Variable Response to Chemotherapeutics by a Subpopulation of MCF-7 Breast Cancer Cells

JULIE MARIE DORLAND

BIOMEDICAL ENGINEERING AND PLAN II HONORS

MAY 17, 2016

ENGINEERING HONORS UNDERGRADUATE THESIS

DEPARTMENT OF BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS AT AUSTIN

AUSTIN, TX 78712

DR. AMY BROCK, PH.D.

DEPARTMENT OF BIOMEDICAL ENGINEERING

FACULTY SUPERVISOR

DR. JANET ZOLDAN, PH.D.

DEPARTMENT OF BIOMEDICAL ENGINEERING

SECOND READER

Table of Contents

List of Figures………………………………………………………………………………..…ii

Abstract………………………………………………………………………………………1

Introduction……………………………………………………………………………………..2

Materials and Methods…………………………………………………………………………8

Cell Culture…………………………………………………………………………..…8

Doxorubicin Treatment………………………………………………………………8

Spiky Cell Induction……………………………………………………………....8

Spiky Cell Characterization……………………………………………….....9

Cell Imaging……………………………………………………………………….....11

Quantification of Proliferation……………………………………………………...11

Invasion Assay…………………………………………………………………….....11

Staining Cells……………………………………………………………………………13

Dose Response Assay……………………………………………………………………15

Results…………………………………………………………………………………………17

Treatment of Cells with Doxorubicin and Imaging Phenotypes During Recovery from Doxorubicin Treatment……………………………………………………………………..17

Proliferation During Recovery from Doxorubicin Treatment…………………………...20

Drug Sensitivity During Recovery from Doxorubicin Treatment………………………22

Invasive Potential of Cells Following Doxorubicin Treatment…………………………25

Staining for Biomarkers Labelling Resistant Cells……………………………………....28

Discussion and Conclusion……………………………………………………………………30

Acknowledgements……………………………………………………………………………37

References……………………………………………………………………………………..38

List of Figures

Figure 1. Schematic of Experimental Design……………………………………………………10

Figure 2. Quantification of Morphology Changes Arising from Doxorubicin Treatment………19

Figure 3. Proliferation During Recovery from Doxorubicin Treatment…………………………21

Figure 4. Drug Sensitivity During Recovery from Doxorubicin Treatment……………………..24

Figure 5. Invasive Potential During Recovery from Doxorubicin Treatment……………….......27

Figure 6. Immunocytochemistry Staining for E-Cadherin……………………………………....29

Abstract

Several different morphologies can be observed in a population of MCF-7 breast cancer cells, but the typical epithelial morphology vastly predominates. After treatment with the chemotherapeutic doxorubicin, the proportion of abnormal morphologies greatly increases. Over the course of recovery from this drug treatment, the population returns back to high proportions of the normal morphologies. However, previous studies in the Brock lab have shown that in rare instances after recovery from doxorubicin treatment, a phenotype termed the "spiky cells" became stable at higher proportions. This population of spiky cells appeared to have characteristics associated with drug-resistant and metastatic cell populations. In this thesis, I tested the aggressive tumor characteristics of this stable spiky subpopulation and whether it responds differently to doxorubicin. I hypothesized that the growth rate, invasiveness, and drug sensitivity of this population would more closely resemble aggressive tumor cells compared with the naïve MCF-7 population. First, I repeated the induction of the spiky subpopulation with a larger sample size, to determine how frequently this spiky subpopulation occurred. Second, I characterized this subpopulation by comparing four groups: untreated controls of both the normal MCF-7 population and the stable spiky population as well as groups from both populations treated with the same concentration of doxorubicin. I performed these comparisons over the 8-week course of recovery from the treatment. These tests included proliferation rate calculations, invasion assays, staining for a critical protein component of cell-cell junctions, and drug sensitivity tests. We found that the spiky subpopulation appeared more aggressive and resistant, with higher invasion and proliferation, fewer surface markers related to cell connections, and decreased drug sensitivity.

Introduction

Breast cancer is a difficult disease to treat. Of patients with early-stage breast cancer, approximately 30% have recurrent disease, and this is often metastatic.[1] The majority of patients receive surgery to remove the primary tumor, and many patients receive a neoadjuvant therapy before surgery to reduce the size of the tumor or an adjuvant therapy after surgery to remove remaining tumor cells.[2] These additional therapies often include cytotoxic chemotherapeutics such as anthracycline drugs.[1] Even patients with a good prognosis may still have an early recurrence of breast cancer. This is due to cancer cell resistance to the chemotherapy drugs, and it has been found that individual cell characteristics create this resistance.[1]

Anthracycline drugs, such as doxorubicin, inhibit DNA replication by intercalating into the DNA.[3] This prevents the synthesis of cellular proteins, leading to cell death. Doxorubicin also prevents the process of DNA repair by ligation of broken strands and inhibits topoisomerase II, making it easier for DNA to be cleaved.[4,5] Doxorubicin is often used, by itself or in addition to other drugs, to treat several forms of cancer, including breast cancer.[6]

For many years, it has been the predominant view in cancer research that tumors are formed by cancer cells that acquire a favorable mutation.[7] This population would then expand clonally and acquire genetic and epigenetic changes to increase its malignancy. However, it has been shown that even cells that are genetically identical can exhibit heterogeneity in their protein expression, and cancer researchers have more recently argued that non-genetic variability could be selected for in cancer cell evolution rather than genetic variability.[7] In order for this non-genetic variability to be selected for by Darwinian evolution, this individuality must be passed on to daughter cells. The heterogeneity of these cancer cells causes them to respond differently to the tumor environment.[7] This heterogeneity can be explained by gene regulatory networks,

which regulate a cell's gene expression. Different cell states are defined by "discrete, stable, self-organizing gene expression patterns," and the stable states, termed "attractor states," are analogous to equilibrium states of minimal energy. Each attractor state corresponds to a gene expression profile, and the genetic network has a large number of these stable attractor states with varying gene expression. Sub-attractor states also exist around a central attractor state, and these sub-attractor states can cause gene expression heterogeneity by trapping a cell on its way to the central attractor state. Heterogeneity in gene expression can manifest in differences in observed phenotype. For example, it has been found that individual cancer cells from the same clonal tumor vary in their malignancy.[7] Because this heterogeneity is dynamic, it could potentially be explained by gene regulatory networks.[7] Studies of melanoma have found that phenotypic heterogeneity in the tumor cells primarily arises from upregulation and downregulation of a range of markers.[8]

Drug resistance is a problem with any type of chemotherapy treatment, and there are two types of resistance: acquired resistance and intrinsic resistance.[9] In acquired resistance, the drug loses effectiveness over time, and in intrinsic resistance the drug is ineffective when the patient first receives it. Breast cancer patients often acquire resistance to chemotherapeutics, which reverses the previous success of their chemotherapeutic treatment.[9] In fact, many different cancers have been found to have this acquired resistance, where patients will respond well to a therapy but, after a break from the treatment, will then experience therapy failure the second time.[10] Studies have found that cancer cell resistance to chemotherapeutics can be due to genetic, epigenetic, or microenvironmental factors.[11] Some researchers have found that there are certain genes for which adjustment in expression can cause increased resistance, and this increased resistance is often related to intracellular pathways related to growth, survival, differentiation,

and apoptosis.[11] It is necessary to understand the mechanisms by which breast tumors become resistant to chemotherapeutics and what cellular changes accompany this resistance in order to combat the problem of resistance when treating breast cancer. This study aims to examine characteristics of tumor aggression, such as resistance, in subpopulations of MCF-7 breast cancer cells in order to understand the effect of doxorubicin on the cells.

Many researchers have argued that chemotherapeutic treatments kill off the majority of cancer cells, leaving a small population of cells which happened to have a resistance-conferring mutation and which then clonally divide to repopulate the tumor.[12] However, the idea of non-genetic heterogeneity calls this theory into question. It has been found that cancer cells shift between different attractor states, some of which are cancer stem-cell-like states, in which cells naturally are more resistant to drugs. The cancer cells can switch between these attractor states spontaneously or due to external perturbation. Spontaneous switching allows for Darwinian selection of cells that happen to be more resistant to chemotherapeutics due to non-genetic heterogeneity. Daughter cells can inherit these resistant cell states even though they are not due to genetic changes, and this allows for the expansion of a subpopulation of the cancer cells, which then increases the probability of genetic mutations. The other mechanism of switching between cell states is by external signals caused by a perturbation. In this method of resistance development, cells enter a resistant cell state due to the introduction of the chemotherapeutic. These non-genetic changes can also be inherited by daughter cells, and this is an example of Lamarckian evolution.[12]

Examples of these stable attractor states in cancer cells include the mesenchymal and epithelial developmental states.[12] Epithelial cells are typically adherent and connected to the basement membrane.[13] The MCF-7 human breast cancer cell line used in these experiments is an

epithelial cell line. Mesenchymal cells are cells that have become mobile and invasive and are more resistant to apoptosis.[13] It is possible for cells to switch between these states, and this is called epithelial to mesenchymal transition (EMT). EMT has been found to correlate with tumor progression, and both EMT and chemotherapeutic resistant cell states have been shown to be reversible transitions.[8]

Proliferation rate is a measure of how quickly the cells are dividing, and a high cancer cell proliferation rate is a mark of an aggressive breast tumor.[14] In vivo, different tumors have different proliferation responses to chemotherapeutics: in one study, some tumors rose in proliferation after treatment, some decreased, and some stayed the same.[15] In another patient study, it was found that cells that are drug-sensitive have repressed growth immediately after treatment, but weeks later, the growth increased again.[16] Studies on the effect of doxorubicin on MCF-7 cells found that at a concentration of 1 μM, cell growth was reduced by 75% when compared to untreated cells, and after 72 hours there were 40% fewer viable cells.[17] However, this study did not assess the long-term effects of the doxorubicin treatment on proliferation.

Researchers in the Brock lab found previously that after treating MCF-7 cells with doxorubicin, high proportions of abnormal morphologies arose in the surviving cell population. They hypothesized that these other morphologies represent different attractor states and the phenotypic changes are due to non-genetic heterogeneity. One of these abnormal phenotypes was termed the "spiky cells" (Figure 1). These cells have long, spindly projections. The typical MCF-7 morphology is epithelial, with cells forming close connections to their neighbors, but the spiky cells do not form as many connections. Normally, after treatment with doxorubicin the cells eventually return to the typical morphology proportions found in untreated cells: the vast majority of cells have the normal phenotype, with a small percentage of cells having the

abnormal morphologies. However, in rare cases, after treatment, rather than recovering back to normal proportions, the spiky cells become stable at higher-than-normal proportions.

In these studies, I compared four treatment groups (Figure 1): untreated controls of both a naïve MCF-7 population (termed untreated normal) and a population that had become stably spiky at around 30% spiky (termed stable spiky) as well as a group from each of these populations which was treated for 24 hours with 500 nM doxorubicin (treated normal and treated spiky). This dose was chosen because previous studies in the lab found that a 500 nM dose of doxorubicin caused higher proportions of morphology changes than did lower doses. Over the course of the cells' recovery from this doxorubicin treatment, I assessed four characteristics pertaining to tumor aggression. I compared the proliferation of the populations because more aggressive tumors proliferate more quickly. I tested dose response, which is a measure of how resistant the cells are to doxorubicin, and more aggressive tumors are more resistant to chemotherapeutics. I tested the invasive potential of the different populations because invasion into the surrounding extracellular matrix is a key aspect of cancer progression. And finally, I used immunocytochemistry to determine the amount and localization of the protein E-cadherin, a cell-cell junction marker related to invasion and mobility. Cells that have fewer cell-cell junctions are typically more mobile and invasive because they are not as tightly connected to the cells around them. In addition to these experiments, I also attempted to repeat the induction of the stable spiky phenotype population, in order to determine at what percentage the cell populations recover to high numbers of spiky cells.

Based on initial observations, we hypothesized that the stable spiky population would show more aggressive tumor characteristics than would the untreated normal MCF-7 population. In comparison with the naïve MCF-7 cells, we believed that the stable spiky cells would be more

proliferative, show increased doxorubicin resistance, have a higher invasive potential, and have fewer cell-cell junctions as demonstrated by lower E-cadherin expression. We expected that the doxorubicin treatment would also have an effect on these characteristics – we hypothesized that the treated groups would be less proliferative at the beginning of the recovery, more resistant, more invasive, and have lower E-cadherin expression than their untreated control.

Materials and Methods

Cell Culture

The MCF-7 human breast cancer cell line is commercially available. The MCF-7 culture media used in these experiments was composed of 89% Minimal Essential Media (MEM, Gibco Lot #1663071), 10% Fetal Bovine Serum (FBS, Gibco Lot #1708457), and 1% Penicillin-Streptomycin (Pen-Strep, Gibco Lot #1601706).

Media was changed every other day. Before replacing the media, the cells were washed with 1X Phosphate Buffered Saline, (PBS, HyClone Lot #AAE202584). Untreated cells were passaged using trypsin-EDTA (Gibco Ref #25300-054) once a week. Treated cells were passaged when they became approximately 80% confluent.

Doxorubicin Treatment

Doxorubicin is a chemotherapeutic, and its handling and disposal adhered to the guidelines set by the University Environmental Health and Safety Department.

Spiky Cell Induction: For the spiky cell induction experiment, we plated 100,000 normal, untreated, low-passage MCF-7 cells into each well of four 6-well tissue culture-treated plates. After allowing the cells to grow for two days, these cells were treated with a 500 nM dose of doxorubicin for 24 hours. The stock solution of 2048 nM doxorubicin was diluted in MCF-7 media, and 3 mL of this doxorubicin-media solution was added to each well. After 24 hours, cells were washed with PBS and further cultured in regular media. These plates were passaged once a week and supplied with fresh media twice a week. Each time they were passaged, three of

the plates were randomly selected to be counted, and these counts were averaged each week. After two weeks, eight of the flasks were left in the incubator and were no longer passaged or replenished with fresh media, and the 16 remaining flasks were still passaged and fed each week. Periodic images were taken each week of several flasks, and after 4 weeks each flask was imaged to assess the proportion of spiky cell morphology.

Spiky Cell Characterization: For the characterization experiments comparing the spiky cells to normal cells (Figure 1), both untreated normal and stable spiky cells were plated on 10 cm and 15 cm cell culture plates. These plates were treated with a 500 nM dose of doxorubicin for 24 hours. The stock solution of 2048 nM doxorubicin was diluted in MCF-7 media, and 10 mL of this doxorubicin-media solution was added to each 10 cm plate and 30 mL was added to each 15 cm plate. After the 24-hour treatment, cells were cultured in normal MCF-7 media. Images were taken of several plates and the media in all plates was replaced three times a week. Each week, several of the plates were passaged in order to use the cells in characterization experiments. Throughout this experiment, we kept untreated controls of both the normal and spiky cells.

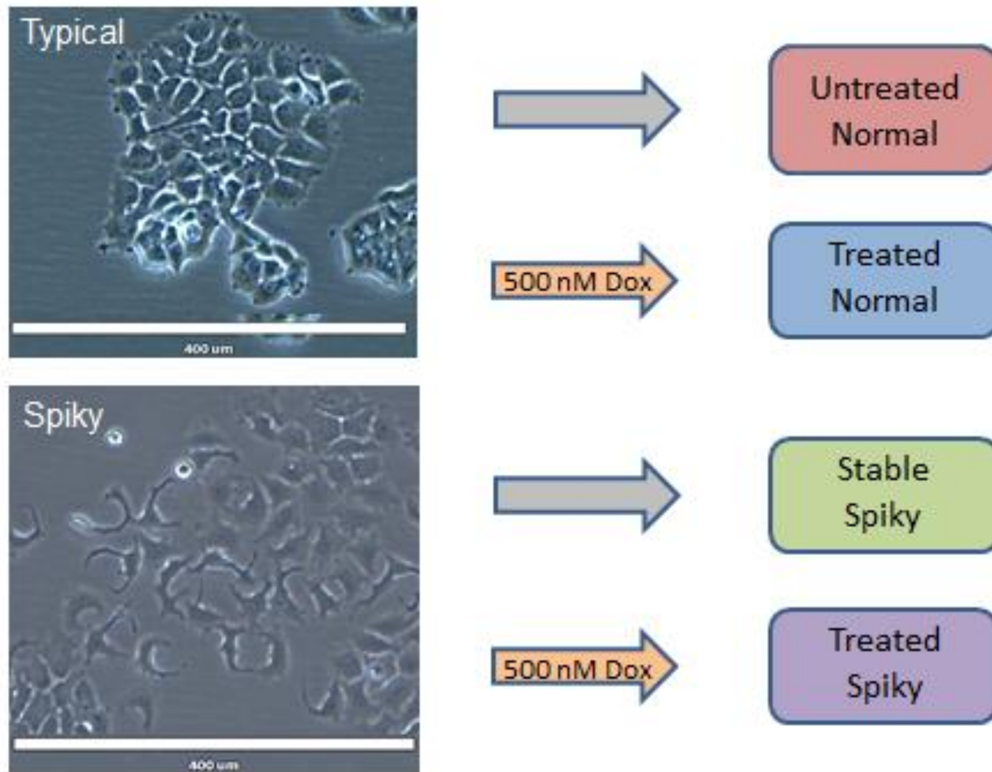

Figure 1 | Schematic of Experimental Design. Typical MCF-7 cells display the cobblestone epithelial phenotype, while the spiky cells form spindle-like projections and do not form connections with neighboring cells. For these experiments, we kept an untreated control of both the naïve MCF-7 cells and the population that had become stably spiky at about 30% spiky cells. To create the treated groups, we took a sample from each of the untreated populations and treated them with 500 nM doxorubicin for a 24-hour period. Over the course of the recovery from this treatment, we assessed the differences between the populations in four characteristics associated with tumor aggression: proliferation rate, dose response, invasive potential, and E-cadherin expression. Scale bars are 400 µm.

Cell Imaging

To image the cells multiple times per week, we used an EVOS light microscope. When imaging, we acquired several images with both the 4X and 10X objectives. To determine total magnification, we multiply the magnification of the objective by the magnification of the monitor by the magnification of the camera-video adaptor. Because there is no glass in the microscope mount, the camera-video adapter magnification is 1X.[18] Assuming a 15-inch display is similar to a 17-inch display, the magnification of the monitor is approximately 54.1X.[19,20] Therefore, the total magnification of the 4X objective is approximately 216.4X and the total magnification of the 10X objective is approximately 541X.

Quantification of Proliferation

To test proliferation rate, we counted the number of cells in the flasks that were passaged each week for the Invasion and Dose Response experiments, two flasks per group. Because these flasks were different sizes and were counted on different days of the week, I could not average the cell counts in order to find proliferation. Instead, I divided the cell count by the amount of cells that had originally been seeded onto that plate and then raised this to 1/(days since seeding). This gave the cell proliferation per day, and this was averaged with the other values of proliferation per day that were found for that cell type that week.

Invasion Assay

Each week of doxorubicin recovery, the invasion assay was performed on cell samples from the four groups – untreated normal, stable spiky, treated normal, and treated spiky – using the Trevigen CultureCoat Low BME Cell Invasion Inserts (Cat #3481-024-01). Each week, two

inserts were used per group, except in weeks seven and eight. In those weeks, I began using three inserts per group in order to calculate standard deviations. We prepared the invasion inserts according to the manufacturer's instructions. Inserts were placed in a tissue culture-treated 24-well plate, and 80 μL of serum-free media was added to the top of each insert. The serum-free media was made by combining 500 mL of MEM (Gibco Lot #1663071), 5.6 mL Pen-Strep (Gibco Lot #1601706), and 0.5 g of Bovine-Serum Albumin (Fisher Scientific Lot #130710). Following an hour incubation at 37°C, 40 μL of cell solution at a concentration of one million cells/mL was placed on the top of each invasion insert. The bottom of each well was filled with 360 μL of complete media, and the plates were placed in the incubator for 24 hours.

After 24 hours, the plate was removed from the incubator and we fixed and stained the cells. We stained the cells with crystal violet because it binds to cellular proteins and stains the cells purple, which made the cells visible for counting under the microscope. To do this, we used the 24-well plate with the invasion inserts and media in eight wells, leaving eight of the wells empty and adding 70% ethanol to the other eight wells. In an additional 24-well plate, we filled eight of the wells with 0.2% Crystal Violet solution in DI water and sixteen wells with DI water. We took the inserts out of the wells with the media and used a sterile cotton-tipped swab to remove the media and cells from the top of the membrane. Being careful not to touch the bottom of the membrane, we then placed the inserts in the 70% ethanol solution and allowed them to soak for 15 minutes. This fixed the cells to the membrane. The tops of the membranes were dried with cotton again, and they were placed in the empty wells to dry for 15 minutes. Then they were placed in the Crystal Violet for 15 minutes, then washed in the two sets of DI water for 10 minutes each, and the membrane was dried with cotton between each of these steps. These rinsing steps washed the excess crystal violet, which had not bound to the cells, off the

membrane. Finally, the inserts were removed from the final wash and the tops of the membranes were dried again, and they were placed in the empty wells until they were completely dry, at least 15 minutes.

Typically, the stained invasion inserts were left overnight to completely dry. At that point, they were imaged for counting the number of invading cells, which appeared as dark purple, roughly spherical shapes. Six images were taken of each insert: one image of the whole insert with the 4X objective (approximately 216.4X total magnification), and five images in a cross pattern with the 10X objective (approximately 541X total magnification). The number of invading cells was counted in each 541X image, and each week the counts for each treatment group were averaged. These average counts were then plotted on a bar graph. Standard deviations were calculated for weeks seven and eight when three invasion wells were used.

Staining Cells

We stained the cells for E-cadherin seven weeks after they had been treated with doxorubicin, and we performed the staining on all four treatment groups: untreated and treated normal and stable and treated spiky. Several days before staining, 100,000 cells were seeded into each well of a 12-well tissue culture-treated plate which had round glass cover slips attached to the bottom. There were two wells of each treatment group. To perform the E-cadherin staining, we followed a general immunostaining protocol. Cells were fixed with 4% paraformaldehyde for 30 minutes, washed twice with 500 µL of PBS for five minutes, and blocked with 800 µL of 3% BSA/0.1% TritonX-100 (Sigma Lot #SLBF4531V)/PBS for 30 minutes at room temperature. We then washed three times with 150 µL of washing solution (0.1% TritonX-100/PBS) for five minutes at room temperature. The next step was to add the primary antibody. Each well was

incubated at room temperature for 90 minutes with 150 μL of rabbit E-cadherin antibody (GeneTex Rabbit monoclonal antibody [EP700Y]) diluted in 1% BSA/PBS at a ratio of 1:500. We then washed three times for five minutes each with the washing solution at room temperature before adding the secondary antibody (Alexa Fluor 568 Donkey Anti-Rabbit Ref# A10042), which was diluted in 1% BSA/PBS at a 1:200 ratio. We added 150 μL to each well and allowed the plate to incubate for 60 minutes in the dark at room temperature. We then washed three times for five minutes each with the washing solution at room temperature. We then stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1 μg/mL for five minutes. DAPI binds to DNA and stains the cell nucleus with blue fluorescence. Next, we rinsed twice for five minutes with PBS at room temperature.

The cover slips were removed from the wells with tweezers, mounted to microscope slides using fluoromount, and sealed with nail polish. Imaging was performed with the EVOS fluorescent microscope using the 60X objective (approximately 4050X total magnification).[18,20,21]

To quantify the E-cadherin levels, I took between eight and 10 4050X images of each treatment group. For each of these images, I counted the total number of cells in each image which were close enough to other cells to form junctions. I then counted the number of cells in each image which formed at least one junction with another cell, and divided this by the total number of cells to find the percentage. I then averaged these percentages from the different images to find the average percentage of cells with at least one junction in each cell population.

Dose Response Assay

Each week of the doxorubicin recovery, a 15 cm plate of each of the conditions (untreated normal, stable spiky, treated normal, and treated spiky) was passaged. These were seeded into 12-well plates, with 100,000 cells of each type plated into six wells. After two days of culturing, we treated the cells with doxorubicin for 24 hours. Each treatment group received six doses, with a different dose to each of the six wells. These doses of 0 µM, 8 µM, 24 µM, 48 µM, 96 µM, and 192 µM were achieved by diluting the 2048 nM doxorubicin with MCF-7 media. After 24 hours, the doxorubicin was removed and the cells were passaged and analyzed using the Cellometer (Nexcelom Cellometer K2 Image Cytometer).

The Cellometer was used to find the percentage of viable cells in each group. Acridine Orange/Propidium Iodide (AO/PI, Nexcelom Lot #1507 13-04-1) was used to stain the live cells green and the dead cells red. After spinning the cells down, the media and trypsin were aspirated, leaving approximately 20 µL of solution in each tube. The MCF-7 Live/Dead Assay was performed with a mixture of 10 µL of AO/PI and 10 µL of cell suspension, and the results were given as number of live cells, number of dead cells, and percent viability. These were graphed for each week.

To obtain the LD50 from the viability percentages at each dose, we fit these values to the following equation:

$$Viability = \frac{Max\ Viability}{1 + e^{slope*(Dose-LD50)}}$$

In this equation, viability is the percentage of cells alive after a 24-hour treatment with doxorubicin, max viability is the viability of that population for the 0 µM doxorubicin dose during that week, slope is the slope of the viability curve, and dose is the variable dose of doxorubicin applied to the cells in µM. To fit the data to this equation, we used the solver

function in Excel to minimize the difference between the actual viability and the estimated viability from the equation. After obtaining the LD50s for each treated population at each timepoint, they were graphed over the weeks of recovery from the original treatment. The week zero timepoint was calculated by averaging the LD50 values for the untreated controls from five timepoints.

Results

Treatment of Cells with Doxorubicin and Imaging Phenotypes During Recovery from Doxorubicin Treatment

After MCF-7 cells have been treated with doxorubicin, many of the cells die off, and they continue to die at high rates through five weeks after treatment. The cells which survive are those we continue to culture, and the survivor cells are the ones which divide in order to repopulate the culture. Of these survivor cells, many take on phenotypes other than the typical MCF-7 epithelial morphology. In the typical MCF-7 population, the majority of cells form a cobblestone pattern, in which the cells form close connections with the cells around them. Other morphologies that arise at high proportions after treatment with doxorubicin include the spiky cell morphology, in which the cells form long, spindly projections and do not form close connections with the neighboring cells. Another phenotype that arises is the "fried egg" morphology. These cells are much larger and flatter than the typical MCF-7 cells. They also do not appear to form close connections with the neighboring cells, and some of them appear to be multi-nucleated.

Figure 2 demonstrates the changes in the proportions of subpopulations within the MCF-7 population after treatment with doxorubicin. Though the non-typical subpopulations are observed in the group without treatment, the majority of the cells have the typical morphology and form the normal cobblestone pattern. In Figure 2, the "Other" category includes the spiky phenotype as well as other non-typical MCF-7 morphologies such as the fried egg cells. These “other” cells also included cells which could not be classified as normal, fried egg, or spiky. These included cells which appeared to be transitioning between the spiky and fried egg morphologies as well as cells which could not be identified because they were not lying flat on

the plate. To identify spiky cells, we looked for cells without close connections, which are not flat to the plate like the fried egg cells and which have a curved shape or thin projections of cytoplasm.

Two weeks after treatment with doxorubicin (Figure 2), the majority of the cells are the other phenotypes, and these other phenotypes persist at higher-than-normal proportions (approximately 40-60%) throughout the 14 weeks tested post-treatment. Although this data is from only one experiment, the entire time course has been reproduced several times in the lab over the past two years and the same pattern emerges.

For the spiky induction experiment, we found that after about eight weeks, all 16 of the flasks which had been passaged each week looked relatively normal in cell morphology. However, we found that the flasks which had been left alone began dividing again after treatment more quickly than did the passaged flasks, and two of these untouched flasks had interesting populations of cells. One of the flasks had many of the big fried egg type cells. After several weeks of passaging these though, the cell population returned back to the normal phenotype. A second flask had a higher proportion of the spiky cells. We used the UnipicK to isolate spiky cells, and we plated these cells into a three wells of a 12-well plate. Two of the wells had approximately 100 cells and one of the wells had approximately 30 cells. We found that after several weeks these cells returned to relatively normal morphologies. In that same time, however, the 10 cm dish with the rest of the population continued to have high proportions of spiky cells.

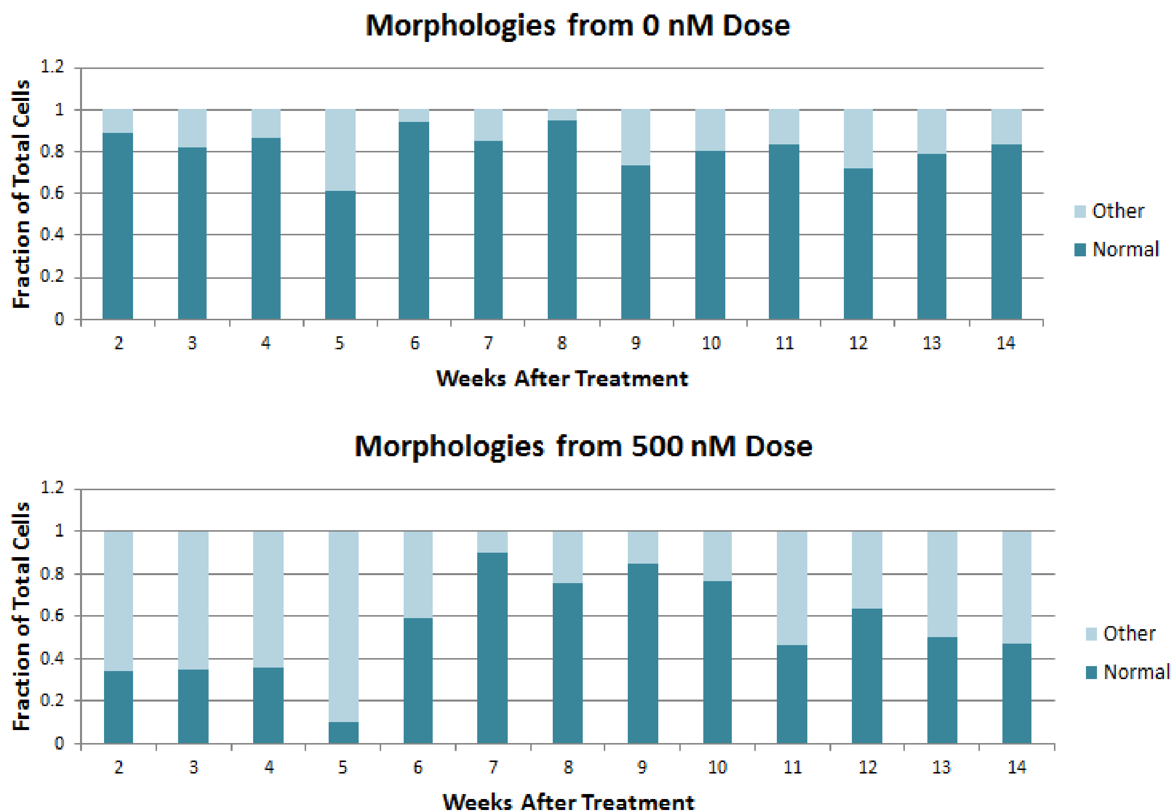

Figure 2 | Quantification of Morphology Changes Arising from Doxorubicin Treatment. In these experiments, three plates of MCF-7 cells were treated with 500 nM of doxorubicin for 24 hours and three plates were left untreated, and the proportions of the different morphologies were quantified over the 14-week recovery period from the treatment. For each time point, one representative image was counted for either two or three plates, and the results from the different plates were averaged.

Proliferation During Recovery from Doxorubicin Treatment

Proliferation rate measures how quickly the cells are dividing, and this value was measured in the four treatment groups over the course of recovery from the doxorubicin treatment by counting several plates of cells each week (Figure 3). The untreated groups had fairly consistent proliferation, with the stable spiky cells having slightly higher (by 1-5% in most weeks) proliferation rates per day. In the treated groups, the cells were still dividing faster than they were dying in the first week, but in weeks two through five they were dying off more quickly than they could divide. Over the course of the recovery from the doxorubicin treatment, the two treated groups had very similar proliferation rates. Because the plotted growth rates are for average growth per day, small differences are magnified over the course of a week. While the stable spiky cells were growing only slightly faster than the untreated normal each day, in many of the timepoints the stable spiky had approximately 1 million more cells in a T-75 flask than the untreated normal cells. In the treated groups, there was a large jump in proliferation between weeks five and six – at this point the cells went from negative net growth rates to proliferation rates similar to the untreated controls. In weeks seven and eight, the proliferation rates continued to approach the untreated controls, demonstrating that the doxorubicin treatment did not appear to have a lasting effect on proliferation.

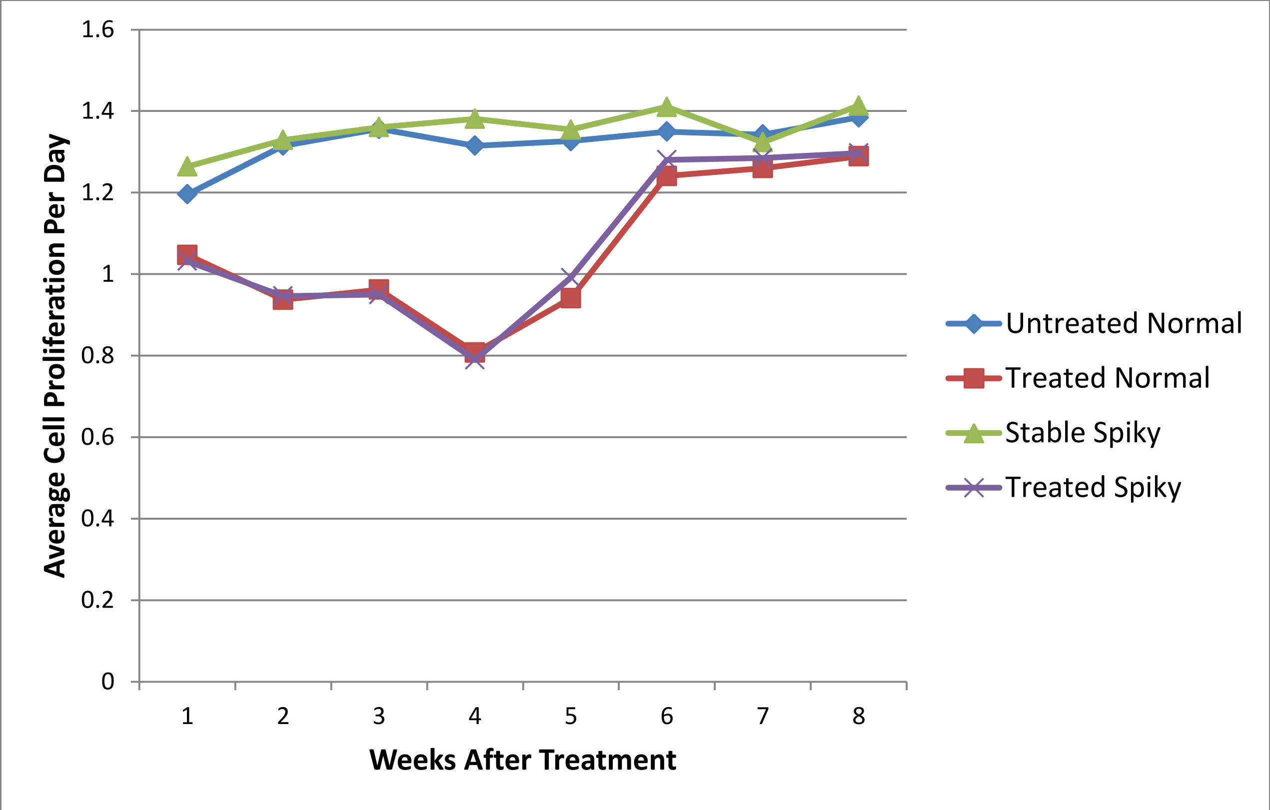

Figure 3 | Proliferation During Recovery from Doxorubicin Treatment. Proliferation rate is a measure of how quickly the cells are dividing. To create this figure, each week we counted at least two plates of cells from each treatment group. We then divided this count by the number of cells that had originally been seeded into the dish and raised this result to 1/(days since seeding). This gave us the proliferation per day, and we averaged the values obtained from two flasks each week.

Drug Sensitivity During Recovery from Doxorubicin Treatment

Drug sensitivity was tested over a wide range of drug concentrations and is displayed in Figure 4 as LD50 over time. LD50 is a measure of the dose of doxorubicin needed to kill 50% of the cells during that week. More resistant cells have higher LD50s, meaning a higher concentration of the drug is required to kill off 50% of the population.

The average LD50 for the untreated normal group was 68.7 µM, and the average LD50 of the stable spiky group was 52.4 µM. These values mean that the stable spiky were actually slightly less resistant than the normal group, which is opposite of what we expected. The standard deviation for the LD50s of the untreated normal population was 5.9 µM, while the standard deviation for the LD50s of the stable spiky population was 10.9 µM. These standard deviations are relatively small, as the LD50s of the treated populations ranged from 37 to 200 µM, demonstrating that the untreated populations both remained relatively consistent over the course of the eight weeks. This is expected because these were the control groups. This result confirms that the assay was performed in a consistent manner over the course of the eight weeks.

The most notable aspect of Figure 4 is that the treated spiky became highly resistant in the first week after treatment, while the treated normal did not become highly resistant until the second week after treatment. The treated normal group responded to the doxorubicin treatment with little change after one week, but in the second week after treatment, the population had become more resistant by more than two times. Over the course of recovery, the resistance of both groups declined back to the untreated value. Because the final LD50s of the treated groups are similar to the average LD50s of the untreated groups, the doxorubicin treatment did not appear to have a lasting effect on the drug sensitivity of the normal population. Additionally,

because the treated spiky group became resistant faster than the treated normal group, the stable spiky cells appear to be primed to become resistant more quickly than the normal MCF-7 cells.

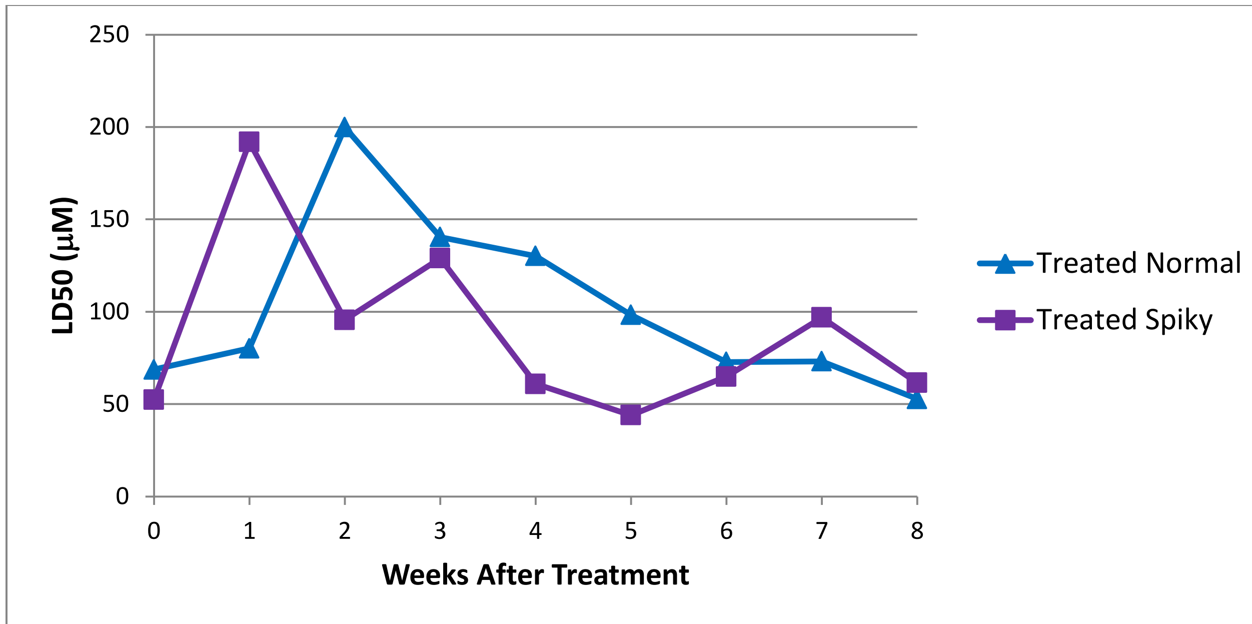

Figure 4 | Drug Sensitivity During Recovery from Doxorubicin Treatment. Resistance to doxorubicin is measured in LD50, which is the concentration of doxorubicin in µM it takes to kill exactly 50% of the cells. Each week of this experiment, each treatment group was given six doxorubicin concentrations over the range of 0 µM to 192 µM. After 24 hours, the viability of the cells in each concentration was assessed, and this curve was used to estimate the LD50.

Invasive Potential of Cells Following Doxorubicin Treatment

The invasive potential of all four treatment groups was evaluated by measuring cell migration through a polycarbonate filter with 8 μm pores coated with a thin basement membrane. The percent of cells migrating across the filter was determined by crystal violet staining of fixed cell populations after 24 hours (Figure 5). As shown in Figure 5, aside from some unusual data points in weeks one and six, the untreated normal cells stayed relatively consistent in their invasive potential over the course of the eight weeks. This is expected, since this was the naïve MCF-7 population which had never received any doxorubicin treatment. The treated normal cells slightly increased in their invasive potential immediately after the doxorubicin treatment, then fell back down below the values of the untreated population for several weeks before settling back in the last two weeks to be very close to the untreated invasion values. The weeks in which the treated normal cells had low invasive potential, weeks three through five, correspond with the weeks in which the cells were dying off most quickly (Figure 3).

The stable spiky population had invasion values that varied across each week. This group was a control group which we did not expect to have large differences in invasion because it had not been treated since the spiky population became stable. However, the stable spiky population was quite consistently more invasive than the untreated normal population. The treated spiky population decreased in invasiveness, compared to both untreated populations, in the first weeks after treatment. Over the weeks, their invasiveness steadily increased, before settling back down to a value similar to the untreated normal population in the eighth week. By examining the spiky cells under the microscope over the course of the eight weeks after treatment, we saw that the treated spiky cells began to approach a population intermediary between the naïve and stable spiky populations. There were more spiky cells in the treated spiky population than in the naïve

untreated population, but the treated spiky population had fewer spiky cells than the stable spiky population. Therefore, the invasion results showing that the treated spiky were often intermediary between the untreated normal and stable spiky populations align with these observations.

Additionally, by examining both treated populations, we see that the doxorubicin treatment does not produce a lasting increase in invasive potential. Though there are several weeks in which the treated population had a higher invasive potential than the corresponding untreated population, particularly at the beginning of the recovery of the treated normal group, by the end of the recovery at eight weeks the treated populations have similar invasion as the untreated population. Treatment with doxorubicin does not appear to have a long-term effect on the invasive potential of the MCF-7 cells.

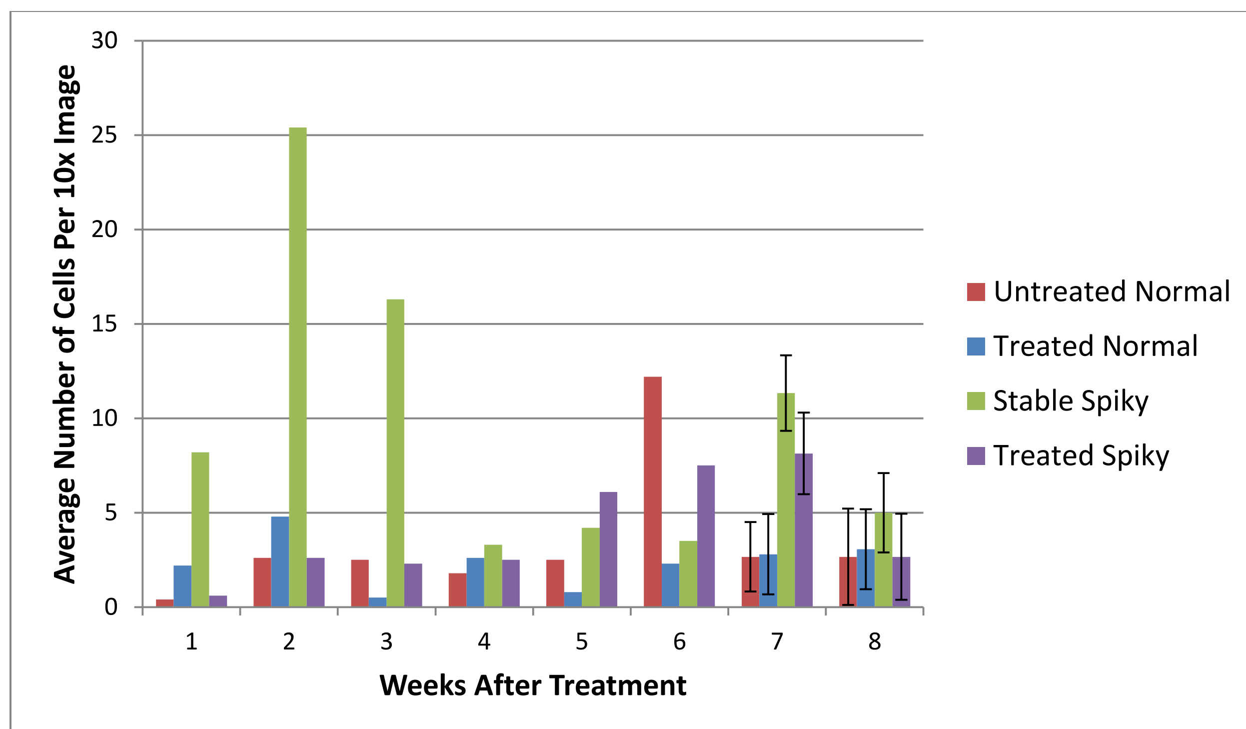

Figure 5 | Invasive Potential During Recovery from Doxorubicin Treatment. Invasive potential was measured using invasion well inserts with a polycarbonate membrane with 8 µm pores coated in thin basement membrane. Each week for weeks one through six, cells of each treatment group were placed in two invasion well inserts with serum-free media which were placed in wells which contained complete media. In weeks seven and eight, three inserts were used for each group. After 24 hours, the membranes were stained with crystal violet fixed cell stain and imaged. To obtain representative images from the wells, we took five images of each well with the 10X objective (541X total magnification) in the same cross pattern: one in the middle, one directly above this, one below the first image, one to the left of the first image and one to the right of the first image. The number of crystal violet-stained cells was counted in each image, and the counts from the 10 images for each treatment group were averaged. The error bars in weeks seven and eight are one standard deviation.

Staining for Biomarkers Labelling Resistant Cells

The cell-cell junction marker E-cadherin was used to examine the differences in cell connections between the treatment groups (Figure 6). This staining was performed seven weeks after the doxorubicin treatment, so in many respects the treated cells were recovered back similarly to the untreated controls. In the images, the blue stain is DAPI, identifying the cell nuclei, and the red stain is the E-cadherin. Close cell connections appear as a solid but thin red line. By examining representative images, we see that the normal groups, both treated and normal, have many solid cell connections. In contrast, the spiky cells have fewer cell-cell junctions, though the treated spiky group appears to be intermediary between the stable spiky population and the normal groups. In the normal groups and even the stable spiky group, the vast majority of the cell junctions that appear are solid red lines. However, in the treated spiky group, most of the cell connections appear as wavy, incomplete lines. It appears that while there is much more E-cadherin in the treated spiky population than in the stable spiky group, the cell junctions are not solid, continuous junctions as they are in the other populations. By quantifying the percent of imaged cells which had at least one junction with another cell, we found that the normal groups and the treated spiky group were quite similar. In the untreated normal population, 82.5% of the cells had at least one junction, in the treated normal population that percentage was 77.9%, and for the treated spiky it was 79%. However, in the stable spiky population, only 1.9% of the cells had a junction with at least one other cell.

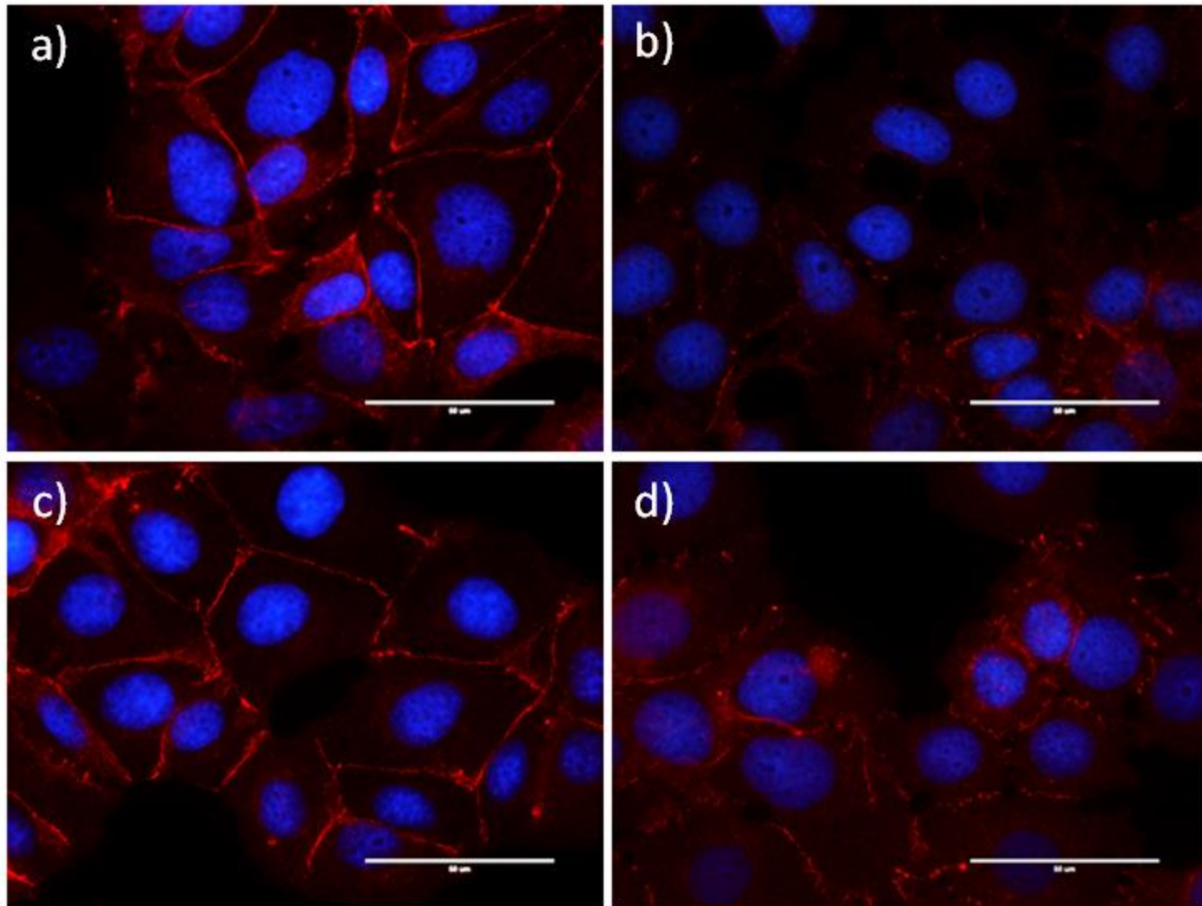

Figure 6 | Immunocytochemistry Staining for E-Cadherin. E-cadherin is a cell-surface marker for cell-cell junctions. In these images with 4050X total magnification, DAPI, a cell nucleus stain, is blue and E-cadherin is red. For this experiment, we stained two slides of each treatment group, 7 weeks after the cells were treated with doxorubicin. To obtain representative images of the staining, we took five random images of slides from each treatment group. For counting, we took between eight and 10 images of each treatment group. A) Untreated Normal B) Stable Spiky C) Treated Normal D) Treated Spiky. Scale bar is 50 µm.

Discussion and Conclusion

From the lab's first studies on drug-induced phenotypic transitions, a key finding was that a short, 24-hour exposure to doxorubicin had lasting results on cell phenotype 14 weeks later. Though the majority of the plates return back to normal phenotype after recovering from treatment, there are some rare cases in which one of the other morphologies stabilizes at a higher proportion, such as in the stable spiky population. In this case, the effects of the doxorubicin lasted even longer than 14 weeks and induced a change in the cells which was maintained over approximately 30 passages. For patients, this implies that even one dose of chemotherapy can have long-lasting impacts on both the tumor and the patient's healthy cells. The first dose of chemotherapy could be altering the composition of the tumor, impacting how it will respond to the next dose, even if that dose is months later, and possibly increasing the resistance of the tumor to the chemotherapeutic agent. Future studies which could improve upon these results could find another way of characterizing the cells that were transitioning between phenotypes and the ones that were difficult to count. Additionally, this data is compiled from my cell counts as well as the counts of two other undergraduates, so the counting method is not completely uniform, which could affect the result.

Though the difference in cell proliferation per day between the untreated normal and the stable spiky population appears small, in many weeks it becomes a difference of about 1 million cells once it is multiplied out over the course of the week. Proliferation is one of the characteristics of tumor aggression, and the fact that these stable spiky cells are growing more quickly shows that they are more aggressive than the typical MCF-7 cells. Notably, however, the treated spiky do not show a considerable difference in proliferation over the treated normal cells, except possibly in two timepoints (Figure 3). Additionally, the proliferation levels of the treated

groups appear to be approaching the pre-treatment levels by the end of recovery. This demonstrates that while the stable spiky population does appear to be more aggressive, a treatment with doxorubicin does not increase this aggressiveness and may decrease it, at least in terms of proliferation.

This data for cell proliferation per day is not completely accurate. Some of the treated plates were left in the same vessel without passaging for several weeks, and to find the cell proliferation per day we used the number of days since seeding the cells. This assumes that the cell proliferation per day was about the same over those several weeks, which we know is not true because the cells were dying off more quickly in the middle weeks of recovery. Future experiments could make this data simpler and more accurate by using the same size vessel for each cell type and passaging the plates each week. Alternatively, future experiments could use an assay to quantify proliferation such as staining for Ki67.

The result that the stable spiky cells are less resistant to the doxorubicin is surprising (Figure 4). However, the LD50 values of the two controls are quite similar to each other. Because the treated spiky population becomes resistant so quickly after treatment, we conclude that the stable spiky cells are primed to become resistant more quickly than the normal cells. From these results we see that the populations return to resistance levels close to those of the controls by the end of recovery. Future experiments could then treat these recovered populations again to determine whether the treated normal population is now primed to become resistant more quickly and whether the treated spiky population becomes even more resistant or if another treatment reverses this resistance.

The invasion assay yielded several interesting results (Figure 5). First, the stable spiky population showed a large amount of variation in invasive potential. This variation implies that

the population may be shifting slightly over time, with varying proportions of the spiky phenotype over the weeks. However, there were no noticeable differences in the stable spiky population morphologies over the course of the experiment. The untreated normal population, which had relatively consistent invasive potential, had incredibly high invasion in week six, and this abnormally high value came from one invasion well. The high rate of invasion could have been due to a flaw in the well or a larger number of cells being seeded into the well. It seems unlikely that this high invasion in one well is a true result, but future studies could confirm this conclusion. For weeks one through six, we used two invasion wells for each population, and in the seventh week we switched to using three invasion wells each. Future experiments should use three invasion wells for each population for each timepoint, so that standard deviations can be found in order to assess the variability of the invasive potential at all timepoints. Taking an average of three wells each week may also help to reduce the variability of the untreated populations between weeks.

The treated normal population became more invasive than the control in the two weeks directly after treatment (Figure 5). This is expected because we predicted that the doxorubicin treatment would increase the aggressive characteristics of the cells. A surprising result was that the doxorubicin treatment had the opposite effect on the stable spiky cells, making them less invasive. By weeks seven and eight, the invasiveness of the treated spiky was beginning to approach that of the stable spiky. This aligns well with the morphological cellular changes I observed, that the treated spiky cells recover to a state in between that of the typical MCF-7 cells and the original stable spiky population (Figure 6).

The E-cadherin results support the hypothesis that the stable spiky cells are more aggressive (Figure 6). As a cell-cell junction marker, high levels of E-cadherin mean the cells are

tightly connected to each other. When cells have less E-cadherin expression, they are less connected to their neighbors and therefore can become mobile more easily. The fact that the stable spiky cells have much less E-cadherin corresponds to the fact that they have a much greater invasive potential and demonstrates that they are more aggressive. Invasion and motility are key aspects of tumor progression, as more advanced tumors are able to spread and eventually become metastatic. Future studies should be done to assess the E-cadherin levels earlier in the recovery from the doxorubicin treatment. By seven weeks, the treated normal are very similar to the untreated normal, and the treated spiky have many of the incomplete junctions that are not found in the stable spiky cells. Performing this staining earlier in the recovery could help us to understand how E-cadherin levels, and by extension mobility and aggression, fluctuate during the recovery from the treatment.

The motivation behind this research is to improve cancer treatments. The goal is to identify a subpopulation of survivor cells which seem to confer tumor aggression and chemotherapeutic resistance and the epigenetic changes that are exhibited by this subpopulation. It has been found that just the up-regulation or down-regulation of certain genes, without any actual mutations, can confer increased resistance to cells.[11] Because these changes are epigenetic, rather than genetic, they are reversible.[8] Then, in order to increase the effectiveness of chemotherapeutics, the gene expression of the subpopulation could be altered back to the gene expression of the normal population before the drug is administered, in order to reduce the likelihood of resistant cells emerging. For example, siRNA could be used to block upregulated genes. Studies of melanoma, another type of cancer, have found that though the majority of tumor cells are the main, fast-growing population, there is also a subpopulation of "slow-cycling, self-renewing" tumor cells.[22] Roesch et al. hypothesized a similar idea to ours – that in order to

have a greater therapeutic effect, it would be necessary to target the slow-cycling subpopulation – though there are differences between their subpopulation and the spiky cells, such as the fact that the spiky cells are highly proliferative.[22]

Another phenotypic state that breast tumor cells have been found to exhibit is that of the stem-like cancer cell.[23] These cells have increased drug resistance and tumor-seeding ability, and Gupta et al. found that it is not only possible for these stem-like cells to transition to other phenotypes but that it is also possible for non-stem cells to transition to the stem-like phenotype. They recommend that to treat breast cancer, it would be necessary to target both the bulk population and the stem-like cells, or to prevent the transition from the non-stem phenotype to the stem-like phenotype.[23] Based on our experiments, it appears that the spiky cells may be exhibiting stem-like characteristics and may have undergone a transition from a non-stem phenotype to a stem-like phenotype.

The epithelial to mesenchymal transition is affiliated with both invasive and metastatic potential, as well as increased aggressiveness, in carcinoma cells such as breast cancer cells.[24] Some of the characteristics of EMT include decreased intercellular adhesion (E-cadherin and occludins), fewer epithelial markers such as cytokeratins, more mesenchymal markers including vimentin, morphological changes to fibroblast-like or spindly morphology due to cytoskeleton reorganization, and an increase in mobility and invasiveness.[24] Several of these characteristics are ones we observed in the stable spiky population: decreased E-cadherin levels, spindle-like projections, and increased invasiveness. Because of these similar characteristics, it may be true that the spiky cells are MCF-7 cells that have undergone EMT. Previous studies in melanoma cells have found that resistance is not only related to EMT but that cells undergo EMT in order to become resistant to chemotherapeutics.[25] This may be what is occurring in the MCF-7 breast

cancer cells. EMT markers have been found in breast carcinoma biopsies, and these markers are associated with increased aggression and poor prognosis.[24] Transitioning to the mesenchymal phenotype could allow the cells to survive the doxorubicin treatment, and because this is a reversible process they could transition back to the epithelial phenotype later in recovery in order to repopulate the normal morphology. The stable spiky population may be a population that has established an equilibrium with a higher proportion of mesenchymal cells. Future studies should further confirm this by examining protein and gene expression related to EMT and the mesenchymal phenotype. Additionally, other experiments in the lab are examining the other phenotypes, such as the fried egg cells, in order to understand what role those cells have in tumor aggression and resistance.

The results from the proliferation, invasion, and E-cadherin experiments (Figures 3, 5 and 6) show that the doxorubicin treatment appears to remove the mesenchymal-like qualities of the spiky cells. This implies that the doxorubicin treatment can push the cells in either direction of the transition – either to mesenchymal cells or in the other direction, toward epithelial cells. Though in these experiments the treated spiky cells began to respond similarly to the normal cells in many of these experiments, morphologically they appeared intermediary between the original stable spiky and the untreated normal. They continued to have higher-than-normal proportions of spiky cells. However, previous experiments in the Brock lab have found that the treated spiky population recovered to a much more normal distribution of morphologies. The reason for this difference in reaction to the doxorubicin treatment is unclear and will be a focus of future follow-up studies in the lab.

In all of my experiments, I was looking at survivor cells that have escaped a substantial dose of a chemotherapeutic drug. Other researchers termed such cells "drug-tolerant persisters"

(DTPs), which are a small group of reversibly resistant cells which are able to survive after most of the population has been killed.[10] Sharma et al. found that cell populations can be reversibly resistant to drugs by keeping a distinct subpopulation of cells of a more resistant phenotype. This subpopulation protects the population by surviving and repopulating after the stress is removed.[10] Other studies of doxorubicin treatments have focused on long-term low doses rather than one pulse of treatment.[26] Fairchild et al. used these long, low doses to create doxorubicin-resistant cell lines. These experiments that we performed, however, characterize a short-term, high dose of chemotherapeutic agent, which more closely mimics the clinical scenario. Rather than examining the effects of a long-term dose on the cells, we are examining how cells adapt or escape from a pulse of treatment.

My experiments characterizing the spiky cells fit into the larger context of other work in the Brock lab. One future direction other researchers will take these studies is to characterize the cells' response to a second dose of doxorubicin. After allowing the treated populations to recover for about eight weeks, they will be treated again and similar experiments characterizing tumor aggression will be performed over the recovery from this treatment. In addition, the epigenetic changes that lead to the spiky phenotype will be characterized, to better understand if the cells did undergo an epithelial to mesenchymal transition or reverted to a stem-like phenotype. These experiments may lead to a better understanding of cancer cell resistance to chemotherapeutics, which could in time help to improve therapeutic treatment of these tumors.

Acknowledgements

First, I would like to thank Dr. Brock for her guidance throughout my time in the lab and during the thesis process. Thank you as well to my second reader Dr. Zoldan. I'd also like to extend a huge thanks to the graduate student I worked most closely with, Grant Howard, for teaching me the necessary experimental work and guiding me throughout the project. Thanks as well to the other graduate students in the lab, Hunter Joyce and Aziz Al'Khafaji, for their willingness to answer questions. I would also like to express my gratitude to the lab technician Millie Clark for her support and friendship throughout my time in the lab. I would also like to acknowledge the undergraduates Sharwin Khot and Daniel Liu, who both assisted in quantifying the data for the morphology quantification figure. Thanks to the Biomedical Engineering Department, the Plan II Honors Program, and the Engineering Honors Program for providing me with this and many other opportunities throughout my undergraduate career. And finally, deepest thanks to my family for convincing me to attend the University of Texas at Austin and for providing me never-ending love and support in all my endeavors.

References

(1) Gonzalez-Angulo, A. M. Overview of Resistance to Systemic Therapy in Patients with Breast Cancer. *Adv. Exp. Med. Biol.* **2007**, *608*, 1–22.

(2) Breast Cancer Treatment http://www.cancer.gov/types/breast/patient/breast-treatment-pdq#section/_185 (accessed Apr 28, 2016).

(3) Hortobágyi, G. N. Anthracyclines in the Treatment of Cancer. An Overview. *Drugs* **1997**, *54 Suppl 4*, 1–7.

(4) NCI Drug Dictionary http://www.cancer.gov/publications/dictionaries/cancer-drug (accessed Apr 28, 2016).

(5) Minotti, G.; Menna, P.; Salvatorelli, E.; Cairo, G.; Gianni, L. Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity. *Pharmacol. Rev.* **2004**, *56* (2), 185–229.

(6) Doxorubicin Hydrochloride http://www.cancer.gov/about-cancer/treatment/drugs/doxorubicinhydrochloride (accessed Apr 28, 2016).

(7) Brock, A.; Chang, H.; Huang, S. Non-Genetic Heterogeneity — a Mutation-Independent Driving Force for the Somatic Evolution of Tumours. *Nat. Rev. Genet.* **2009**, *10* (5), 336–342.

(8) Quintana, E.; Shackleton, M.; Foster, H. R.; Fullen, D. R.; Sabel, M. S.; Johnson, T. M.; Morrison, S. J. Phenotypic Heterogeneity among Tumorigenic Melanoma Cells from Patients That Is Reversible and Not Hierarchically Organized. *Cancer Cell* **2010**, *18* (5), 510–523.

(9) Florea, A.-M.; Busselberg, D. Breast Cancer and Possible Mechanisms of Therapy Resistance. *J. Local Glob. Health Sci.* **2013**, *2*.

(10) Sharma, S. V.; Lee, D. Y.; Li, B.; Quinlan, M. P.; Takahashi, F.; Maheswaran, S.; McDermott, U.; Azizian, N.; Zou, L.; Fischbach, M. A.; et al. A Chromatin-Mediated Reversible Drug-Tolerant State in Cancer Cell Subpopulations. *Cell* **2010**, *141* (1), 69–80.

(11) Wood, K. C. Mapping the Pathways of Resistance to Targeted Therapies. *Cancer Res.* **2015**, *75* (20), 4247–4251.

(12) Pisco, A. O.; Brock, A.; Zhou, J.; Moor, A.; Mojtahedi, M.; Jackson, D.; Huang, S. Non-Darwinian Dynamics in Therapy-Induced Cancer Drug Resistance. *Nat. Commun.* **2013**, *4*, 2467.

(13) Kalluri, R.; Weinberg, R. A. The Basics of Epithelial-Mesenchymal Transition. *J. Clin. Invest.* **2009**, *119* (6), 1420–1428.

(14) Collett, K.; Eide, G. E.; Arnes, J.; Stefansson, I. M.; Eide, J.; Braaten, A.; Aas, T.; Otte, A. P.; Akslen, L. A. Expression of Enhancer of Zeste Homologue 2 Is Significantly Associated with Increased Tumor Cell Proliferation and Is a Marker of Aggressive Breast Cancer. *Clin. Cancer Res.* **2006**, *12* (4), 1168–1174.

(15) Rasbridge, S. A.; Gillett, C. E.; Seymour, A. M.; Patel, K.; Richards, M. A.; Rubens, R. D.; Millis, R. R. The Effects of Chemotherapy on Morphology, Cellular Proliferation, Apoptosis and Oncoprotein Expression in Primary Breast Carcinoma. *Br. J. Cancer* **1994**, *70* (2), 335–341.

(16) Sun, X.; Bao, J.; Shao, Y. Mathematical Modeling of Therapy-Induced Cancer Drug Resistance: Connecting Cancer Mechanisms to Population Survival Rates. *Sci. Rep.* **2016**, *6*, 22498.

(17) Fornari Jr, F. A.; Jarvis, W. D.; Grant, S.; Orr, M. S.; Randolph, J. K.; White, F. K. H.; Gewirtz, D. A. Growth Arrest and Non-Apoptotic Cell Death Associated with the Suppression of c-Myc Expression in MCF-7 Breast Tumor Cells Following Acute Exposure to Doxorubicin. *Biochem. Pharmacol.* **1996**, *51* (7), 931–940.
(18) The Leading Investigator https://www.leica-microsystems.com/fileadmin/downloads/Leica%20FS%20C/Newsletters/eforensic_1_2005.pdf (accessed May 13, 2016).
(19) EVOS All-In-One Digital Microscope https://www.emsdiasum.com/microscopy/products/digital/evos.aspx (accessed May 13, 2016).
(20) Total Magnification (Eyepiece Observation, Video Monitor Observation) http://www.olympus-ims.com/en/microscope/terms/total_magnification/ (accessed May 13, 2016).
(21) EVOS FL Auto Cell Imaging System https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cellular-imaging/cell-imaging-systems/evos-fl-auto.html (accessed May 13, 2016).
(22) Roesch, A.; Fukunaga-Kalabis, M.; Schmidt, E. C.; Zabierowski, S. E.; Brafford, P. A.; Vultur, A.; Basu, D.; Gimotty, P.; Vogt, T.; Herlyn, M. A Temporarily Distinct Subpopulation of Slow-Cycling Melanoma Cells Is Required for Continuous Tumor Growth. *Cell* **2010**, *141* (4), 583–594.
(23) Gupta, P. B.; Fillmore, C. M.; Jiang, G.; Shapira, S. D.; Tao, K.; Kuperwasser, C.; Lander, E. S. Stochastic State Transitions Give Rise to Phenotypic Equilibrium in Populations of Cancer Cells. *Cell* **2011**, *146* (4), 633–644.
(24) Sarrió, D.; Rodriguez-Pinilla, S. M.; Hardisson, D.; Cano, A.; Moreno-Bueno, G.; Palacios, J. Epithelial-Mesenchymal Transition in Breast Cancer Relates to the Basal-like Phenotype. *Cancer Res.* **2008**, *68* (4), 989–997.
(25) Kemper, K.; Goeje, P. L. de; Peeper, D. S.; Amerongen, R. van. Phenotype Switching: Tumor Cell Plasticity as a Resistance Mechanism and Target for Therapy. *Cancer Res.* **2014**, *74* (21), 5937–5941.
(26) Fairchild, C. R.; Ivy, S. P.; Kao-Shan, C.-S.; Whang-Peng, J.; Rosen, N.; Israel, M. A.; Melera, P. W.; Cowan, K. H.; Goldsmith, M. E. Isolation of Amplified and Overexpressed DNA Sequences from Adriamycin-Resistant Human Breast Cancer Cells. *Cancer Res.* **1987**, *47* (19), 5141–5148.