp-3 can inhibit the expression of the major histocompatibility complex (MHC) II B cell proliferation and dendritic cell maturation (Lamkanfi et al. 2007). This indicates that Casp-3 is located in normal tissues at variable levels. Casp-3 can be effective in the apoptosis process and in the proliferation, differentiation, and survival of normal cells and malign tissues. Casp-3 interacts directly with intracellular signal pathways, and finally, the gene expression profile generates changes in stem differentiation and proliferative activity (Eskandari and Eaves 2022).

Sublethal mitochondrial activation of Casp-3 and Caspase-activated DNAse (CAD) in addition to being able to promote invasion and metastasis, resulting in increased aberrations and micronucleus formation. Casp-3 is indicated to be important for facilitaing the initiation of cancer cell transformation and aggressiveness, and has a negative impact on fully transformed cell responses to conventional treatments (Eskandari and Eaves, 2022; Feng et al. 2015).

1.5.3. Caspase-6

Caspase-6 (Casp-6) is an effector caspase that breaks down substrates involved in the cell cycle, survival, or development, and affects B cell activation (Watanabe et al. 2008). During apoptosis, Casp-6 is an effector caspase like Casp-3 and is usually cleaved and activated by an initiator caspase such as Casp-2 (Watanabe et al. 2008). Casp-6 is described as a short-term pro-domain and sequence-identifying executor caspase, but activation of Casp-6 alone is not enough to cause apoptosis in all cells (Julien and Wells 2017). Furthermore, Casp-6 direct activation does not control apoptosis in *in vitro* human embryonic kidney cells (HEK293 cell line), unless Casp-6 is combined with the lower-lethal inhibition proteins (Julien and Wells 2017).

Casp-6 expression is observed in the cytoplasm of cancer cells; it is expressed heterogeneously in the cytoplasm and nucleus of ovarian carcinoma (Capo-Chichi, Cai and Xu

2018). Atypical ovarian cancer cells detected high levels of Casp-6 activity in the cell, while normal ovarian epithelial cells had no or low Casp-6 expression (Cowling and Downward 2002; Capo-Chichi, Cai and Xu 2018). Capo-Chichi et al. reported on the interaction between activated Casp-6 and the loss of the nuclear structural protein Lamin A in ovarian cancer cells. The loss of Lamin A/C causes nuclear structural changes before the absorption of the nucleus, structural defects in the nucleus body, aneuploidity, and chromosomal instability, causing carcinogenesis (Capo-Chichi, Cai and Xu 2018; Watanabe et al. 2008).

1.6. CELL VIABILITY DETECTION

1.6.1. NADPH

Nicotinamide adenine dinucleotide hydrogen phosphate-diaphorase (NADPH-d) is a coenzyme that can reduce nitro blue tetrazolium (NBT) dye to the visible reaction product formazan (Scherer-Singler et al. 1983); the reaction involves hydrogen transfer from the substrate in the presence of NADPH, to a hydrogen acceptor.

NADPH performs its duties as an antioxidant substrate for thioredoxin and glutathione antioxidant systems, thus reducing the level of hypoxia in a cell (Klener and Klanova 2020; Pribluda, De La Cruz, and Jackson 2015). Paclitaxel, as an anticancer drug, is not only involved in the hyperstabilization of microtubules and the inhibition of cytoskeletal restructuration, but an increase in metabolic oxidative stress and NADPH oxidase is also associated with Paclitaxel’s anticancer effect (Hadzic et al. 2010b). NADPH, as a reducing agent, is required for antioxidant defense systems—it is a universal electron donor in reductive biosynthesis and detoxification of the cell (Agledal, Niere and Ziegler 2010).

1.6.2. Neutral red uptake

A neutral red (NR, 3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) assay is used to determine cell viability (Gordon, Brown, and Reynolds 2018; Repetto, del Peso, and Zurita 2008). The NR assay is based on the flow of cationic and noncationic cell fluids, penetrating cell membranes by passive diffusion and accumulating in lysosomes by binding to electrostatic hydrophobic boundaries (Repetto, del Peso, and Zurita 2008). The NR assay feature in cells depends on the ability of healthy cells to take up NR in lysosomes after toxic substance submission. Cells' ability to take up NR into lysosomes is because of treatment, drugs or toxic substances can cause cell integrity, resulting in reduced NR uptake (ICCVAM 2006;

Singh, Khanna and Pant 2018; Gordon, Brown, and Reynolds 2018). Therefore, the loss of NR uptake in cells, in lysosomes, coincides with the loss of cell viability (Jain et al. 2018).

1.6.3. Endocytosis

Endocytosis is the cell's ability to take up ingredients from the extracellular environment. Endocytosis is a phenomenon that occurs by incorporating 60–120 nm vesicles into the plasma membrane and regulating the internalization of liquids, extracellular components such as proteins, lipids, metabolites, small molecules, ions, and other elements (Wu et al. 2022; Iversen, Skotland and Sandvig 2011). Endocytosis and cell signaling have functional connections in the morphogenetic stages and cell proliferation, metabolism, motility, differentiation, and immunity (Wu et al., 2022; Wang et al. 2020). As endocytosis is related to cellular signalling and transmission, it can be involved in phenotypic changes, especially those related to abnormal cellular specifications. The mechanism of endocytosis in tumor heterogeneity may highlight the specific characteristics of tumors and response to therapeutic drugs targeting signaling receptors (Wu et al., 2022; Wang et al. 2020). Cancer cells can avoid being detected and caught by the immune system. Blindness of the immune system to cancer cells may be related to the regulation of surface antigens and tumor-induced immunosuppression through tumor immunity endocytic pathways (Wu et al., 2022; Mellman and Yarden 2013; Wang et al. 2020).

2. MATERIALS AND METHODS

2.1. Cell Cultures

All cell lines were obtained from the American Type Culture Collection (ATCC) which cooperated with the Latvian Biomedical Research and Study Center in this study. Human cervical carcinoma (*HeLa)* was grown at 37 ^{0}C, 5% CO_2 and maintained in adhesive culture an initial density of ~ 1×10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM; Thermo Scientific, IL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), 10 mg/mL a solution of penicillin-streptomycin mix (Sigma-Aldrich, MO, USA). Cells were grown in an air incubator at 37 ^{0}C, 5% CO_2.

Melanoma cells Sk-Mel-28 are melanocytes that are isolated from the skin tissues of a male patient with malignant melanoma by T. Thang and associates (Thang et al. 2015; "Sk-Mel-28 (ATCC ® Htb-72 TM)" 2020). Cells were grown at 37 ^{0}C, 5% CO_2, and maintained an initial density of ~ 1×10^5 cells/mL in DMEM (Thermo Scientific, IL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), 10 mg/mL of a penicillin-streptomycin mix solution (Sigma-Aldrich, MO, USA).

Human skin fibroblasts (Hs-68) isolated from the foreskin of a white male patient with aspartoacylase deficiency (ATCC 2003; Messina et al. 2016). The cell line is considered a normal diploid cell line with a limited life span (Messina et al. 2016). Cells were grown at 37 ^{0}C, 5% CO_2, and maintained an initial density of ~ 1×10^5 cells/mL in DMEM (Thermo Scientific, IL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), 10 mg/mL of a penicillin-streptomycin mix solution (Sigma-Aldrich, MO, USA).

Human lung carcinoma A549 (CRM-CCL-185™) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 37 ^{0}C, 5% CO_2, and maintained an initial density of ~ 1×10^5 cells/mL in DMEM (Thermo Scientific, IL, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), 10 mg/mL of a penicillin-streptomycin mix solution (Sigma-Aldrich, MO, USA).

All cell cultures were harvested in 24 hours to reach 80% confluency before examination. Two groups of cell samples were made: control and treated cells.

2.2. Stress Factors Applied to Cells

Stressors were used to induce microcell formation in the treated cell sample group. *HeLa*, Hs-68, Sk-Mel-28 cells were processed for 24 h with doxorubicin (DOX; 50 mg; TEVA) at a final concentration of 2.5 μM or with Paclitaxel (PTX; 6 mg/mL; TEVA) at a final concentration of 0.7 μM in DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution and grown at 37 °C in a 5% CO_2 atmosphere. DOX and PTX were not used together in the same sample. After treatment, the medium was decanted and replaced with fresh DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution, and the cells were cultivated for another 24 and 48 h at 37 °C in a 5% CO_2 atmosphere. Control cell samples were cultivated in DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution and under the same conditions as the treated cells. Subsequently, after 24 hours, the control cells were given the same fresh medium as the treated cells and continued cultivation at 24 and 48 h at 37 °C in a 5% CO_2 atmosphere. Furthermore, all cells were processed according to subsequent experiments: microscopy, antigen detection, or evaluation of metabolic activity. The *HeLa* cells were treated with methanol (Sigma Aldrich, USA) at a final concentration of 7.5% in DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution for 1 h at 37 °C in a 5% CO_2 atmosphere. After treatment, cells were cultivated in fresh DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution for 6 h. The cultured cells were fixed with 4% formaldehyde solution for 10 min at room temperature.

2.3. The Neutral Red Uptake Assay

The neutral red (NR) uptake assay was used for the detection of cytotoxicity. The principle of this assay is based on the detection of viable cells through the uptake of the dye neutral red (ICCVAM 2006; Testing, Assessment and Testing, n.d.). NR is a faintly cationic supravital dye pierced cell membrane by nonionic passive diffusion way (Singh, Khanna and Pant 2018; Gordon, Brown, and Reynolds 2018). Sk-Mel-28 cells were harvested into the 24-well plate on a coverslips at the concentration of 1×10^5 cells/mL. Reaching 80% of confluency cells were treated with PTX (6 mg/mL; TEVA) at a final concentration of 0.7 μM in DMEM supplemented with 10% FBS and 10 mg/mL of a penicillin-streptomycin solution and grown at 5% CO_2 at 37 °C for 24 h. After the treatment, the DMEM was replaced with fresh DMEM medium, and the cells were cultivated for another 24 h at 37 °C in a 5% CO_2 atmosphere.

Sequentially neutral red (NR, Sigma-Aldrich, Taufkirchen, Germany) was added to control and treated cells for 3 h incubation. After 3 h the staining solution was removed, and cells were rinsed three times with PBS. The cells were fixed with 4% formaldehyde solution for 10 min at room temperature and rinsed three times with PBS. Coverslips were removed from the 24-well plate, dried, and coated with CV ultra-mounting medium (Leica Biosystems Newcastle, United Kingdom) and analyzed under a transmitted light microscope.

2.4. NADPH test for Cell metabolic Activity

Nicotinamide adenine dinucleotide hydrogen phosphate-diaphorase (NADPH-d) is a coenzyme that can reduce nitro blue tetrazolium (NBT) dye to the visible reaction product formazan (Scherer-Singler et al. 1983); the reaction involves hydrogen transfer from the substrate in the presence of NADPH, to a hydrogen acceptor. Cell oxidation processes related to biosynthesis take place in two systems, from mitochondria to the endoplasmic reticulum. The hydrogen ion is transferred through the cytochrome system (cytochrome 450) and cell viability is shown (Kluchová et al. 2001). In this experiment, NADPH activity was detected. NADPH is a coenzyme that can reduce nitro blue tetrazolium (NBT) dye to the visible reaction product formazan - the reaction involves hydrogen transfer from the substrate, NADPH, to the hydrogen acceptor (Scherer-Singler et al. 1983; Hope et al. 1991).

Initially, Sk-Mel-28 cells were cultivated for 24 h with 0.7 μM of PTX, then the medium was changed and the cells were cultivated for another 24 h. The NADPH test started with pre-fixation for 1 min with a 4% formaldehyde solution (Sigma-Aldrich, MO, USA), then cells were carefully washed two times with 1.5 M TRIS buffer saline and incubated for 30 min in the incubation medium (2.5 mL of 1.5 M TRIS buffer saline, 1.5 g of sucrose, 0.5 mL of nitro blue tetrazolium (Sigma- Aldrich, MO, USA), and 0.9% NaCl to 10 mL). The NADPH substrate (β-Nicotinamide adenine dinucleotide phosphate reduced sodium salt hydrate; SERVA, Germany) was administered to the solution shortly before incubation. The reaction was stopped with a 4% formaldehyde solution fixation for 10 min at room temperature, washed with distilled H_2O, and covered with coverslips using a CV ultra-mounting medium (Leica Biosystems, Nussloch, Germany). NADPH diaphorase activity was observed as dark blue or black spots in the cell cytoplasm. The NADPH activity was semi-quantitatively scored by two independent observers using ZEISS microscopy Camera Axiocam 202 mono, where 0 points- activity was not

detected; 1 point- low activity; 2 points- activity was detected at average level; 3 points high activity, but not in a whole cell or sample; 4 points strong activity in the entire cell or sample.

2.5. Cell transfection and GFP expression time-lapse

Initially, Sk-Mel-28 cells were seeded on glass-bottom 24-well plates (Cellvis, CA, USA) at a concentration of ~ 1×10^5 cells/mL and grown in a humidified atmosphere containing 5% CO_2 at 37 °C. The DMEM cultivation medium was supplemented with 10% fetal bovine serum and 10 mg/mL of a penicillin/streptomycin solution (all Sigma-Aldrich, MO, USA). When Sk-Mel-28 -melanoma cells reached 70–80% confluence, the medium was replaced with fresh growth medium containing 10% FBS (Sigma-Aldrich, MO, USA).

The cells were grown for 24 h in the presence of the plasmid; after the incubation period, the growth medium was replaced with DOX (50 mg; TEVA) at a final concentration of 2.5 μM into each well, and cultivated for 24 h at 37 °C in 5% CO_2, normoxia, using a confocal laser microscope (Leica SP8 Confocal, Leica, Wetzlar, Germany) cell cultivation chamber. Then the medium was changed to a fresh medium without DOX.

A transfection mix for one sample was prepared using 1 μg of plasmid pcDI-EGFP (6837 bp) containing GFP gene diluted in 100 μL of serum-free DMEM with an additional 2 μL of TurboFect™ reagent (Thermo Scientific, IL, USA), according to the manufacturer's protocol (Thermo Scientific 2011). A plasmid pcDI-EGFP allows GFP expression in viable cells. The plasmid and TurboFect™ complex were incubated for 15 min at room temperature. Then, 100 μL of the TurboFect™/plasmid mixture drop was used to transfect each well of the plate.

The confocal laser microscope transmitted light and the fluorescent light detectors were equipped at the argon laser line of 488 nm, the time-lapse function was used for time-lapse imaging. Images were taken every 120 min after Doxorubicin administration and examined for 72 hours. GFP was excited by a 488 nm laser line, and the emission was detected at 510 nm.

2.6. ANS-ethidium Bromide staining

The 8-anilinonaphthalene-1-sulfonic acid (ANS) – ethidium bromide (EtBr) staining technique was used to detect the presence of proteins and nucleic acids in cells. ANS is a staining protein at the hydrophobic site of a protein, fluoresces in a blue light (Ali et al. 1999; Qadeer et al. 2012), while EtBr fluoresces in red light, thereby revealing DNA and RNA (Zhao et al. 2018). *HeLa* cells were seeded in monolayers on a coverslip at a density of ~ 1×10^5

cells/mL and grown in a humidified atmosphere containing 5% CO_2 at 37 °C. DMEM growth medium was supplemented with 10% fetal bovine serum and 10 mg/mL of a penicillin/streptomycin solution (all from Sigma Aldrich, USA). When the cell density was 80–100% on the monolayer, a final concentration of 7.5% methanol (Sigma-Aldrich, MO, USA) was added. Cells were incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. After treatment, cells were cultivated in fresh DMEM for 6 h. The cultivated cells were fixed with a 4% formaldehyde solution for 10 min at room temperature, then stained with 5 µg/mL of ANS (Sigma-Aldrich, MO, USA). The dye solution was kept in the dark, poured over the cover slide with cells, and incubated for 10 min at room temperature, before being drained without washing. EtBr (3 µg/mL; Sigma- Aldrich, MO, USA) was poured on the cover slide with the cells, incubated for 10 min at room temperature, kept in the dark, and then drained without washing. Cells were dried in the air, and covered with coverslips using CV ultra-mounting medium (Leica Biosystems, Nussloch, Germany). ANS–EtBr fluorescence was visualized using a three-band blue, red and green (Leica BRG) optical filter and a Leica DM1000B microscope qualitatively scored blinded by two independent observers as negative or positive staining.

2.7. RNA Synthesis Detection

For RNA synthesis detection, the RNA synthesis assay kit (ab228561, Abcam, Cambridge, UK) was used. Sk-Mel-28 cells were seeded onto a 24-well plate at a density of 1 x 10^5 cells/mL and cultivated in DMEM with 10% FBS and 1% penicillin/streptomycin at 37 ^{o} C in a 95% humidified, 5% CO_2 atmosphere. Cells were cultivated onto coverslips. When cells reached 80% confluence, two groups were distributed as the control and PTX samples. For the control samples, the cultivation medium was changed, while for the PTX samples, the cultivation medium was changed and PTX was added at a final concentration of 0.7 µM and the cultivation continued for 24 h. The 1X RNA label was added to the control and PTX cells. After incubation for 24 h, cells were fixed and processed according to the manufacturer's instructions (Abcam 2020). Cells were analyzed by microscopy.

2.8. DNA Synthesis Detection

The detection of DNA synthesis is used to assess the ability to proliferate after added anticancer drugs. DNA synthesis detection was used with Click-iT® EdU Flow Cytometry

Assay Kits (Cat.no. C10419, Molecular probes by life technologies) that is, BrdU (bromo-deoxyuridine) assay analog (Technologies Molecular probes by life 2009). 5-ethynyl-2 -deoxyuridine (EdU) is a nucleoside analog to thymidine and is involved in DNA during active DNA synthesis. EdU is in the ethynyl moiety and the visualization agent is azide coupled to Alexa Fluor® 647 dye to be detected by flow cytometer. SK-MEL-28 cells were seeded onto a 24-well plate at a density of 1 x 10^5 cells/mL and cultivated in DMEM with 10% FBS and 1% penicillin/streptomycin at 37 ^oC in a 95% humidified, 5% CO_2 atmosphere. When the cells reached 80% confluence, two groups were distributed as the control and PTX samples, and subgroups – control with and without EdU and PTX group – with and without EdU. For the control samples, the cultivation medium was added. For the PTX samples, the cultivation medium was changed and PTX was added at a final concentration of 0.7 µM. Both groups were cultivated for 24 hours. Sequentially, the medium was replaced with the fresh cultivation medium with EdU at the final concentration of 10 mM, and the cultures were maintained for another 24 h. The cells were then trypsinized and fixed in 4% formaldehyde. The incorporation of EdU was determined with the Click-iT EdU Alexa Fluor 647 flow cytometry assay kit (Molecular Probes) according to the manufacturer's instructions (Technologies Molecular Probes by Life 2009; Lin et al. 2011).

2.9. Immunocytochemistry

The immunocytochemistry method was used to detect cells expressing proteins. Cells were cultivated on coverslips and fixed with a 4% formaldehyde solution for 10 min at room temperature, then washed twice in 1X PBS for 5 min. Slides were subsequently permeabilized for 10 min in 0.02% Triton X-100 (X100; Sigma-Aldrich, MO, USA) and rinsed three times in 1X PBS for 5 min. For slide nonspecific binding blocking, 2% bovine serum albumin (BSA)/Tris-buffered saline (TBS) containing 0.2% Tween 20 (E108; Nordic BioSite, Finland; TBST) was used for 1 h at room temperature. The cells were incubated overnight with a monoclonal antibody, according to the manufacturer's recommendation, in a humidified chamber at +4 ^0C. Sequentially, the incubation solution was decanted from samples and samples were not washed. Subsequently, the samples were covered with secondary antibody diluted in 1%BSA/TBS and incubated in the dark for 1 h at room temperature in a humidified chamber. For staining sequences with multiple antigens, nonspecific fluorescence blocking is performed after incubation with the secondary antibody; subsequently, primary and secondary antibodies are added. Cell nuclei were counterstained with 1 µg/mL DAPI (02157574-CF; MP Bio-

medicals, Solon, OH) for 1 min and finally embedded in CV Ultra Mounting Media (14070936261; Leica Biosystems, Nussloch GmbH, Germany). The expression was examined by two independent observers, as negative control and positive staining. Primary and secondary antibodies are listed in Table 1.

Table 1. Primary and secondary antibodies.

Antibody	Description	Antibody emission and excitation	Dilution/ used concentration	Product No and manufacturer
ALDH2	Primary antibody; Rabbit anti human Rabbit polyclonal		1:75	158-201549-T08-200, Sino Biological/ Nordic BioSite
SOX2	Primary antibody; Mouse anti human Mouse monoclonal		1:100	TA302025, Origene
Anti-PCNA	Primary antibody; Rabbit anti human, Rabbit recombinant monoclonal		1:100	ab92552, Abcam
Nanog	Primary antibody; Rabbit anti human, Rabbit polyclonal		1:100	14295-1-AP, Proteintech Europa
Caspase-2	Primary antibody; Rabbit anti Human, Rabbit Polyclonal	FITC Conjugated; Ex.494nm/Em.518nm	1:100	BS-5802R-FITC, Bioss antobodies/ Nordic BioSite
Caspase-6	Primary antibody; Rabbit anti Human, Rabbit Polyclonal	ALEXA FLUOR® 555 Conjugated Ex.553nm/Em.568 nm	1:100	BS-0151R-A555, Bioss antobodies/ Nordic BioSite
Caspase-3	Primary antibody; Mouse anti human, Mouse monoclonal		1:100	GTX13586, Biolegend/ GeneTex Inc.
Alexa fluor 488	Secondary antibody; Goat Anti-Rabbit IgG H&L	Ex: 495nm, Em: 519nm	1:1000	ab150077 Abcam
	Secondary antibody; Goat Anti-Mouse IgG H&L	Ex: 495nm, Em: 519nm	1:1000	ab150113 Abcam
Alexa fluor 594	Secondary antibody; Goat Anti-Mouse IgG H&L	Ex: 590nm, Em: 617nm	1:1000	ab150116 Abcam
	Secondary antibody; Goat Anti-Rabbit IgG H&L	Ex: 590nm, Em: 617nm	1:1000	ab150088 Abcam

2.10. Determination of the Cell Number

Cell count was determined using a Leica DM1000B microscope (Leica Microsystems) with a 40× objective (dry, plan apochromatic, with a numeric aperture of 0.85). The microcell count and the whole number of *HeLa*, Hs-68, and Sk-Mel-28 cells were obtained in the three experiments in at least ten fields of view. The total number of cells counted was 1060 cells at each time point: 24, 48, and 72 h after applied therapy.

2.11. Microscopy and Image Evaluation

A Leica DM1000B microscope (Leica Microsystems, Wetzlar, Germany) with 63× objective (oil, plan apochromatic, with a numeric aperture of 1.40) equipped with a Leica DFC400 (Leica Microsystems, Wetzlar, Germany) digital camera and a ZEISS Axiolab 5 microscope with 63×/0.85 objective (oil, N-Achroplan, Ph3 M27; ZEISS, Jena, Germany) with ZEISS Microscopy Camera Axiocam 202 mono (ZEISS, Jena, Germany) was used for

microscopy of the samples. For image analysis and processing, Image-Pro® plus the Proven Solution™ software, version 4.0, and LAS X lite (Leica Microsystems, Wetzlar, Germany) and ZEN 3.0 Blue lite (ZEISS, Jena, Germany) were used.

2.12. Transmission electron microscopy

Transmission electron microscopy was performed in previous pilot experiments inducing microcells by stress factors. The cell line 4/21 was seeded in 24 cm^2 Karell flasks with a density of ~ 3×10^5 cells/mL and grown in Eagle medium supplemented (Sigma-Aldrich, MO, USA) with 10% FBS and 1% penicillin/streptomycin (growth medium) in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were treated with Thio-TEPA (triethylenethiophosphoramide) at a final concentration of 20 μg/mL for 24 h. After treatment, the medium was removed and washed with serum-free medium, and fresh growth medium was added and incubated for 24 h. The incubation medium was removed and a 2.5% glutaraldehyde solution prepared at 4 ° C in PBS was added for the preparation of the cell sample for electron microscopy. Cells were fixed for 15 minutes in the 2.5% glutaraldehyde solution. Sequentially, the cell monolayer was gently using a cell scraper collected in a 5 mL test tube and centrifugated for 10 min at 200 g. The cells were washed twice with PBS at 4 ° C and postfixed with 2% osmium acid solution prepared in PBS for 10 min. The pellet was dehydrated in 70% ethanol and embedded in Epon (Epoxy embedding medium; Sigma-Aldrich, MO, USA). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined using an electron microscope JEM- 100B (TEM, Japan).

2.13. Statistical Analysis

The number of microcells in the control (untreated) and treated cells was compared using a two-tailed Student's t-test for unpaired samples. The statistical significance in the difference of means was calculated where appropriate. The differences were considered significant at $p \leq 0.05$. The percentages were counted, assuming a total cell count of 1060 to be 100%.

2.14. Formalin Fixed Paraffine Embedded Samples

Permission for this study was received from the Research Ethics Commission of the Riga East University Hospital Oncology Centre and Experimental and Clinical Medicine Institute, University of Latvia. Primary surgical material of women's breast cancer was collected from

September 2011 till February 2012. There were two groups of breast cancers – luminal (32 histological samples) and triple-negative breast cancer (11 histological samples). The mean patient age in the luminal breast cancer group was 59 years and, in the triple,-negative breast cancer group – 52 years. The material was prepared for sectioning in paraffin blocs. For each sample, six cuts (1.5 μm) of the paraffin section were performed using a microtome, at the Pathology Centre of the Riga East University hospital.

2.15. Immunohistochemical staining

In the first steps in immunohistochemical staining deparaffination: xylol for 4 min two times, 96% ethanol for 5 min, 70% ethanol for 5 min, distilled water (dH_2O) for 3 min, and dried samples at room temperature. Primary antibodies against CD44 (the cluster 44, clone: DF1485, mouse monoclonal antibody, NovocastraTM, Leica Biosystems Newcastle, United Kingdom), CD24 (the cluster of differentiation 24, clone: ML5, mouse monoclonal antibody, BioSite, Finland), Oct3/4 (homo- domain transcription factor of the POU family, expressed in embryonic stem cells and germ cells, clone: N1NK, mouse monoclonal antibody, NovocastraTM, Leica Biosystems Newcastle, United Kingdom) and polyclonal antibody against ALDH (aldehyde dehydrogenase, ALDH1A1, clone: 5A11, mouse monoclonal antibody, BioSite, Finland) were used to differentiate cancer cell populations. The nuclei were counterstained with hematoxylin. For both breast cancer, slide samples were stained with hematoxylin-eosin. Slides were immunohistochemically stained according to the manufacturer's recommendations. For each biological marker, a slide was analyzed under a microscope and 16 images were taken for each sample.

2.16. Feulgen method

The Feulgen method was used to determine the heterogeneity of the breast cancer cell population, the integrated optical density (IOD), the mean optical density of cell nuclei, and the size of cell nuclear area. As a control for IOD, human lymphocyte cell samples were used, known as diploid cells. In the first step, deparaffination is needed: xylol for 4 min two times, 96% ethanol for 5 min, 70% ethanol for 5 min, distilled water (dH_2O) for 3 min, and dried samples at room temperature. The breast cancer histological sections (FFPE samples) were subjected to hydrolysis for 50 min at 37 °C in 3N HCl and rinsed in 1N HCl. Then, samples were transferred to Schiff's reagent (pH 3.5 to 4.0) and incubated for 1.5 h at 37 ^{0}C for 1h at 37

°C in a hermetically sealed container. After that added H_2S is prepared *ex tempore:* 0.5 g $Na_2S_2O_3$ (sodium meta bisulfide) dissolved into 100 mL dH_2O, sequentially added 5 mL 1N HCl (hydrochloric acid) for 3 min three times, taped water 5 min two times and dH_2O for 3min.

Integrated optical density (IOD) is used in microscopy and cell research for DNA quantification in cells. IOD is obtained by deducting the background pixel value of a certain field of interest from the value of a single pixel. IOD was taken from the green channel of the Leica DFC400 camera color image (Farrell, 2010). The green channel of the Leica DFC400 camera was calibrated to optical density units (Figure 3).

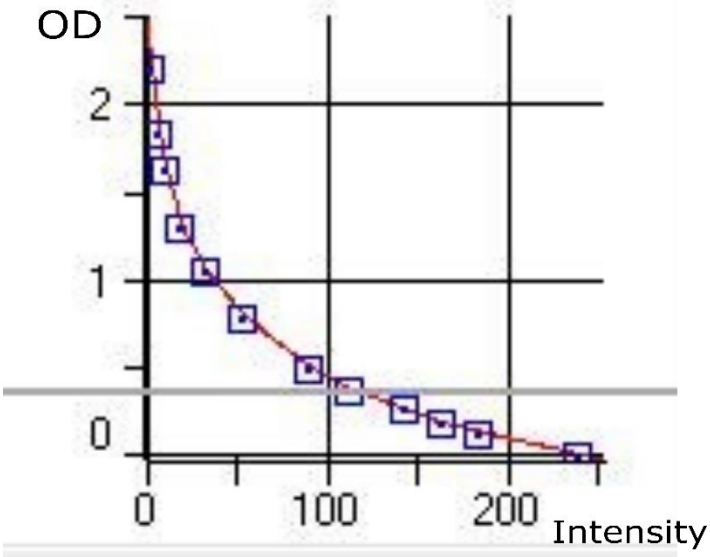


Figure 3. Leica DFC400 calibration curve, ImagePro Plus 6.0 software.

2.17. Flow cytometry and data analyses

Flow cytometry was done using a three-laser BD FACS Aria II flow cytometer (BD - Becton Dickinson, Biosciences, USA). The analysis was performed with IDEAS 6.2. software. Singlets were analyzed by size using the FCS scatter plot (forward scatter) vs SSC (side scatter) to determine the population of small cell populations, all cells and populations by size were compared using PI (propidium iodide; PI flow cytometry kit 200 test, Ex.493/Em. 636 nm, Cat.no ab139418, Abcam) and EdU (Click-iT EdU Alexa fluor 647 flow Cytometry Assay kit, 100assays, Cat. No C10419, Ex.650⁄Em.670 nm Flow Cytometer Laser Lines, Molecular Probes life Technologies) median fluorescence intensity. Cell cycle phases were determined on PI staining intensity. EdU fluorescence was determined using an APC laser.

2.18. Data Evaluation

The expression of biological markers (CD44, CD24, ALDH, Oct3/4, Casp-2, Casp-6, SOX2, Nanog, Anti-PCNA) was detected by semi-quantitative method in histological samples in our previous work, where 0, expression was not detected; 1, low expression; 2, expression was detected at the average level; 3, high expression, but not in the whole sample; 4, strong expression in the entire sample. Those results were used to compare both cell culture and tissue sample results.

Furthermore, both types of cancer histological samples were stained using the Feulgen method to estimate the amount of DNA in the nuclei of cancer cells. The integrated optical density, proportional to the amount of DNA, and the mean optical density, proportional to the concentration of DNA in the nucleus, were measured using ImagePro Plus 6.0 software. Optical density (OD) detection and analyses allow for a variety of measurements. OD detection is a method using light absorption to detect the amount of a substance in a sample (Farrell 2010). IOD is obtained by deducting the background pixel value of a certain field of interest from the value of a single pixel. IOD is produced from a green spectral image from RBG (red, blue, and green color image) (Farrell 2010).

3. RESULTS

In the present investigation, we choose the Sk-Mel-28 melanoma cell line for immunohistochemical characterization of microcells formed after the administration of chemotherapeutic agents. The Sk-Mel-28 cell line is resistant to both DOX and PTX drugs (Ko et al. 2019; Xiao et al. 2018). On the other hand, in some studies, other cell cultures, *HeLa*, Hs-68, and A549 were used for the comparison of the results.

Many studies have been done on cancer development and resistance. However, there are few studies on the population of cancer cells after the application of therapy (McGranahan and Swanton 2017; Rekha, Sunil, and Rathy 2018; Risom et al. 2018; Salmina et al. 2017; Bema et al. 2008; Zhao et al. 2023). In our research, we determined a number of microcells developing and characterizing them during applied therapy. Microcells are small, round cells whose formation induces anticancer drugs such as triethylenethiophosphoramide (Thio-TEPA) (Freivalds, Buikis, and Harju 1996; Buiķis, Harju, and Freivalds 1999). In this study, PTX was a microtubule inhibitor that stops mitotic division to induce microcells (Farrar and Jacobs 2020).

3.1. Induction of Microcell Formation Using Paclitaxel and Doxorubicin

Microcells are round or oval cells with thin cytoplasm. The microcells were above noticed in human cervical carcinoma and human fibrosarcoma (HT-1080) cell lines (Buiķis, Harju, and Freivalds 1999; Buiķis et al. 2002). These cells have been described as a natural tumor component, but interestingly their relative numbers increase after cancer therapy has been initiated (Buiķis, Harju, and Freivalds 1999; Freivalds, Buikis, and Harju 1996; Bema et al. 2008).

In this part of the morphological study, melanoma (Sk-Mel-28), *HeLa* and human fibroblast (Hs-68) cell lines were investigated *in vitro* after anticancer chemo preparations were used. Paclitaxel (PTX) and Doxorubicin (DOX) or methanol were used as stress factors and to initiate microcell formation. Cells were cultivated for 48 hours until investigation. After that, the cell culture samples were examined.

Sk-Mel-28 cells were evaluated after culture for 48 h following PTX (Figure 4). The untreated/control sample cells were spindle-shaped with a round and elongated nucleus, without small-sized, intensively stained cells (Figure 4, A). On the contrary, small, round, and

intensively stained cells – microcells were observed in Sk-Mel-28 samples after PTX therapy (Figure 4, B) (Simsone et al. 2021).

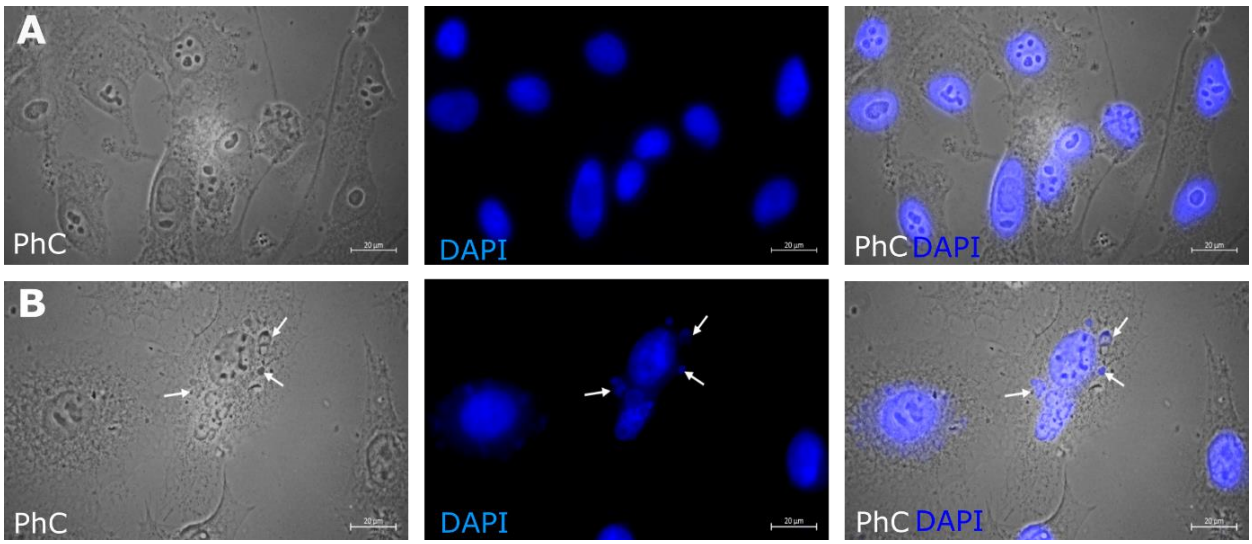


Figure 4. Microcell formation in Sk-Mel-28 cells after Paclitaxel (PTX) treatment for 48 h. (**A**) Untreated Sk-Mel-28 cells (control sample) and phase contrasts (PhC); cell nuclei are stained with DAPI (blue) and overlaid (PhC/DAPI). (**B**) SK-MEL-28 PTX-treated cells, PhC. The white arrows show the formation of microcells. The cell nuclei are stained with DAPI (blue) and are overlaid (PhC/DAPI). The scale bar is 20 μm (Simsone et al. 2021).

In the three evaluated cell lines the count of microcells was performed in 24 h, 48 h and 72 h after treatment, overall, 1060 counted cells per each cell line (Figure 5).

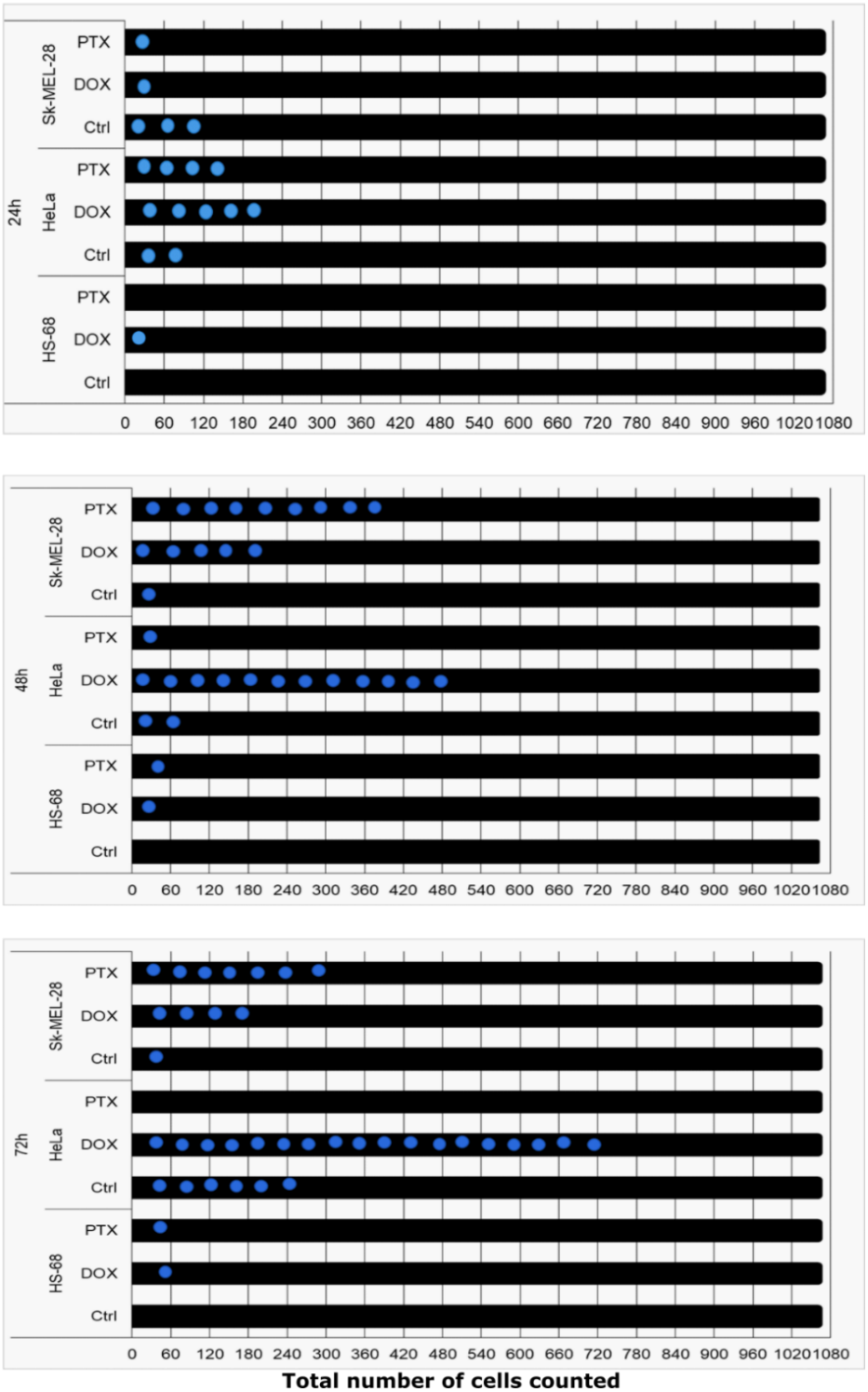


Figure 5. Increase in the number of microcells after applied therapy. Cells were counted after 24, 48 and 72-hours following Doxorubicin (DOX) and Paclitaxel (PTX). A total of 1060 cells were counted after PTX and DOX treatment. Microcells—blue dots representing each cell. In HS-68, the fibroblast

cells, the increase in the number of microcells was small. Only in cancer cell lines (*HeLa* and Sk-Mel-28) were microcells evaluated in the control cells (Ctrl) untreated cells and 48 hours after applying PTX and DOX, there was a tendency for microcell count to increase (Simsone et al. 2021).

Microcells were mostly observed in both PTX and DOX treated and control samples in *HeLa* and Sk-Mel-28 cells. In the control samples the number of cells observed was scarce, one or few cells. The microcell increasing tendency was observed in the *HeLa* cell line and the highest number of these cells was observed 72 h after DOX was applied: 1.57% from all cells counted, and the percentage increase was statistically significant (Table 2). In turn, the highest number of microcells in Sk-Mel-28 cell samples was observed 48 h after PTX treatment (Figure 5), which is up 1% ($p<0.016$) of the total cell count (Table 2).

Table 2. Microcells count 24, 48, and 72 h after treatment with DOX (doxorubicin) and PTX (Paclitaxel) treatment in Hs-68, *HeLa* and Sk-Mel-28 cell lines.

Cell Line	24 h					48 h					72 h				
	Ctrl (%)	DOX (%)	*p*-Value	PTX (%)	*p*-Value	Ctrl (%)	DOX (%)	*p*-Value	PTX (%)	*p*-Value	Ctrl (%)	DOX (%)	*p*-Value	PTX (%)	*p*-Value
HS-68	NA	0.09	0.324	NA	NA	NA	0.09	0.324	0.09	0.324	NA	0.09	0.324	0.09	0.324
HeLa	0.28	0.46	0.437	0.37	0.684	0.19	1.11	**0.013**	0.09	0.560	0.56	1.57	**0.047**	0.00	**0.023**
SK-MEL-28	0.28	0.19	0.700	0.09	0.302	0.09	0.65	**0.037**	0.83	**0.016**	0.09	0.37	0.157	0.56	0.151

Significant microcell count increase p values (≤ 0.05) are highlighted in bold. p values obtained by Student's t-test. Ctrl (control); NA - no microcells detected (Simsone et al. 2021).

No microcells were observed in the non-cancerous Hs-68 cell line in control samples compared to *HeLa* and Sk-Mel-28 cells (Figure 4). The increase in the number of microcells was small: one microcell of the total counted cells. These results show that microcells are a subpopulation of natural cancer cells with a tendency to increase the count during and after applying the chemotherapeutic drugs DOX and PTX (Simsone et al. 2021).

3.2. Small-sized Cell Identification in FFPE Breast Cancer Samples

In the research, in characterization of the breast cancer cell population, small size was observed in formalin-fixed paraffin-embedded (FFPE) breast cancer samples. The small size cell population was noticing investigation samples with the Feulgen assay. The Feulgen assay experiment labeled nucleic acids with Schiff's reagent in red. The luminal breast cancer contained cells with low and high nucleic acid concentrations, while large cells exhibited only low nucleic acid concentrations (Figure 6, A). In the population of triple negative breast cancer

cells, polyploid cells and microcells contained high and low concentrations of nucleic acid, respectively (Figure 6, B). Cell populations with a larger nucleus area and lower average optical density were dominant in luminal breast cancer samples. On the triple-negative breast cancer cell population was shown. cells with a smaller nucleus area and higher average optical density (Figure 5).

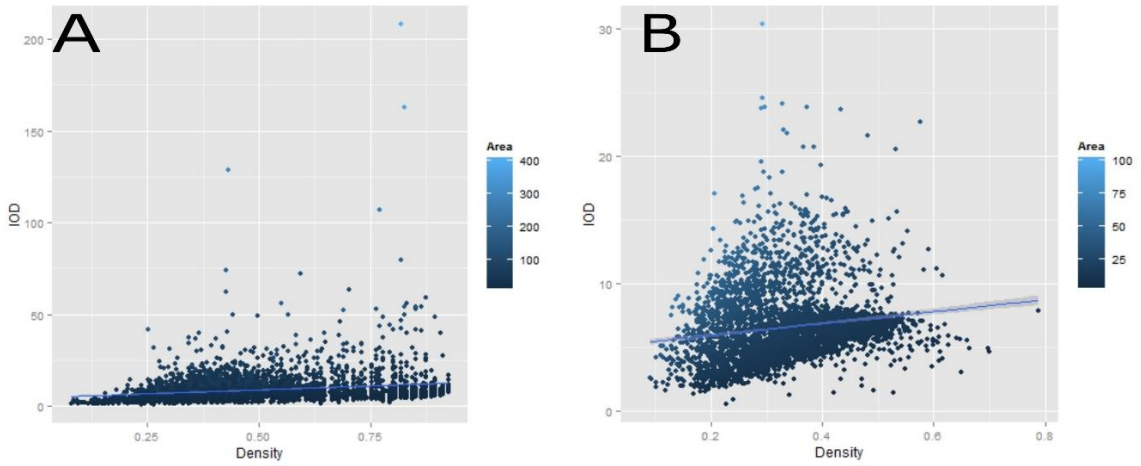


Figure 6. Integrated optical density versus cell nucleus average optical density and nucleus area in the cancer cell population. (A) in triple negative breast cancer histological samples; (B) in luminal breast cancer histological samples (Simsone et al. 2015).

3.3. Characterization of Microcell Morphology Using TEM

For microcells, ultrastructure morphological characterization and determining the presence of cell organelles were used using the transmission electron microscope (TEM). The Dzungaria hamster cell line 4/21 was treated with Thio-TEPA for 48 h. In Figure 7, microcells are seen with an electron-dense cell nucleus, nuclear envelope, cell membranes, cell cytoplasm, and organelles. In the cell cytoplasm, ribosomes are seen as dark or black spots.

The early stage of young microcells is characterized by the increasing relationship between the cell nucleus and the cytoplasm (Figure 7, A). In this young cell nucleus, a large part of it is heterochromatin, known as inactive chromatin, to indicate a partly differentiated cell. Additionally, the microcell is seen in Figure 7, B of the increased ratio of the cell nucleus to the cytoplasm. A microcell is characterized by nucleoli and euchromatin that occupies most of the cell nucleus, suggesting a new cell with acquired stem cell characteristics (Simsone et al. 2021).

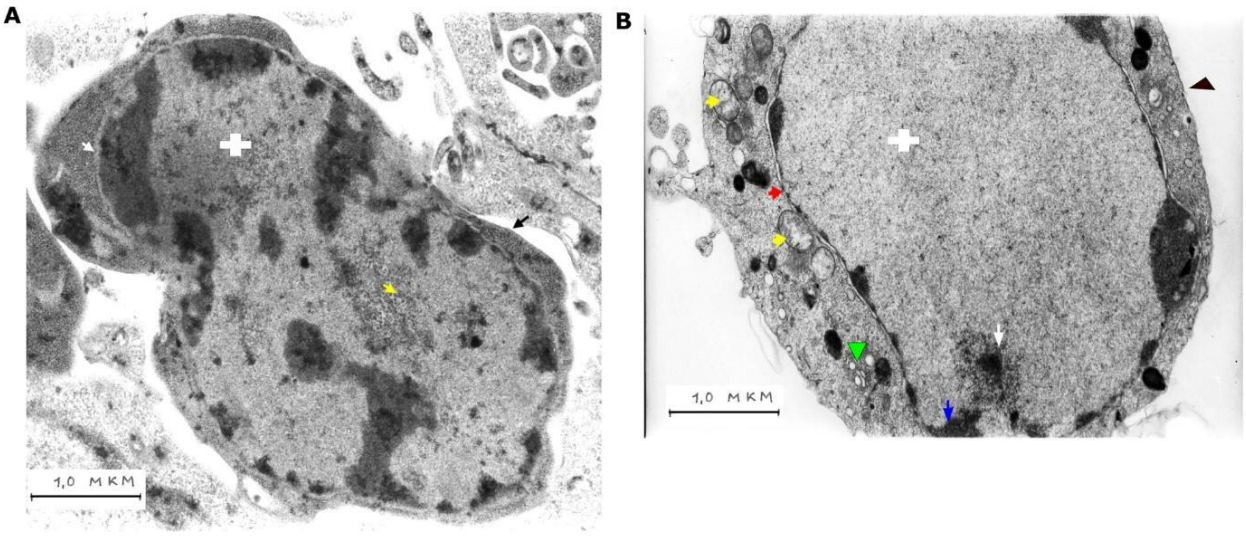


Figure 7. The microcell formed after 48 h Thio-TEPA treatment. (**A**) Microcell with the cell nucleus (white cross), the nuclear envelope (white arrow), the cell membrane (black arrow) and nucleolus (yellow arrow). (**B**) Microcell with the cell nucleus (white cross), the nuclear envelope (red arrow), the cell membrane (black arrowhead), heterochromatin (blue arrow), mitochondria (yellow arrows), the Golgi apparatus (green arrowhead), and nucleoli (white arrow). The scale bar is 1 μm (Simsone et al. 2021).

3.4. Microcell initiation and formation in time after Doxorubicin treatment *in vitro*

Microcells were observed in fixed-cell samples. The next step was to identify and catch under the microscope in a live-in time-lapse experiment. To observe a microcell, a GFP (green fluorescent protein) transfection assay was used, and Sk-Mel-28 cell culture was used. This assay also shows the cell's ability to proliferate and be viable. The time-lapse experiment in general was 72 h, but 24 h after chemotherapy, the morphology of melanoma cells behaved similarly to apoptotic cells. The cells were round (Figure 8), their cytoplasm produced blebs (Figure 8, 10 h), and then the cell formed into apoptotic-like bodies (Figure 8, 12-14 h). However, after that, it was observed that a microcell classified as an apoptotic cell was capable of self-renewal in every 2 h time-lapse image. Visually, this process appears to be similar to active endocytosis, when cells take substances from the extracellular space and form as a cell expressing GFP (The video of the microcell formatting process is seen in the article Simsone et al. 2021; Cancer microcell initiation and determination in additional file 1: Supplementary file 1: GFP expression in the microcell after 48 h of Doxorubicin treated Sk-Mel-28 cells. Leica SP

8 confocal microscope, objective 63x. https://doi.org/10.1186/s12885-021-08813-5). The newly formed microcell is a roundish, small-sized cell (approximately 3-5 µm) with endocytic ability and GFP expression (Figure 8, 16 – 20 h). The expression of GFP indicating viability and cell proliferation activity leads to live cell machinery activity.

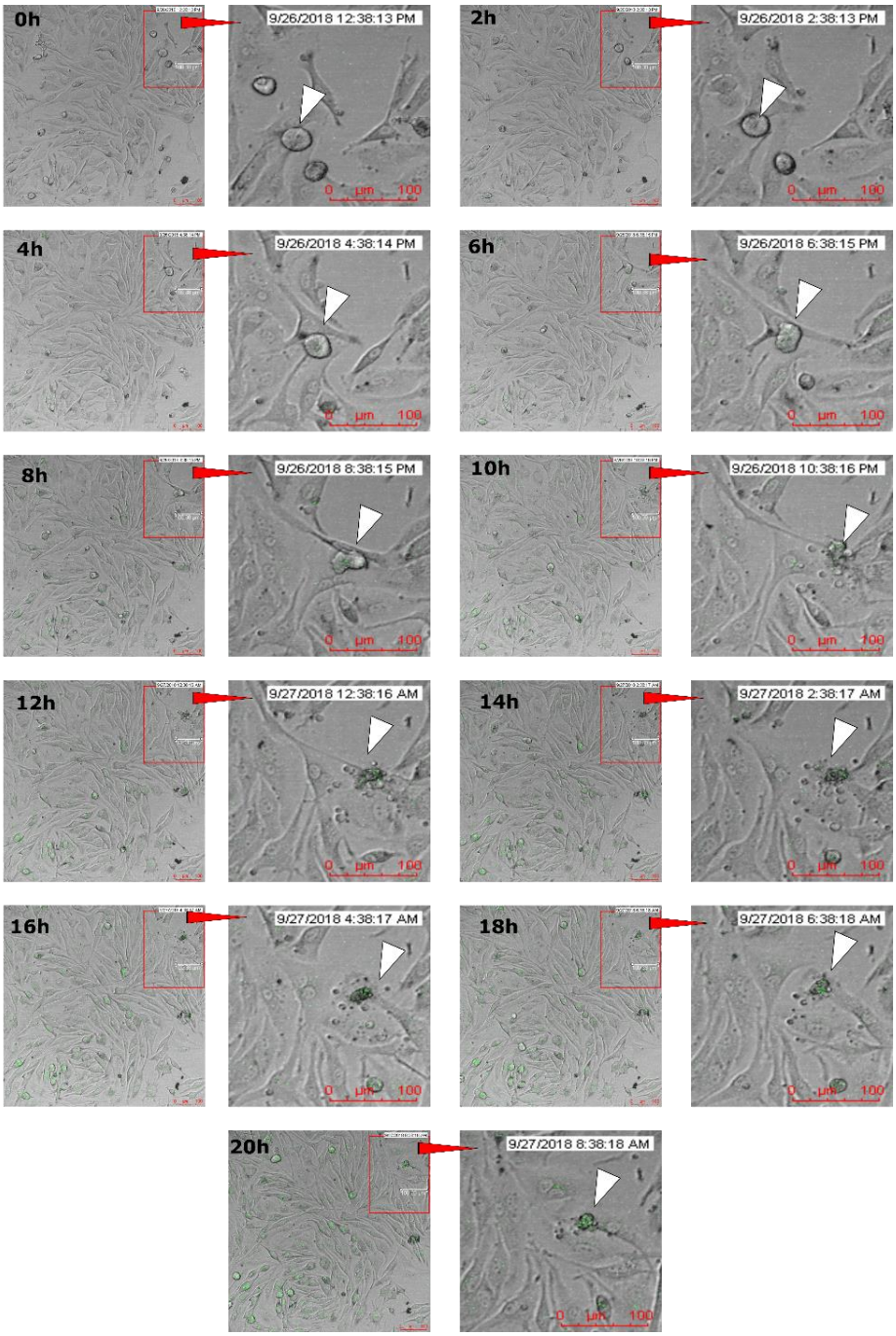


Figure 8. Microcell development and GFP expression after 24 h of doxorubicin (DOX) treatment in Sk-Mel-28 cells. The images were taken every 2 hours; the red squares mark the region of interest (ROI). At 0, 2, 4, 6, 8, and 10 h, the cell was round, morphologically similar to the beginning of apoptosis

(white arrow); at 12, 14, 16, and 18 h, the start of microcell development of microcells (16–18 h, white arrow) and the expression of GFP could be observed. At 20 h (white arrow), the microcells were fully developed and expressed GFP. The scale bar is 100 µm (Simsone et al. 2021).

3.5. Determination of Microcell Viability and Metabolic Activity

In the opinion of our research group, one of the most important microcell features is endocytosis. These cells could uptake from different substances as Neutral red uptake (NRU). This assay is used for cytotoxicity detection. The principle of this assay is based on the detection of viable cells by uptake of the NRU. Untreated Sk-Mel-28 cells (Figure 9, A) showed uptake of NR via active transport–endocytoses, meaning these cells are viable. Otherwise, Sk-Mel-28 (Figure 9, B) cells presented lack or no NRU at all, which means that the cells are non-viable. In turn, the microcell (Figure 9, B, red arrow) presented intensive NR uptake. As seen in phase contrast (Figure 9, B, PhC) around the microcell there is an empty area that indicates an increase in endocytosis and cell viability (Simsone et al. 2021).

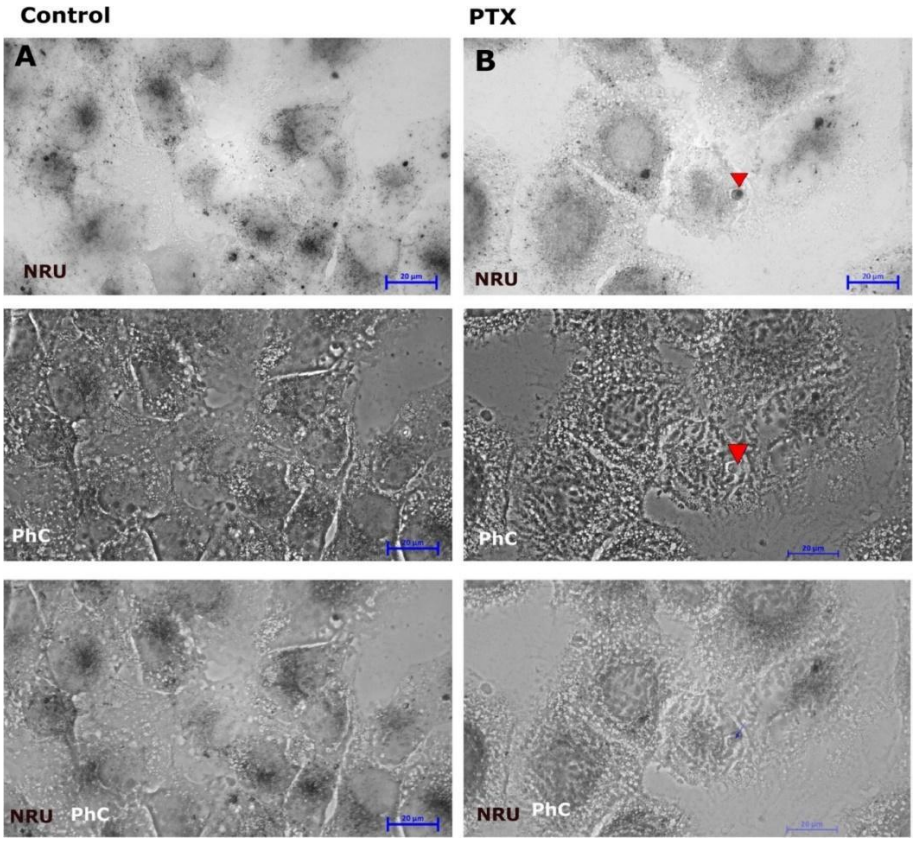


Figure 9. Microcell viability detection using the NRU assay in Sk-Mel-28 cell line after applying PTX for 48 h. (**A**) Untreated Sk-Mel-28 cells with positive NR uptake, NRU – NR uptake, phase contrasts, NRU/PhC - NRU and PhC overlay; (**B**) SK-MEL-28 cells treated with PTX with significant

NR uptake in microcells (NRU, red arrow), NRU – NR uptake, PhC – phase contrast, NRU/PhC - NRU and PhC overlay. The microcell showed intensive NR uptake, and this small single cell is localized near the microcell debris (PhC). The scale bar is 20 μm (Simsone et al. 2021).

To assess the viability of cells after anticancer treatment, we used an *in vitro* NADPH test. PTX, as an anticancer compound, and the Sk-Mel-28 cell line were chosen for this experiment. The NADPH test shows the metabolic activity of the cell. Untreated Sk-Mel-28 cells exhibited metabolic activity, as expected (Figure 10, A). However, the macrocells had decreased metabolic activity, except for the microcells, which exhibited high NADPH activity (Figure 10, B). Microcells can breathe, meaning that these cells are not apoptotic and thus cannot die. Consequently, microcellular protein expression is not excluded; This metabolic activity indicates that microcell cells resist treatment (Simsone et al. 2021).

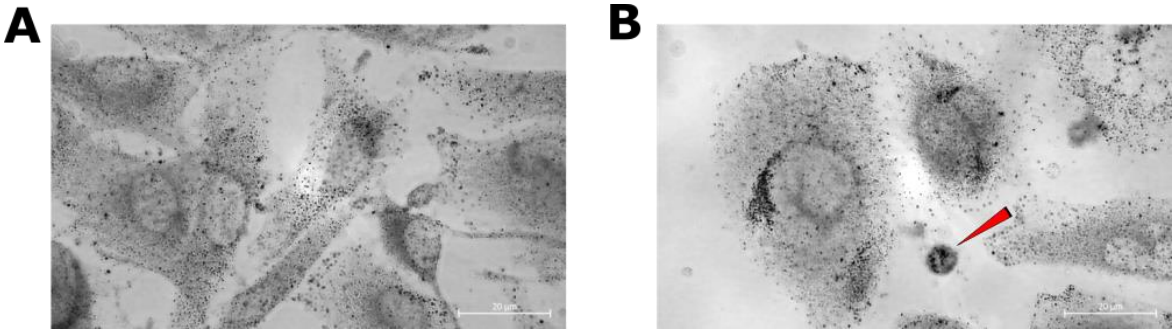


Figure 10. Microcell metabolic activity was determined by a NADPH test using Sk-Mel-28 cells 48 h after Paclitaxel (PTX). (**A**) Untreated cells, all NADPH positive, dark spots; (**B**) PTX-treated cells, microcell (red arrow), NADPH positive cell. The scale bar is 20 μm (Simsone et al. 2021).

3.6. Ability of Microcells to Regeneration of DNA and RNA

3.6.1. Proteins and nucleic acids in early formed Microcell

ANS-EtBr cell staining methods were used to identify proteins and nucleic acids in the microcell. ANS stains proteins (Figure 11, A) and EtBr stains nucleic acids (Figure 11, B). In this experiment, methanol was used as a stress factor to induce microcell formation. Methanol is known as an apoptosis stimulant (Toumia et al. 2020).

Proteins are expressed stronger in microcells after anticancer therapy. Therefore, they are more remarkable than other cancer cells.

Although the microcells contain both proteins and nucleic acid, it has not yet been indicated that the cells are viable. Therefore, it is important to determine microcell regenerative properties and RNA and DNA synthesis ability (Simsone et al. 2021).

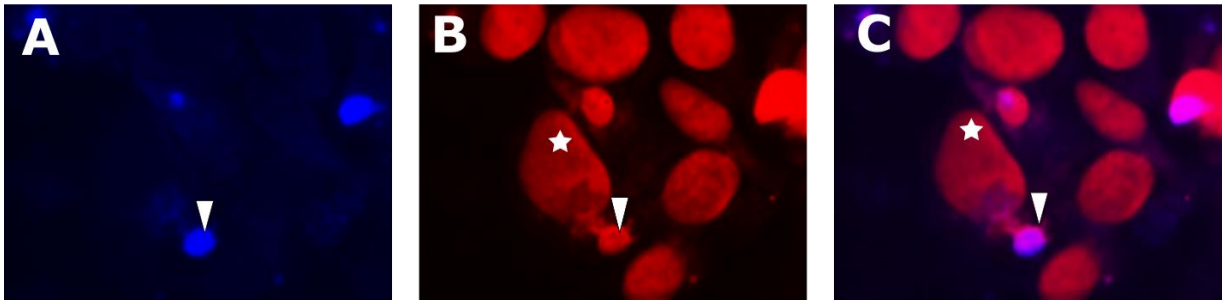


Figure 11. Microcell formation in the *HeLa* cell line after treatment with 7.5% methanol. (**A**) Microcell formation (blue protein body (ANS) and white arrow); (B) EtBr-stained microcell (white arrow) in contact with a macrocell (white star); (**C**) overlay (**A**, **B**). ANS–EtBr staining 6 h after treatment. The scale bar is 25 μm (Simsone et al. 2021).

3.6.2. RNA Synthesis in Microcells After Paclitaxel Treatment

During RNA labeling, it is possible to detect newly synthesized RNA (Abcam 2020). *The de novo* synthesized RNA demonstrates the cell's ability to survive after PTX treatment, as RNA is crucial for gene translation and protein expression in living cells. RNA synthesis is observed in all control cell nuclei (Figure 12, A, control). However, RNA synthesis is detected in various cell nuclei in PTX-treated cells (Figure 12, PTX-treated). RNA synthesis is not equal in all cells; it is observed in polyploid cell nuclei (Figure 12, B, PTX treated I), microcells (Figure 12, B, PTX-treated II), and macrocell nuclei (Figure 12, B, PTX-treated III). It appears that there are defined microcell subpopulations characterized by RNA+ synthesis and RNA synthesis. Macrocells that developed after PTX treatment were also characterized by two subpopulations: RNA+ synthesis and RNA− synthesis.

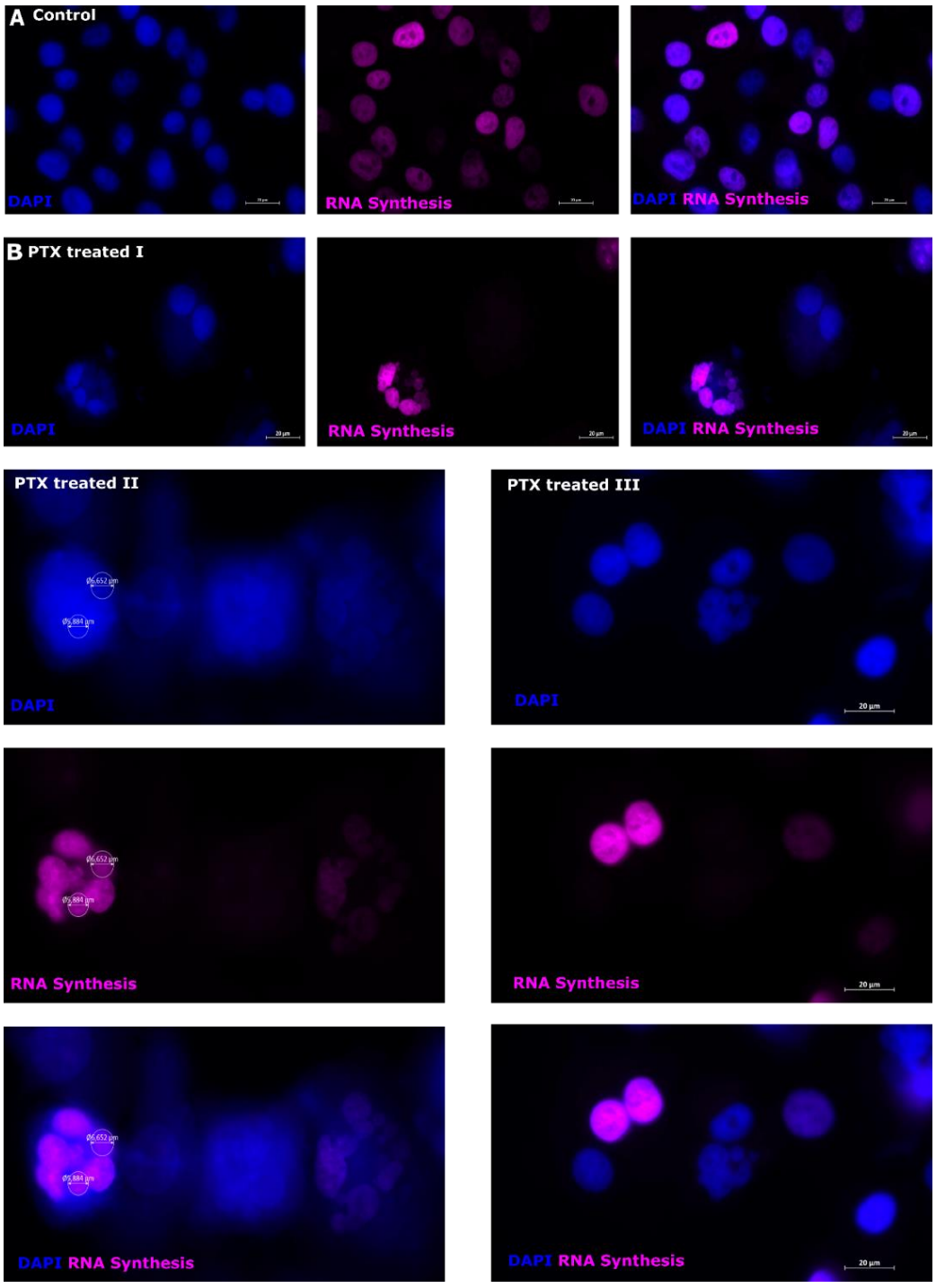
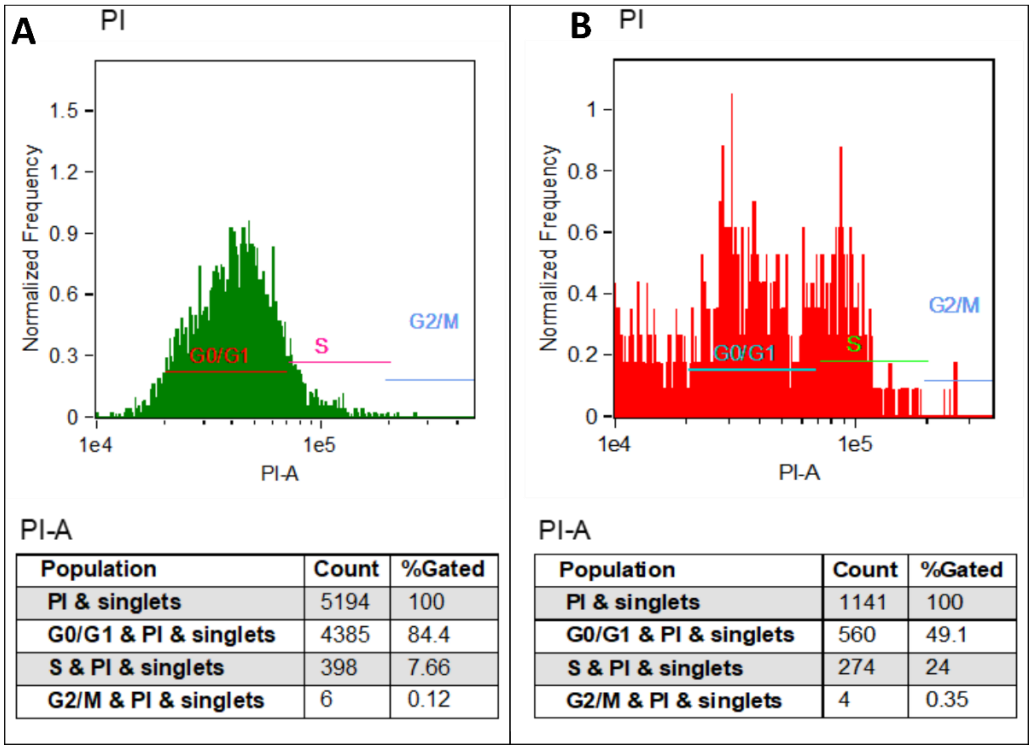


Figure 12. RNA synthesis in cells treated with PTX. (**A**) Control Sk-Mel-28 cells show RNA usynthesis labeling (purple) in all cell nuclei; cell nuclei are counterstained with DAPI (blue). (B) Sk-Mel-28 treated cells indicate RNA synthesis (purple) in polyploid cell nuclei (I), microcells (II) and macrocell nuclei (III); cell nuclei are counterstained with DAPI (blue).

3.6.3. Ability of Microcells to Regenerational DNA

Previous experiments have shown the formation of microcells and cells intensely stained with basic dyes (Freivalds, Buikis, and Harju 1996; Buiķis et al. 2002). Propidium iodide (PI) dye was used to assess the distribution of DNA abundance in the Sk-Mel-28 cell population under the influence of PTX. Figure 13 shows the distribution of cellular DNA in a histogram. In the control sample, a distribution is observed clustered around the diploid region of the DNA (Figure 13, A). After 48 h under the influence of PTX, both cells with increased amount of DNA and small cells with decreased amount of DNA are observed in the Sk-Mel-28 cell population (Figure 13, B). The results of subsequent studies using the EdU marker confirmed the viability of microcells in the small-cell population.



PI-A

Population	Count	%Gated
PI & singlets	5194	100
G0/G1 & PI & singlets	4385	84.4
S & PI & singlets	398	7.66
G2/M & PI & singlets	6	0.12

PI-A

Population	Count	%Gated
PI & singlets	1141	100
G0/G1 & PI & singlets	560	49.1
S & PI & singlets	274	24
G2/M & PI & singlets	4	0.35

Figure 13. DNA distribution in melanoma cell line 48 h after PTX treatment. DNA content histograms. (**A**) Sk-Mel-28 cells control sample, nuclei stained with PI (propidium iodide), (**B**) Sk-Mel-28 cells were treated with PTX and nuclei stained with PI.

Microcell proliferation was examined using flow cytometry. The EdU substrate was added to both Sk-Mel-28 control and PTX-treated cells. EdU is a thymidine nucleoside analog and is activated during DNA synthesis (Technologies Molecular Probes by Life, 2009). By analyzing the dot plot of the flow cytometry data, a small region of cells can be distinguished with high levels of DNA synthesis (Figure 14). It can be seen that the microcells have a high

level of DNA synthesis. The number of microcells in the control group is 0.84% and increased to 4.42% under PTX treatment (Table 3).

Additionally, the ratio of DNA synthesis to the amount of nucleic acids (EdU/PI) of positive microcells is significantly higher than that of the cell population (Table 3).

The ratio indicates an increased accumulation of DNA in the microcell compared to the rest of the population. They are metabolically more active cells with high proliferative potential.

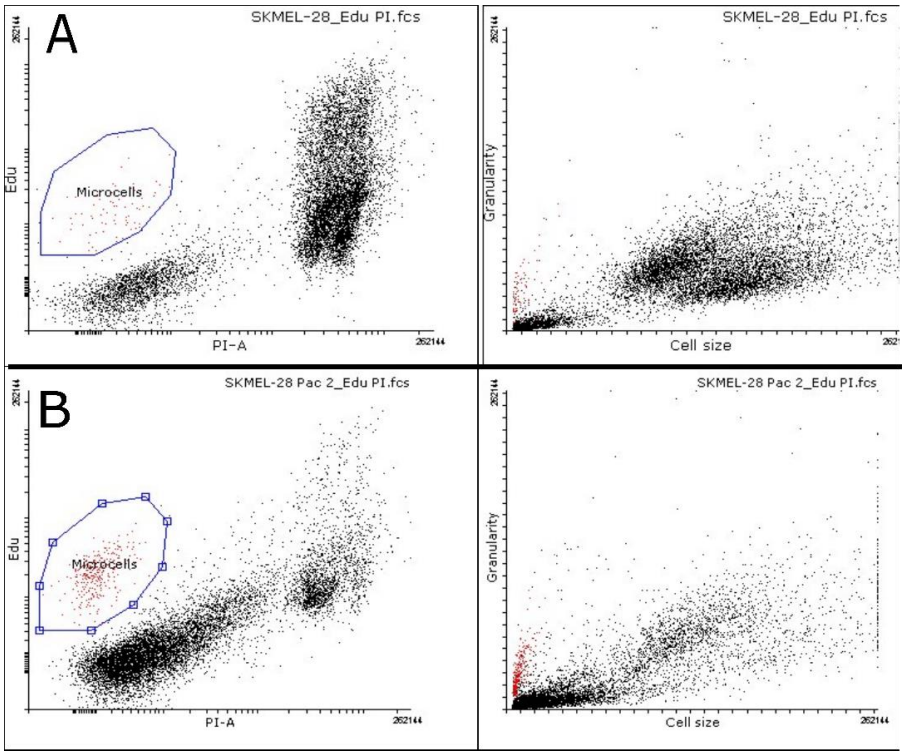


Figure 14. Flow cytometry data showing DNA synthesis in melanoma cells after PTX treatment. (**A**) Sk-Mel-28 control cells, stained with propidium iodide, and marked EdU. (B). Sk-Mel-28 PTX treated cells, stained with propidium iodide and marked to EdU.

Table 3. Cell fluorescence intensity ratio EdU/PI

Microcells		
	Microcell count	Mean (EdU/PI)
SKMEL-28_EdU/PI (control)	84	20.6
SKMEL-28 PTX 2_EdU/PI.	442	18.7
All cells		
SKMEL-28_EdU/PI.	10000	0.329
SKMEL-28 PTX 2_Ed_ PI.	10000	0.937

3.7. Identification of Microcells in the Cell Membrane Using Different Cell Markers

3.7.1. Microcells do not expressing TSG101 Antigen

TSG101 is a biogenesis factor associated with extracellular vesicles (Gebara et al. 2020). In this study, the expression of TSG101 is evaluated in microcells to differentiate microcells from extracellular vesicles. Sk-Mel-28 cells untreated (Figure 15, A) expressed TSG101. The expression of TSG101 is seen as a collection of small green dots (Figure 13, A, TSG101). Antigen is expressed in the cell cytoplasm (Figure, 15, A, PhC) near the nucleus (Figure 15, A, DAPI/TSG101/PhC). In turn, PTX treatment induced microcell formation in the SK-MEL-28cell line (Figure 15, B, DAPI). However, TSG101 (Figure 15, B, TSG101) is not expressed in microcells, but in the cell cytoplasm (Figure 15, B, PhC) and is located near the nuclei (Figure 15, A, DAPI/TSG101/PhC). This experiment showed that microcells are not extracellular vesicles that express TSG101.

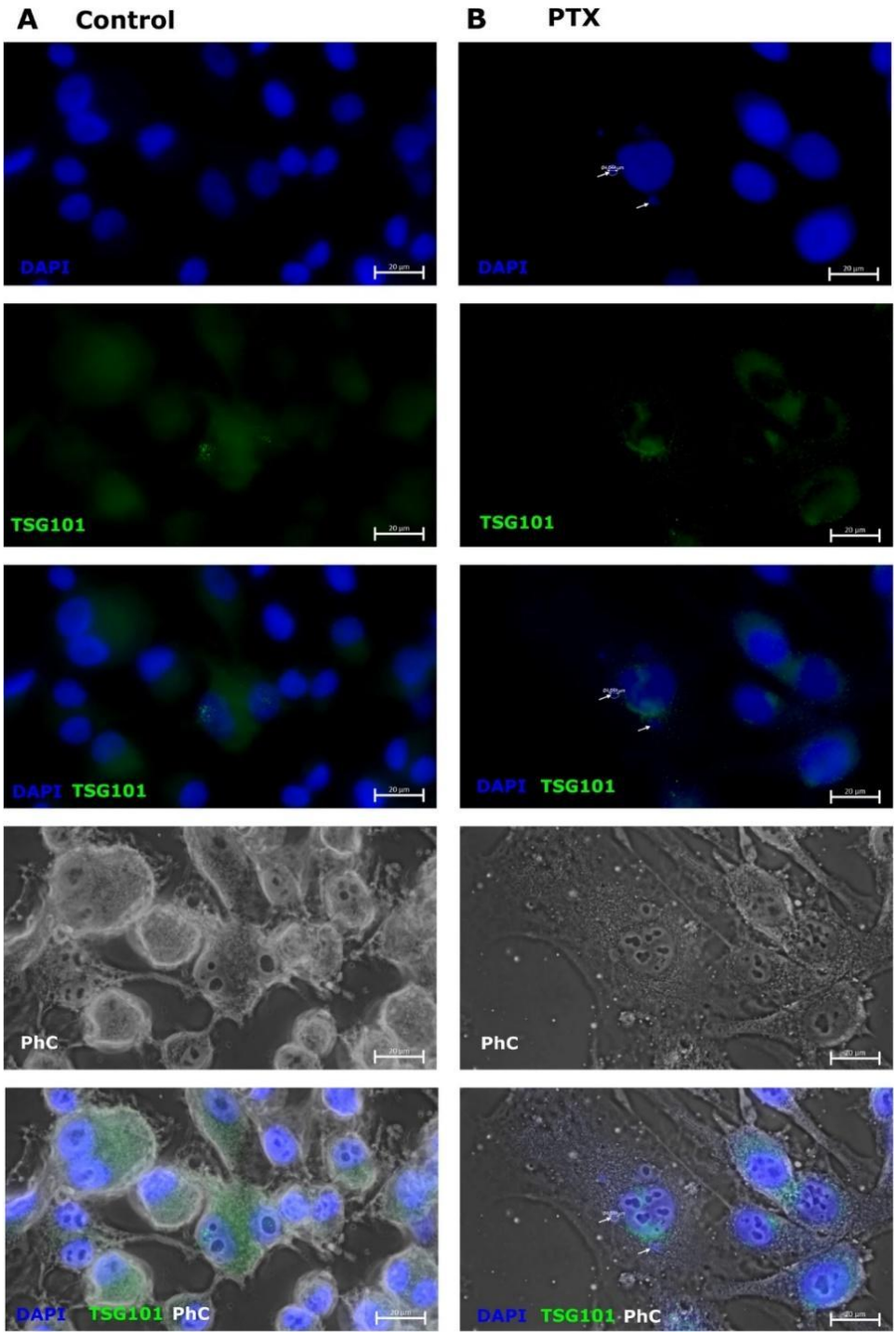


Figure 15. TSG101 expression in Sk-Mel-28 cell. (**A**) Untreated Sk-Mel-28 cells expressed the TSG101 (green) marker in their cytoplasm (PhC- phase contrast) located near the nucleus (DAPI, blue). Cell nuclei are stained with DAPI (blue) and the last figure in the column is overlaid (DAPI/TSG101/PhC); (**B**) Sk-Mel-28 treated with paclitaxel (PTX) after 48 h. Cell nuclei are stained with DAPI (blue), and cells are captured in phase contrast (PhC) and overlaid (DAPI/TSG101/PhC). After PTX treatment, microcell formation was induced (DAPI, blue); nevertheless, TSG101 (green) is not expressed in microcells, but nearby nuclei. The scale bar is 20 μm (Simsone et al. 2021).

3.7.2. Caspase-2 Expression After Paclitaxel Treatment in Microcells

Caspase-2 (Casp-2) functions as an initiator caspase of the caspase family of proteases, which responds by stimulating the initiation of the apoptotic cascade (Ando et al. 2017; Brown-Suedel and Bouchier-Hayes 2020). Casp-2 is expressed in the cell nucleus in the control sample (Figure 16, A) with green fluorescence. Cell nuclei were counterstained with DAPI in fluorescent blue (Figure 16). Casp-2 expression is equal and smooth in the nucleus of the control cell, while in the treated cells, the expression is different (Figure 16, B). Casp-2 expression in the nucleus of the PTX-treated cell is not smooth, and it is stronger in large polyploid cells with multiple nuclei (Figure 16, B I, II). The nuclei are of different sizes and contain various amounts of DNA amounts that show staining with DAPI (Figure 16, B I, II). The microcell nuclei are 2.5, 3.37, and 4.61 μm in diameter (Figure 16, B I, II). The microcell nuclei strongly expressed Casp-2 (Figure 16 B I, II), and the amount of DNA (counterstained with DAPI) in the microcell is high, as shown by DAPI.

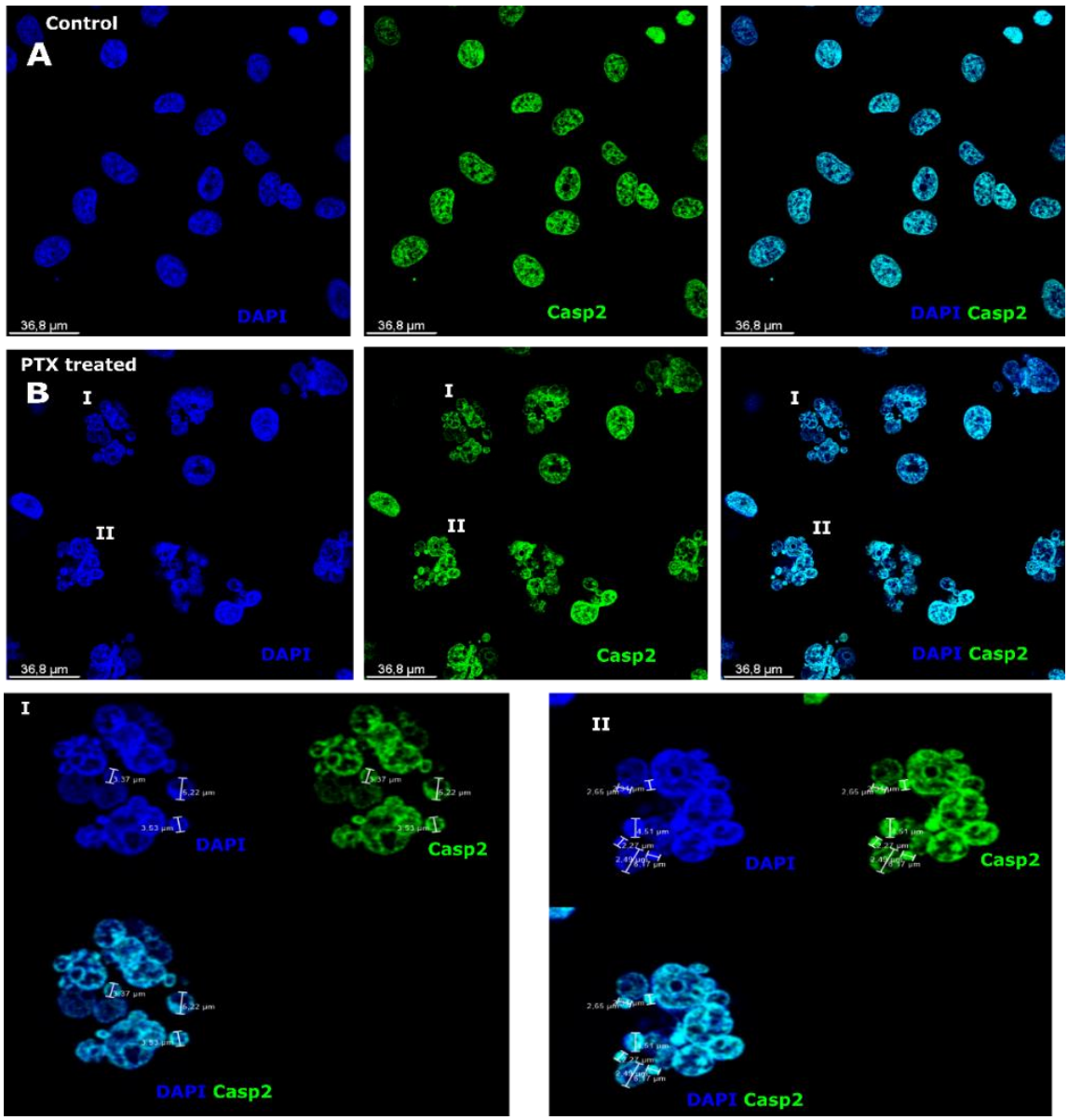


Figure 16. Caspase-2 expression in PTX-induced microcells. (**A**) Untreated Sk-Mel-28 cells (control cells). Cell nuclei are counterstained with DAPI (blue). Caspase-2 (green) is expressed in cell nuclei. (B) Sk-Mel-28 cells. Cell nuclei are counterstained with DAPI (blue) and Caspase-2 (green) is expressed in cell nuclei, though stronger expression of Caspase-2 is evaluated in microcells (B I and BII). I and II are enlarged to observe the PTX-treated B I and II macrocells and adjacent microcells.

3.7.3. Caspase-6 Expression After Paclitaxel Treatment in Microcells

During apoptosis, Caspase-6 (Casp-6) is an effector caspase like Caspase-3 and is usually cleaved and activated by an initiator caspase such as Casp-2 (Watanabe et al. 2008). Casp-6 was found in the cytoplasm of Sk-Mel-28 control cells, but was more abundant in the cell periphery and cell membrane (Figure 17, A). In turn, Casp-6 from Sk-Mel-28 cell Casp-6 was expressed in cell nuclei (Figure 17, B) and microcells (Figure 17, B, white arrowhead). The nuclei are counterstained with DAPI (blue).

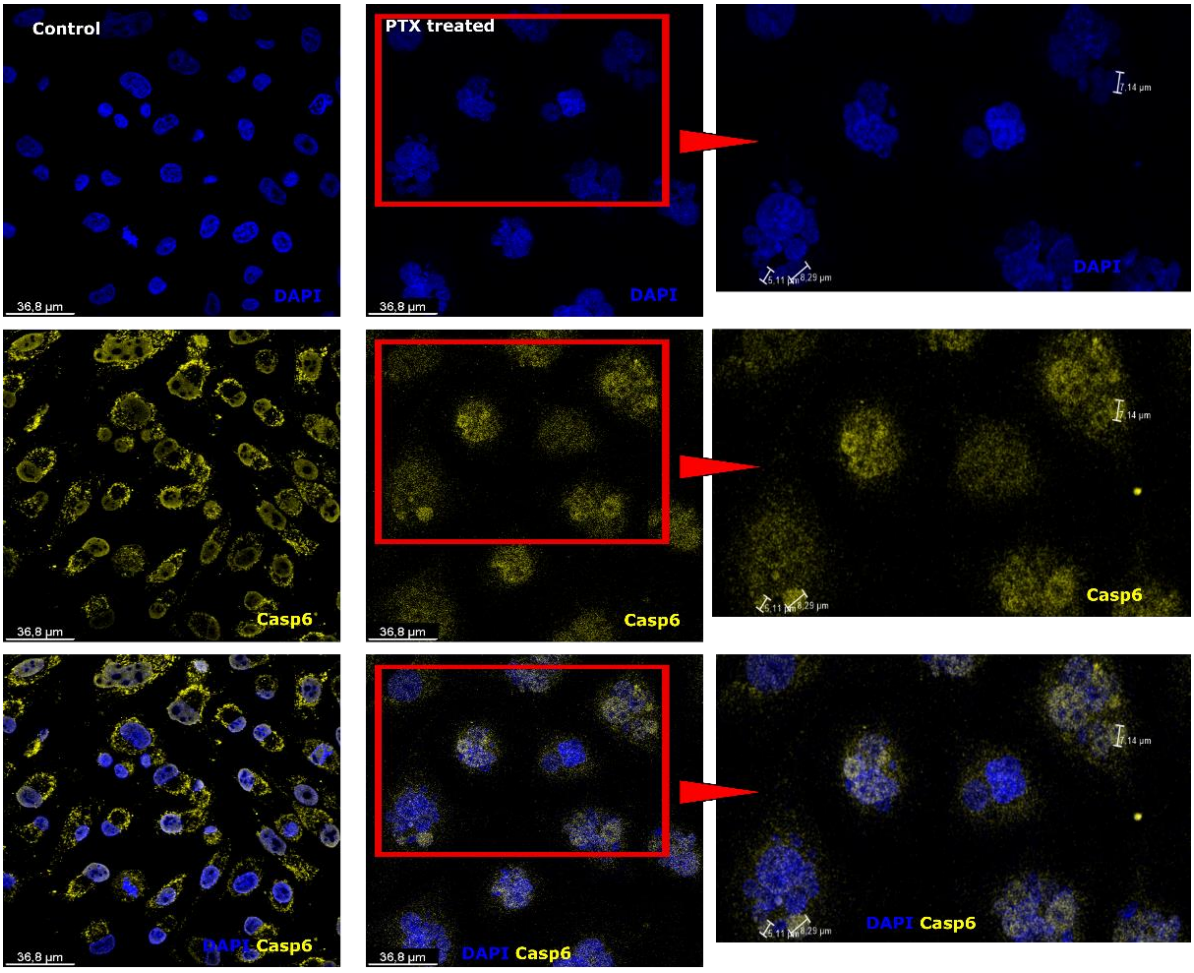


Figure 17. Caspase-6 expression in microcells. (A) Control, untreated Sk-Mel-28 cells expressed Caspase-6 (Casp-6; yellow) in the cell cytoplasm; cell nuclei are counterstained with DAPI (blue). (B) Sk-Mel-28 cells expressed Caspase-6 (Casp-6; yellow) in the nucleus area and microcells (white arrowhead); Cell nuclei are counterstained with DAPI (blue), the region of interest (red square) enlarged on the right side.

3.7.4. Expression of aldehyde dehydrogenase and caspase-3 after Paclitaxel Treatment in microcells

ALDH2 (aldehyde dehydrogenase-2) is a co-enzyme that characterizes cell metabolic activity (Yan and Wu 2018), indicating the ability of cells to resist treatment. ALDH2 is expressed in the cytoplasm of control and PTX-treated melanoma cells (Figure 18). However, in cells treated with PTX, the expression of ALDH2 is stronger (Figure 18, B).

Caspase-3 (Casp-3) is involved in the execution of apoptosis and plays a role in the evaluation of tumor regression (Toumia et al. 2020). Casp-3 expression is not observed in control cells (Figure 18, A, green), while expression in cells is observed in the cell cytoplasm (Figure 18, B, green).

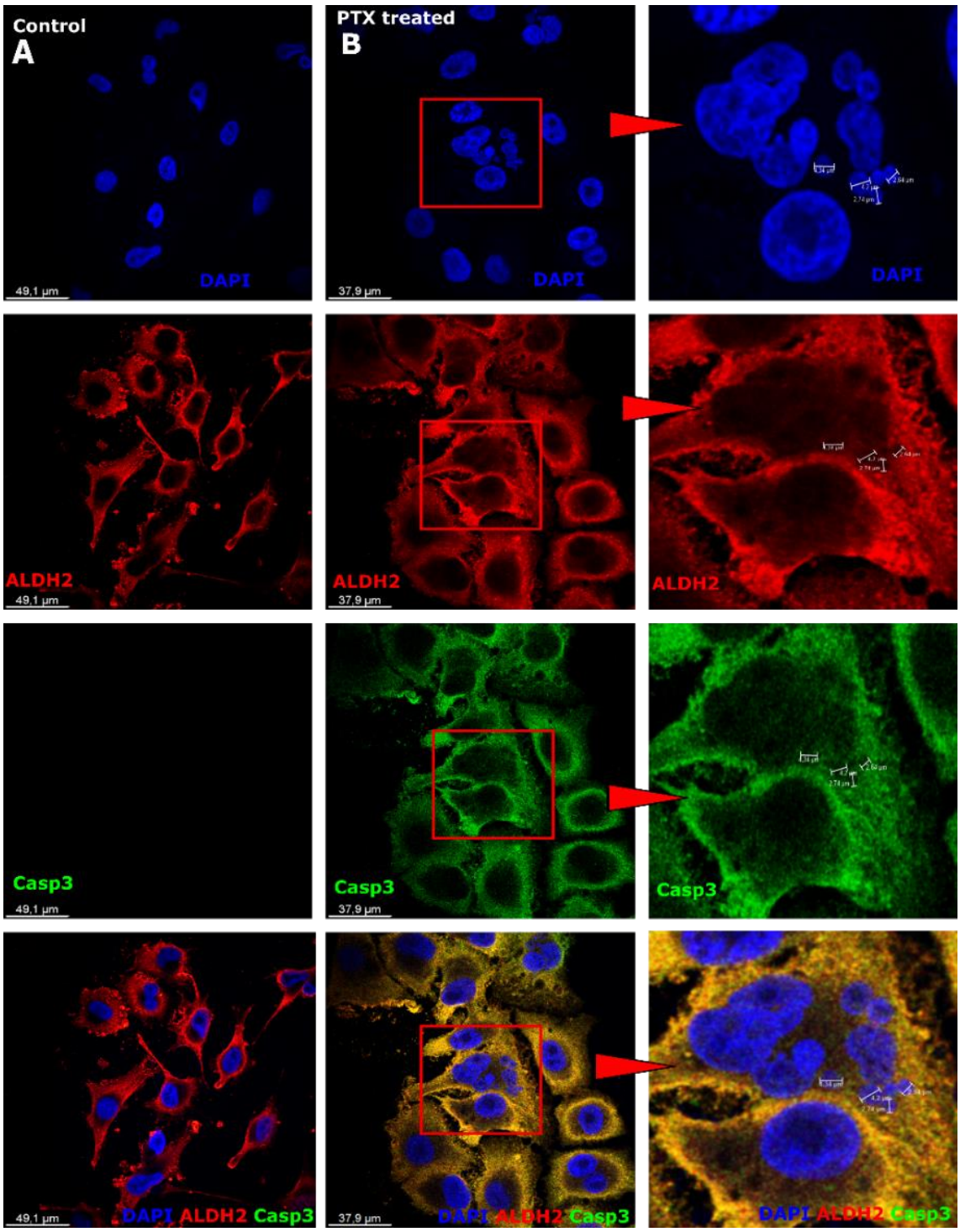


Figure 18. Expression of aldehyde dehydrogenase and caspase-3 in melanoma cells. (**A**) Control cells SK-MEL-28 expressed aldehyde dehydrogenase (ALDH2; red) in the cell cytoplasm; caspase-3 (Casp-3; green) expressed in the cell cytoplasm; cell nuclei are counterstained with DAPI (blue). (B) SK-MEL-28 cells with aldehyde dehydrogenase (ALDH2; red) in cell cytoplasm; caspase-3 (Casp-3; green) expressed in cell cytoplasm; cell nuclei are counterstained with DAPI (blue), region of interest (red square) enlarged on the right side.

3.7.5. SOX2 and Nanog Expression after Paclitaxel Treatment in Microcells

SOX2 and Nanog are stem cell markers associated with pluripotency, cell regulation, and reprogramming (Shuchen Zhang 2014; P. Yu et al. 2018). SOX2 is expressed in the cell nuclei (Figure 19, A, green) and Nanog is expressed in the cell cytoplasm (Figure 19, A, purple) in control cells. SOX2 was expressed strongly in cell nuclei but weakly in the cytoplasm in cells (Figure 19, B, green), while Nanog was expressed in the cytoplasm (Figure 19, B, purple). However, in PTX-induced microcells (Figure 19, B, white arrows), the expression of SOX2 and Nanog is observed in the cell region between small nuclei.

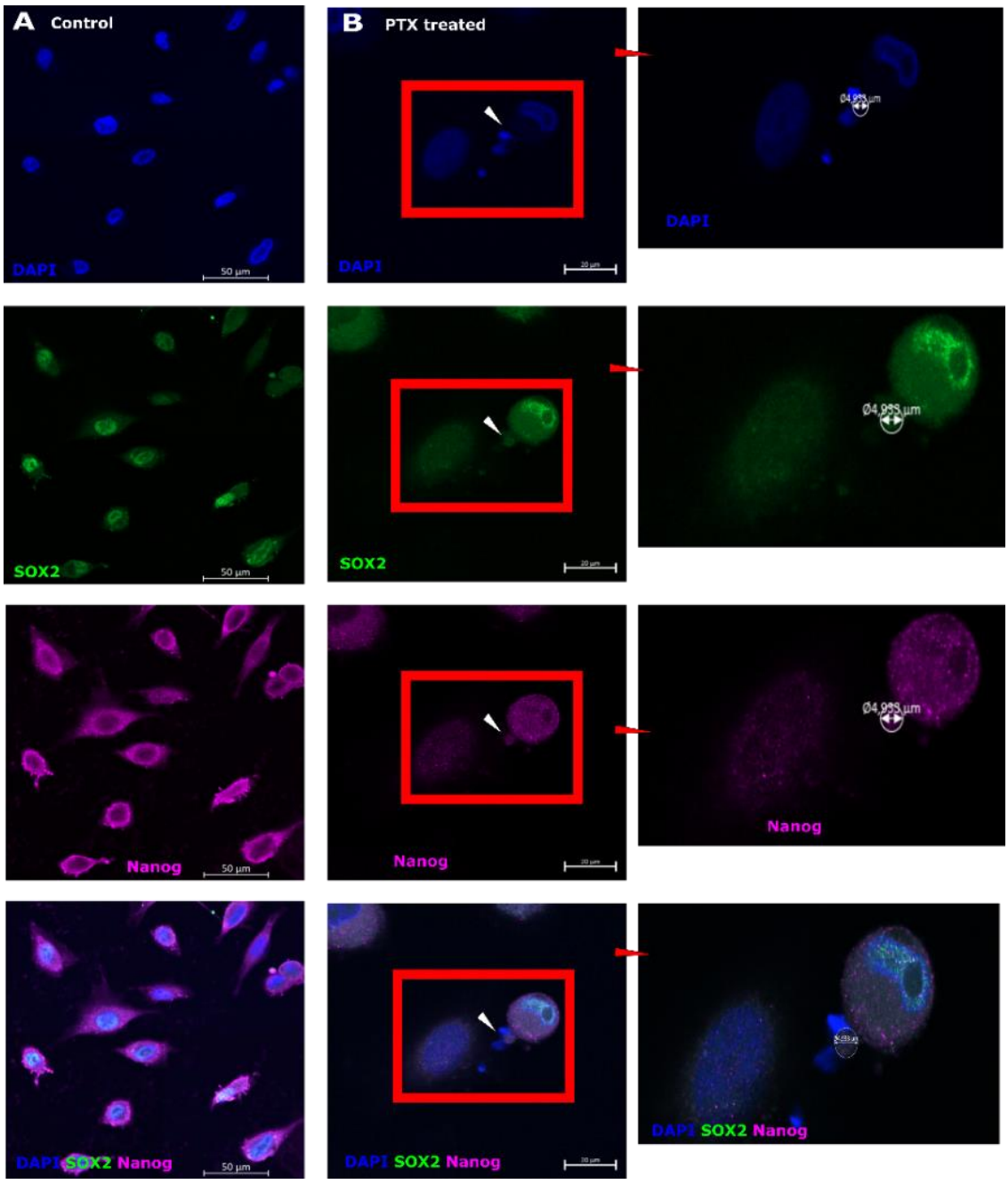


Figure 19. SOX2 and Nanog expression in microcells. (**A**) Control Sk-Mel-28 cells expressed SOX2 (green) in cell nuclei; Nanog (purple) expressed in the cell cytoplasm; cell nuclei are counterstained with DAPI (blue). (**B**) PTX-treated Sk-Mel-28 cells SOX2 expressed (green) in cell nuclei and cytoplasm; Nanog (purple) expressed in cell cytoplasm; cell nuclei are counterstained with DAPI (blue). Microcells (white arrows) expressed both SOX2 and Nanog; the region of interest (red square) is enlarged on the right side.

3.7.6. Proliferating cell nuclear antigen expression after Paclitaxel Treatment in microcells

The proliferation cell nuclear antigen (PCNA) plays an essential role in normal DNA synthesis and replication (Peng et al. 2018). This experiment was carried out on two different cell lines: melanoma (Sk-Mel-28) and human lung carcinoma (A549) to show that the microcells are not specific to any particular cancer cell line. Microcells are observed in *HeLa* and the human sarcoma cell line HT-1080 (Buiķis, Harju, and Freivalds 1999; Buiķis et al. 2002; Bema et al. 2008). PCNA expression is observed in cell nuclei in Sk-Mel-28 control cells (Figure 20, A, control). However, this expression is weak and is also observed in the cell cytoplasm. In cells treated with PTX, PCNA is expressed only in cell nuclei (Figure 20, B, PTX treated). The expression of PCNA is not equal in all nuclei. There are round nuclei where PCNA expression is moderately strong compared to multinuclear cells. In multinuclear cells, there are a few microcell nuclei that strongly express PCNA (Figure 20, B, PTX treated, red square, white arrow). Microcells can be separated by PCNA expression region. PCNA is expressed in all cells, but not in all microcells. PCNA is expressed in the periphery of the nucleus, nucleus, and cell cytoplasm of microcells. The A549 control cells (Figure 20, C, control) show different expressions of PCNA. Expression is evaluated in the cell nuclei, but parts of cells show expression in the periphery of the nucleus. Microcell formation (Figure 20, D, A549) is observed in the A549 cell line after treatment with PTX. The antigen of the PCNA expression in microcells is observed in the periphery of the nucleus (Figure 20, D, A549 treated with PTX, white arrow).

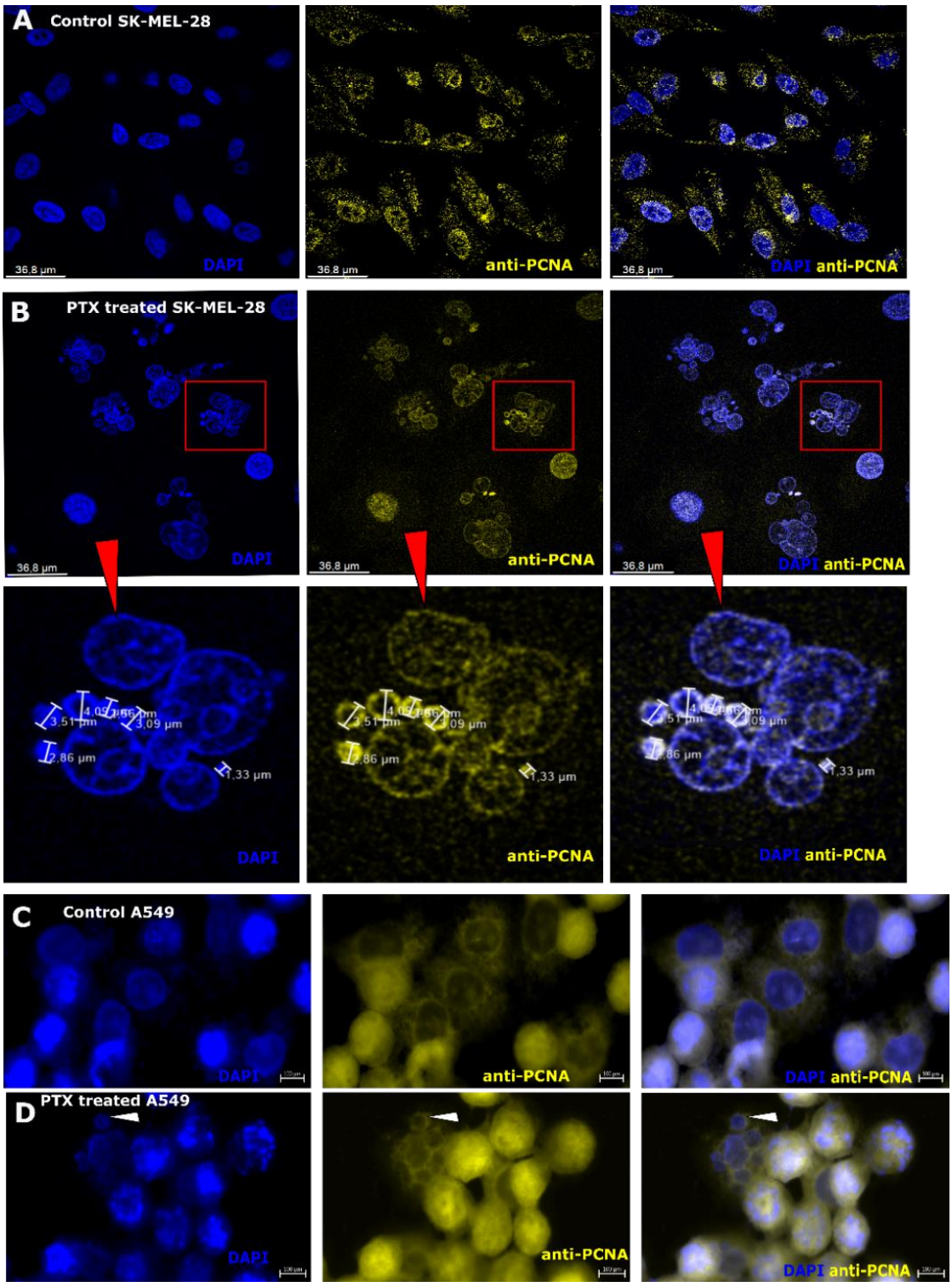


Figure 20. Anti-PCNA expression in melanoma and human lung carcinoma cells. (**A**) Control Sk-Mel-28 cells weakly expressed anti-PCNA (yellow) in the cell nuclei; cell nuclei are counterstained with DAPI (blue). (B) Sk-Mel-28 cells expressed anti-PCNA (yellow) in the cell nuclei and strongly expressed it in the microcells; cell nuclei are counterstained with DAPI (blue). Microcells (white arrow) strongly expressed anti-PCNA (yellow); region of interest (ROI; red square) is enlarged below. (**C**) Control, A549 cells express anti-PCNA (yellow): part of the cells shows strong expression, while the

other part of the cells shows weak expression; cell nuclei are counterstained with DAPI (blue). (**D**) PTX treated A549 cells expressed anti-PCNA (yellow) in cell nuclei and microcells; cell nuclei are counterstained with DAPI (blue). Microcells (white arrow) expressed anti-PCNA (yellow) in the periphery.

Summarizing these results, microcells are characterized by the expression of Casp-2, Casp-6, Casp-3, ALDH2, SOX2, Nanog, PCNA, and RNA synthesis (Table 4) in cell nuclei as well as cell cytoplasm.

Table 4. Marker expression in control cells treated with PTX.

The examined markers	Expression in control cells	Expression in PTX-treated cells	Expression in PTX-induced microcells induced by PTX
Casp-2	Nucleus	Strongly expressed in the nucleus	Strongly expressed in the nucleus
Casp-6	Cytoplasm	Cytoplasm and nucleus	Nucleus and nucleus periphery
Casp-3	No expression	Cytoplasm	Cytoplasm
ALDH2	Cytoplasm	Cytoplasm	Cytoplasm
SOX2	Nucleus	Strongly expressed in the nucleus	Cytoplasm and nucleus
Nanog	Cytoplasm	Cytoplasm	Cytoplasm and nucleus periphery
PCNA	Nucleus and weakly in cytoplasm	Nucleus	Strongly expressed in the nucleus and nucleus periphery
RNA synthesis	Nucleus	Nucleus	Nucleus

4. DISCUSSION

In this study, the chemotherapeutic drugs DOX and PTX acted on cells for 24 hours to generate microcells in human cancer cell lines (Sk-Mel-28, A549 and *HeLa*) and human fibroblasts (HS-68). After 6 hours of 7.5% methanol treatment on *HeLa* cells, we saw that microcells also appeared. We have demonstrated how apoptotic macrocells, which themselves evolved from apoptotic bodies, give rise to microcells. Microcell development in this study was observed after exposure to chemotherapy to cancer cells or as indicated by morphological changes in cells. Our results are consistent with those of Buiķis et al. (Buiķis et al. 2002), who demonstrated that the sporosis mechanism may successfully produce microcells from injured or fatally damaged macrocells. Newly formed microcells have endocytosis activity and are autonomous and migratory. Microcells with a diameter of 2.5 to 5 μm that form by the sporosis mechanism can resist anti-cancer treatment (Simsone et al. 2020; Golubnitschaja et al. 2020). Following anticancer therapy, Bonghan microcells (BH-MCs), Raju cells, nucleolar aggresomes (NoAs), and microcells have also been seen in cancer cell lines. After being exposed to etoposide (VP-16) or X-rays, Sundaram et al. noticed the formation of cell mitotic colonies on the monolayer in cell culture (Sundaram et al. 2004). The number of Raju cells increased to approximately 10, with a diameter of 6-10 μm, on day 14 after the start of the treatment, from the mother cell through a process known as neosis; nevertheless, only 8% of them failed to survive (Sundaram et al. 2004). After a mitotic crisis and the creation of fresh Raju cells from polyploid cells, Raju cells can survive for up to 8 weeks (Sundaram et al. 2004). Another study demonstrated that NoAs form five days after receiving VP-16 therapy, and these NoA cells contained fibrillarin, rDNA, and pericentric heterochromatin (Salmina et al. 2017). In contrast to Raju cells and microcells, BH-MCs have been seen in healthy tissues, including the rat small intestine and blood cell leukocytes (Baik et al. 2009; Baik, Dobberstein, and Soh 2007). A BH-MC is a tiny cell that resembles adult stem cells in size, ability to divide, and pluripotent differentiation characteristics (Baik et al. 2009). The distinction between BH-MCs and microcells is that BH-MCs form from micronucleation during typical physiological processes, whereas microcells are produced from cancer cells (Buiķis, Harju, and Freivalds 1999; Baik et al. 2009). Raju cells and NoAs grow from the Raju cell line, which is different from microcells and Raju cells because microcells develop from macrocells 24 to 48 hours after anticancer therapy (Buiķis, Harju, and Freivalds 1999; Simsone et al. 2021), whereas Raju cells and NoAs are formed from the polyploid or multiply-sized cell group up to one week after

anticancer treatment. Using carmine red and Indian ink, it was found that the ability of microcells to engulf and ingest particles increased, indicating metabolic activity of cells is increased (Bema et al. 2008). Microcell formation is a rare occurrence that occurs in less than 1% of cases. The number of small cells in the group of cell lines studied was only 1%. Studies have found that after erlotinib treatment, a small percentage (around 0.3-5%) of cancer cells become drug-tolerant and can survive. DTP cell are a type of cancer cells that is resistant to anti-cancer drugs, specifically those used in chemotherapy (Sharma et al. 2011; Rehman et al. 2021). Numerous anticancer medications, including those used in this work, Paclitaxel, and doxorubicin, as well as methanol, generate cell stress, resulting in DNA damage and cellular membrane, disrupt cell homeostasis, and induces apoptosis (Krysko et al. 2008; Matczak et al. 2009; Toumia et al. 2020). Following treatment with UV radiation, DOX, or PTX, the cell membrane becomes permeable (Matczak et al. 2009; Yao et al. 2008), making it simpler to transfect plasmid DNA into the cells. During mitosis, cells modify their architecture to a spherical shape to protect themselves from harm; this action is regulated by modifications to the actin cytoskeleton. Repairing DNA damage during mitosis is risky for cells and may result in erroneous chromatid separation during anaphases, which can lead to genomic instability and the development of cancer (Burgess, Rasouli, and Rogers 2014). Apoptosis, a fundamental and intricate biological process that kills unwanted or damaged cells, is made possible by an organism's development and homeostasis (Porter and Ja 1999). Plasma membrane blebbing, nuclear fragmentation, chromatin condensation, which includes chromatin parts on the nuclear membrane, and the development of apoptotic bodies are all common features of an apoptosis scenario (Repetto, del Peso, and Zurita 2008). Many concerns remain unresolved even though a vast amount of data suggests that cytotoxic medications stimulate and activate the cellular stress response, as well as the apoptosis initiation machinery. For instance, as previously shown by Herr and Debatin (2001), the belief that apoptosis constitutes the fundamental process by which tumor cells are eliminated as a result of cancer therapy may not always be correct (Herr and Debatin 2001). Small, round, or oval cells with little cytoplasm are known as microcells. The maximum number of microcells was observed 48 hours after chemotherapy, with the microcells generally increasing after chemotherapy, irradiation, or immunotherapy (Buiķis, Harju, and Freivalds 1999). Microcell count after 24 hours of anticancer drug treatment. According to one theory, a microcell develops from a dead macrocell (Buiķis, Harju, and Freivalds 1999). We demonstrated that proteins predominate in the early stages of microcell development (Figure 4). For the study of proteins, the ANS solution is an ubiquitous and often

used substance (Labieniec and Gabryelak 2006). Nucleic acid can also be seen when the microcells have fully developed (Figure 10, B). In another study, it was discovered that the microcells formed during therapy contained ribosome-like particles and a nucleus with strong functional activity (Simsone et al. 2020; Buiķis, Harju, and Freivalds 1993). The microcell has particular organelles that are typical of live cells, as can be observed in the electron microscopy image (Figure 6). Oxidative stress leads to DNA damage, which promotes cancer (Valko et al. 2006). A NADPH test can show that these cells are metabolically active. To reduce the degree of hypoxia in a cell, NADPH serves as an antioxidant substrate for the antioxidant systems of thioredoxin and glutathione (Klener and Klanova 2020; Pribluda, De La Cruz, and Jackson 2015). In addition to hyperstabilizing microtubules and inhibiting cytoskeletal reorganization, Paclitaxel also increases metabolic oxidative stress and NADPH oxidase, which are both linked to Paclitaxel's anticancer action (Hadzic et al. 2010a). NADPH is a universal electron donor in reductive biosynthesis and cell detoxification, making it an essential reducing agent for antioxidative defense mechanisms (Agledal, Niere, and Ziegler 2010).

After anticancer therapy, we saw a decrease in NADPH activity and found microcells with high NADPH activity. Metabolically active cells create NADPH, a co-enzyme that signals cell viability (Jacobsson et al. 1987; Vercelli, Boido, and Jhaveri 2012). Furthermore, evidence suggests that following PTX therapy, microcells are metabolically active. Due to the high activity of NADPH in the microcell, we employed a 1 minute prefixation with 4% formaldehyde solution before performing the NADPH test (Simsone et al. 2021). This shows that the microcell's metabolism has increased as a result of the stress. NADPH oxidase is necessary for endothelial cell proliferation and migratory activity, according to Abid et al. (2000). NADPH is involved in DNA synthesis because it is crucial for the process by which ribonucleotide reductase converts ribonucleotides into deoxyribonucleotides (Agledal, Niere and Ziegler 2010). Microcells that have just developed are simpler to transfect since they express EGFP similarly to other cells. According to certain studies, cancer cells absorb more DNA molecules from the cultivation medium than noncancer cells (Kong et al. 2017; Sieni et al. 2020). There was a propensity for large cells with low DNA concentration and tiny cells with low DNA content to predominate in the luminal breast cancer cell population. However, the population of breast cancer cells included large and small cells as well as cells, with high and low DNA concentrations. Flow cytometry data show that PTX-treated melanoma cells are small with a high EdU content, indicating DNA synthesis (Figures 7 and 8). DNA synthesis is

indicative of a cell's ability to develop and proliferate (Zhang et al. 2021; Gu, Hickey, and Malkas 2023).

According to research to date, cancer stem cells can repair their DNA, which allows them to resist the effects of treatment (Islam, Gopalan, and Lam 2018; Yosefzon et al. 2018). PCNA and RNA production are markers of cell proliferation and protein synthesis (Lu, Ratnayake, and Rich 2019; Peng et al. 2018; Abcam 2020). PCNA expression was observed in the nucleus and cytoplasm of control cells. We noticed that microcell nuclei expressed PCNA after PTX treatment, indicating that microcells contain DNA (Simsone et al. 2020; Golubnitschaja et al. 2020). Increased expression of PCNA in microcells implies that they have a strong potential for renewal and high levels of replication activity. All control cell nuclei exhibit RNA production, while PTX-treated cells do not. Through RNA production, subpopulations of cells could be identified. Microcell subpopulations lacking RNA synthesis exist alongside macrocell subpopulations and microcells with enabled RNA synthesis. This suggests that the cells are active and can respond to or avoid cancer treatments. Microcells with PCNA expression and RNA production can survive treatment using the characteristics of cancer stem cells.

Cancer cells treated with PTX express ALDH2 more strongly than untreated cells. This enzyme illustrates how cells defend themselves and resist the use of alkylating chemicals in cancer treatment. Microcells can differentiate into pluripotent cells and have a better capacity for renewal. When cancer treatment is administered, microcells develop. Even though microcells contain apoptotic markers that signify planned cell death, these markers may also be a sign of the cell's tolerance to treatment. Undifferentiated cells are indicated by cell self-renewal and progeny. Cell metabolic activity is shown by the synthesis of ALDH, PCNA, and RNA, which encourages resistance to the treatment being used. To assess the efficacy of anticancer therapy, it is crucial to continue research and create specialized methods of microcell formation detection after treatment.

Microcells have a connection to tolerance to cancer treatment or tumor therapeutic resistance. Antigens and markers distinguish these microcells. Among other substances, markers include proteins, nucleic acids, antibodies, and peptides (Peng et al. 2018; Freivalds, Buikis, and Harju 1996; Henry and Hayes 2012; Aponte and Caicedo 2017). Cancer cells that are resistant to drugs but do not exhibit a phenotypic mutation can multiply in response to treatment. The actions of anticancer medications can cause certain cells in a subpopulation to grow, and these cells can carry a rare mutation that results in resistance to treatment (Balderstone et al. 2019; Fiorillo, Sotgia and Lisanti 2019). In our study, we saw microcell

induction and 48 hours after chemotherapy, we saw an increase in the number of microcells. It is crucial to focus on this type of cell in future studies as a characteristic of the mechanism of drug resistance or drug-tolerant cells. Endocytosis is a mechanism that occurs in cancer cells but not in normal cells, as reported by Kong et al. (2017). Other studies have demonstrated that cancer microcells collect nanoparticles and have enhanced endocytosis abilities (Bema et al. 2008; Simsone et al. 2021). High levels of metabolic activity in a cell, as seen in the cytosol or mitochondria, which are differentiated by their electrophoretic motilities, are correlated with the expression of ALDH2. PTX treated cells exhibit higher levels of cytoplasmic ALDH2 expression than control cells. Alcohol derivatives are metabolized by ALDH2 to support aldehyde dehydrogenation (Yan and Wu 2018). Chemotherapy drugs such as paclitaxel, doxorubicin, and gemcitabine are ineffective against cancer cells that have been found to produce ALDH. Tumor precursor cells are also related to the presence of ALDH in cells (Islam, Gopalan, and Lam 2018; Yan and Wu 2018). The family of initiator caspases is made up of Casp-2 (O'Byrne and Richard 2017). When cells are exposed to the microtubule inactivator vincristine, Casp-2 expression is seen in the cytoplasm rather than the typical cell nucleus (Paroni et al. 2002). All control cell nuclei showed Casp-2 expression, which was weaker in the nuclei of PTX-treated cells but strong in their microcells. Casp-2 is strongly expressed in nuclei, suggesting that the cell can resist anticancer therapies (Paroni et al. 2002; Capo-Chichi, Cai, and Xu 2018). In the current work, microcells that arise after PTX treatment nuclei also showed significant expression of Casp-6. Hpwever, Casp-6 is expressed in the cytoplasm of control cells near the nucleus. Casp-6 expression has been associated with nuclear laminas in cancer cells, which is a characteristic of tumor initiation, according to the literature (Balderstone et al. 2019). According to recent research, Casp-2, Casp-3, and Casp-6 are linked to cancerogenesis and erythroblast activation during differentiation rather than apoptosis (Lamkanfi et al. 2007; Feng et al. 2015; Balderstone et al. 2019; Moras, Lefevre, and Ostuni 2017). Erythrocyte progenitor cells are erythroblasts (Bareiss et al. 2013).

Nanog and SOX2 are markers that show stemness. During embryogenesis, SOX2 is crucial for the preservation of stem cells. It plays an important role in the process of cell regeneration, reprogramming, and homeostasis and encourages cell proliferation and survival (Mamun et al. 2018). An essential oncogenic protein called SOX2 promotes tumor growth by acting in a dysfunctional manner. Numerous tumor forms have been found to overexpress and amplify SOX2, causing cancer cells to migrate, proliferate, invade, and metastasize more quickly and ultimately resist apoptosis (Mamun et al. 2018). In our study, we found that control

cell nuclei showed SOX2 expression, while PTX-treated cell cytoplasm and microcell showed SOX2 expression (Simsone et al. 2020; Golubnitschaja et al. 2020). Cancer cell aggression and the ability to withstand stress are indicated by SOX2 cytoplasmic expression (Mamun et al. 2018; Gu, Liu, and Zheng 2012). In the cytoplasm of both control and PTX treated cells, as well as in microcells, Nanog expression was observed (Simsone et al. 2020; Golubnitschaja et al. 2020). Cancers with a poor prognosis have been found to express Nanog (Mamun et al. 2018; Gu, Liu, and Zheng 2012). Another embryonic stem cell marker Oct3/4, in our previous work, was demonstrated in triple negative breast cancer and displayed more expression than the luminal breast cancer group (Simsone et al. 2015). Additionally, Oct3/4 expression was found in both large and small cell populations of triple negative breast cancer. Furthermore, tiny cells that expressed Oct3/4 made up the majority of the luminal breast cancer cell population (Simsone et al. 2015). Oct3/4+ cells can form conglomerates and can spontaneously produce differentiated cells *in vitro* outside of the bone marrow, in contrast to Oct3/4, which was associated with cell plasticity or a subgroup of passive stem cells in studies involving bone marrow cells (Pallante et al. 2007). According to Martelotto et al. (2014), intratumor heterogeneity refers to the coexistence of cancer cell subpopulations that differ in their genetic, phenotypic, or behavioral properties both inside the original tumor and between the primary tumor and its metastasis. Due to intratumor genetic variability, many medical failures can be attributed to the emergence of clones that have particular resistance mechanisms and existed before the start of therapy (Martelotto et al. 2014).

As a result, our research revealed that macrocells and microcells with large DNA concentrations could both contribute to resistance. However, it appears that the progenitors of the resistant cell population could only be microcells. Here, we describe the morphology of microcells. Following the administration of medication, the number of microcells increased; these cells were inherent parts of malignancies. A subset of malignancy known as microcells is distinguished by resistance to medication. Therefore, analysis of the microcell population in a tumor receiving anticancer therapy may be a powerful predictor of a patient's prognosis for survival, as well as a possible source of cancer cell regeneration after the tumor itself has died.

5. CONCLUSION

1. Microcells are natural components of cancer tissues and cells. The number of microcells increases after 24 h during chemotherapy against cancer. Microcells are not cancer-specific. These cells are observed in multiple cancer cell lines *in vitro*, such as *HeLa*, Sk-Mel-28, A549, 4/21, and HT-1080.

2. Microcells are truly live cells and have all active and proliferative cell organoids and cell compartments: dense cell nucleus, nuclear envelope, cell membranes, cell cytoplasm, mitochondria, and Golgi apparatus. In the cell cytoplasm, ribosomes are seen as dark or black spots observed by transmission electron microscopy.

3. Endocytic ability is an important microcell distinguishing sign from cancer stem cell cells, such as cells. Microcells take up ingredients from an extracellular environment such as plasmids with the cloned GFP gene, which is seen as GFP expression in microcells.

4. Microcells could be differentiated from other cancer cells using SOX2+, Nanog+, anti-PCNA+, ALDH+, TSG101-, Casp-2+, and Casp-6+ cell antigen markers.

5. Microcells can actively proliferate, as shown by EdU incorporation into DNA in the active synthesis phase, as well as RNA synthesis detected by the RNA synthesis assay kit and ANS-EtBr staining showing the amounts of protein and nucleic acids in cells.

6. THESIS

1. SOX2+, Nanog+, anti-PCNA+, ALDH+, TSG101-, Casp-2+, Casp-6+ expression, and EdU, RNA synthesis can be used as microcell identifiers.

2. Microcell development is an indicator of resistance to tumor therapy. The presence of microcells as a natural subpopulation of the tumor prior to therapy indicates a low efficacy of therapy.

3. Microcells can be used as an important prognostic factor for anticancer therapy and thus can be used as a characterizing factor for individual therapy because changes in the number of microcell populations occur during therapy.

4. The endocytosis property of microcells can be the basis for the development of a new type of anticancer drug by stopping the emergence of new resistant clones in the cancer subpopulation.

7. PUBLICATION

The results are presented in three original publications and one problem-solving article. The author's contribution to the included original publications is listed below each publication.

Paper I. Simsone, Z., Freivalds, T., Harju, L., Gudra, D., Kudaba, I., Liepniece-Karele, I., Bērziņš, J., Buiķis, I. Evaluation of morphological differences of breast cancer cells using various biological markers and the Feulgen method. 2015, Environmental and Experimental Biology, 13: 133–138 pp.

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Contribution: participated in experimental design, performed immunohistochemistry, microscopy, and analysis of results, and participated in the preparation of the manuscript draft and its final version.

Paper II. Simsone, Z., Freivalds, T., Bema, D., Harju, L., Bērziņš, J., Buiķis, I. Microcells as a possible predictive factor for tumor treatment efficiency. 2020. EPMA Journal, 11(Suppl. 1): S78-79 pp). https://doi.org/10.1007/s13167-020-00206-1

Contribution: participated in the experimental design, performed immunocytochemistry, microscopy, and analysis of results, and participated in the manuscript draft and its final version.

preparation.

Paper III. Simsone, Z., Freivalds, T., Bēma, D. et al. Cancer microcell initiation and determination. BMC Cancer 21, 1087 (2021). https://doi.org/10.1186/s12885-021-08813-5

Contribution: participated in experimental design, performed immunocytochemistry, time-lapse experiment, microscopy, and analysis of results, and participated in manuscript draft and its final version.

Problem-solving article: Freivalds, T, **Simsone, Z,** Kudaba, I, Bērziņš, I. Prognostic and predictive significance of breast cancer stem cells. Acta Chirurgica Latviensis, 2011, 11: 122-124. DOI: 10.2478/v10163-012-0023-7

8. APROBATION OF RESEARCH

1) Oral presentation: “Cancer microcells as prognostic predictive marker”, **Simsone Z**., Freivalds T., Bēma D., Harju L., Bērziņš J., Buiķis I. 7th World Congress on Cancer Research & Therapy, Oct 20-21, 2022, Las Vegas, USA. Abstract book, Innovinc: 54.-55. pp.

2) Oral presentation: “Microcell Functional Activity Differences Based on the TEM”, **Simsone Z.,** Freivalds T., Harju L., Groma V., Buiķis I. Riga, 25-26 March 2022.; 58, (Supplement 1):152. The International Scientific Conference on Medicine was organized within the frame of the 80th International Scientific Conference of the University of Latvia.

3) Thesis: “Predictive biomarkers for therapy-resistant cells”, **Simsone, Z.,** Freivalds, T., Bēma, D., Harju, L., Bērziņš, J., Buiķis I., Rīga. Medicina (Kaunas) 2020;56(Supplement 1):102. ISSN 1648-9233

4) Web news: LU Kardioloģijas un reģeneratīvās medicīnas institūta Eksperimentālās onkoloģijas grupa veic pētījumu. 11.02.2022. **Zane Simsone** https://www.lu.lv/en/about-us/ul-media/news/single/t/70237/

5) Oral presentation: EPMA World Congress 2019. “Anti-cancer agent resistant cell population characterization using multiple biomarkers”, **Simsone, Z.,** Freivalds, T., Harju, L., Bērziņš, J., Buiķis I. September 19-22, 2019, Check Republic, Pilsen.

6) Poster presentation: Rīga Stradiņš University International Conference on Medical and Health Care Sciences «Knowledge for Use in Practice» with poster presentation “Microcell formation in various cell lines due to the stress” **Simsone, Z.,** Freivalds, T., Petrovska, R, Harju, L., Buiķis I. April 1-3, 2019, Riga, Latvia.

7) Pilot experiment of UL researchers brings new hope for improving the effectiveness of cancer therapy, 25.09.2019, https://www.fonds.lv/en/about-us/news/t/60803/

8) Poster presentation: Personalized Medicine - International Symposium 2017. Heidelberg, Germany, 03.06.- 04.06.2017. “Cancer cell viability detection and microcell formation after chemotherapy in various cell lines”, **Simsone, Z.,** Freivalds, T., Petrovska, R,

Harju, L., Bēma, D., Buiķis I. Abstract book; International symposium on personalized medicine, July 3-4, 2017, Heidelberg, pp 54.

9) Oral presentation: 24th Wilhelm Bernhard Workshop on the Cell Nucleus: "Induction of microcell formation in HeLa cells using methanol in vitro" **Simsone Z.,** Freivalds T., Harju L., Buikis I., Zeidaka A. August 17-22, 2015, Vienna, Austria

10) Poster presentation: „Ki-67 and oct3/4 expression in two types of breast cancer histological samples", **Simsone Z.,** Freivalds T., Harju L., Gudrā D., Kudaba I., Liepniece-Karele I., Bērziņš J., Buiķis I. Riga, Latvia 18-20 September 2014, VII International meeting "From Molecular to Cellular Events in Human Pathologies".

11) Poster presentation: „Evaluation of morphological differences of breast cancer cells using various biological markers", **Simsone Z.,** Freivalds T., Harju L., Gudrā D., Kudaba I., Liepniece-Karele I., Bērziņš J., Buiķis I., LS-2. Structure and function of cells and organelles, LS-2-P-2507, Proceedings ISBN: ISBN 978-80-260-6720-7, September 7-12, 2014, 18th International Microscopy Congress; Prague, Czech Republic.

12) Poster presentation: „Aoumnt of DNA in luminal and triple-negative breast cancer cells", **Simsone Z.,** Freivalds T., Harju L., Kudaba I., Liepniece-Karele I., Buikis I., Berzins J." August 19-24, 2013, 23rd Wilhelm Bernhard Workshop on the cell nucleus, Hungary, Debrecen. http://wbw23.unideb.hu/docs/abstract_book.pdf

13) Oral presentation: „Identification of cancer stem cells in histological breast cancer sections using CD44, CD24, ALDH, Oct3/4", **Simsone Z.,** Freivalds T., Harju L., Kudaba I., Liepniece-Karele I., Buiķis I, Bērziņš J., 7th -8th September, 2012, Riga, University of Latvia, International Medical Meeting.

14) Oral presentation: "Chromosome structural changes as the cause of development and progression of cancer" **Simsone Z**, T. Freivalds, L. Harju, I. Kudaba, I. Buiķis, J. Bērziņš February 13th Riga, University of Latvia, 73rd Scientific conference of University of Latvia, Biology, Human and Animals Physiology section. http://eeb.lu.lv/EEB/201503/EEB_XII_1_abstracts.pdf

15) Oral presentation: „DNA amount in triple-negative and luminal breast cancer cell population", **Simsone Z**, T. Freivalds, L. Harju, I. Kudaba, I. Liepniece-Karele, I. Buiķis, J. Bērziņš. February 8th, 2013, Riga, University of Latvia, 70th Scientific conference of University of Latvia, Biology, Human and Animals Physiology section.

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