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Establishing in vitro systems addressing the clonality of drug tolerant persister cells

Bachelor 's thesis

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1 Introduction and Question

1.1 Breast cancer cell lines

Cancer is a leading cause of death, accounting for about one in six deaths worldwide in 2022. (Bray et al. 2024) Cancers of the female breast have been second most frequently diagnosed, with 11.6% of the nearly 20 million new cases of cancer that year. With 6.9% of all cancer deaths, breast cancer is the fourth leading cause of cancer mortality. Among women, this type of cancer is responsible for almost a quarter of all cases as well as for one in six cancer deaths, which makes it the cancer type with both, the highest incidence, and mortality for female patients. Those numbers show the urgent need of more in-depth research on cancer, in particular on breast cancer. (Bray et al. 2024)

To gain further knowledge at the molecular level, cell lines as *in vitro* models are crucial for cancer research. Over 70 years ago the first human cell line, called HeLa, was established. The first breast cancer cell line was established in 1958. (Holliday and Speirs 2011) When *in vitro* experiments are successful, often *in vitro* to *in vivo* extrapolation is performed. Human cancer cells can be transplanted into mice, resulting in a so-called xenograft cancer model, however, the mice are required to be immunocompromised. Since several anticancer agents influence the anti-tumor immune response, models with an intact immune system are vital for developing new therapeutic strategies. Therefore, the use of mouse cell lines, which can be transplanted into immunocompetent mice, is essential. (Hámori et al. 2020, Holliday and Speirs 2011)

1.2 The chemotherapeutic drug doxorubicin

There is a variety of options to treat breast cancer, including local treatment with surgery or radiation and systemic treatment with hormonal therapy, chemotherapy or biological therapy. (Ben-Dror et al. 2022) For tumors lacking molecular targets, for instance triple negative breast cancer, neoadjuvant chemotherapy is the method of choice. One of the most widely used chemotherapeutic drugs to treat breast cancer is doxorubicin, which was discovered in the 1960s and approved by the FDA in 1974. Doxorubicin enters cells through rapid diffusion and carrier-mediated uptake. Intracellular doxorubicin acts on multiple targets and mechanisms including reactive oxygen species generation, DNA intercalation and adduct formation, topoisomerase II inhibition, histone eviction, calcium and iron homeostasis regulation as well as ceramide overproduction. (Sritharan and Sivalingam 2021) Apart from the cytotoxic effect, doxorubicin aids immune-mediated clearance of tumor cells. In general, doxorubicin targets

dividing cells while non-dividing cells have limited drug diffusion, poor drug accumulation and sensitivity. A major issue with doxorubicin-treatment is the development of chemoresistance. (Kciuk et al. 2023, Nicoletto and Ofner 2022, Sritharan and Sivalingam 2021)

Drug resistance is one of the biggest issues to tackle in order to improve patient survival, thus, it is crucial to understand the evolution of treatment resistance. Cells can be intrinsically resistant, which means that those cells do not respond to initial treatment. Resistance can also be acquired, meaning that resistance develops after prolonged treatment. Alternatively, a subpopulation of cancer cells has shown a transient ability to enter a non-genetically induced state through which cells persist drug treatment (Figure 1). This mechanism could provide cells with

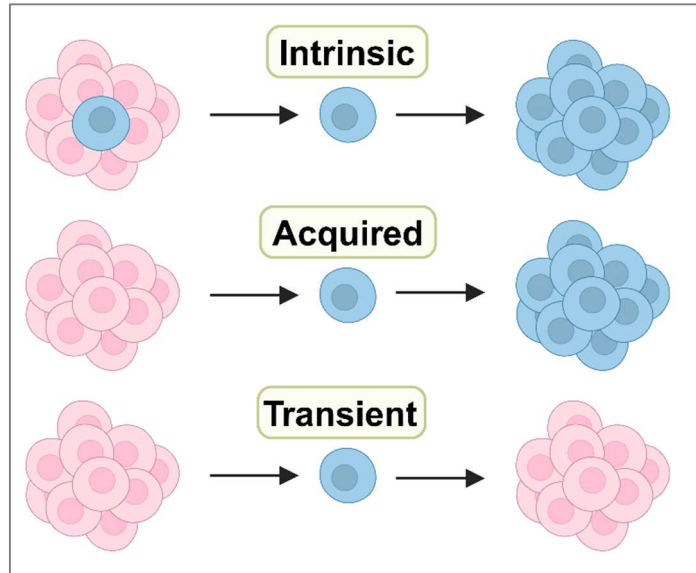


Figure 1: Forms of resistance, Created with BioRender.com

This figure shows three different forms of resistance. Resistance can be intrinsic i.e., existing prior to treatment. An acquired resistance can develop after prolonged treatment. Moreover, cells can enter the DTP state and survive drug treatment through a transient resistance.

additional time to possibly acquire resistance. (Cabanos and Hata 2021)

1.3 Cellular barcoding

To address the relevance of clonal changes in resistance evolution, cellular barcoding is used. Cellular barcoding is a powerful strategy to track clonal dynamics by tagging individual cells with unique heritable labels. DNA barcodes can be introduced into a cell population to serve as molecular tags marking the progeny of a particular cell. Lentiviral vectors can be used to integrate barcodes into genomic DNA. (Chhouri et al. 2023, Walens et al. 2020) The integrated sequence often consists of more than just a DNA-barcode. Often a promotor is integrated to allow detecting the barcode, not only at the DNA level, but also at the RNA level. Resistance genes as well as reporter genes are also commonly parts of the construct. Following experiments, genomic DNA has to be isolated from cells and barcodes have to be amplified and purified in order to be used in next generation sequencing. (Cellecta 2018)

1.4 Drug-tolerant persister cells

Chemoresistance comes in many forms, and it can be caused, for example, by the selection of pre-existing cell clones that exhibit genetic resistance mechanisms linked to mutations or gene amplification. Recently, a new mechanism leading to therapy resistance has been discovered. (Chhouri et al. 2023) A transient form of resistance can arise from so-called drug-tolerant persister (DTP) cells. DTP cell populations were originally discovered in 1944 in antibiotic-resistance of bacteria. Over 60 years later, research by Settleman and his group found DTP cells in a cancer cell population. (Pu et al. 2023) Since then, DTP cells have been found in various cancer types including breast cancer. (Dhanyamraju et al. 2022) DTPs represent a minor population of cancer cells acquiring a temporary state of tolerance following anticancer treatment. (Pu et al. 2023) The DTP phenotype is characterized as a transient, low-proliferative state that is based on adaptive changes, rather than genomic alterations. Multiple processes, including epigenetic modifications, transcriptional processes, metabolic remodeling and interactions with the tumor microenvironment seem to regulate the persister state. (Mikubo et al. 2021) When exposed to initial systemic drug treatment, a subset of cancer cells enter a state of dormancy, while the majority of cancer cells die. Following drug removal, a subset of DTP cells reenter the cell cycle, reverting to a drug-sensitive state similar to the drug naive cells. (Swayden et al. 2020) DTP cells are believed to exhibit stem cell-like characteristics including a high level of plasticity, leading to rapid changes in the phenotype. DTP cells are a potential therapeutic target to create new therapies that might have advantages over traditional approaches and potentially improve patient life quality. (Dhanyamraju et al. 2022)

1.5 Overall aim

The overall aim of the project, of which this thesis is a part, is to gain more insights into the underlying mechanisms leading to the DTP state in breast cancer. It is not yet proven and still debated whether DTP cells derive from a pre-determined population of drug naive cells existing prior to treatment or if they stochastically undergo this phenotypic transition. There have been multiple experiments, some hinting at the clonal selection of drug naive cells exhibiting DTP features and others suggesting a drug-induced phenotypic transition. (Cabanos and Hata 2021, Mikubo et al. 2021) To address this question in *in vitro* experiments, replicates of the so-called repopulation assay of barcoded cells can be used. Repopulation assays use a lethal dose of drug to model acute drug response for the introduction of the DTP state. Following dormancy of the DTP cells, some cells reenter the cell cycle and proliferate. Barcode analysis has the power to evaluate whether DTP cells are pre-determined or if the process is stochastic.

If surviving DTP cells are selected stochastically barcodes will be selected randomly. If cells are predisposed to be resistant, they will appear at a higher than chance rate across replicates of repopulation assays. Predisposition can either mean that cells are intrinsically resistant or that they are genetically poised to undergo phenotypic changes associated with the DTP state. Cells that survived drug treatment by entering the DTP state would be sensitive to a second round of treatment while resistant cells would not. (Szebényi et al. 2023)

Several genes are believed to be involved in drug tolerance due to their upregulation in DTP cells across multiple experiments recently performed in the host laboratory. To establish the functional relevance of these genes, knock-down experiments should be run. In the planned experiment repopulation assays of a mixture of cells with and without expression of the proposed genes will be run. This will show if cells with or without the expression of those genes are favored to enter the DTP state. To distinguish between cells expressing the target genes, which serve as a negative control, and cells showing downregulation of the target genes, two cell lines labeled with different fluorescent tags, e.g., green fluorescent protein (GFP) and mCherry, are required.

1.6 Hypothesis

The hypothesis of this thesis is that the use of 250 nM and 125 nM doxorubicin in repopulation assays for MCF-7 and CSTR cells respectively, causes the majority of cancer cells to die and at the same time lets DTP cells survive.

Another hypothesis of this thesis is that two phenotypically equivalent populations, which have been labeled with two different fluorescent proteins, show the same characteristics leading to neither the selection of one nor the other cell type.

2 Material and Methods

2.1 Cell culture

In this thesis two different cell lines were used: the human cell line MCF-7 and the mouse cancer cell line CSTR. The MCF-7 cell line was established in 1973 and named after the Michigan Cancer Foundation, where Dr. Soule and colleagues isolated those cells from the pleural effusion of a 69-year-old female patient. She had undergone a mastectomy of a benign tumor in her right breast in 1963, as well as a radical mastectomy to remove a malignant adenocarcinoma in her left breast four years later. Due to the fact that the tumor was estrogen receptor positive, hormone therapy was used alongside of radiotherapy to control local recurrence for three years before widespread nodular recurrence occurred. In 1970 samples of a pleural effusion were taken, which were then used to establish the MCF-7 cell line. (Comşa et al. 2015) Besides MCF-7 cells, the mouse mammary tumor cell line CSTR was used in this thesis. This cell line shows features of triple negative breast cancer, meaning there is a lack of expression of the estrogen receptor, the progesterone receptor and the epidermal growth factor receptor 2. The CSTR cell line was derived from a genetically engineered mouse model with a conditional knockout of BRCA1 and p53. Since germline mutations in BRCA1 are linked to an inherited predisposition to breast cancer, this mouse model allows studying hereditary breast cancer. (Hámori et al. 2020, Liu et al. 2007)

To ensure a sterile environment, all work was performed in a laminar flow hood (Safemate ECO+ Class II (Type A2) Microbiological Safety Cabinet, BioAir).

2.1.1 Media changes

To ensure the presence of sufficient nutrients, the cell culture medium was changed every few days, depending on cell confluency. For all cell lines used in this thesis, RPMI (RPMI-1640 Medium, Merck, Germany) with 10 % fetal bovine serum (Fetal Bovine Serum South America, Heat Inactivated, Biowest, France) and 1 % penicillin-streptomycin (Penicillin-Streptomycin (with 10,000 units penicillin and 10 mg/ml streptomycin), Merck, Germany) was used as cell culture medium. For different cell culture plates or flasks different volumes of media were used as can be seen in Table 1 below. The medium was always prewarmed in a water bath (VWB 18, VWR)

Table 1: Required medium volumes for different cell culture vessels

This table shows how much media is required for a certain cell culture flask or plate in order to cover all the cells in the vessel to provide them with nutrients.

Cell culture flask/plate	Volume of medium [ml]
T25-flask (Falcon® 25cm ² Rectangular Canted Neck Cell Culture Flask with Vented Cap, Corning Incorporated)	4
T75-flask (Falcon® 75cm ² Rectangular Canted Neck Cell Culture Flask with Vented Cap, Corning Incorporated)	10
12-well plate (Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific)	1

2.1.2 Trypsinization

In order to split adherent cells for maintenance, to expand them or to use them in certain experiments, they have to be trypsinized. To accomplish that, firstly, the medium was removed and the cells were washed with phosphate buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline, Merck, Germany) As a next step 1× trypsin (Trypsin (1:250), powder, Thermo Fisher Scientific, USA) was pipetted into the culture vessel and equally distributed across the whole surface by tilting the vessel before being put into the incubator (Heracell™ 150i CO₂ Incubator, Thermo Fisher Scientific) for two minutes. The incubator used in this thesis was set to 37 °C and 5 % carbon dioxide. To stop trypsinization, medium was poured into the vessel and a pipette was used to rinse the surface. The cell suspension was poured into a 15 ml reaction tube (Falcon® 15 mL High Clarity PP Centrifuge Tube, Conical Bottom, with Dome Seal Screw Cap, Sterile, 50/Bag, 500/Case, Corning Incorporated) and centrifuged (Megafuge™ 8 Small Benchtop Centrifuge, Thermo Fisher Scientific) at 300 × *g* for three minutes. The supernatant was discarded, and cells were resuspended in 1 ml of medium. The different volumes needed for the trypsinization of different vessels can be seen in Table 2.

Table 2: Volumes required for trypsinization of different vessels

This table shows how many ml of phosphate buffered saline (PBS), trypsin and medium are required when trypsinizing different cell culture flasks or plates.

Cell culture flask/plate	PBS [ml]	Trypsin 1× [ml]	Medium [ml]
T25-flask	4	1	4
T75-flask	10	2	10
12-well plate	1	0,25	1

2.1.3 Thawing cells

The cryovial (VWR® Cryogenic Vials, 2,00 ml, Round bottom, 12,5×48 mm, Avantor) was held into the water bath until the cell suspension was completely thawed. The suspension was quickly pipetted into a 15 ml reaction tube containing 3 ml of warm medium since the freezing agent dimethyl sulfoxide (DMSO) is cytotoxic. The tube was then centrifuged at 300 ×g for three minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of medium.

2.1.4 Seeding cells

After trypsinization or thawing, cells can be seeded in different vessels. When thawing cells, the whole cell suspension was used. For splitting cells, a certain proportion, for example taking 100 µl of 1 ml for a 1:10 split, was used. For experiments, a 1:10 dilution of an aliquot of the cell suspension with medium was made and this dilution was mixed 1:2 with trypan blue (Trypan Blue, Bio-Rad, USA) to stain dead cells. Live cells, which were not stained blue, were counted in a counting chamber (Counting chambers Neubauer-improved, dark lines, double net ruling, with 2 cover glasses, 1 mm, Paul Marienfeld) under the microscope (Primovert und AxioCam Erc 5s, Zeiss). The received cell concentration was used to calculate the needed volume to aim for a desired cell-number required for the experiment. For seeding, prewarmed medium was put into the vessel before adding the cell suspension. Then the vessel was moved in a figure-eight motion to achieve an even distribution of cells.

2.1.5 Cryopreserving cells

450 µl of a cell suspension with two million cells were pipetted into a cryovial. Next, 50 µl of DMSO (Dimethyl sulfoxide Cell culture grade, PanReac AppliChem ITW Reagents, Spain, Germany, Italy) were added and the vial was transferred to a -80 °C freezer (TSX Series Ultra Low Temperature Freezer TSX600V 230V/50Hz, Thermo Fisher Scientific) in a freezing container (Corning® CoolCell® LX, Cell Freezing Container, for 12 x 1 mL or 2 mL Cryogenic Vials, 4-Pack, Purple, Orange, Green, and Pink, Corning). This way the temperature dropped 1 °C per minute. A few days later the vials were transferred into a liquid nitrogen tank.

2.1.6 Flow cytometry and Fluorescence activated cell sorting

To prepare cells for flow cytometry (BD LSRFortessa™ X-20 Cell Analyzer, BD Biosciences) or fluorescence activated cell sorting (FACS) (BD FACSMelody™ Cell Sorter, BD Biosciences), cells were trypsinized. The resulting cell pellet was washed twice with 2 ml of PBS and the cells were then resuspended in 1 ml of PBS.

2.2 Lentiviral transduction

2.2.1 Virus quantification

In each well of a 12-well plate, 100.000 MCF-7 cells were seeded. The next day, three wells were trypsinized and counted. The medium of the remaining wells was exchanged for different ratios (see Table 3) of medium containing 8 µg/ml polybrene (Polybrene Infection / Transfection Reagent, Merck, Germany) mixed with a GFP-lentivirus that was made in the host-laboratory prior to this project (Cellecta, Inc. 2023). The plate was incubated for 24 hours before the medium was changed. The following day, the medium was removed, and the cells were washed twice with 1 ml of PBS before receiving fresh medium. One day later, cells were analyzed by flow cytometry.

Table 3: GFP-lentivirus and polybrene-medium volumes

This table visualizes how many ml of polybrene-containing medium and GFP-lentivirus each well received.

Number of wells	Volume Polybrene-medium [ml]	Volume GFP-lentivirus [ml]
1	437,5	0
2	432,5	5
2	427,5	10
2	387,5	50
2	337,5	100

2.2.2 Engineering barcoded CStr, GFP+ MCF-7 and mCherry+ MCF-7 cells

800.000 CStr cells were seeded into six T75-flasks and two million MCF-7 cells were seeded into three T75-flasks each. The next day one T75-flask of CStr cells was trypsinized and counted to estimate the desired virus volume given a viral titer of about 1,4 million transduction units per ml and a desired MOI of about 0,05. Four of the remaining flasks received a mixture of 9,3 ml of medium including 8 µg/ml polybrene and 125 µl of the lentiviral barcode library “Cellecta’s CloneTracker XP library”, see Figure 2. As a negative control, a flask of CStr and MCF-7 cells received the same polybrene-containing medium without the addition of virus. Each one flask of MCF-7 cells received a mixture of 9,3 ml of polybrene-medium with 100 µl of GFP-lentivirus or mCherry-lentivirus. All of the three viruses were made in the host-laboratory prior to this project (Cellecta, Inc. 2023). After 24 hours of incubation, the medium of all flasks was exchanged. One day later, fluorescence was checked using a fluorescence microscope (Eclipse Ti2, Nikon) to see if transduction was successful. The GFP+ MCF-7 cells (MCF7-GFP) should exhibit green fluorescence and the mCherry+ MCF-7 cells should show

red fluorescence. Since not only a DNA-barcode but also an RFP was introduced into the CSTR cells (CSTR-bar), they are expected to show red fluorescence.

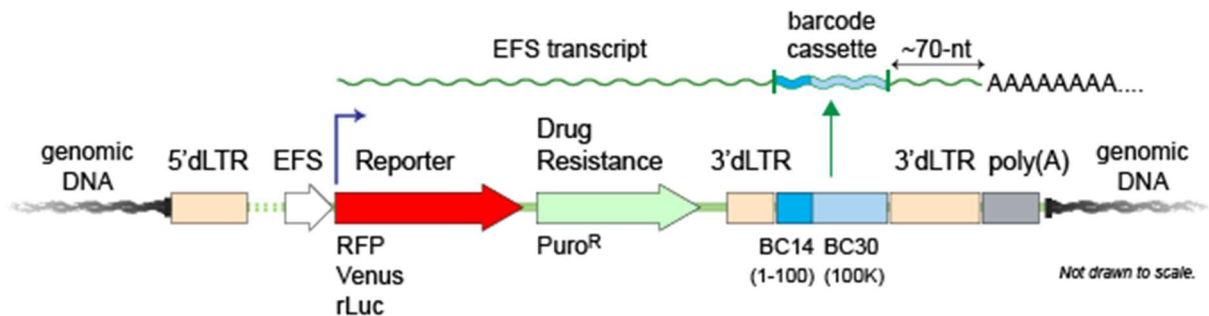


Figure 2: Design of CloneTracker XP lentiviral libraries in linearized form. (Cellecta 2018)

This graph shows the design of the CloneTracker XP lentiviral library. The sequence is introduced into genomic DNA by lentiviral transduction. The sequence consists of deleted long terminal repeats (dLTR), an elongation factor 1 α short promoter (EFS), either red fluorescent protein (RFP), Renilla-luciferin 2-monooxygenase (rLuc) or Venus as reporter, a puromycin resistance gene (*Puro^R*), a barcode cassette consisting of a 14-nucleotide long barcode (BC14, from 1 to 100 different sequences) and a 30-nucleotide long barcode (BC30, 100.000 different sequences) and a polyadenylation sequence (poly(A)). The construct used in this thesis uses RFP as a reporter.

After fluorescence microscopy all flasks were split 1:2 with the PBS-washing step being performed twice. Furthermore, the CSTR-bar cell suspensions of all flasks were pooled before reseeding. The following day all cells were analyzed by FACS. The CSTR-bar samples were pooled prior to analysis. Sorted cells (50.000 MCF7-GFP cells and 100.000 CSTR-bar cells) were collected in 5 ml of medium and they were centrifuged for three minutes at 300 $\times$ g. The cell pellets were resuspended in 1 ml of medium and seeded into a well of a 12-well-plate. MCF7-GFP and CSTR-bar cells were expanded and selected by adding 2 μ g/ml Puromycin (Puromycin dihydrochloride, Merck, Germany) into the medium. After three passages a flow cytometry analysis on the CSTR-bar cells was performed to confirm that all cells are fluorescent, and they were cryopreserved. Aliquots of MCF7-GFP cells were cryopreserved after six passages.

2.3 Cytotoxicity Assays

To test how much medium evaporates when incubating a 12-well plate over 24 hours, triplicates of different volumes (1000, 900, 800 and 700 μ l) were pipetted into a 12-well plate and the same volumes were pipetted into 1,5 ml reaction tubes (Eppendorf Safe-Lock Tubes 1,5 ml, Eppendorf) and weighed (1712MP8, Sartorius) After 24 hours of incubation, the medium of each well was transferred into 1,5 ml reaction tubes and weighed.

Next, four 12-well-plates per cell line with 20.000 cells per well, but with different volumes of medium (see Table 4 and Table 5), were seeded. The following cell lines were tested: CSTR-

bar, barcoded and RFP+ MCF-7 cells (MCF7-bar) that were made in the host laboratory prior to this thesis as well as MCF7-GFP. After 24 hours of incubation, medium was added to replace the evaporated volumes, see Table 4 and Table 5. Different volumes (volumes listed in Table 4 and Table 5) of medium containing 1000 nM doxorubicin (Doxorubicin, Hydrochloride Salt, >99%, LC Laboratories, USA) were added into the wells. Work involving doxorubicin was always performed in the dark due to the light sensitivity of the drug. The CSTR and MCF-7 plates were incubated for five and seven days, respectively.

After the incubation, the medium was replaced with 0,5 ml of 5 % prestobblue (PrestoBlue™ Cell Viability Reagent, Thermo Fisher Scientific, USA) solution in PBS. This step was performed in the dark as prestobblue is light sensitive. Following a 90-minute incubation the liquid of all wells was transferred into wells of a 48-well-plate (Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes, Thermo Fisher Scientific). This plate was then analyzed by measuring fluorescence intensity (Spark® Multimode Microplate Reader, Tecan) at an excitation wavelength of 555 nm and an emission wavelength of 585 nm.

Table 4: Cytotoxicity Assay of MCF-7 cells

This table visualized the volumes of medium each well received and how much medium was added to correct for the evaporation of the medium. Moreover, this table shows the different volumes of 1000 nM doxorubicin which were added into the wells. This volume equals the doxorubicin concentration in the well in nM.

Number of wells	Volume of medium [μl]	Volume of evaporated medium [μl]	Volume of doxorubicin 1000 nM [μl] / final drug concentration in the well [nM]
12	1000	50	0
3	995	49,75	5
3	990	49,5	10
3	975	48,75	25
3	950	47,5	50
3	850	42,5	150
3	825	41,25	175
3	800	40	200
3	775	38,75	225
3	750	37,5	250
3	725	36,25	275
3	700	35	300
3	675	33,75	325

Table 5: Cytotoxicity Assay of CSTR cells

This table visualized the volumes of medium each well received and how much medium was added to correct for the evaporation of the medium. Moreover, this table shows the different volumes of 1000 nM doxorubicin which were added into the wells. This volume equals the doxorubicin concentration in the well in nM.

Number of wells	Volume of medium [μ l]	Volume of evaporated medium [μ l]	Volume of doxorubicinorubicin 1000 nM [μ l] / final drug concentration in the well [nM]
12	1000	50	0
3	1000	50	0,2
3	999	49,95	1
3	995	49,75	5
3	990	49,5	10
3	975	48,75	25
3	950	47,5	50
3	925	46,25	75
3	900	45	100
3	875	43,75	125
3	850	42,5	150
3	825	41,25	175
3	800	40	200

2.4 Repopulation assays

2.4.1 Repopulation assays of MCF-7, MCF7-bar and MCF7-GFP cells

To perform a repopulation assay, 600.000 cells were seeded into six T25-flasks per cell line. The day on which seeding was performed is referred to as day zero. The following cell lines were used: MCF-7, MCF7-bar as well as MCF7-GFP. The next day, the medium of each three flasks of every cell line was exchanged for medium containing 250 nM or 200 nM doxorubicin. The drug was removed after seven days of incubation and replaced with fresh medium. The medium was changed weekly and every few days the flasks were examined microscopically. On day 50, two flasks of MCF7-bar which were treated with 200 nM doxorubicin were trypsinized and each 600.000 cells were reseeded.

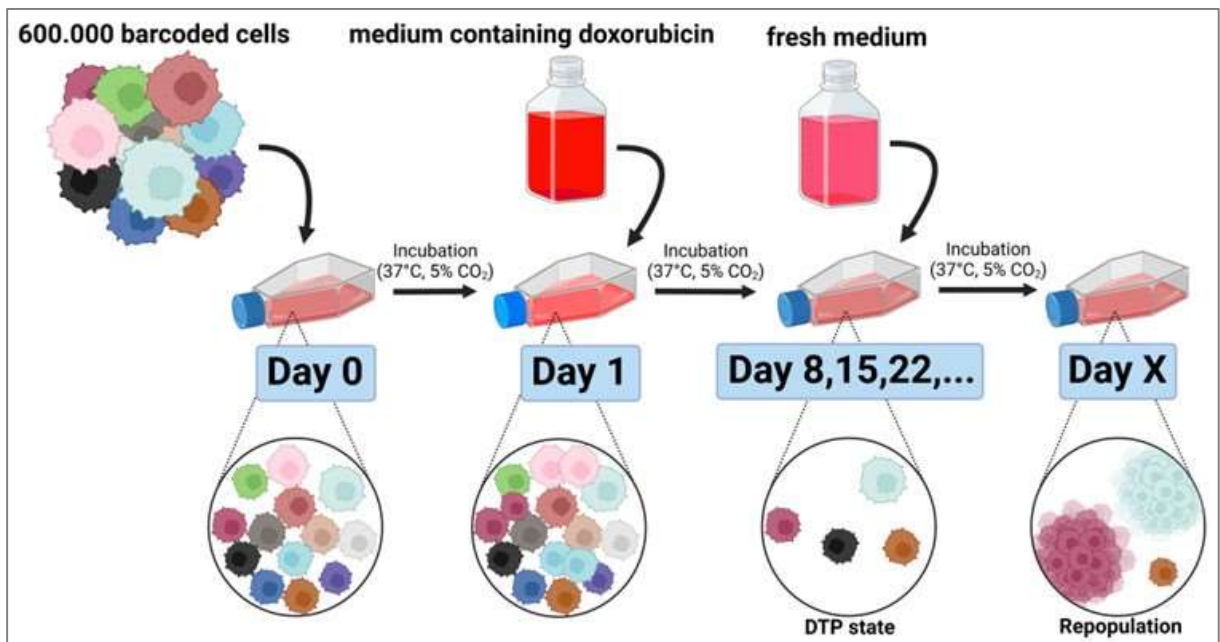


Figure 3: Repopulation assays of barcoded cells, Created with BioRender.com.

This graph shows the concept of repopulation assays. On day zero 600.000 barcoded cells (either murine CSTR-bar cells or human MCF7-bar cells) are seeded into a T25-flask. After a day of incubation doxorubicin treatment is started. Following the removal of the drug on day eight, medium changes are performed weekly. Drug treatment causes cells to either die or enter the DTP state. Cells remain dormant for several weeks before giving rise to colonies (repopulation).

2.4.2 Testing different ways of reseeding MCF7-bar repopulation assays

On day 64, the three flasks of MCF7-bar which were treated with 250 nM doxorubicin were trypsinized and pooled, resulting in 2 ml of cell suspension. 0,5 ml of the suspension were filtered (Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap, Corning Incorporated), 0,5 ml were pipetted up and down for ten minutes and to 1 ml 2 µl of DNase (DNase I (RNase-free), New England Biolabs, USA, 50 mg/ml → final concentration of 100 µg/ml) was added and incubated for 15 minutes at room temperature. Half of the DNase treated suspension was filtered after the incubation. All of the four differently treated cell suspensions were seeded into T25-flasks, incubated for a week and microscopically analyzed.

2.4.3 Repopulation assays of CSTR-bar cells

On day zero, 600.000 CSTR-bar cells per flask were seeded into six T25-flasks. The following day, the medium of each three flasks was replaced with medium containing 75 nM or 125 nM doxorubicin. One week later the drug was removed and from then on, the medium was changed weekly. Every few days, the flasks were analyzed microscopically. As soon as colonies started to appear, they were counted. Drug treatment of all three flasks which had been treated with 75 nM doxorubicin on day one was repeated using the same drug

concentration after 17 days. A second round of treatment with again 125 nM was performed on the remaining three flasks after 22 days. After 43 days one flask that had been treated with 125 nM doxorubicin was trypsinized, counted and 600.000 cells were reseeded into a new flask. Drug treatment was repeated with 125 nM doxorubicin the following day.

2.4.4 Repopulation assays of a mix of MCF7-GFP and MCF7-bar (GFP-RFP-mix)

MCF7-bar and MCF7-GFP cells were trypsinized, counted and a cell suspension with an equal number of GFP+ and RFP+ cells was prepared. Partially this suspension was analyzed with flow cytometry and a part was used to seed each 600.000 cells into five T25-flasks and 20.000 cells into three wells of a 12-well-plate. The next day three T25-flasks were analyzed by flow cytometry to determine the ratio of RFP+ and GFP+ cells. The remaining two flasks were treated with 250 nM doxorubicin for one week after which the medium was changed weekly. Four days after seeding, the three wells were analyzed with flow cytometry and on day 24 both of the T25-flasks were analyzed as well.

2.4.5 Cell number variation experiments

To investigate the influence of cell number on repopulation assays, three T25-flasks with each 200.000 cells of MCF7-bar cells were cultured for a day before a seven-day-drug treatment with 250 nM doxorubicin. Following drug removal, the medium was changed weekly.

Two flasks containing 200.000 MCF7-GFP cells each were seeded in order to be trypsinized and counted the following day to ensure that the intended cell number was seeded. Moreover, duplicates of T25-flasks with 200.000, 600.000 and 1.200.000 MCF7-GFP cells each were seeded. Starting 24 hours after seeding, those six flasks were treated with 250 nM doxorubicin over the course of one week. After that, cells were analyzed with a flow cytometer.

2.5 Statistics

The GraphPad Prism 10.2.3 software was used to statistically test whether there is a significant difference between the mean time until repopulation between the barcoded and RFP+, the GFP+ and the parental MCF-7 cells and if so, between which. Therefore, a one-way Analysis of Variance (ANOVA) and a Tukey's test was performed.

3 Results

3.1 Origin of DTP-cells

To assess whether DTP cells are pre-determined or if they stochastically undergo this phenotypic transition in *in vitro* experiments a combination of cellular barcoding and repopulation assays can be used. In this thesis the experimental setup for this future experiment is optimized.

3.1.1 Establishing the CSTR-bar cell line

CSTR cells were transduced with a lentiviral barcode library. To ensure that each cell has no more than one barcode, transduction must be performed at a low multiplicity of infection (MOI). Cells having multiple barcodes would lead to misleading results. To guarantee a low MOI, cells were counted prior to viral exposure. Since all flasks were seeded the same way, one flask was counted, and it was assumed that the remaining flasks showed the same cell number. 800.000 cells were seeded and after one day of incubation cell counting was performed. The counting indicated approximately three million cells, which is more than expected considering that CSTR cells are believed to have a doubling time of 34 hours. (Hámori et al. 2020) Virus production, performed by the host laboratory prior to this experiment, resulted in a titer between 1,4 and 2 million transduction units per ml. Since the barcoding experiment aims for an MOI between 0,05 and 0,1, it was decided to use 125 µl of the available virus stock for transduction, corresponding to an MOI between 0,06 and 0,08.

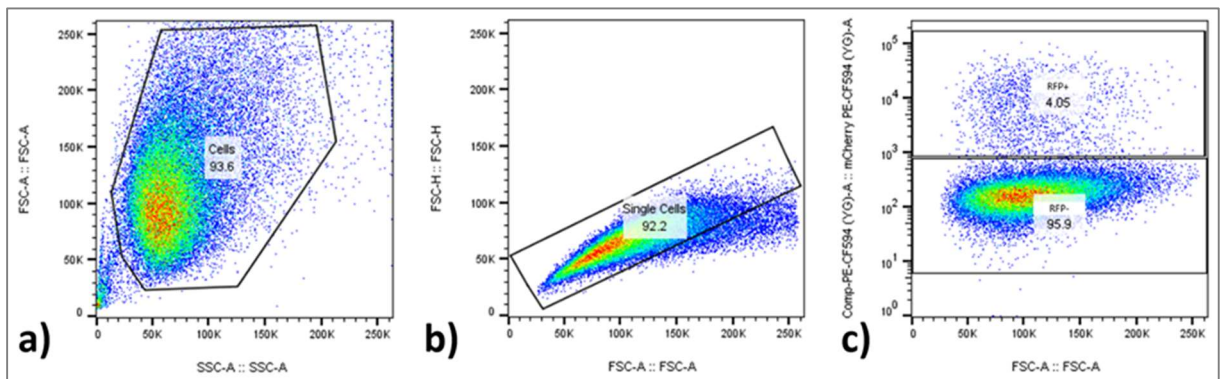


Figure 4: FACS-analysis of CSTR-bar cells after viral transduction.

This figure shows the FACS-analysis of CSTR cells after the transduction with a lentiviral barcode library. a) This graph plots the front scatter area (FSC-A) against the side scatter area (SSC-A). Every dot represents a particle, and the gating was used to exclude all particles that are debris rather than cells based on their size. b) This panel plots FSC-A against front scatter height (FSC-H) and gating was used to exclude duplets and cell clumps. This was made possible by the fact that single cells show a linear relationship between FSC-A and FSC-H whereas cells clumped together do not and can be excluded. c) In this graph RFP fluorescence was plotted against FSC-A. Gating was used to differentiate between RFP+ and RFP- CSTR cells.

Transduced CSTR cells (CSTR-bar) were expanded and sorted by FACS, enabled by an RFP that was introduced along with the barcode. Figure 4 shows the analysis of the FACS-results. This analysis showed, that 4,05 % of all single cells were RFP+, meaning that they were successfully transduced, and they now contain a DNA-barcode. The percentage of transduced cells corresponds to the expected MOI of 0,06-0,08.

As a next step the sorted CSTR-bar cells were expanded in the presence of puromycin, since the transduced cells should show a resistance against it. To double check that none of the cells have lost their introduced sequence, including the barcode, the puromycin resistance and an RFP, a fraction of cells was analyzed with flow cytometry. The result of this analysis was that 99,5 % of CSTR-bar cells were RFP+. Finally, cryopreservation of the CSTR-bar cells was performed to preserve this newly established cell line for future experiments.

3.1.2 Assessing the IC₅₀ of the CSTR-bar cell line using the cytotoxicity assay

In order to characterize the newly established CSTR-bar cell line, a cytotoxicity assay was performed. Cells were seeded into 12-well plates and 24 hours later drug treatment with a range of concentrations was performed. Following a five-day incubation, cell viability was assessed with prestobblue.

Unexpectedly, during initial experiments, cells did not survive the medium exchange performed 24 hours after seeding. To overcome this issue, instead of changing the medium 24 hours post seeding, the drug was added to the initial medium. This required the determination of how much medium evaporates in 24 hours in order to readd the proposed medium volume. Through this step achieving precise drug concentrations is possible. Therefore, different volumes of medium were weighted before and after 24 hours of incubation. In Figure 5 the calculated percentage of evaporated media was plotted against the different volumes. This visualizes that on average 5,0 % of medium evaporate, regardless of the absolute volume. These results allow correcting for medium evaporation as the loss can be compensated by adding the evaporated volume.

Initially cells were supposed to be incubated for one week with the range of different drug concentrations, however, cells in the lower concentrations grew to 100 % confluency on day five, therefore the assay was evaluated two days earlier.

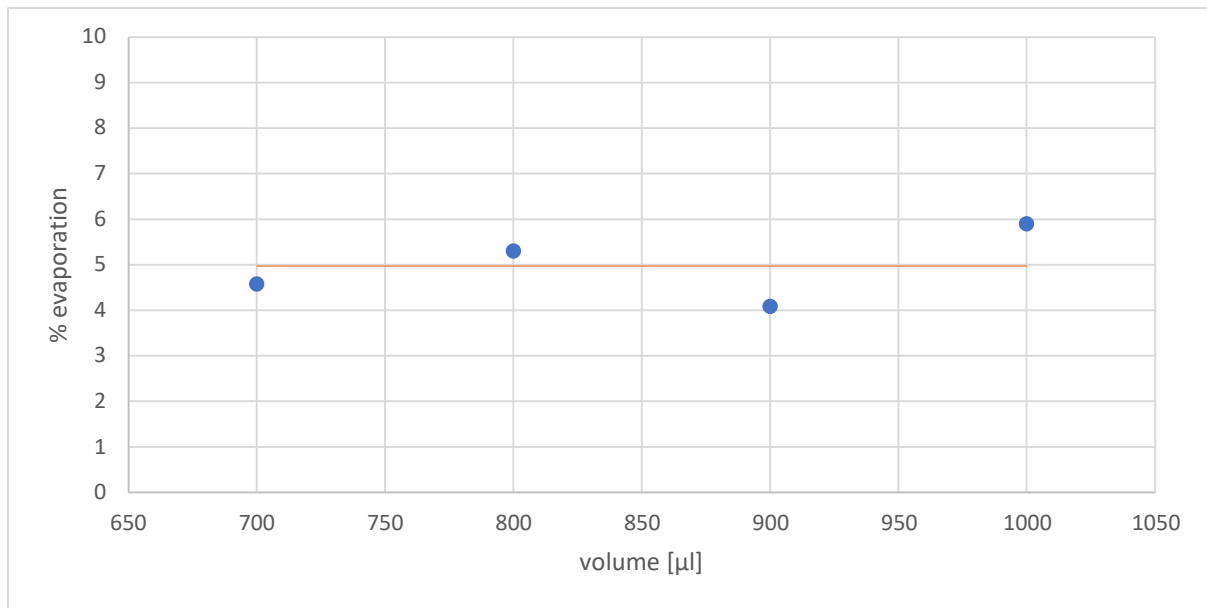


Figure 5: Medium evaporation of different volumes over 24 hours.

It was tested, how much medium evaporates when different volumes are incubated for 24 hours. The x-axis marks the volumes used and the y-axis shows how many percent of this volume evaporated. The orange line marks the average percentage of evaporation of all samples (5 %).

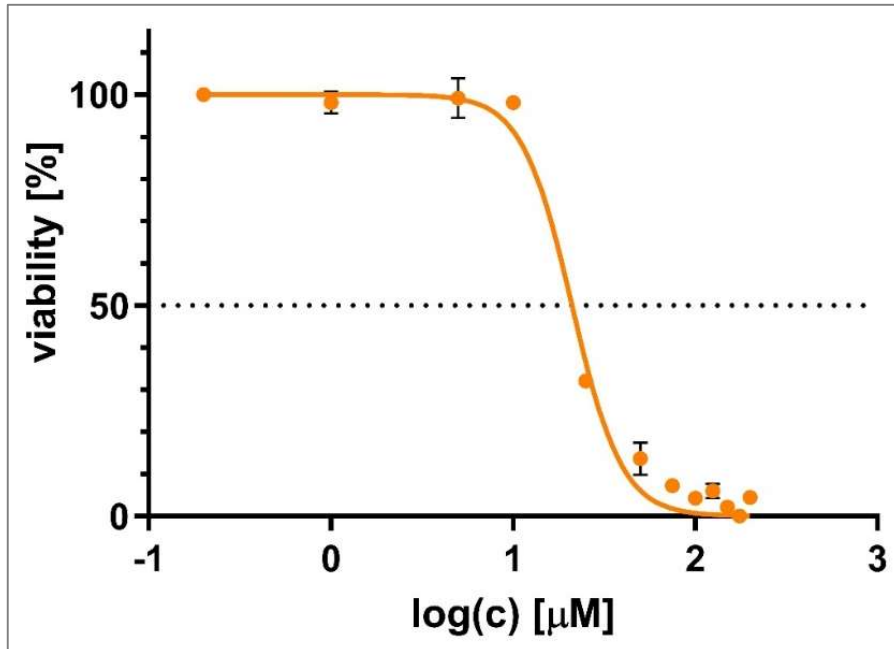


Figure 6: Dose-response curve of CSTR cells treated with doxorubicin.

The graph visualizes the fitted dose-response curve of CSTR cells treated with the chemotherapeutic drug doxorubicin. The cell viability is plotted against the logarithm of the concentration ($\log(c)$). The dotted line at 50 % cell viability marks the half maximal inhibitory concentration. It can be seen that cell viability decreases when increasing the concentration of doxorubicin.

To determine the half maximal inhibitory concentration (IC_{50}), cell viability was assessed with prestobblue by measuring fluorescence intensity. Fluorescence intensity values were normalized and plotted against the logarithm of the concentration. The curve was fitted by the GraphPad Prism 10.2.3 software using the "log(inhibitor) vs. normalized response – variable slope" equation, see Figure 6. Curve fit statistics allowed the determination of the IC_{50} value of CStr-bar, which was estimated to be 21,07 nM.

3.1.3 Optimizing repopulation assays of CStr-bar

Triplicates of repopulation assays of CStr-bar cells were performed using two different doxorubicin concentrations: 75 nM and 125 nM, which both are below the IC_5 value. These concentrations were tested since recent experiments in the host laboratory suggested to use concentrations in this range. The goal of this assay was to determine how long it takes until repopulation takes place and to suggest the optimal concentration for following experiments. Moreover, the most efficient way to perform repeated drug treatment in repopulation assays was determined.

On day eight, a cell confluency of 5-10 % was observed. As expected, only a small population of cells survived drug treatment and entered a state of dormancy. The three flasks of CStr-bar cells treated with 75 nM doxorubicin took on average 13 days until the first colony formed and those treated with 125 nM doxorubicin took on average two days longer. This suggests the use of 125 nM doxorubicin in future repopulation assays since it facilitates a later repopulation than using 75 nM doxorubicin, which is preferred.

Cells that survived the first round of drug treatment by entering the DTP state should be sensitive to treatment. Cells that acquired resistance on the other hand would not respond to a second round of treatment. An experiment that aimed to evaluate if cells are still sensitive to doxorubicin treatment was performed. The flasks previously treated with 75 nM doxorubicin formed a high number of colonies in a short period of time (Figure 7). On day 17 the flasks containing the colonies were retreated by exchanging the medium for doxorubicin-containing medium with the same concentration (75 nM) as the initial treatment. Although some colonies died, the number of colonies continued to increase despite the repeated drug treatment. Colonies started to fuse making counting impossible from day 22 on. After only two days of the repeated doxorubicin treatment colonies detached and cells died.

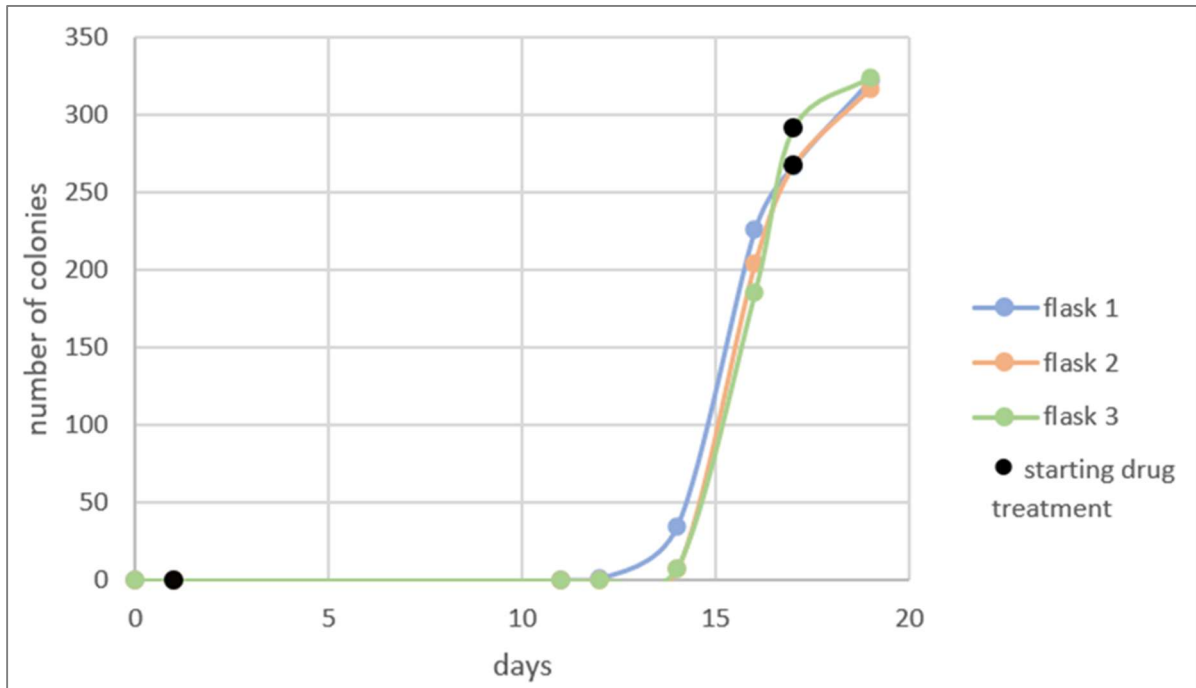


Figure 7: Outgrowth curves of CStr-bar cells treated with 75 nM doxorubicin.

This figure shows the number of colonies in repopulation assays of CStr-bar cells that were treated with 75 nM doxorubicin over time. Black dots mark the beginning of a one-week-long drug treatment. It can be seen that it took on average 13 days until colonies formed. On day 19 over 300 colonies formed in each of the three flasks. Despite repeated drug treatment more colonies formed, and counting was not possible anymore.

The other three flasks of CStr-bar cells, which were treated with 125 nM doxorubicin, formed colonies a bit slower than those treated with a lower concentration, consequently, cells were retreated with again 125 nM doxorubicin on day 22. Two of the flasks (flasks one and two) had reached over 200 colonies at the time of retreatment. Those flasks continued to show more colonies despite being retreated. After two days of repeated doxorubicin treatment colonies in flask one and two started to detach and die. One flask (flask three), however, had approximately only a quarter of colonies compared to the other two flasks. In this flask it was visible that some colonies started to die and a repeated response to drug treatment could be seen, see Figure 8 (green line). This indicates that the DTP stage was reached rather than that resistance was acquired.

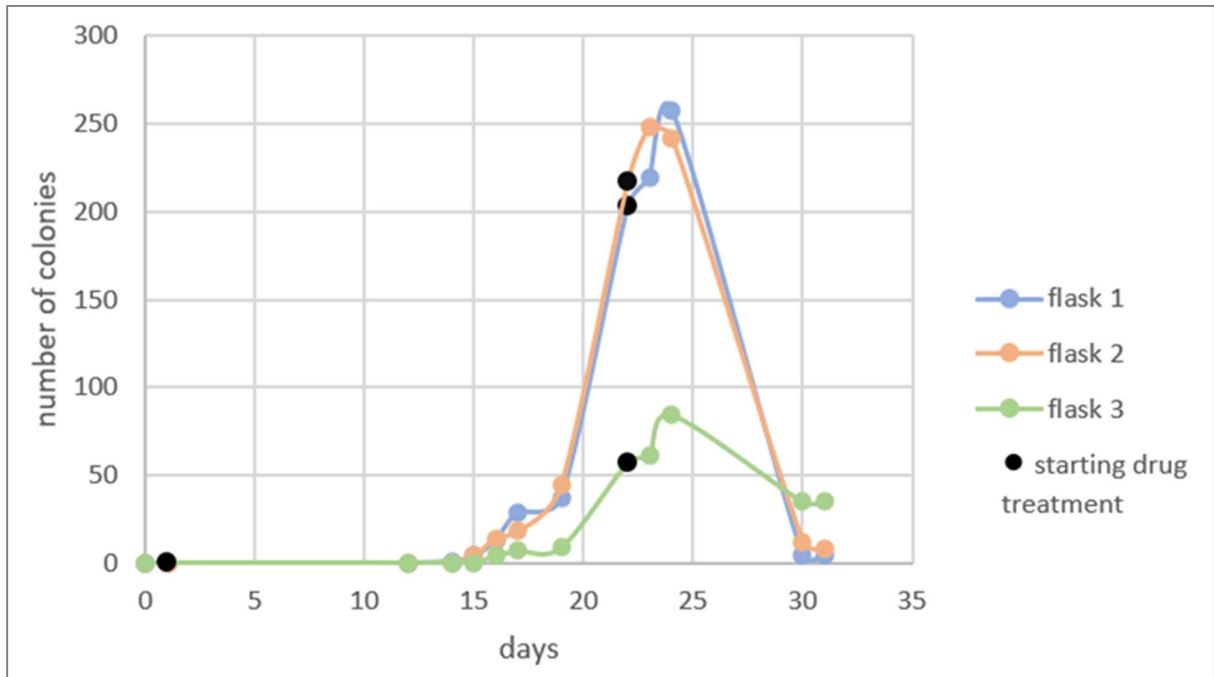


Figure 8: Outgrowth curves of CSTR-bar cells treated with 125 nM doxorubicin.

The graph visualizes the formation of colonies over time in repopulation assays of CSTR-bar cells which were treated with 125 nM doxorubicin. It took on average 15 days until colonies were visible. The one-week-long drug treatment (marked with a black dot) was repeated. While flask three responded to the second round of treatment, the other flasks died.

It could be observed that repeated drug treatment was not successful when it was started after over 200 colonies were visible. When starting retreatment at only about 50 colonies, a reduction of colonies could be seen, however, not all colonies died. As a result, another attempt of retreatment was performed on the last remaining flask (flask three) of CSTR-bar cells treated with 125 nM doxorubicin. This flask was left to grow about 200 colonies and on day 43 the flask was trypsinized. 600.000 cells, the same number as when initially seeding this assay, were seeded into a new flask and the next day treatment with 125 nM doxorubicin was repeated for one week. This resulted in cell death and only 5-10 % of cells were alive after removal of the drug. These cells remained in a state of dormancy before reinitiating proliferation and giving rise to new colonies, see Figure 9.

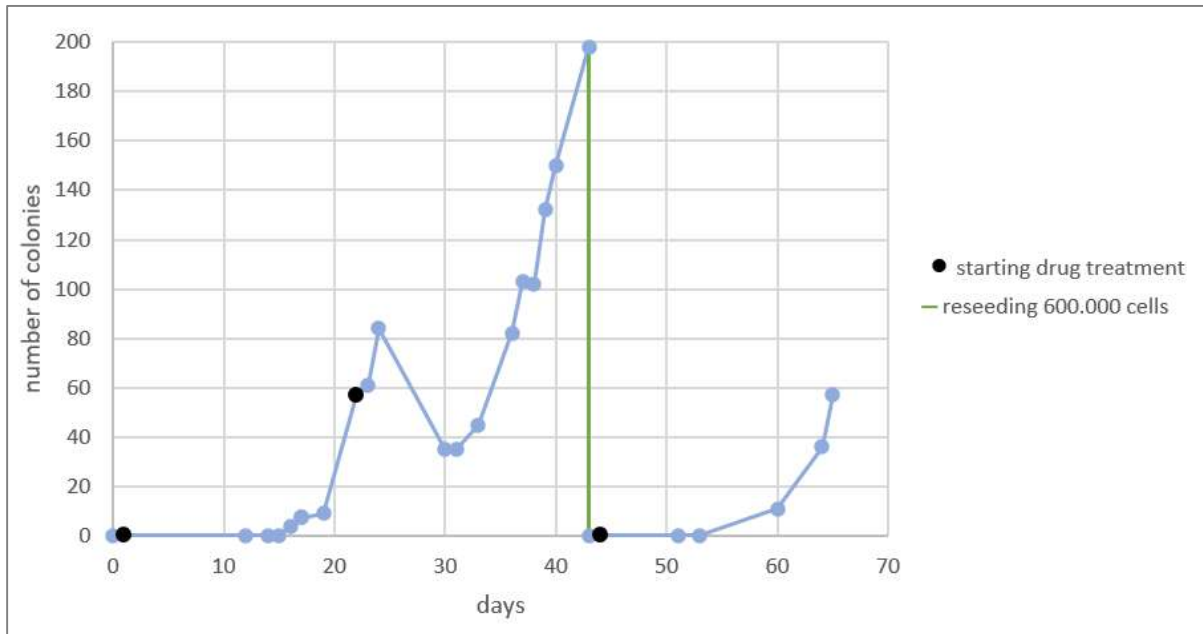


Figure 9: Outgrowth curve of flask 3 of CSTr-bar cells treated with 125 nM doxorubicin.

The graph visualizes the colony formation in a repopulation assay of CST-bar cells over time in flask 3. Drug treatment was performed three times using 75 nM doxorubicin in the course of one week. One day before the third round of treatment, cells were trypsinized and 600.000 cells were reseeded. Thus, the number of colonies jumped from almost 200 to zero.

3.1.4 Optimizing repopulation assays of MCF7-bar

Prior to this thesis an MCF7-bar cell line was established in the host laboratory. In addition to the barcoded CSTr cells, this cell line was used in repopulation assays to assess the time until repopulation and to determine which of the two drug concentrations that were tested should be used for future experiments.

Triplicates of repopulation assays using either 200 nM or 250 nM doxorubicin were performed. Those concentrations were suggested in previous experiments which were performed in the host laboratory. After drug removal it was visible that the flasks showed a similar confluency than before the treatment. In the following two weeks cells started to die until a remaining confluency of about 10 % was visible across all flasks. The flasks treated with 200 nM doxorubicin took an average of 36 days until the first colony formed and those treated with 250 nM took on average 45 days. For future experiments 250 nM doxorubicin is the suggested drug concentration since it causes a longer dormancy state.

The MCF7-bar cells pose the problem that they tend to form cell clumps. The seeding of cell clumps resulted in colonies showing on day eight, where we would expect cells to die or to enter dormancy. Colonies are expected to be seen weeks after the drug treatment. Those

“initial colonies”, which resulted from seeded cell clumps partially died over time, partially stayed the same size and some colonies proliferated and expanded. To overcome this issue, seeding was improved by properly resuspending the cell pellet in medium when making the cell suspension. Moreover, the cell suspension was not added into a flask containing medium and then moved in a figure-eight motion anymore. From now on, cells were resuspended in the culture medium and this cell suspension was added into the bottom of the vessel and then the vessel was tilted to make the suspension cover the whole surface.

3.1.4.1 Attempting repopulation assays with a lower cell number

To overcome the issue of MCF7-bar cells tending to clump together and to evaluate the influence of cell number on repopulation assays, triplicates of MCF7-bar repopulation assays using only 200.000 cells were performed. Those assays were treated with 250 nM doxorubicin. The assays initially responded alike to assays with 600.000 cells: after drug removal a similar confluency as before drug treatment was visible and in the course of the next weeks cells continued to die. In contrast to the assays with 600.000 cells seeded, cells in these assays continued to die over a longer period of time. On day 57 no signs of repopulation were detectable and a cell confluency below 5 % was reached. Since there were only very few cells left per flask and since the flasks did not show any signs of repopulation, those assays were terminated. Although seeding only 200.000 cells results in less clumping of cells, these results suggest that using 600.000 cells is the better approach for repopulation assays. The results show that only very few cells survive drug treatment and repopulation takes either a lot longer or does not take place at all.

3.1.4.2 Testing the influence of cell number on drug treatment

To further investigate the influence of the number of seeded cells on drug treatment, duplicates of three different cell numbers (1.200.000, 600.000, 200.000) were seeded and treated with 250 nM doxorubicin the following day. After one week of drug treatment, cells were analyzed by flow cytometry. Doxorubicin exhibits a fluorescence signal detectable in the RFP-channel. The flasks with 1.200.000 cells in it contained on average 13,6 % non-fluorescent cells, the flasks with 600.000 cells exhibited 4,35 % non-fluorescent cells and the flasks with 200.000 cells showed 0,72 % non-fluorescent cells, see Figure 10. These cells seemingly did not take up doxorubicin. The relationship between the percentage of cells not exhibiting doxorubicin fluorescence and the cell number appears to be linear, see Figure 11. In summary, a higher cell number resulted in a higher percentage of cells not showing doxorubicin fluorescence.

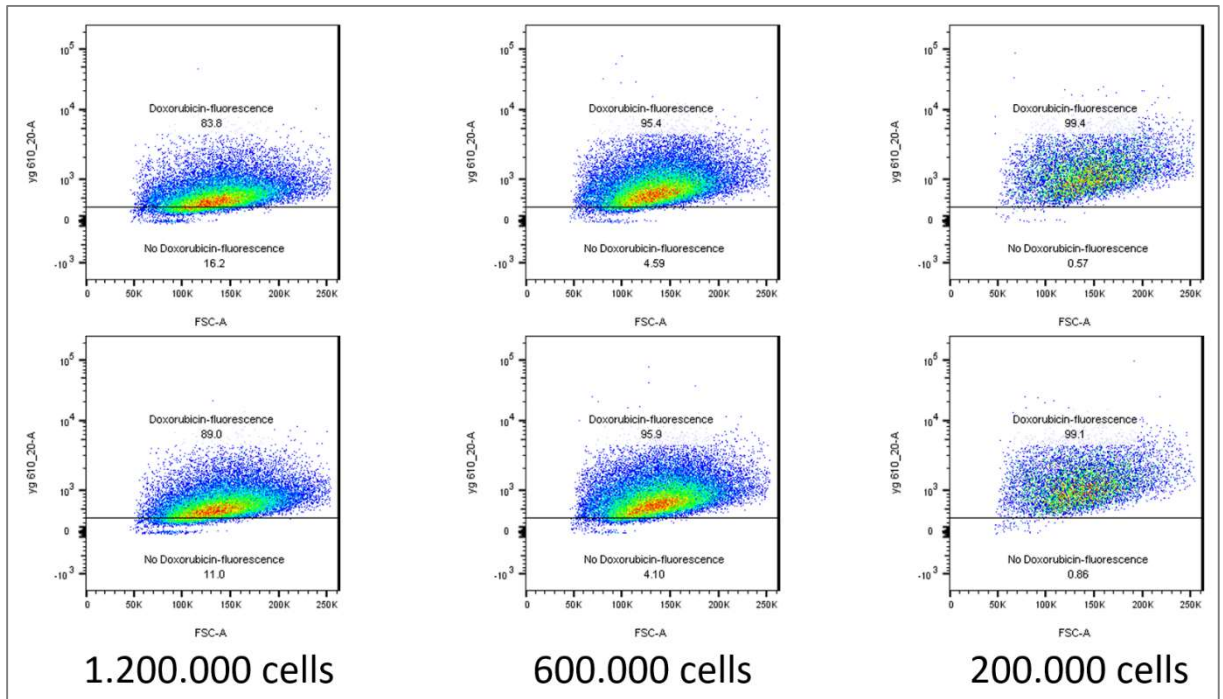


Figure 10: Flow cytometry results of repopulation assays performed with different cell numbers.

The panels plot the front scatter area (FSC-A) against the doxorubicin fluorescence (yg610_20-A). Every dot represents a single cell. The graph differentiates between cells with and without doxorubicin fluorescence. The samples show an increase of non-fluorescent cells when increasing the number of cells.

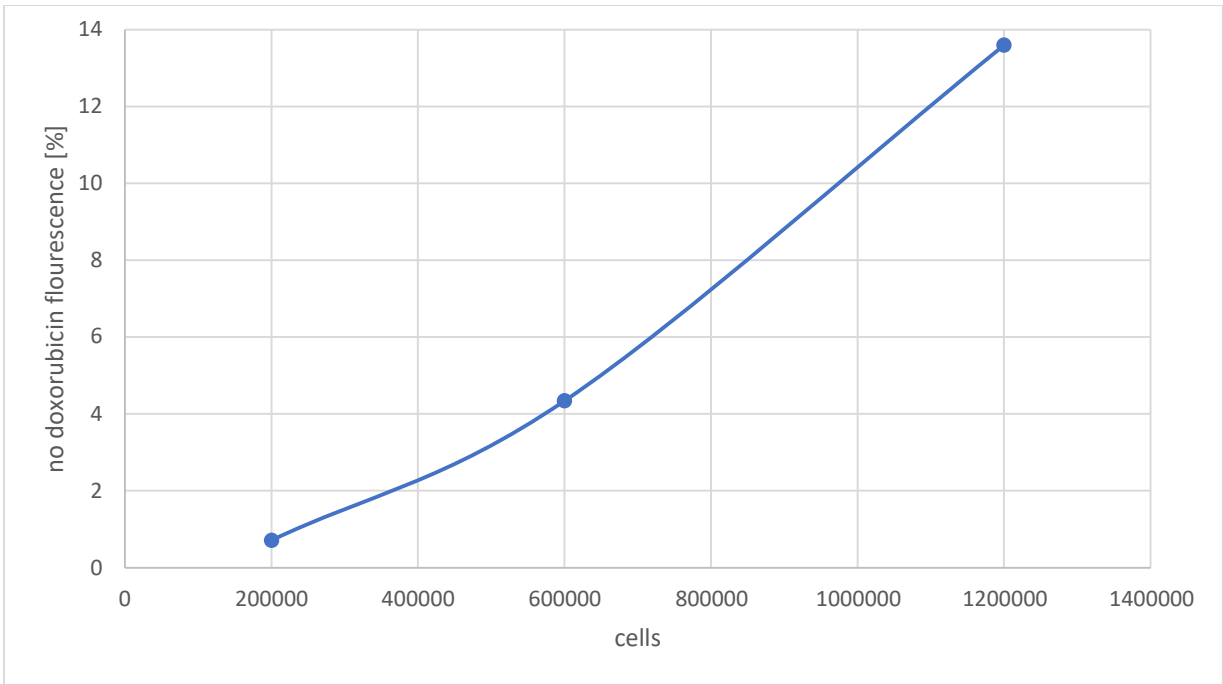


Figure 11: Influence of cell number on drug treatment.

This graph visualizes the relationship between the cell number and the percentage of cells not showing doxorubicin fluorescence. It can be seen that the relationship is approximately linear, meaning that the percentage of cells without doxorubicin fluorescence increases when increasing the number of cells.

3.1.4.3 Optimizing reseeding of cells for multiple rounds of drug treatment

Next, some repopulated flasks were trypsinized and 600.000 cells were reseeded. When seeding was checked, it was visible that the MCF7-bar cells had clumped together and instead of evenly spread single cells the flask showed patches with many cell clumps. To overcome this issue of MCF7-bar cells sticking together four possible attempts were tested. Trypsinized cells were either pipetted up and down for ten minutes, exposed to DNase, filtered or exposed to DNase prior to filtering. Those cell suspensions were then seeded and incubated before microscopically analyzing them. As shown in Figure 12 using DNase in combination with a filter resulted in the best separation of cells. Filtering and DNase on its own did improve the seeding, however, there were still cell clumps. Pipetting the cell suspension up and down for ten minutes showed the least improvement and many clumps were seeded. The result of this experiment suggests that when a second round of drug treatment is necessary for MCF7-bar repopulation assays, cells have to be trypsinized, treated with DNase, filtered and counted. Lastly 600.000 cells should be seeded. Although a way to prevent MCF-7 cells from clumping together when being reseeded was found, reseeding and retreatment of MCF-7 repopulation assay was not performed due to the lack of time.

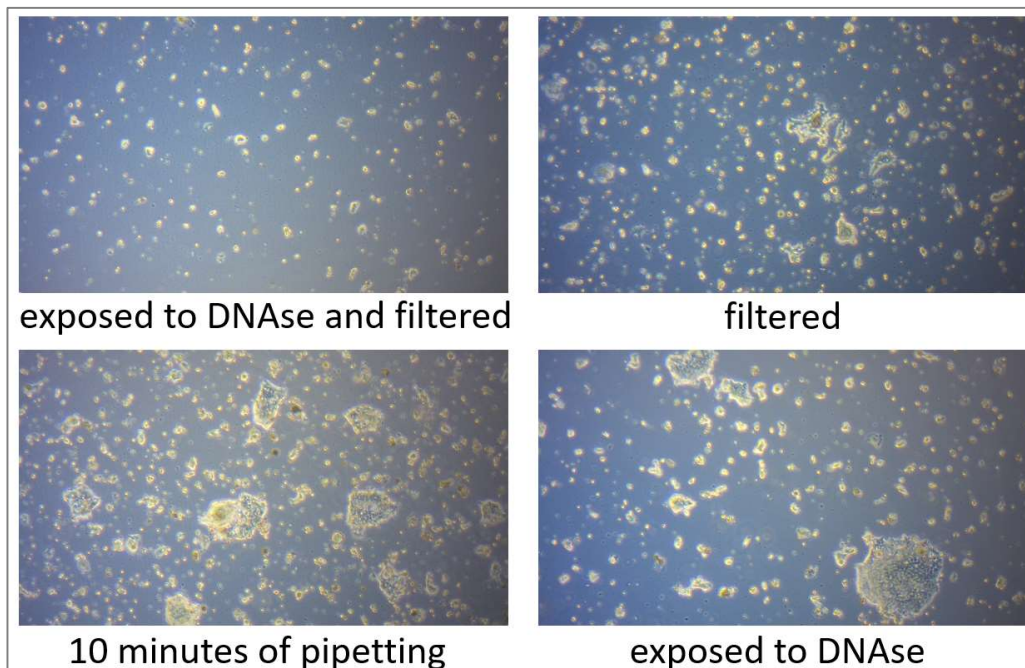


Figure 12: Optimizing reseeding of MCF7-bar repopulation assays.

The pictures show MCF7-bar cells that were trypsinized and reseeded at 4×. Before being reseeded the trypsinized cells were treated in four different ways with the goal of removing cell clumps. Exposure to DNase followed by filtering is the most efficient way to remove cell clumps since there are no remaining clumps visible. The other three attempts all showed remaining cell clumps.

3.2 Establishing and characterizing two fluorescently labeled cell lines for future gene knockdown experiments

Multiple genes are believed to be involved in drug tolerance due to their upregulation in DTP cells across multiple experiments recently performed in the host laboratory. To establish the functional relevance of these genes, knock-down experiments in repopulation assays are planned. In a future experiment a mixture of cells with and without expression of the proposed genes will be used in repopulation assays to establish if the downregulation of the particular gene has any effect on the formation of colonies. To do so, two cell lines labeled with different fluorescent tags, e.g., green fluorescent protein (GFP) and mCherry, will be used to differentiate between the cells with and without expression of the target genes. In this thesis two MCF-7 cell lines with two different fluorescent proteins were established and characterized.

3.2.1 Viral quantification

To establish two cell lines with different fluorescent tags, two viruses were required. Both viruses were produced according to the same protocol in the host laboratory prior to this thesis. The viral production aimed for a titer between 1,4 and 2 million transduction units per ml. To assess if that is the case, virus quantification the GFP-virus was performed. To do so, 100.000 MCF-7 cells were seeded per well and viral transduction with different volumes of virus was performed the next day. A flow cytometry analysis was then used to quantify how many cells had been transduced.

Before viral transduction, three wells were trypsinized and counted, because the cell number at the time of transduction is required to calculate the viral titer. Although 100.000 cells were supposed to be seeded the day before, only an average of 45.000 cells per well was counted.

Figure 13 visualizes the results of the flow cytometry. A linear relationship between the used virus volume and the percentage of GFP+ transduced cells was found. However, the percentage of GFP+ cells reached a plateau at a virus volume of 50 µl.

According to the instructions (Cellecta, Inc. 2023), a lentiviral titer of about 1,2 million transduction units per ml was calculated.

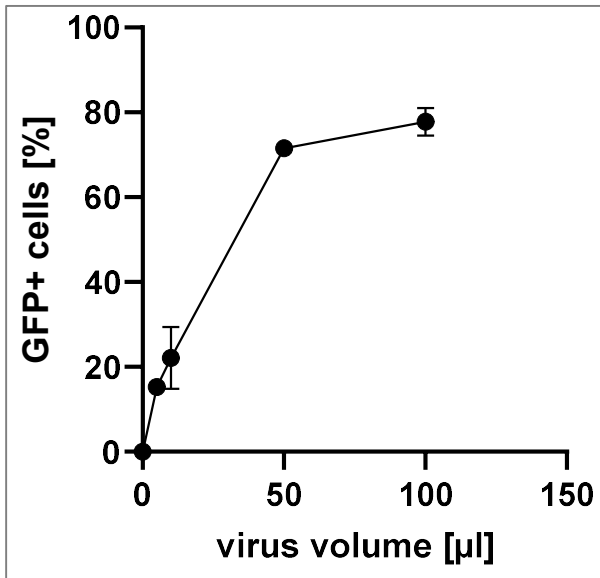


Figure 13: Lentiviral transduction: how the used virus volume influences the percentage of positive cells.

This graph plots the used virus volume against the percentage of GFP+ MCF-7 cells. A linear increase in successfully transduced cells can be seen, however, at about 50 µl the amount of successfully transduced cells reaches the plateau.

3.2.2 Establishing the cell lines MCF7-GFP and MCF7-mCherry

Lentiviral transduction of MCF-7 cells was performed to establish a GFP and a mCherry labeled cell line.

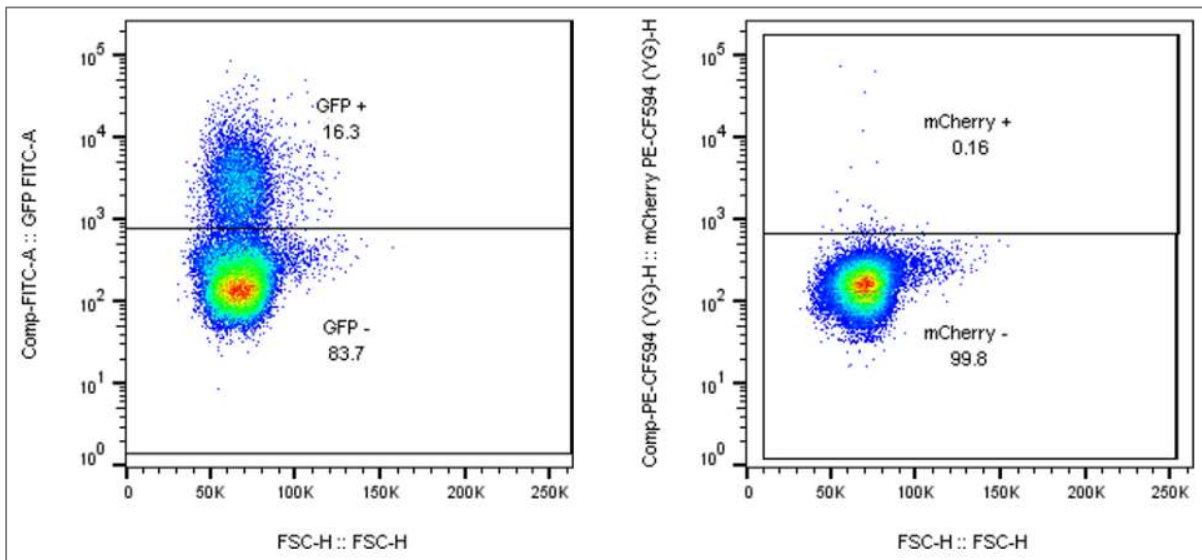


Figure 14: FACS-analysis of transduced MCF-7 cells.

The graphs plot the front scatter height (FSC-H) against the GFP or mCherry fluorescence. The left panel shows that 16,3 % of MCF-7 cells were successfully transduced with a GFP lentivirus. The right panel shows that the mCherry lentivirus failed to transduce MCF-7 cells.

As seen on the left panel in Figure 14, the transduction of MCF-7 cells with a GFP-virus resulted in 16,3 % of all MCF-7 cells being MCF7-GFP cells. With FACS 50.000 MCF7-GFP cells were sorted and then cultured. Following expansion, the newly established MCF7-GFP cell line was cryopreserved for future experiments. The right panel of Figure 14 shows that the transduction with the mCherry-virus failed to produce MCF7-mCherry cells. Further experiments require a second fluorescently labeled cell line, thus the MCF7-bar cell line, which was established in the host laboratory prior to this thesis, was used for following experiments. This cell line is fluorescently tagged with RFP.

3.2.3 Testing the influence of fluorescent proteins on drug sensitivity

Both, the MCF7-bar cells which have an RFP, and the MCF7-GFP cell line derive from the same parental MCF-7 cells with the same characteristics. Cytotoxicity assays were performed on both cell lines to analyze if the introduction of the two different fluorescent proteins has an influence on the IC_{50} values.

MCF7-bar (RFP+) or MCF7-GFP cells were seeded, incubated for 24 hours, evaporated medium (see 3.1.2 for details) and different amounts of doxorubicin was added the next day and after one week of incubation prestoblue was used to assess cell viability by measuring fluorescence intensity. The measured fluorescence intensity was normalized and plotted against the logarithm of the concentration. The curve was fitted by the GraphPad Prism 10.2.3 software using the "log(inhibitor) vs. normalized response – variable slope" equation Figure 6. Curve fit statistics allowed the determination of the IC_{50} values.

The MCF7-GFP cell line showed an IC_{50} of 78,70 nM and the IC_{50} of the MCF7-bar (RFP+) cell line was 61,05 nM. Figure 15 is a visual indicator that drug sensitivity does not seem to be significantly different between MCF7-GFP and MCF7-bar. This however could not be tested statistically due to the cytotoxicity assay only being performed once and not in replicates.

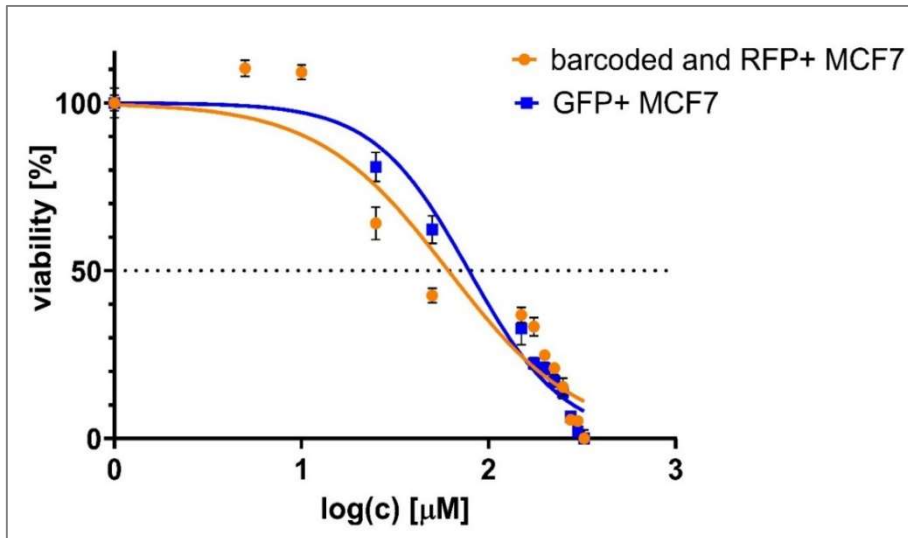


Figure 15: Dose-response curve of MCF-7 cells.

This graph visualizes the fitted dose-response curve of two MCF-7 cell lines treated with doxorubicin. It plots the cell viability against the logarithm of the concentration ($\log(c)$). The drug sensitivity of the two cell lines does not seem to be drastically different. The dotted line marks the IC_{50} values.

3.2.4 Evaluating the influence of fluorescent proteins on repopulation assays

Furthermore, replicates of repopulation assays of MCF-7, MCF7-bar and MCF7-GFP cells were run to estimate the influence of introducing fluorescent proteins on the duration of dormancy. Two different drug concentrations (200 nM and 250 nM) were used to find the optimal conditions for running repopulation assays.

On day zero for each of the three cell lines (MCF-7, MCF7-bar and MCF7-GFP) six flasks with 600.000 cells were seeded. Three flasks of each cell line were drug treated with either 250 nM or 200 nM from day one to eight. After drug removal, weekly media changes were performed and flasks were monitored microscopically.

On day eight, all flasks showed a similar confluency as before drug treatment. The following weeks doxorubicin caused cells to die until the flasks had only 5-10 % of cells left. This phenomenon was visible in all flasks regardless of the used cell line or drug concentration.

After weeks of dormancy some DTP cells resumed proliferation and gave rise to colonies. Colonies formed in all flasks, except for one (MCF7-GFP cells, treated with 250 nM doxorubicin). That flask showed no colonies forming on day 64, whereas all other flasks reached such a high confluency that most cells died due to the lack of nutrients given that media changes were only performed weekly. Parental MCF-7 cells repopulated on average on day 32 when treated with 200 nM doxorubicin and on day 44 when treated with 250 nM

doxorubicin. The MCF7-bar cells took on average 36 days after treatment with 200 nM doxorubicin and 45 days when exposed to 250 nM doxorubicin. The MCF7-GFP cells took on average 42 days when treated with 200 nM doxorubicin and 41 days when treated with 250 nM doxorubicin to form colonies. Overall, the time until colonies appeared is not significantly different between MCF-7 cells without a fluorescent protein, with a GFP or with an RFP expression, see Figure 16 Table 6. This result allows the use of a mixture of MCF-7 cells with RFP or GFP in repopulation assays since the introduction of those proteins did not result in a statistically significant different time of dormancy.

Table 6: Results of the repopulation assays of different kinds of MCF-7 cells

This table shows the results of the performed repopulation assays. Three different MCF-7 cell lines, which either have a green fluorescent protein, a red fluorescent protein or no fluorescent protein (parental), were used. Three flasks of each cell line were treated with 200 nM doxorubicin and three more flasks received 250 nM doxorubicin. This table shows after how many days colonies started to form in each flask and the average of the each three flasks of every cell line treated with the same doxorubicin concentration.

	parental		GFP+		RFP+	
concentration [nM]	200	250	200	250	200	250
flask 1: day of first colony formation	31	43	36	38	36	38
flask 2: day of first colony formation	36	43	38	44	36	43
flask 3: day of first colony formation	30	45	51	-	36	53
average number of days	32	44	42	41	36	45

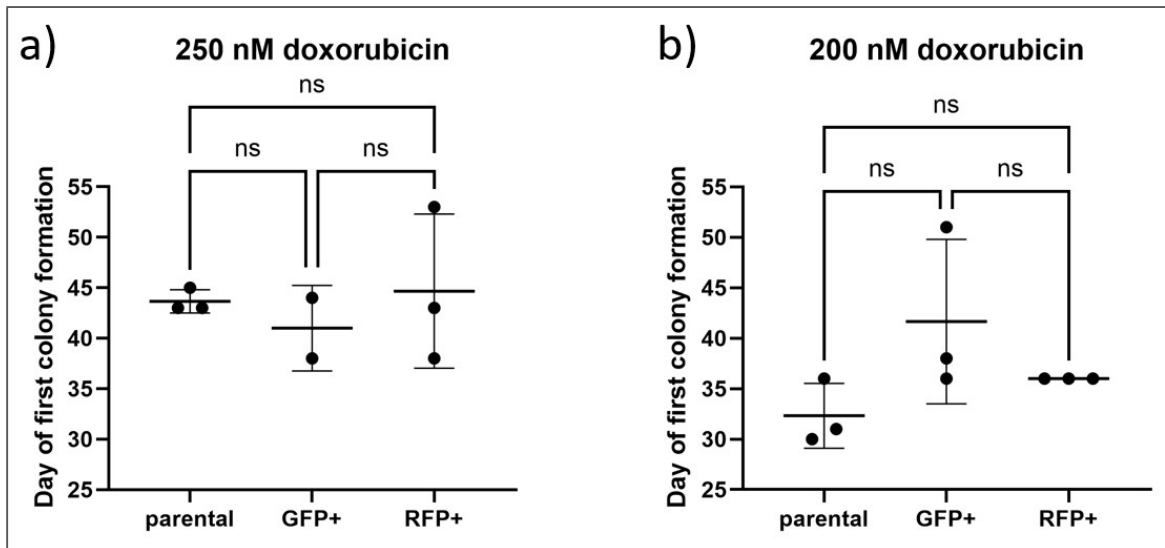


Figure 16: Repopulation assays of different types of MCF-7 cells do not significantly differ in repopulation time.

Repopulation assays of different types of MCF-7 cells (parental, GFP+ and RFP+) have been performed using either 250 nM doxorubicin or 200 nM doxorubicin. A one-way ANOVA and a Turkey's test were performed on the repopulation times. This showed that no significant difference in the time until repopulation of the different cell lines (MCF7 parental, GFP+ and RFP+/barcoded) was found, independent of the used concentration of doxorubicin.

3.2.5 Testing the stability of the ratio of a mixture of cells with two different fluorescent proteins

The next experiment aimed at determining if the ratio of a mix of RFP and GFP labelled MCF-7 cells was stable when performing repopulation assays or if there was a selection for one of the cell types. Therefore, a mix of cells with equal numbers of MCF7-bar and MCF7-GFP was made. This mix was analyzed with flow cytometry on day zero (seeding), on day one (start of drug treatment) four days after seeding without drug treatment and on day 24 after a week of doxorubicin treatment.

The flow cytometry analysis of the mixture used for seeding (day 0) was expected to show each 50 % RFP+ and GFP+ cells. 52 % of the MCF-7 cells were GFP+ and 47,5 % were RFP+. This is almost the ratio, that has been aimed at. On day one we saw a higher number of GFP and a lower number of RFP than in the seeding mixture. Four days after seeding, without drug treatment of the cells, a highly similar ratio was obtained. On day 24 fewer GFP+ and more RFP+ cells showed. All samples showed to some extent double positive cells. Moreover, an increase of non-fluorescent cells was observed over the whole period of time (Table 7). Seemingly one or both cell lines tend to lose their introduced protein over time, which is an issue that requires further investigation. Summarized, these results suggest that there is no strong selection for either cells having an RFP or a GFP.

Table 7: Average flow cytometry results at different timepoints

This table shows the average flow-cytometry results at four different timepoints (day 0,1,4 and 24). It shows each the percentage of non-fluorescent, red, green as well as red and green fluorescent cells. Green fluorescent protein (GFP) is responsible for the green fluorescence whereas red fluorescence can be caused by a red fluorescent protein (RFP) as well as by the autofluorescence of doxorubicin which can be detected at the red channel as well.

day	GFP+ cells [%]	RFP+ cells [%]	GFP+ and RFP+ cells [%]	Non-fluorescent cells [%]
0	52,0	47,5	0,091	0,50
1	58,9	39,9	0,73	0,50
4	58,7	38,5	2,09	0,67
24	49,0	44,8	1,13	5,11

4 Discussion

4.1 Origin of DTP cells

4.1.1 Establishing the CStr-bar cell line

This experiment, which aimed to establish the CStr-bar cell line, was successful and cryopreserved aliquots of the cell line were produced.

In the first step of the experiment 800.000 CStr cells should have been seeded and cells were counted the next day right before viral transduction. This showed that there were approximately three million cells, which is a higher number than expected since CStr cells are believed to have a doubling time of 34 hours. (Hámori et al. 2020) The high cell number might have been caused by pipetting errors or fluctuations in manual cell counting. It is also possible that this batch of CStr cells has a faster growth rate and thus more cells were visible.

The experiment showed that the percentage of transduced cells corresponded to the expected MOI. Literature states that the number of integrations is approximately equal to the number of transduced cells, when the percentage of infected cells is at or below 20 %, which aligns with this finding. (Cellecta, Inc. 2023)

4.1.2 Assessing the IC₅₀ of the CStr-bar cell line

To characterize the new CStr-bar cell line a cytotoxicity assay using doxorubicin was performed, which showed that the IC₅₀ of this cell line is 21,07 nM. In contrast, CStr cells have been shown to have an IC₅₀ of 200 nM doxorubicin. (Hámori et al. 2020) Potentially the CStr batch available in the host laboratory is more sensitive than the originally established cell line. Furthermore, the transduction of the cells possibly influenced the drug sensitivity of the cells.

Assays were already analyzed after five days and not after seven days as planned, because cells got confluent by then. This might result from pipetting errors, fluctuations in cell counting or a faster growth rate than expected.

Initially, media changes were supposed to be performed 24 hours after seeding cells into the 12-well plates. However, after these media changes all cells were dead. By trying many different approaches, it was ruled out that handling the cells e.g., exposing cells to air for too long or pipetting not gently enough, was the cause. Plates of three different brands were tested and all of them showed the same phenomenon. This indicated that the plate was not the cause, although there is a minor possibility that e.g., by wrong storage all of these plates, especially their coating, were damaged. When the same cells were cultured in T25- or T75-flasks they

survived media changes perfectly fine, which suggests that cells are not the cause. It was tested if potentially cells were too stressed by the media changes being performed only 24 hours after seeding the cells by performing the media changes two days later. The later media changes also resulted in complete cell death, showing that the point of time when media changes were performed did not make a difference. The cells could have potentially secreted growth factors into the medium and their sudden disappearance due to the media change caused the cells to die. This issue could have been fixed by performing a partial media change, however, since cells grew without any problems in T25- and T75-flasks, this scenario is most likely not the cause. (Kolkmann et al. 2020) Presumably multiple factors contributed to this phenomenon, however, it was not possible to determine which ones exactly played a role.

4.1.3 Optimizing repopulation assays of CStr-bar

The results of this experiment suggest that a higher drug concentration is associated with a longer phase of dormancy and a later start of repopulation. Additionally, this suggests the use of 125 nM doxorubicin in future repopulation assays. The use of this drug concentration facilitates a later repopulation than using 75 nM doxorubicin. This is preferred due to the fact that a later repopulation is rather caused by DTP cells than by resistant cells. Taking an even higher drug concentration might prolong the phase of dormancy even more, but it could possibly cause cell death of all cancer cells and fail to induce the DTP state.

When attempting a second round of treatment on repopulation assays showing 200 colonies or even more, regardless of the used drug concentration, it was seen that after two days the majority of cells had detached or died. The indicator of the medium showed that the nutrients were completely metabolized by that day, which is most likely the reason why all the cells died. One flask showed fewer colonies when the second round of treatment was started. The repeated treatment of this flask resulted in the decrease of colonies, however, there were still colonies that remained. Most probably, cells growing in colonies are less accessible for doxorubicin. The first round of drug treatment showed changes in cell numbers in accordance with the literature. (Szebényi et al. 2023) The second round of treatment, however, did not clearly show those changes, thus a third round of drug treatment was performed on reseeded cells. This showed that these cells were definitely not resistant cells since the cells were shown to be sensitive to doxorubicin as they showed a similar response as to the first drug treatment. Keeping this in mind, only a reduction of cells and not complete cell death would be expected to be seen if doxorubicin was the cause of cell death making the lack of nutrients the most

probable cause. Moreover, this time we again saw the characteristic changes in cell number over time.

To summarize, the results suggest using 125 nM doxorubicin for drug treatment in CStr-bar repopulation assays. To perform multiple rounds of drug treatment, these results suggest doing the following: First, let over 200 colonies form after the first round of treatment. Then trypsinize all cells and reseed 600.000 cells. Lastly, repeat doxorubicin treatment the following day.

4.1.4 Optimizing repopulation assays of MCF7-bar

Repopulation assays of MCF7-bar cells showed that when the drug was removed a similar confluency than before drug treatment was visible. Over the next weeks more and more cells died until a confluency of 5-10 % was reached. This suggests that doxorubicin inhibits the proliferation of MCF7-bar first and it induced cell death later. Doxorubicin acts on multiple intracellular targets, which is possibly the reason for the delayed cell death. (Kciuk et al. 2023, Nicoletto and Ofner 2022, Sritharan and Sivalingam 2021)

These repopulation assays again suggested that treatment with a higher drug concentration is associated with a longer period of dormancy. 250 nM doxorubicin was found to be the better drug concentration since it resulted in a later repopulation than treatment with 200nM doxorubicin. This is preferred because a later repopulation is more likely to be caused by DTP cells than by resistant cells.

It was tested if seeding fewer cells (200.000) than usual (600.000) worked in repopulation assays to help with the issue of MCF7-bar cells clumping together. This resulted in the survival of only very few cells and no signs of repopulation were visible on day 57. Potentially the assay would have shown repopulation later on or repopulation would not have taken place at all and doxorubicin would have caused the remaining cells to die as well. The assay was terminated because a cell confluency far under 5 % was reached. This is not desirable since more than 200 colonies should form in the assays, which requires the availability of enough DTP cells.

The influence of the cell number on drug treatment was tested and it was found that a higher cell number is associated with a higher number of cells showing no doxorubicin fluorescence. One reason for this could be that the drug was not able to access cells due to denser seeding. Another possible scenario is that those cells are DTP-cells since these cells are characterized by slow proliferation and doxorubicin only targets dividing cells.

4.2 Establishing and characterizing two fluorescently labeled cell lines for future gene knockdown experiments

Several genes are believed to be involved in the DTP state and knockdown-experiments can be used to determine their functional relevance. These planned experiments require two cell lines with different fluorescent labels. This thesis found that MCF-7 cells with the expression of RFP or GFP, derived from the same parental MCF-7 cells, show similar characteristics enabling planned knockdown experiments.

4.2.1 Establishing the cell lines MCF7-GFP and MCF7-mCherry

The MCF7-GFP cell line was established successfully, and cell aliquots were cryopreserved. The transduction with the mCherry-virus failed to produce MCF7-mCherry cells. Most likely something in the process of the viral production went wrong and no viral particles were present at the time of transduction. Since the same batch of MCF-7 cells was transduced with the GFP-virus, which resulted in transduced cells, it is unlikely that the cells were the cause of failed transduction.

4.2.2 Testing the influence of fluorescent proteins on drug sensitivity

There was a 22 % deviation of the IC_{50} values of MCF-7 cells with an RFP and a GFP. The introduction of the fluorescent proteins must not necessarily be the only cause for the deviation. Fluctuations in cell counting and the seeding itself as well as fluctuations in drug concentration can also contribute to the difference.

4.2.3 Evaluating the influence of fluorescent proteins on repopulation assays

On day eight, all flasks, regardless of which cell line, showed a similar confluency as before drug treatment, suggestive of the fact that doxorubicin inhibits proliferation in that time frame. The following weeks doxorubicin caused cells to die until the flasks had only 5-10 % of cells left. This phenomenon was the same, regardless of the MCF-7 cells having GFP, RFP or no fluorescent protein, suggestive of the fact that the proteins do not influence drug treatment. This experiment showed changes in cell number in accordance with the literature, except for the fact that cell death occurred delayed. (Szebényi et al. 2023)

One of the flasks (MCF7-GFP cells, treated with 250 nM doxorubicin) failed to repopulate, which was potentially caused by the seeding of fewer cells in that flask or by a higher than intended drug concentration.

The repopulation times of the MCF-7 and MCF7-bar cells suggest that a higher drug concentration is associated with later colony formation. The MCF7-GFP assays in contrast showed that the higher drug concentration does not seem to extend the time until repopulation, however, that result might be influenced by the fact that one of the flasks treated with 200 nM doxorubicin repopulated about two weeks later than the other two.

4.2.4 Testing the stability of the ratio of a mixture of cells with two different fluorescent proteins

The seeding mixture which aimed to show a 50:50 ratio was found to have 52 % GFP+ cells and 47,5 % RFP+ cells. The slight deviation might be caused by pipetting errors or not accurate cell counting. On day one we saw a higher ratio of GFP and a lower ratio of RFP than in the seeding mixture. A possible cause could be insufficient mixing of the two cell suspensions. On day 24 less GFP+ and more RFP+ cells showed. It was observed that denser regions, caused by uneven seeding, were more likely to survive the process of drug treatment. Possibly more RFP+ cells were present in those regions which then caused a shift in the ratio. All samples show to some extent double positive cells which potentially resulted from wrong gating of the data and thus inclusion of cell clumps. Yet there is no literature on the use of different fluorescent proteins in repopulation assays.

A potential limitation that was found is that over time the percentage of non-fluorescent cells increased. This suggests that after several weeks some cells seem to not express the fluorescent protein anymore. This issue has to be overcome, otherwise no analysis of the results from the knockdown experiment is possible since it would not be clear which cells exhibit gene expression and which do not.

4.3 Outlook

Cellular barcoding allows analyzing clonal relations, thus, it can be used to study the clonality of DTP cells. Replicates of repopulation assays can show if cells that survive drug treatment are selected stochastic or if they are predisposed to be resistant. Predisposed cells are either intrinsically resistant or they are genetically poised to undergo phenotypic changes associated with the DTP state. To differentiate between those two possible scenarios, multiple rounds of drug treatment can be used.

This thesis established the conditions for these replicate repopulation assays using barcoded cells. Seeding 600.000 barcoded cells, either MCF7-bar (human cell line) or CStr-bar (mouse cell line), was the cell number which worked best in repopulation assays. The results of this

thesis indicate that drug treatment on day one should be performed with two different concentrations for the different barcoded cell lines. 125 nM doxorubicin is optimal for CStr-bar cells and 250 nM doxorubicin for MCF7-bar cells. The CStr-bar cells will repopulate approximately one week after drug removal and the MCF7-bar cells will start forming colonies on average five weeks after the doxorubicin removal. The optimal way to repeat drug treatment is to trypsinize repopulated flasks as soon as they show about 200 colonies and to reseed 600.000 of those cells. MCF7-bar cells must be exposed to DNase and be filtered before being reseeded since they clump together. Doxorubicin treatment is repeated the next day using the same concentration as in the first round of treatment.

Multiple samples have to be collected at different points of time, meaning cells have to be harvested in order to isolate their genomic DNA. It is vital to collect samples on day one and after that samples should be collected at different points of time, for example on day eight (right after drug treatment), fifteen (state of dormancy) and at the time of repopulation. Additionally, samples should be collected after each of multiple rounds of drug treatment. The genomic DNA of harvested cells has to be isolated, the barcodes must be amplified by polymerase chain reaction and the barcodes must be purified before being analyzed by next generation sequencing.

Those sequencing results will reveal the origin of DTP cells. Tracking the clonal survival of cells will reveal if breast cancer cells surviving treatment are selected by chance or not. On day zero we expect all repopulation assays to exhibit a high number of different barcodes with a similar abundance. The results could possibly show that drug treatment selects for certain clones. If replicate assays show that the same clones are selected and repopulate, this can be the result of two scenarios: Either cells were intrinsically resistant or cells entered the DTP state to survive treatment. To ensure that the second scenario is the case, multiple rounds of treatment are used. Cells responding to repeated drug treatment would indicate that DTP cells survived the drug treatment. If that is the case, observing the same cell clones across multiple assays would suggest that DTP-cells are pre-determined. Furthermore, barcode analysis could show if multiple rounds of treatment lead to a further enrichment of clones. However, cells not responding to repeated drug treatment indicate that certain clones had an intrinsic resistance. Possibly the results show that the cells are selected randomly. In this case different barcodes would be observed across the replicate repopulation assays. This is suggestive of cancer cells stochastically undergoing the phenotypic transition.

Possibly this experiment can also be performed *in vivo* by transplanting barcoded cells into mice. This future experiment can reveal more of the underlying mechanism of the evolution of DTP cells, which potentially reveals a new target for new therapies in the future.

A limitation of this approach is that a barcode labeling a certain clone does not necessarily imply a genetic or epigenetic difference. Clones are all cells marked by a particular barcode, which reflects the fact that they arose from the same cell. A clone, however, does not mandatorily distinguish between genetically or epigenetically distinct cells. It is possible that two cells with the exact same genetic and epigenetic background are labeled by two different barcodes.

In summary, DTP cells are a proven but yet poorly characterized concept enabling drug sensitive cancer cells to survive and relapse despite drug treatment. This relapse is believed to provide cancer cells with the additional time required to acquire resistance. It is of utmost importance to study how DTP cells emerge and what characterizes them in order to establish new therapies targeting DTP cells. Potentially therapeutic approaches could hinder DTP cells from leaving dormancy, awake DTPs and kill them due to their drug sensitivity or use specific regulators of DTP cells as target.

5 Abstract

Among women, breast cancer is the type of cancer with the highest mortality and incidence. Despite treatment with a combination of therapies and surgery patients often show signs of incomplete response and eventual relapse. Lately a temporary state of drug resistance known as drug tolerant persistence was found. Those so-called drug-tolerant persister (DTP) cells and their mechanisms require further research.

Currently, it is not known whether DTP cells are randomly selected for survival or are derived from a pre-determined sub-population of drug-naive cells, and whether such “pre-DTP” cells would be clonally determined. To address these possibilities in *in vitro* experiments, this thesis assessed an experimental setup using lentiviral tagging of cells and so-called repopulation assays where modeling acute drug response induces the DTP state. It was shown that 250 nM for MCF-7 cells and 125 nM of doxorubicin for CSTR cells was the best concentration to induce the DTP state. CSTR cells were shown to repopulate after about two weeks while MCF-7 cells took in average approximately five weeks.

Several genes are believed to be involved in the phenotypic transition. Their functional relevance can be assessed in knockdown experiments requiring two cell lines with different fluorescent tags to distinguish cells with and without gene expression. This thesis tested if the introduction of red and green fluorescent proteins drastically influences drug sensitivity of MCF-7 cells, time of dormancy of DTP-cells and whether there is a selection for either one of the cell types when running repopulation assays. It was found that MCF-7 cells with two different fluorescent proteins show similar drug sensitivity and that the time until repopulation is not significantly different. Moreover, it was shown that the ratio of RFP+ and GFP+ cells does not drastically change over time, thus allowing planned knockdown experiments.

6 Zusammenfassung

Brustkrebs ist unter weiblichen Patienten jener Typ Krebs mit sowohl der höchsten Mortalität als auch Inzidenz. Trotz der Behandlung mit einer Kombination von Therapien und Operation sprechen Patienten oft nicht vollständig auf diese an und erkranken eventuell erneut. Kürzlich wurde ein vorübergehender Zustand der Arzneimittelresistenz, bekannt als arzneimitteltolerante Persistenz (DTP), entdeckt. Diese sogenannten DTP-Zellen und ihre Mechanismen erfordern weitere Forschung.

Derzeit ist es unbekannt, ob DTP-Zellen zufällig zum Überleben ausgewählt werden oder ob sie aus einer vorbestimmten Subpopulation unbehandelter Krebszellen stammen und ob solche „Prä-DTP“-Zellen klonal bestimmt werden. Um diese Möglichkeiten in *in vitro* Experimenten zu testen, wurde in dieser Arbeit ein Versuchsaufbau, basierend auf dem lentiviralen Markieren von Zellen und auf sogenannten Repopulationstests, in welchen der DTP-Zustand durch Modellierung einer akuten Arzneimittelreaktion hervorgerufen wird, entwickelt. Es wurde gezeigt, dass 250 nM für MCF-7 Zellen und 125 nM Doxorubicin für CSTR Zellen die besten Konzentrationen sind, um den Zustand der Arzneimittelresistenz zu induzieren. Während CSTR Zellen bereits nach rund zwei Wochen Repopulation vorweisen konnten, brauchten die MCF-7 Zellen etwa fünf Wochen dafür.

Es wird vermutet, dass gewisse Gene an diesem phänotypischen Übergang beteiligt sind. Die funktionelle Relevanz der Gene kann in Knockdown-Experimenten ermittelt werden, welche zwei mit verschiedenen fluoreszenten Proteinen markierte Zelllinien erfordern, um Zellen mit und ohne Genexpression voneinander zu unterscheiden. In dieser Arbeit wurde untersucht, ob durch die Expression dieser fluoreszierenden Proteine die Empfindlichkeit der Zellen gegenüber Arzneimitteln und die Dauer der Ruhephase der DTP-Zellen drastisch beeinflusst und ob in Repopulationstests eine Selektion für einen der beiden Zelltypen stattfindet. Es wurde festgestellt, dass MCF-7 Zellen mit zwei verschiedenen fluoreszierenden Proteinen eine ähnliche Empfindlichkeit gegenüber Arzneimitteln aufweisen und, dass die Zeit bis zur Repopulation sich nicht signifikant unterscheidet. Außerdem wurde gezeigt, dass das Verhältnis von RFP+ und GFP+ MCF7 Zellen sich über die Zeit nicht drastisch verändert, was zukünftige Knockdown-Experimente ermöglicht.

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10 List of abbreviations

Abbreviation	Meaning
A	area
ANOVA	Analysis of Variance
CSTr-bar	barcoded and RFP+ CSTr cells
DMSO	dimethyl sulfoxide
FACS	fluorescence activated cell sorting
FSC	forward scatter
GFP	Green fluorescent protein
H	height
IC50	half maximal inhibitory concentration
MCF7-bar	barcoded and RFP+ MCF-7 cells
MCF7-GFP	GFP+ MCF-7 cells
MOI	multiplicity of infection
PBS	phosphate buffered saline
RFP	Red fluorescent protein
SSC	side scatter