Identification of Inhibitors for Tangocytosis Through High-Throughput Screening

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Abstract

Breast cancer is the most common cancer in women, affecting 2.3 million women globally. However, no identified drugs effectively block tumor movement to another site, rendering metastatic breast cancer a chronic and incurable disease. Previously, the Liu lab discovered a process called "tangocytosis," in which an epithelial cell engulfs a tumor cell and transfers the reporter gene from the tumor cell to the epithelial cell. Blocking direct cell-cell interaction prevents subsequent marker gene transfer, which provides a useful tool for studying the cell entrapment process via high-content screening methods. In this thesis, high-content screening using FDA-approved small molecule inhibitors revealed that DNA damage repair drugs can effectively block the gene transfer between the donor and recipient cells.

Following the screening, potent inhibitors of tangocytosis, Clofarabine and Teniposide, were identified and selected for further analysis. It was discovered that these drugs effectively blocked reporter gene transfer and minimized the formation of cell-in-cell structures, suggesting that inhibiting such structures can prevent gene transfer. Additionally, the drugs had polarizing effects on both donor and recipient cells. Pretreating RPE1 cells with the drugs resulted in a decrease in gene transfer upon co-culture while pretreating MDA-MB-231 cells with drugs increased gene transfer upon co-culture. The polarizing effect indicates that gene transfer may be mediated by the upregulation of different proteins in each cell line.

To investigate whether ROCK1, an essential kinase in cell tumor migration, plays a role in the polarization, immunofluorescence staining of ROCK1 was conducted

on MDA-MB-231 and RPE1 cells. The results showed that ROCK1 intensities were higher in regions of cell-in-cell structures compared to free cells. In the DMSO control group, ROCK1 intensity was significantly higher in cell-in-cell regions than in absent regions. However, when Clofarabine was added to the co-culture, the difference was no longer significant. The findings suggest that DNA damage repair drugs may reduce gene transfer by reducing the polarization of ROCK1. Due to the high number of hits related to DNA damage repair and the reduction of polarization, the DNA damage repair pathway presents as an essential pathway for successfully blocking gene transfer through the inhibition of cell-in-cell structures.

Introduction

I. Migration of primary tumor cells through metastatic cascade

The metastatic cascade is the sequential process by which cancer cells spread from the primary tumor site to distant organs in the body. While the exact mechanisms involved in each step are still being elucidated, it is widely accepted that the first step in the metastatic cascade involves detachment of the tumor from the primary site and entry into the bloodstream or lymphatic system while resisting the high sheer stress in the bloodstream. [1,2,3] The circulating tumor cells acquire epithelial-mesenchymal transition properties that help them navigate through the circulation environment, evade immune surveillance, and extravasate into a secondary site where they can colonize and form a new tumor.[4]

One proposed mechanism for cancer cell entry into the circulation is through breaching the endothelial walls of blood or lymphatic vessels. While the exact process by which cancer cells breach the endothelial layer is not fully understood, it has been hypothesized that cancer cells may use a similar process as leukocyte diapedesis. [5] During leukocyte diapedesis, white blood cells use adhesion molecules and pseudopods to attach to and move through the endothelial cell layer of blood vessels. Leukocyte movement is characterized by rapid changes in their shape that allow them to move transcellularly and paracellularly through the endothelial layer as they slip between joined epithelial cells or through them.[6] Similarly, cancer cells exhibit transendothelial migration by making direct physical contact with the endothelium and using adhesion molecules and pseudopods to move through the layer.

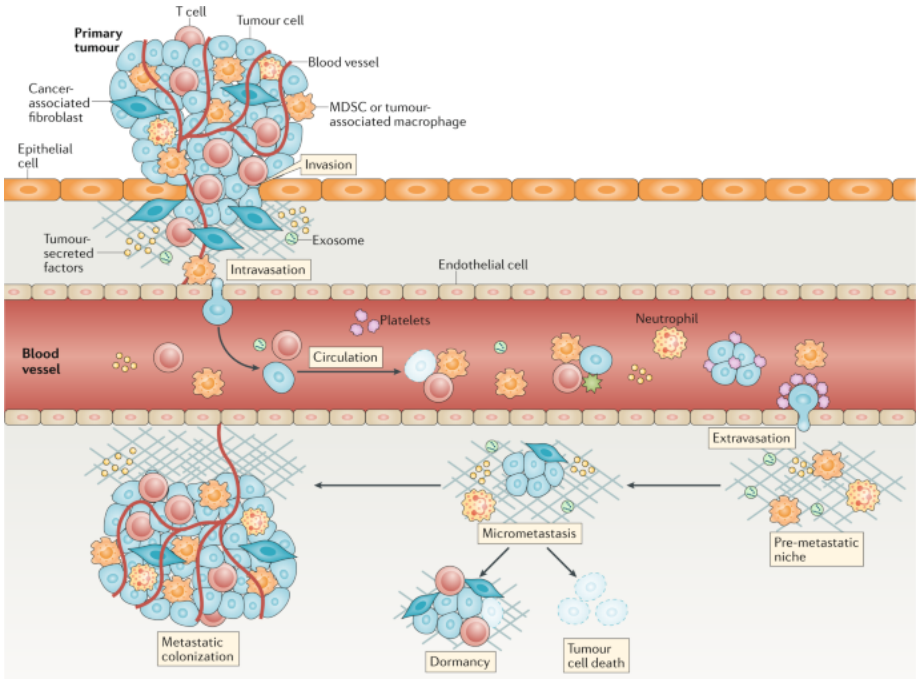

Figure 1: The primary tumor cell breaches the epithelial barrier and intravasates into the circulatory system before it breaches the barrier again to perform extravasation to its secondary site[7]

During the migration, the tumor cell undergoes a series of morphological changes to slip through the narrow gaps between endothelial cells. Actin-myosin contraction plays a pivotal role in remodeling the cell's cytoskeleton for invasion and generating contractile force. RhoA and its downstream effector, Rho-associated protein kinase 1 (ROCK1) signaling promotes actin-myosin contraction and the formation of invadopodia, which are necessary for cancer cells to invade through the extracellular matrix and basement membrane.[8] Due to its vital role in tumor migration, ROCK1 expression is significantly higher in cancer tissues compared to normal tissues, and high expression of ROCK1 is associated with a poor prognosis and progression of various types of cancer, including lung cancer, breast cancer, prostate cancer, and colon cancer.[9] [10,11] Consequently, inhibition of ROCK1 leads to reduced cell mobility and

invasion ability through the inhibition of the PTEN/P13K/FAK pathway.[12] In regard to breast cancer, ROCK-inhibited treatment through Y-27632 exhibited diminished migratory and invasive behavior.[13]

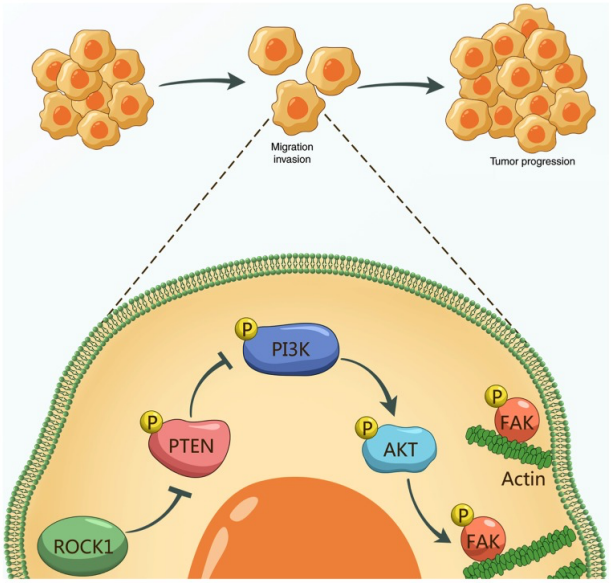

Figure 2: ROCK1 inactivates PTEN which allows phosphorylation of P13K/AKT, leading to association of FAK with actin, promoting tumor cell migration, invasion, and progression.[14]

Following circulation in the bloodstream, the cancer cells extravasate and enter the parenchyma of distant tissues which require the tumor cell to once again breach the endothelial wall to escape the bloodstream.[15] Far from the primary site, the tumor cells establish growth and colonization at the secondary site while resisting host tissue immune system defenses.[16]

II. Intracellular gene transfer in cancer

Intracellular gene transfer occurs between two cells that are in direct physical contact with each other. Vertical gene transfer is a common paradigm in cancer

progression where the initial tumor cell can propagate its genes to its subsequent offspring.[17] Horizontal gene transfer (HGT) is pervasive in prokaryotes which results in recombination of the bacterial genome with its host through methods of transformation, transduction, and conjugation.[18] In eukaryotes, HGT can also transfer DNA between tumor and normal cells through phagocytosis of apoptotic bodies, exosomes, and circulating cell free DNA (cfDNA).[19] Although the exact source is not known, a large majority of cfDNA originates from apoptotic bodies which originate from dying tumor cells and can transport diverse cargoes such as DNA, RNA, and protein. Their transport ability suggests that they have the potential to induce transformation in neighboring cells by integration of the tumor DNA within the recipient's genome. Changes in the outer membrane of the apoptotic body occur which induces neighboring cells to become phagocytic and engulf the apoptotic body.[20] After engulfment, exchange of genetic material occurs which leads to the adoption of new traits in the engulfing cell.

Additionally, cfDNA levels are elevated in cancer patients compared to healthy patients, reflecting increased turnover in cancer cells. Studies have shown that lateral transfer of tumor-derived cfDNA can induce genomic instability in normal cells to develop into a malignancy.[21] A proposed mechanism is through the horizontal transfer of oncogenic mutations from tumor cells to non-tumor cells, leading to cancer progression.[22] Additionally, tumor cell DNA can be transferred through the use of small vesicles that package the DNA or RNA, known as exosomes. These exosomes can contain oncogenic DNA elements or RNA retrotransposon elements that become

integrated into the host cell's genome, leading to phenotypic changes that promote cancer cell growth and proliferation.[23]

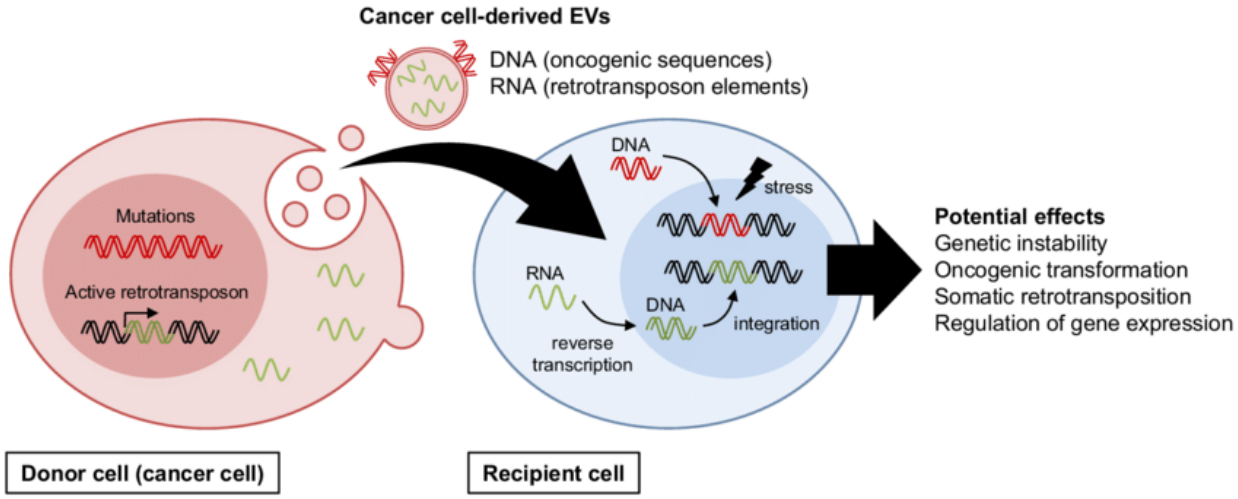

Figure 3: Representation of sEV-mediated HGT among tumor/microenvironmental cells from the donor cell and recipient cell perspective[24]

III. Small molecule inhibitors in cancer treatment

Chemotherapy has been the conventional cancer treatment but its inability to distinguish between cancerous and normal cells results in toxicity and significant side effects. One of the main advantages of small molecule drugs is their ability to target specific cancer cell signaling pathway. By selectively inhibiting specific molecules or pathways involved in cancer cell growth, small molecule inhibitors can minimize damage to healthy cells and reduce the risk of side effects.[25, 26]

High throughput screening (HTS) is a method used in drug discovery that enables the rapid screening of a large number of compounds for their ability to interact with a target of interest. It can be used in conjunction with drug repurposing which allows identification of new therapeutic uses for existing drugs.[27] The approach is attractive

since many of these drugs have already undergone clinical testing for safety and toxicity, which can reduce the time and cost required for drug development. Small molecules have several benefits over larger biologics such as peptides or monoclonal antibodies, as they have a well-defined structure and the ability to be administered orally for in vivo applications. These properties make small molecule drugs advantageous in a clinical setting as therapeutics. Additionally high throughput screening evades the pitfalls of CRISPR KO and siRNA as they require removal of the protein target, which can leave out other genes and cause off target effects. [28]

IV. Project Aims

Our previous result showed that horizontal gene transfer would occur when H2B-mCherry labeled breast cancer cells, MDA-MB-231, were cocultured with Venus-Parkin labeled epithelial cells RPE1 or endothelial cells HUVEC. The coculture will result in the third cell population which are double positive for both Venus-Parkin and H2B-mCherry. Live cell imaging revealed that the cells were transiently entrapped and entangled with each other throughout the 24 hr co-culture, indicating that the transient entrapment of breast cancer cells within the epithelial cell contributes to the transfer of genetic material. This transient entrapment process is also accompanied with H2B-mCherry gene transfer from MDA-MB-231 cells to RPE1-Venus-Parkin. This phenomenon is referred to as tangocytosis and in summary requires (1) direct cell-cell interaction (2) transfer of genetic material. To better understand this pathway, small molecule inhibitors were utilized to identify signaling pathways associated with tangocytosis and to reveal improved drug mechanisms to prevent cancer metastasis.

The overarching goal is to utilize high-throughput screening to identify targets that prevent cell-cell interaction between breast cancer and normal cells. Prestwick Chemical Library®, which contains 1520 FDA- approved & EMA-approved drugs, was pursued to develop an understanding of how current clinically used drugs could also be used to block metastasis of a primary tumor. From the screening, drugs that have a similar mechanism were prioritized in further analysis as they could reveal a key regulatory mechanism in mediating gene transfer.

Materials and Methods

Cell Culture

MDA-MB-231 and RPE1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen), penicillin, streptomycin (100 IU/ml and 100 mg/ml, respectively), and L-Glutamine in a humidified incubator at 37°C with 5% CO2.

Sub-Library preparation

The chemical drugs from Prestwick Chemical Library® were transferred from 96-well plate to a 384-well plate using an integra pipetting robot. Each well contains 5ul of 100uM of drugs. Each 384-well plate will have DMSO in column12 and Stavudine in column 24 as a positive control and negative controls accordingly.

Primary screening of hits

$5x10^4$ of MDA-MB-231 and RPE1 were seeded in a 1:1 ratio in each well of a PerkinElmer 386 well plate. DMSO was added to an entire column, and Stavudine was added at 10µM in a separate entire column to serve as controls. Drugs were added to each well, excluding the control columns, utilizing Integra Assist. After incubation at 37°C, with 5% CO_2 for 48 hr, the plates were analyzed under the Opera Phenix.

Secondary screening of hits

$5x10^4$ of MDA-MB-231 and RPE1 were seeded in a 1:1 ratio in each well of a PerkinElmer 386 well plate. The plate was divided into six 8 x 8 regions to group drugs based on similar mechanisms. Drugs started at 10 µM, followed by eight successive

serial dilutions utilizing Integra Assist. After incubation at 37°C, with 5% CO_2 for 24 hr, the plates were analyzed under the Opera Phenix.

Live cell imaging

MDA-MB-231 and RPE1 cells were seeded at a 1:1 ratio on glass-bottomed dishes (Mattek, Ashland, MA) in 150 μL complete DMEM. Live microscopy on the Opera Phenix (PerkinElmer) using a 20X air objective was performed in an incubation chamber at 37°C, with 5% CO_2. Images were acquired every 20 minutes over 24 hours.

Dose response assay

RPE1 cells were seeded in a 12-well plate at a density of $5x10^4$ cells/mL and incubated for 24 h. The culture medium was then replaced with fresh DMEM containing serial dilutions of Clofarabine (0, 0.125, 0.25, 0.5, 1, and 2 μM). The cells were incubated for 24 h at 37°C in a CO2 incubator. After 24 h, the DMEM was removed, and MDA-MB-231 cells were seeded in a 12-well plate at a density of 50,000 cells/well and incubated for 48 h. After 48 h of incubation, the cells were digested with trypsin/EDTA and washed with PBS. The cells were then resuspended in DMEM and analyzed using a flow cytometer (BD Accuri C6, BD Biosciences). The following was repeated for pretreatment of MDA-MB-231 with Clofarabine.

Immunofluorescence Staining

Primary antibodies against protein of interest, ROCK1 (ProteinTech, Rosemont, IL, USA), and secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse IgG, were purchased from Thermo Fisher Scientific. For drug treatment, the cells were

seeded in a 6-well plate at a density of 2.5 x 10^5 cells per well and treated with 1 μM of either Actinomycin D, Clofarabine, or DMSO for 24 h. The cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. The cells were then incubated with the primary antibodies against ROCK1 (1:500 dilution) overnight at 4°C. After washing with PBS, the cells were incubated with the secondary antibodies, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000 dilution), for 1 h at room temperature in the dark.

The stained cells were imaged using the Opera Phenix high-content screening system equipped with a 20x objective. Four fields per well were imaged. The acquired images were analyzed using the Harmony software. The nuclei and cytoplasmic regions were segmented, and the fluorescence intensity of ROCK1 were quantified.

Statistical Analysis

Statistical analysis of MDA231:RPE1 ratios was done through unpaired t-tests to calculate *p*-values. These data were analyzed in GraphPad Prism using one-way ANOVA plus Post Hoc analysis. Other calculations, including average, SD, and P values, were performed using GraphPad Prism software (GraphPad Software, Inc.).

Results

I. High-throughput screening reveals tangocytosis inhibitors

To prevent the direct interaction between breast cancer cells MDA-MB-231 and RPE1, current FDA-approved drugs were repurposed to see they could potentially block this process. Since direct cell-cell interaction is required for intercellular gene transfer, tangocytosis inhibitors were identified by screening inhibitors for intercellular marker gene transfer. However, the possibility that the drugs allowed cell-cell interaction but blocked the subsequent process for the gene transfer cannot be fully excluded. To identify small molecule inhibitors of tangocytosis, a high throughput screening approach was utilized, wherein 1520 FDA-approved cancer drugs were screened using the PerkinElmer Opera Phenix microscope with high content image-based analysis. For the screening process, RPE1 and MDA-MB-231 were seeded in a 386 well plate, with DMSO used as a negative control and Stavudine as a positive control. Stavudine, a reverse transcriptase inhibitor, had previously been shown to decrease the amount of gene transfer within the co-culture with an IC50 value of 1.83µM. Using these controls, individual drugs were added at a concentration of 10µM to each well to undergo high throughput screening.

To ensure robustness of the high throughput screening, the Z’ factor was used as a statistical measures to confirm that the assay was reliable and accurate. The Z' factor provides a measure of the separation between the positive and negative controls in an assay. Its value ranges from -1 to 1, with a value of 1 indicating a perfect separation between the positive and negative controls, and a value of 0 indicating no separation. Z'

factor of 0.5 or higher indicates that there is a clear separation between the positive and negative controls in the assay. A protocol was generated to identify the number of MDA-MB-231 nuclei within RPE1, which revealed the ratio of double-positive cells within a well. The ratio of MDA-MB-231 nuclei within RPE1 under the positive control of Stavudine and the negative control of DMSO was used to generate the Z' factor for the screening assay. The preliminary screening revealed a Z' factor of 0.52, indicating reliability and robustness of the assay (Fig. 4).

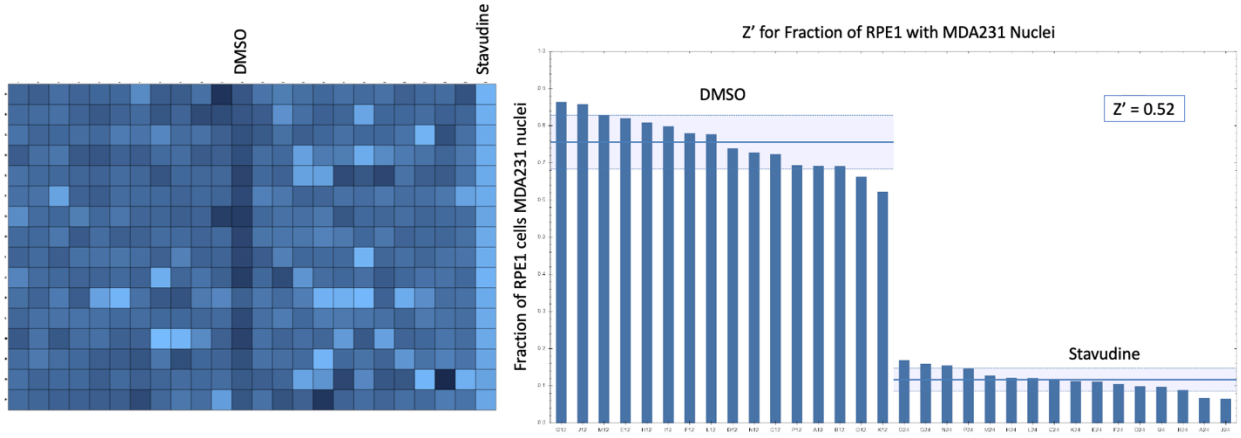

Figure 4: Preliminary small molecule inhibitor screening through using automated high-content screening platform of Opera Phenix. 5x10⁴ RPE1 and MDA-MB-231 were co-cultured for 48 hours followed by addition of drugs. Z' of 0.52 determined through using DMSO as the negative control and Stavudine as the positive control from the screening assay.

The fraction of RPE1 cells containing MDA-MB-231 nuclei in the remaining wells was normalized to the negative control, DMSO. Compounds that inhibited gene transfer equal to or greater than 3 standard deviations were identified as hits and selected for further analysis (Fig. 5). Hits that exceeded a value of 1.5 enhanced gene transfer, while those below 0.5 decreased gene transfer. Compounds that fell below the ratio of 0.5 were examined using cell imaging of each well to verify that the drug blocked gene transfer while maintaining a healthy cell population. Any hits that caused a reduction in

cell numbers or significant changes to cell morphology due to cytotoxic effects were excluded. It was observed that some hits provided a false positive reading, as the absence of gene transfer was a result of a decrease in the number of cells in the well rather than an effective blockage of cell-cell interactions.

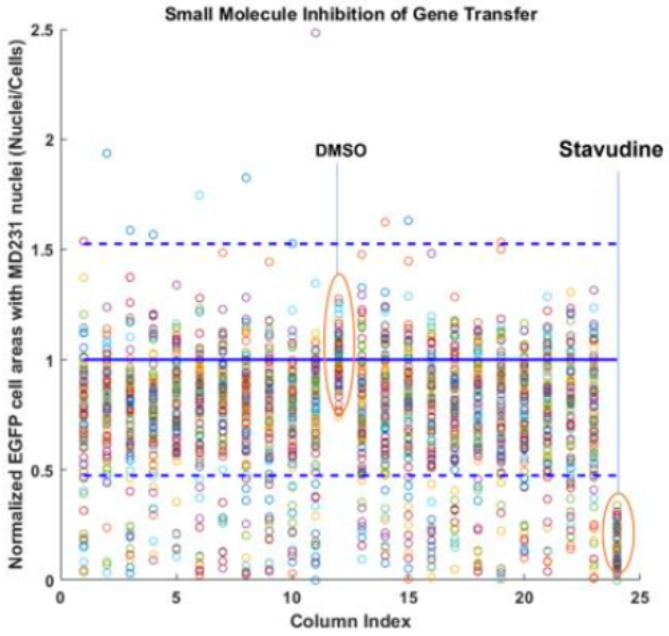

Figure 5: Data points indicate the fraction of RPE1 cells with MDA231 nuclei in a single well. Hits that are equal to or below 3 standard deviations of the normalized DMSO control were of interest for the secondary screening.

Drugs that effectively blocked tangocytosis from the primary screening were then subjected to a dose response assay, which started at a concentration of 20μM to assess their potency and cytotoxicity across a range of concentrations. The drugs that successfully inhibited tangocytosis were classified into five major groups based on their mechanisms of action: virology, MAPK signaling, tyrosine kinase, metabolism, and DNA damage repair (Fig. 6). The majority of the inhibitors belonged to the DNA damage repair group, suggesting that the mechanism of gene transfer is closely linked to the DNA damage repair pathway. Among the DNA damage repair inhibitors, Clofarabine,

Teniposide, and Raltitrexed were found to effectively block gene transfer without causing any cytotoxicity to the cells.

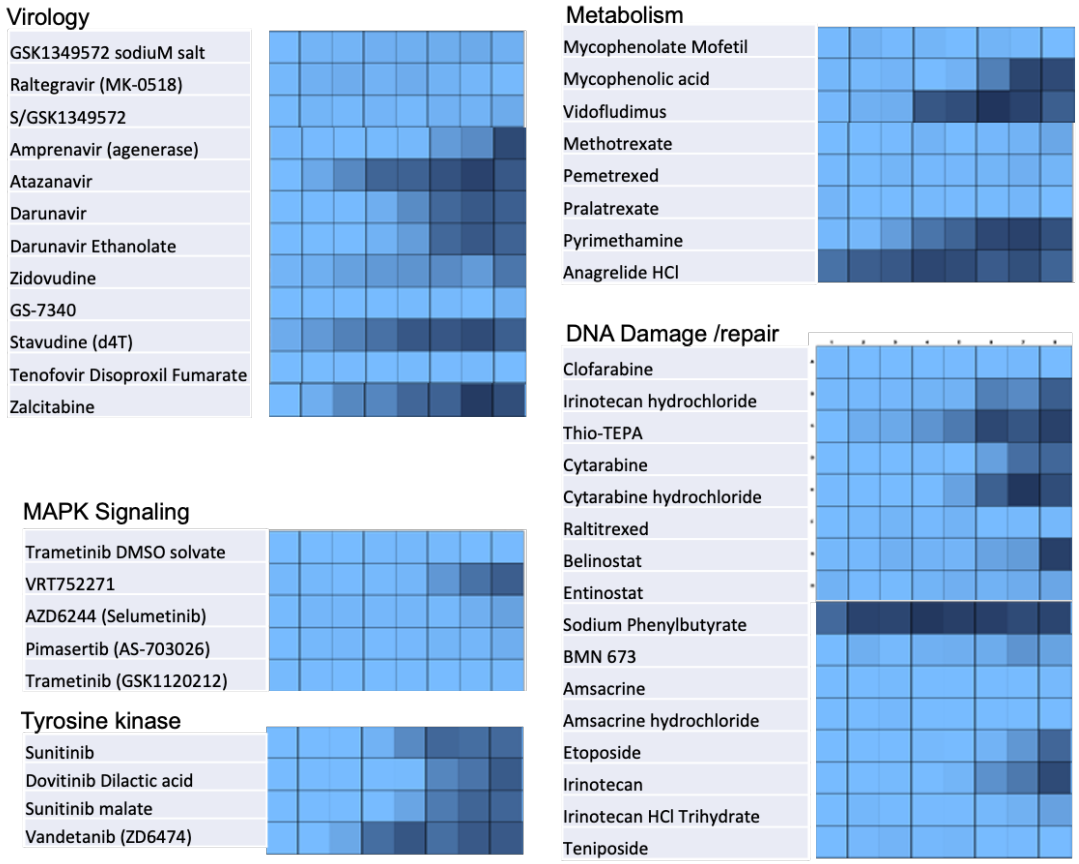

Figure 6: Serial dilutions of each hit from the primary screening. Selected drugs were added at 10uM followed by 8 successive dilutions. Darker boxes indicate a higher number of MDA-MB-231 inside of RPE1 cells

After the initial dose response, all three drugs demonstrated inhibition of gene transfer at all concentrations tested. To determine the concentration at which 50% of gene transfer is inhibited, a secondary dose response was conducted, starting at a lower concentration of 10μM. However, Raltitrexed did not exhibit a logarithmic inhibition of gene transfer with the serial dilutions, which led to its exclusion from further analysis. The EC50 values of Clofarabine and Teniposide were found to be 0.03518uM and 0.0806uM, respectively (Fig. 7).

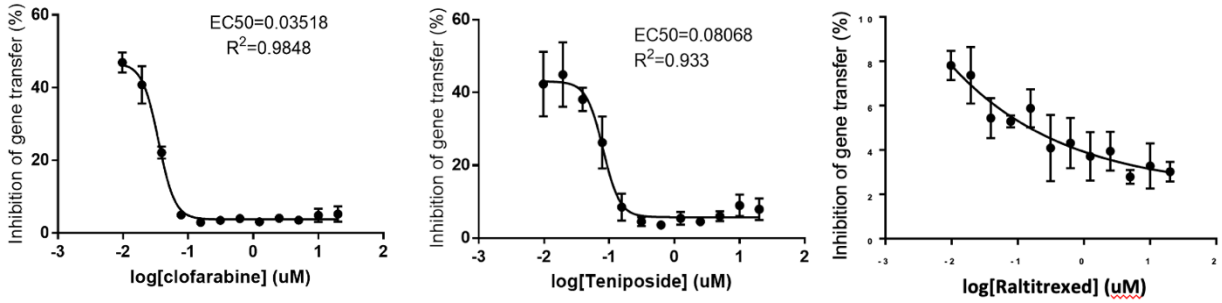

Figure 7: Dose response assay generated from incubation of drugs with 5x10⁴ of RPE1 and MDA231 in a co-culture. The drugs were added at a starting concentration of 20uM, followed by 12 successive dilutions.

To investigate potential differences in drug response between MDA-MB-231 and RPE1 cells, a pretreatment assay was conducted using the EC50 values of Clofarabine and Teniposide. MDA-MB-231, the gene transfer donor cells, were treated with the drugs for 24 hours prior to co-culturing with recipient RPE1 cells for an additional 24 hours. The final co-culture was analyzed by flow cytometry to determine the percentages of RPE1, MDA-MB-231, and gene transfer cells. The donor-to-recipient cell ratio was calculated as the number of donor cells (Q3 + Q4) divided by the number of recipient cells (Q1 + Q2) (Fig. 8).

The results showed that gene transfer decreased logarithmically when the recipient cells were treated with Clofarabine for 24 hours before co-culture (Fig. 9a), while gene transfer increased logarithmically when the donor cells were pretreated with Clofarabine for 24 hours before co-culture (Fig. 9b). Teniposide and Pemetrexed were also subjected to the same pretreatment assay.

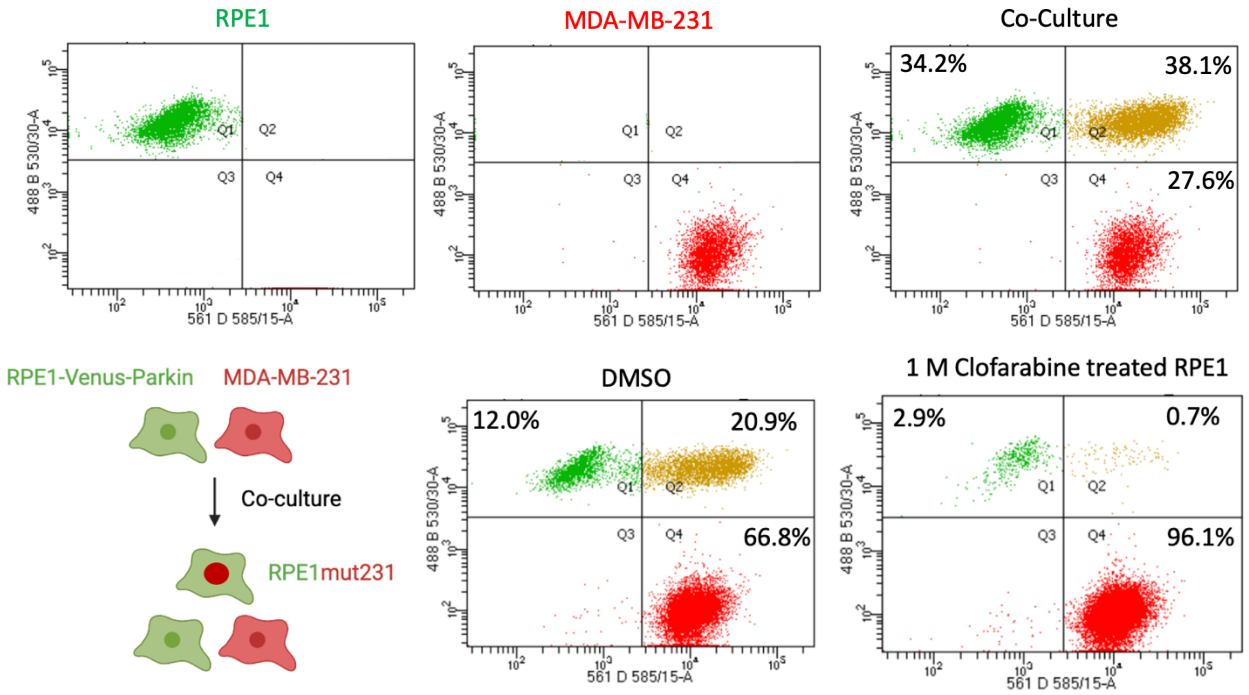

Figure 8: Schematic of the coculture experiment and flow cytometric analysis of RPE1-Venus-Parkin, MDA-MB-231-H2B-mCherry, and recipient cells showing both Venus-Parkin and H2B-mCherry signals with DMSO compared to Clofarabine

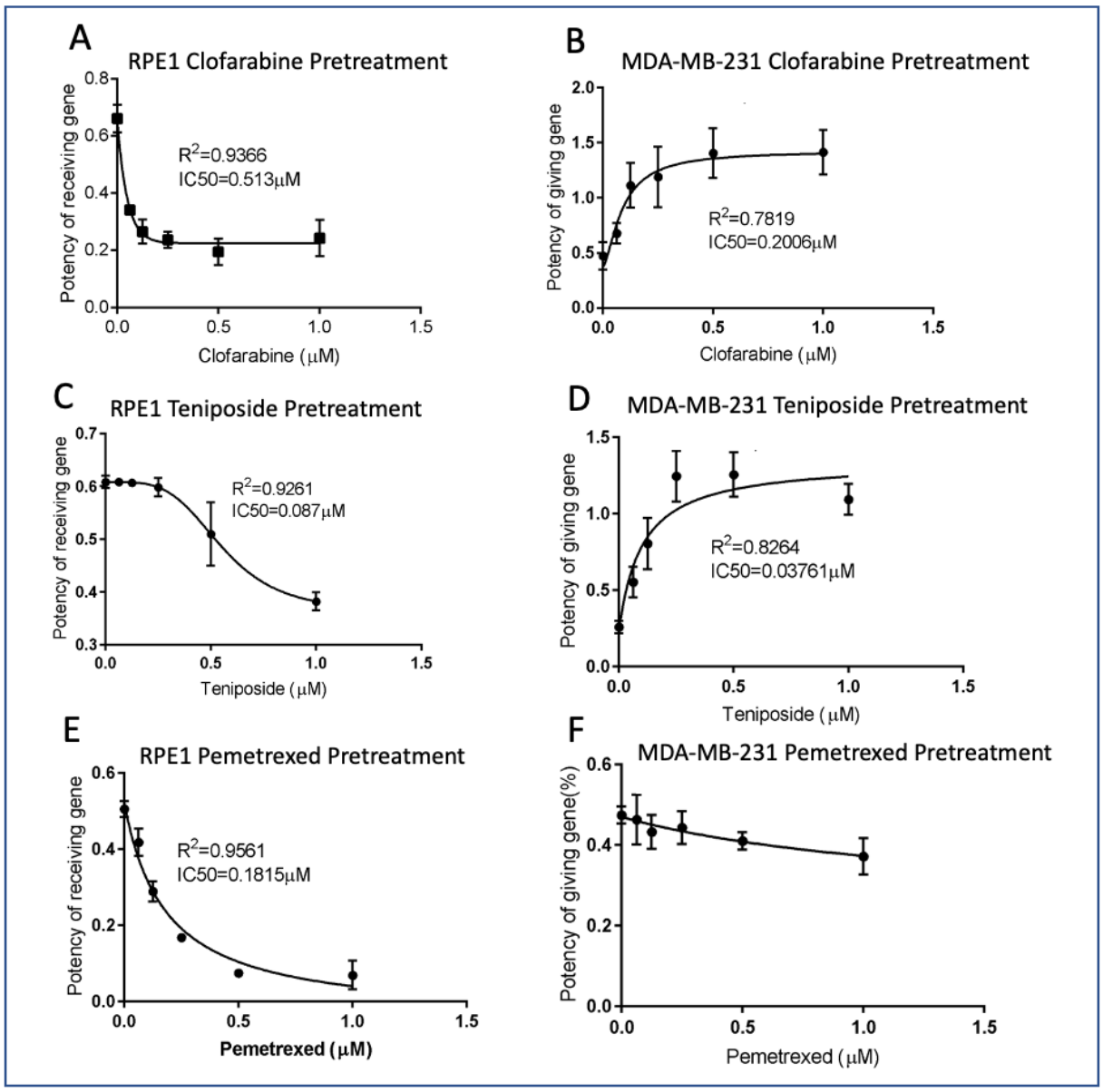

*Figure 9: **a** RPE1 pretreated with Clofarabine for 24 h followed by MDA-MB-231 co-culture for 48 h **b** MDA-MB-231 pretreated with Clofarabine for 24 h followed by RPE1 co-culture for 48 h **c** RPE1 pretreated with Teniposide for 24 h followed by MDA-MB-231 co-culture for 48 h **d** MDA-MB-231 pretreated with Teniposide for 24 h followed by RPE1 co-culture for 48 h*

II. Actinomycin D allows cell entrapment but prevents subsequent gene transfer

During the initial screening, a hit was discovered that significantly increased gene transfer. However, further manual imaging analysis revealed an intriguing phenomenon: Actinomycin D could lock the RPE1/MDA cell-in-cell structure for more than 48 hours. This resulted in RPE1 cells maintaining a distinct MDA-MB-231 entrapment area (Fig. 10). To better comprehend this process, a co-culture of MDA-MB-231 and RPE1 cells were treated with varying lengths of time of 10uM Actinomycin D to optimize the conditions that increased the number of cell-in-cell structures. Cells were visualized under the Opera Phenix for 24 hours, with time points captured every 20 minutes after treatment for 12 or 24 hours, or upon imaging. In all treatment groups, the number of cell-in-cell structures increased throughout the 24-hour imaging period, with the most significant increase observed when Actinomycin D was added at the beginning of the co-culture (Fig. 11). To determine if tangocytosis could occur in the absence of any drugs, RPE1 and MDA-MB-231 cells were co-cultured in a 1:1 ratio and imaged for 24 hours. Live cell imaging revealed the clear entrance and exit of MDA-MB-231 cells into RPE1 cells without dismantling their respective membranes (Fig. 12). The Actinomycin D result confirmed out hypothesis that multiple steps are involved in intercellular marker gene transfer between MDA-MB-231 and RPE1 cells.

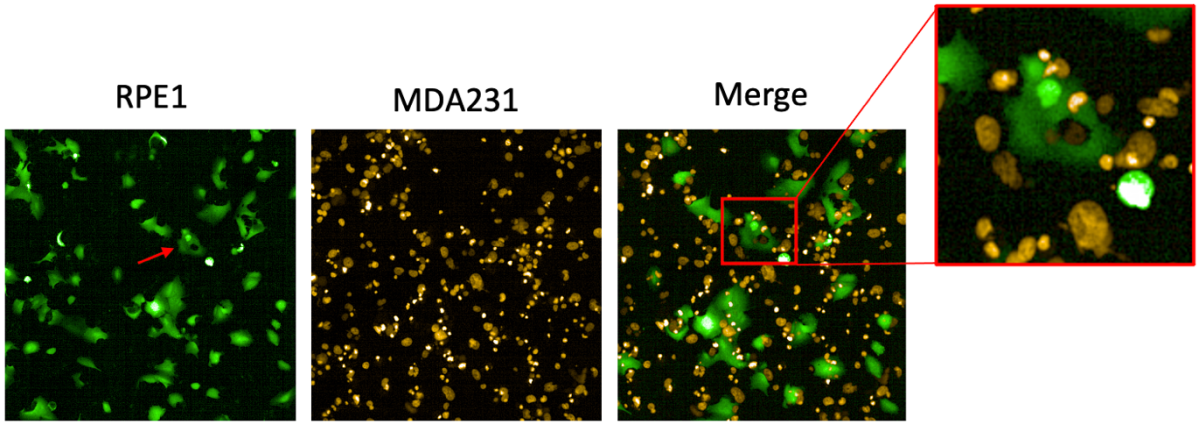

Figure 8: Live cell imaging taken of cell entrapment between RPE1 and MDA-MB-231-H2B-mCherry with the addition on 1uM Actinomycin D.

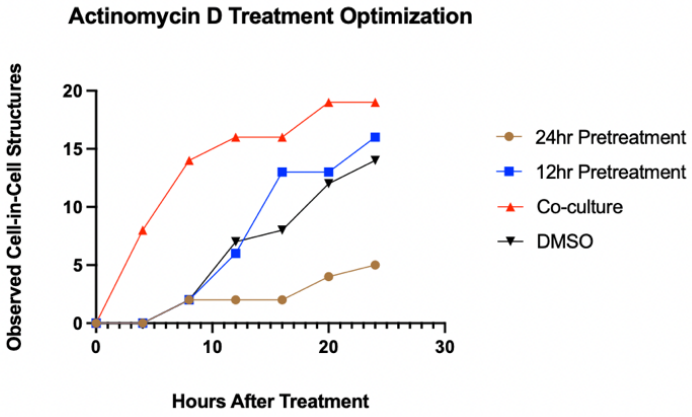

Figure 9: The number of cell-in-cell structures were recorded every 4 hours during live cell imaging of RPE1 and MDA-MB-231 treatment of 1uM Actinomycin treatment.

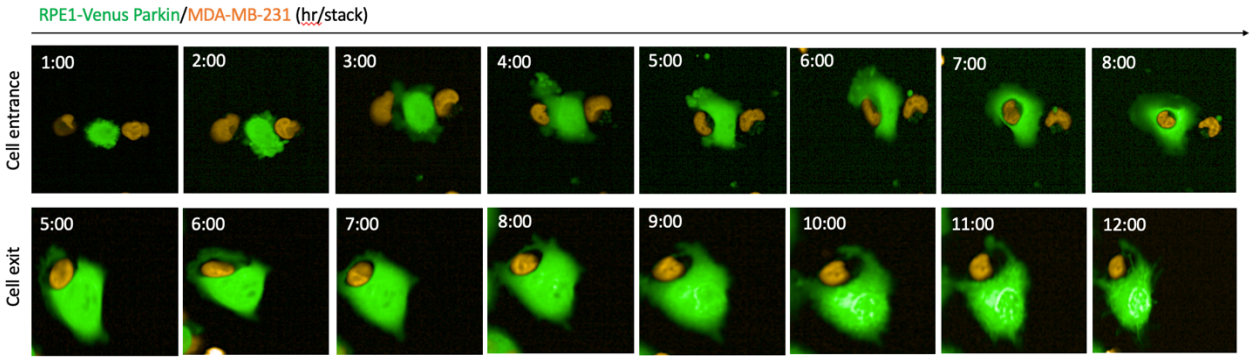

Figure 10: Live cell imaging entrapment of RPE1-Venus-Parkin and MDA-MB-231-H2B-mCherry. The upper panel shows engulfment of the donor cell by the recipient over eight hours; the bottom panel shows the exit of the donor cell from the recipient over eight hours.

III. DNA damage repair drugs block intracellular gene transfer

To investigate whether a single MDA-MB-231 cell can transfer its genes to multiple RPE1 recipient cells, a co-culture experiment was conducted using increasing ratios of RPE1 and a control of 1:1 co-culture without Clofarabine. If MDA-MB-231 can transfer its genes to multiple RPE1 cells simultaneously, there would be an increase in the percentage of double-positive cells. The potency of the giving gene was calculated by the number of donor cells (Q3 + Q4) divided by the number of recipient cells (Q1 + Q2). Both the percentage and the individual counts showed that the increased ratios of RPE1 did not result in an increase in the number of double-positive cells, indicating that MDA-MB-231 does not engage in multiple interactions with different RPE1 cells to transfer its genetic material. (Fig. 13).

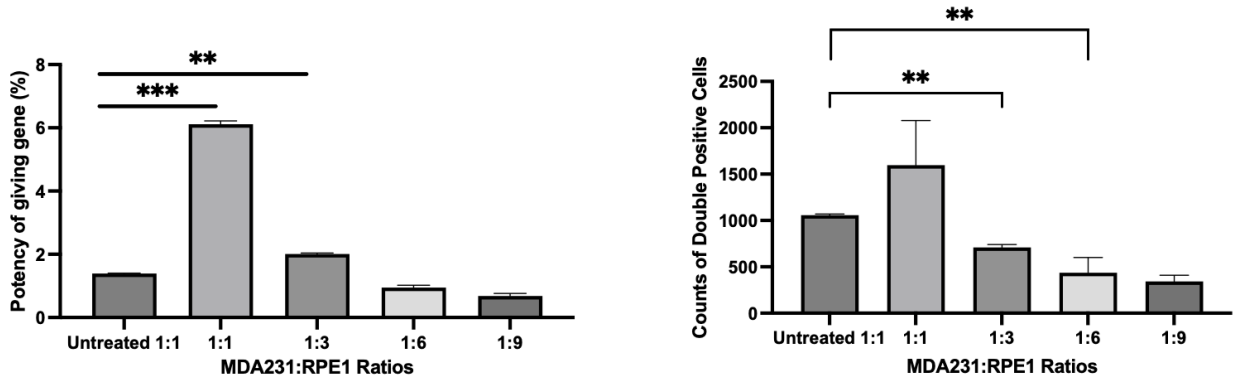

Figure 11: MDA-MB-231 co-cultured with increasing ratios of RPE1 over 24 hours followed by flow cytometry analysis assessing double positive cells.

Live cell imaging over a time course of 16 hours was conducted on a co-culture treated individually with Clofarabine, Teniposide, Pemetrexed, and Stavudine. Selected timepoints of the treatment show that MDA-MB-231 and RPE1 engage in both intracellular and paracellular interactions throughout all treatments as MDA-MB-231 cells can be seen sliding through two RPE1 cells. Opera Phenix software was utilized to

visualize the membranes of the cells to ensure that MDA-MB-231 cells were slipping between two cells and not creating the cell-in-cell structure. With the exception of Stavudine, cell-in-cell structures were not observed in the other drug treatments. Although there a few cells with gene transfer are present in Clofarabine, Teniposide, and Pemetrexed, it is reduced compared to the control DMSO.

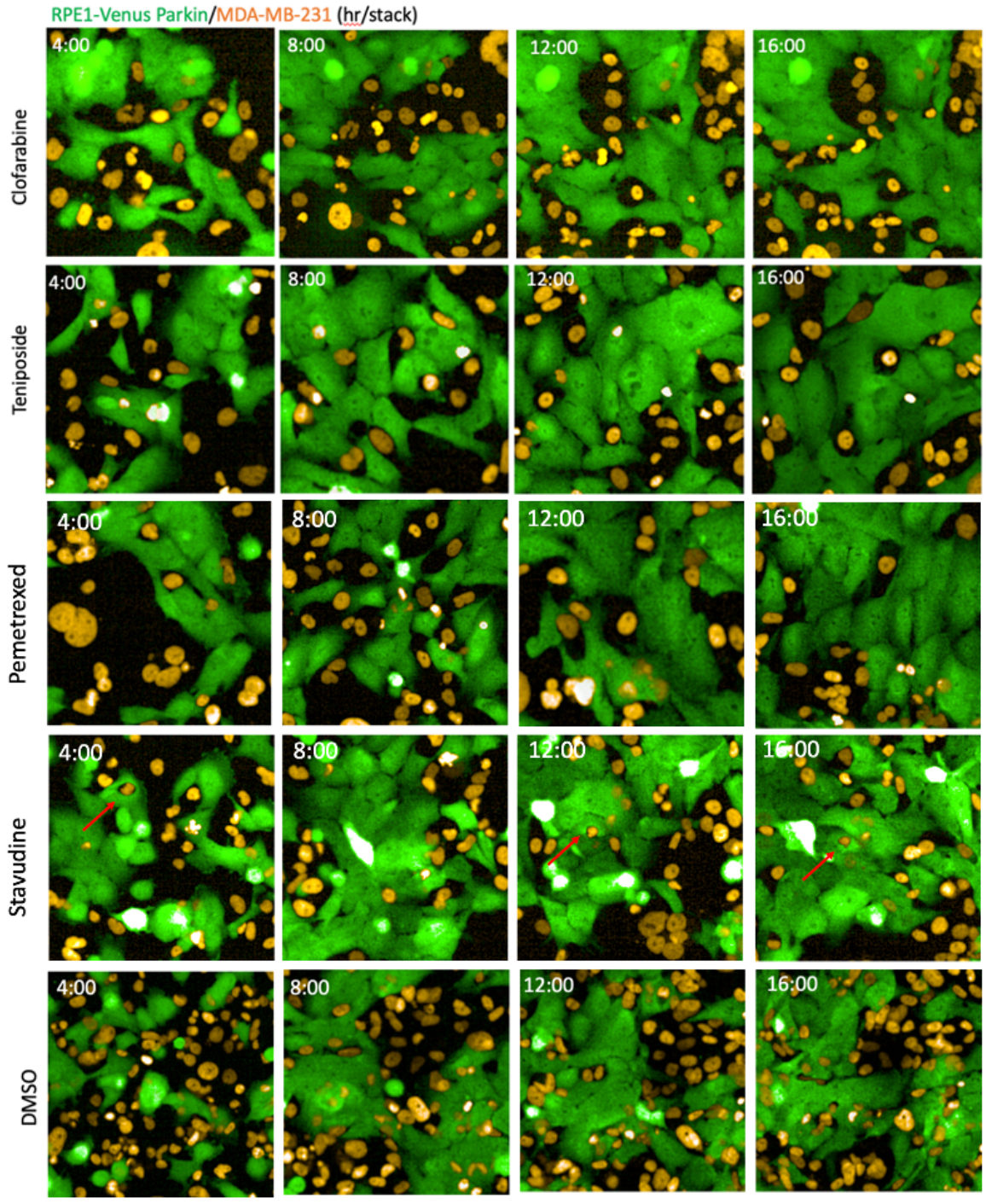

Figure 12: Live cell imaging of 5x10^4 cell/mL MDA-MB-231 and RPE1 co-culture over 16 hours with 1μM concentrations of drugs

The lab previously demonstrated a polarizing effect of ROCK1 kinase on donor and recipient cells[29]. To examine the impact of ROCK1 intensity levels on cell-in-cell

structures, immunofluorescence staining was performed on DMSO, 10μM Actinomycin D, and 1μM Clofarabine-treated cells (Fig. 15). H2B-mCherry was used as a marker for MDA-MB-231 nucleus to identify cells experiencing gene transfer, while Venus-Parkin was used as a marker to identify RPE1 cells. While the phosphorylated substrate level is not known, the ROCK1 intensity is highly correlated with its kinase activity. The intensity of the ROCK1 channel was measured in cell-in-cell structures of both RPE1 and MDA231 to assess its correlation with gene transfer. In MDA-MB-231, regions of cell-in-cell structures in the DMSO control and Actinomycin D-treated co-culture exhibited significantly higher ROCK1 intensities compared to regions without such structures. In RPE1, the polarization is not as distinct but is still present in the DMSO treatment where cell-in-cell regions exhibit higher ROCK1 intensities. For both MDA-MB-231 and RPE1, in the Clofarabine-treated co-culture, the difference disappeared as both regions displayed similar ROCK1 intensities (Fig. 16).

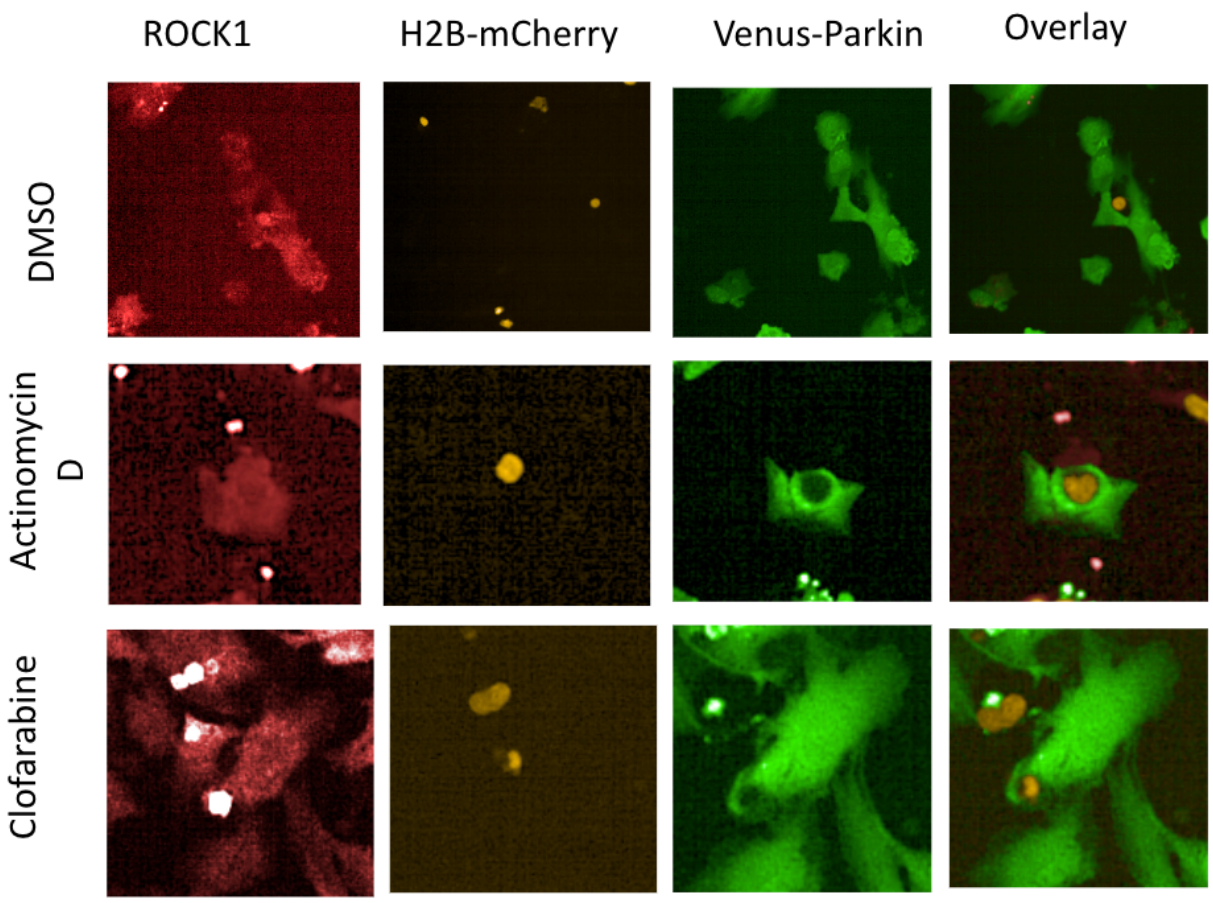

Figure 13: Immunofluorescence staining of RPE1 and MDA-MB-231 co-culture with ROCK1 primary antibody.

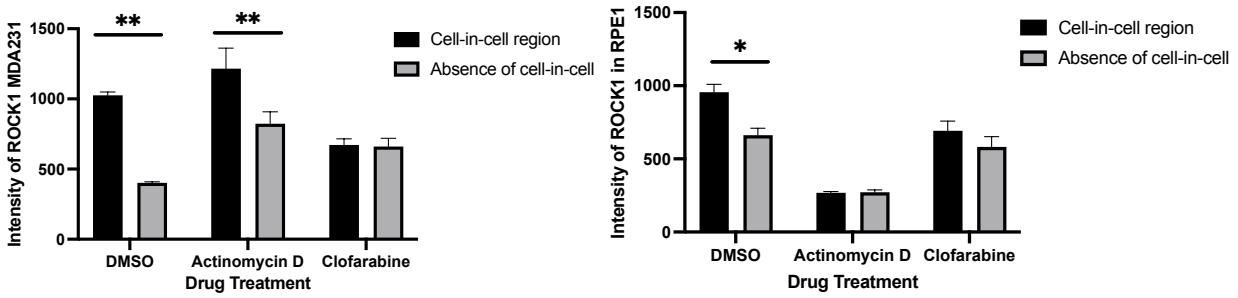

Figure 14: The ROCK1 intensity was obtained through the Alexa 647 channel at regions of cell-in-cell structure and at the absence for each treatment.

Discussion

I. Polarization of gene transfer between donor and recipient cells

The pretreatment assay exhibited a polarizing effect of Clofarabine and Teniposide on the recipient and donor cells. When MDA231 cells were treated with the drug first, gene transfer increased, yet RPE1 pretreatment yielded a decrease of gene transfer. The overall decrease of gene transfer that occurs in a co-culture with the added drug indicates that RPE1 has a greater effect on the inhibition of gene transfer which overrides the enhancing features of MDA-MB-231.

A similar polarizing phenomenon is exhibited in entosis, an elimination process whereby one cell engulfs another cell. This process is known to be driven by the RhoA-ROCK1 signaling pathway, which polarizes the ROCK1 activity between the engulfing and engulfed cells.[30] One study concluded that inhibition of ROCK by Y-27632, a specific inhibitor of ROCK, decreased the frequency of entotic cell death in breast cancer cells. The study also demonstrated that ROCK inhibition did not affect other forms of cell death, such as apoptosis, suggesting that entosis is a distinct cell death process that is regulated by Rho-ROCK signaling.[31] The different ROCK1 levels in recipient and donor cells before engulfment indicate that polarization is an essential step in mediating the cell engulfment. The Liu lab has previously shown that during engulfment the ROCK1 levels decreased in MDA-MB-231 while it increased in RPE1. Although cell-in-cell structures may produce polarizing effects, the absence of the polarizing effect in ROCK1 intensity in the Clofarabine treatment of the immunofluorescence staining shows that gene transfer is not likely mediated through

ROCK1 levels but through a different protein. It would be interesting to further investigate the genes that are upregulated or downregulated in the cells to observe what processes may be responsible for tangocytosis.

II. DNA damage drugs inhibit cell-in-cell interactions

Tangocytosis involves both the formation of a cell-in-cell structure along with gene transfer from the donor cell to the recipient cell. The live cell imaging paired with flow cytometry results of Actinomycin D show that formation cell-in-cell structures do not immediately indicate gene transfer. Although Actinomycin D increases the number of cell-in-cell structures, the flow cytometry data at the same concentration indicates high gene transfer. However, the gene transfer is elevated due to MDA-MB-231 and RPE1 creating aggregates within each other rather than true gene transfer occurring. When cells were visualized through live cell imaging, the number of cells with the MDA231 nuclei embedded within it did not correlate to the high percentage of gene transfer that flow cytometry analysis showed.

The live cell imaging of the selected hits show that the DNA damage repair drugs allow for cell-cell interaction as MDA-MB-231 cells are seen moving both transcellularly and paracellularly through the RPE1 cells. However, the co-culture exhibits a very low amount of cell-in-cell formation structures. At 1μM concentration, Clofarabine and Teniposide have a gene transfer percentage of 4% within a co-culture. Likewise, the live cell images do not show cell-in-cell structures, indicating that these structures may be responsible for gene transfer. However, presence of gene transfer does not always

indicate that a cell-in-cell structure formed beforehand, indicating that the pathway to gene transfer is not fully understood.

III. Proposed Mechanism of Gene Transfer

From the identification of inhibitors, it is likely that the integration of the tumor cell's RNA into the host cell is essential in gene transfer. When the tumor cell mRNA enters the cell, it would have to be transcribed into DNA. Stavudine likely inhibited gene transfer due to prevention of endogenous reverse transcriptase activity. Following this, the DNA would have to be incorporated into the host genome where dNTPs and topoisomerase would play a crucial role in repairing and reannealing the DNA breaks. Clofarabine is a nucleoside analogue that blocks the activity of the enzyme ribonucleotide reductase, which is essential for the synthesis of deoxyribonucleotides, the building blocks of DNA.[32] In the tumor DNA integration, dNTP pools would have to be accessed in ligating the broken ends of the host's genome to insert the tumor DNA. Along with this, topoisomerase would have to relieve torsional strain that forms ahead of the breakage points. To do this, topoisomerase makes double stranded and single stranded breaks within the DNA and seals them after the DNA has been unwound. However, Teniposide was likely a potent gene transfer inhibitor because it blocked topoisomerase from resealing the DNA breaks, leading to the accumulation of DNA breaks that eventually cause cell death.[33]

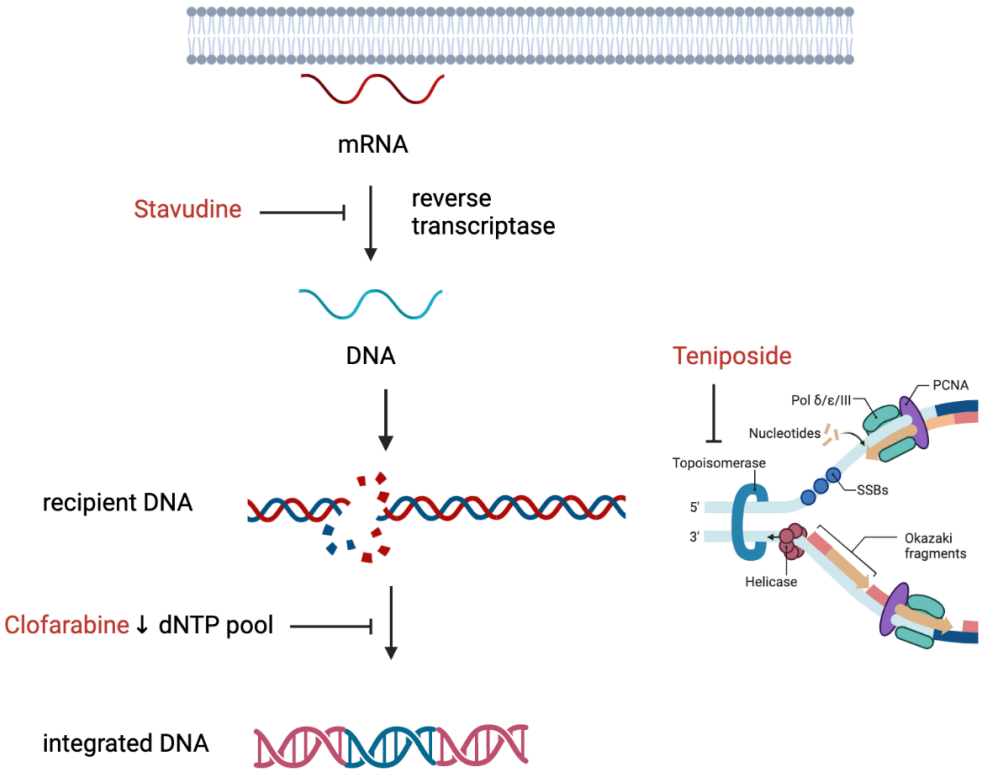

Figure 15: Schematic of the potential pathway that leads to the gene transfer from the identification of tangocytosis inhibitors.

IV. Future Directions

DNA damage repair drugs, Clofarabine and Teniposide, present as potent blockers of tangocytosis with minimal cytotoxic effects. Along with these drugs, multiple hits of the high throughput screening were related to the DNA damage repair pathway, indicating this pathway may reveal the mechanisms that gene transfer occurs through. To elucidate the pathway involved in tangocytosis, further experiments related to the DNA damage repair pathway can be conducted. γH2AX is a histone protein variant that is phosphorylated at a specific site in response to DNA double-strand breaks, and measuring γH2AX levels is a widely used technique to assess DNA damage[34]. γH2AX can be used to assess if low concentrations of Clofarabine and Teniposide can trigger

the DNA damage response. After co-culturing RPE1 and MDA-MB-231, the presence of γH2AX can be determined through both immunofluorescence staining and Western blot.

Additionally, CRISPR can be utilized to induce double-stranded DNA breaks within the donor and recipient cell to observe if triggering the DNA damage pathway would block gene transfer. If DNA damage is indeed responsible for the pathway, the breaks should prevent gene transfer from taking place. Additionally, a siRNA can be designed to deplete the targets of Clofarabine and Teniposide, which would help determine if the targets of the DNA damage repair pathway are essential to blocking gene transfer.

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