The Role of Cell Compaction in Radiation Therapy for Breast Cancer

A thesis presented by

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Statement of Research

My thesis project was performed in the Professor Donald Ingber’s laboratory in the Vascular Biology Department of the Boston Children’s Hospital in the Wyss Institute for Biologically Inspired Engineering at Harvard under the guidance of Dr. Akiko Mammoto and Dr. Tadanori Mammoto. I joined the Ingber Lab in the summer of 2014 as a part of the Harvard College Program for Research in Science and Engineering (PRISE). I continued this project in the fall and spring of the 2014/2015 academic year while enrolled in ES91r.

My research was conducted in collaboration with and under the close supervision of Akiko Mammoto and Tadanori Mammoto, and with the immense assistance of the lab technicians, Elisabeth Jiang and Amanda Jiang. The experiment was designed based on prior findings from scientists including Don Ingber, Akiko Mammoto and Tadanori Mammoto; I have included a selection of these findings in this write-up. Cell cultures and microcontact printing systems were prepared by Tadanori Mammoto, Elisabeth Jiang and me. All irradiation of cells and tumours was performed by Elisabeth Jiang and Amanda Jiang. Fixing of cells and tumours and staining of cells were done by Elisabeth Jiang, Amanda Jiang and me. Confocal imaging was done by Tadanori Mammoto and me. Analysis of data was done by me with assistance and guidance from Akiko Mammoto. qRT-PCR preparation was performed by Elisabeth Jiang and me. All ELISA testing was conducted by Amanda Jiang. Preparations and injections of mice were performed by Elisabeth Jiang and me. Cryosectioning of tumours was done by Amanda Jiang and me. Cage changes, BAPN treatment and tumour measurements were conducted by Tadanori Mammoto, Elisabeth Jiang and me.
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I am incredibly indebted to Professor Donald Ingber, Dr. Akiko Mammoto and Dr. Tadanori Mammoto for their willingness to accept me so graciously into their lab and to allow me to participate in their oncology research, something I have yearned to do for so long. I cannot even begin to fathom how much of a risk this was as I showed no concrete proof that I would be a resourceful addition to their world-class research, and for that I am extremely humbled. I am incomprehensibly appreciative of this opportunity to witness how the compassion of such esteemed scientists for those burdened by such a horrible disease translates into their drive and devotion to finding ways to make a patient’s journey easier. This is something I will forever look back on for inspiration when I become a practicing physician. My words cannot begin to express my immense gratitude to them for allowing me under their wings to learn research techniques first-hand.

My sincerest thanks go to Akiko and Tadanori Mammoto for being so patient with me when I faltered and when I grew nervous. It has been a great journey with them and I feel so blessed to have been granted this chance to study under such skilled and naturally gifted scientists, with an eye for detail that I didn’t know could be achieved. Huge thanks go to Akiko Mammoto for her consistent and exceptional research advice and for always keeping a smile on her face without fail; she was a constant joy to be around and her laughter always made me feel very welcome and comfortable.

Upon joining the Ingber lab, I was particularly pleased to meet the lab technicians, Elisabeth and Amanda Jiang. This pair of sisters was so very welcoming and so quick to engage me in their conversations. I am beyond grateful for the help and assistance they offered me throughout my time in the lab, completing experiments for me when I had to make it back to school in time for my next class and willingly teaching me the tricks of the trade in the lab. I owe my rapid burst of comfort and confidence in the lab to these two, and the other student, Sarah Scalia ’15, who conversed and laughed with me regularly and served as great resources for me to talk to about science and life in general.

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Lastly, I am honoured to thank my parents and siblings for their consistent thoughtful prayers in my journey before and throughout my Harvard career. Specifically, I am appreciative of my brother, Kojo Welbeck’s encouragement for me to write a senior thesis. I am 100% sure that if I had not spoken to him about his senior thesis and he had not questioned and prodded at my decision to not write a thesis, I would have missed out on such a fulfilling experience. A huge shout-out goes to my amazing roommates Qaren, Adiam, Kimberly, D’joy and Ope, my wonderful thesis fairy, Elise, and my other friends for their support during this time. Finally, I’d like to dedicate this thesis to my late Grandpapa and my late Grandmama, whose passing from cancer stirred in me a desire to go into medicine and oncology.
Abstract

Physical compaction and accompanied collagen remodelling are required for normal organ development, while tumour cell compaction induces tumour angiogenesis and growth by changing expression of an angiogenic factor, Vascular Endothelial Growth Factor. Tumour cell compaction is involved in the resistance to chemotherapy in ovarian and breast cancer, and to radiotherapy in colon cancer. Here, we show that the modification of physical cell compaction, which changes the tumour microenvironment, is able to improve the response and to decrease resistance to radiotherapy in breast cancer. To investigate whether mechanical compression of breast cancer cells alters their response to irradiation, we first examined the effects of irradiation on 4T1 breast cancer cells in vitro. Irradiation of 4T1 cells causes DNA fragmentation 4 h after irradiation, which is partially recovered 24 h later. The expression of Platelet Derived Growth Factor-b (PDGF-b) decreases in the cells 4 h post-irradiation, while this decrease is attenuated 24 h after irradiation, suggesting that PDGF-b may mediate this recovery from radiation-induced DNA damage by increasing resistance. We then explored whether mechanical compression and subsequent changes in ECM structure contribute to these effects by plating breast cancer cells at different densities or by compressing tumour tissues in vitro. Tumour compression inhibits DNA fragmentation in vitro by stimulating a rise in PDGF-b expression, and treating the cells with a PDGF inhibitor hinders this resistance. These findings suggest that manipulation of mechanical forces such as tumour cell compaction could improve tumour response to radiotherapy through PDGF-b signalling.
Key Terms

BAPN - β-aminopropionitrile
BME – β-mercaptoethanol
BRCA – Breast Cancer Susceptibility Gene
cDNA – complementary Deoxyribonucleic Acid
DEPC – Diethylpyrocarbonate
DSB – Double Strand Breaks
ELISA – Enzyme-Linked Immunosorbent Assay
EtOH – Ethanol
FBS – Foetal Bovine Serum
FCS – Foetal Calf Serum
H&E – Haematoxylin and Eosin
LOX – Lysyl Oxidase
MAPK – Mitogen Activated Protein Kinase
PBS – Phosphate Buffer Solution
qRT-PCR – Quantitative Reverse Transcriptase Polymerase Chain Reaction
PDMS – Polydimethylsiloxane
PFA – Paraformaldehyde
p53BP1 – p53-binding protein 1
RIPA – Radio-Immunoprecipitation Assay
RNA – Ribonucleic Acid
SEM – Standard Error of Mean
VEGF – Vascular Endothelial Growth Factor
Introduction

Breast Cancer Epidemiology

After 2008, The International Agency for Research on Cancer (IARC) released data as part of their GLOBOCAN project showing that the most common cancer amongst women worldwide was Breast Cancer. Their research estimated that 1.4 million new Breast Cancer cases were identified solely in the year 2008; contrary to the notion that Breast Cancer is a disease of the Western developed world, they recorded a surprising 50% of these in developing nations, making Breast Cancer one of the main causes of cancer death in these countries (American Cancer Society 2011) ¹. In a 2013 Press Release, The World Health Organization (WHO) published data showing the rise in incidence of the disease by more than 20% since 2008; the year 2012 produced about 1.7 million more breast cancer diagnoses in women, making this cancer a quarter of all cancers in women worldwide, as well as the most commonly diagnosed cancer amongst females in 140 of 184 countries. In the United States specifically, slightly less than 30% of all female cancer cases are Breast Cancer cases making the disease the second most frequently diagnosed cancer among women, after skin cancer². In fact, it is estimated that about 12.5% of all women in the United States will develop invasive Breast Cancer during their lifetimes².

The IARC has recorded an increase of 14% in mortality rates since 2008, positioning breast cancer as the leading cause of death by cancer among the female sex. In the United States, Breast Cancer has one of the highest cancer mortality rates in women, second to only Lung Cancer. According to the 2013 WHO Press Release, the developed world still records higher incidence rates than the developing world, while mortality in the less developed nations is relatively greater.
than that in the Western World. This rise in cancer mortality and incidence rates is the joint result of lifestyle changes and the inability to access clinical advances from these parts of the world.

The financial burden of cancer in the United States was predicted to reach $124.5 billion in 2010\(^3\). Of this sum, the cost for Breast Cancer was estimated to amass up to $16.5 billion, the highest compared to all other cancers\(^4\). It is projected that, due to a population increase, total cost of cancer care would rise to $157.77 billion by the year 2020, if all else, including incidence and survival rates and costs of care, remain the same; this would also result in a rise in breast cancer costs\(^3\). Furthermore, the lifetime cost per patient for treatment of breast cancer in the United States is estimated to lie between $20,000 and $100,000\(^5\), placing a huge burden on affected women and their families.

**Existing Treatments**

There are a number of different measures that are currently being used as treatment for breast cancer. The most invasive treatment yet is surgery. Breast cancer patients can either undergo a Mastectomy, which involves removal of all breast tissue, or a Lumpectomy, which is the removal of the tumour itself plus some surrounding tissue. Unfortunately, surgery comes with its risks; along with the many problems that could arise during the actual procedure including risk of death of the patient, although rare, surgery does not provide 100% certainty of full recovery. The Lumpectomy is known to have a slightly higher rate of breast cancer recurrence after treatment than the Mastectomy\(^6\).
Chemotherapy is another common choice of therapy for many cancers. Medication is administered, which works to weaken and kill the cancerous cells at the site of the tumour and any strays around the body. Many of the cytotoxic drugs used inhibit the process of mitosis in the cell cycle; this is achieved in many ways, including by binding DNA and preventing the cellular machinery from participating in cell division. Chemotherapeutic drugs are often alkylated and thus, are able to use their alkyl groups to form covalent bonds with the DNA. When the cell tries to repair the DNA, breaks in the DNA strands can form leading to apoptosis, a form of programmed cell death. Often times, chemotherapy is strategically employed before surgery to decrease tumour size and/or after surgery to prevent recurrence or metastasis of the disease. Although some can be up-taken orally, most chemotherapeutic drugs are administered intravenously at a high enough dosage for treatment to be effective. At this point, they target cells that are rapidly dividing via the bloodstream. However, this poses a problem for the progression of other essential and rapidly proliferating cells like blood cells and hair cells as, largely, the medication is not specific to the carcinogenic cells. This can often result in hair loss, a low blood cell count, which can lead to memory loss, and a loss of bone density, leading to osteoporosis. A disruption in the proliferation of white and red blood cells and platelets could also result in the weakening or depression of the immune system, which could lead to Typhlitis, a deadly gastro-intestinal impediment caused by chemotherapy. Chemotherapeutic drugs could also cause gonadal dysfunction, which might manifest as Premature Ovarian Failure (POF) or even infertility. In addition, chemotherapy is not 100% effective and may not always kill all the malignant cancer cells.
Patients commonly undergo radiotherapy, the use of high-energy ionizing radiation to kill malignant cells, as another treatment option. The radiation damages the DNA by ionizing the atoms that comprise the DNA with photons, protons or other charged particles, either directly or indirectly. This causes both single- and double-strand breaks (DSBs) in the DNA molecule’s sugar-phosphate backbone as well as cross-links between DNA strands and chromosomal proteins. Indirect ionization works by ionizing water with electromagnetic radiation (EMR) to produce transient free hydroxyl radicals that subsequently affect the DNA. Radiotherapy is often used in conjunction with chemotherapy. Unlike chemotherapy, radiotherapy is regarded as highly targeted and is also often used after surgery to prevent recurrence. Like the other treatments, radiotherapy comes with its side effects, including but not limited to breast tissue shrinkage, breast oedema and radiation fibrosis. It can also result in hair loss, fatigue and damage to the skin (radiation dermatitis) due to breakdown of epithelial cells around the region of treatment. Although this may be less common, patients may end up with heart problems after a few years.

Hormone therapy is yet another treatment option for breast cancer. Exposure to oestrogen is known to be connected to the development and progression of breast cancer. Thus, hormone therapy decreases oestrogen secretion in the body by working as either a competitive inhibitor (e.g., Tamoxifen) or an inhibitor of aromatase, an enzyme that works to convert androgens to oestrogens. However, this form of treatment also carries the risk of women developing vaginal bleeding, diarrhoea, thromboembolisms and arthralgia, amongst other conditions.
In addition to the side-effects listed above, these forms of therapy often face resistance \(^\text{21}\). This poses a serious problem for all kinds of cancer treatment as it effectively counteracts the work done to kill the cells and prevent/hinder tumour growth. An increase in resistance of the carcinogenic cells results in the death of a smaller number of these cells and the survival of a larger number upon treatment and thus, continued tumour progression. This leads to a considerable decrease in the efficiency of the previously mentioned therapeutic measures.

These issues with current treatments, paired with the pain and discomfort of the disease as well as the financial costs patients are continuously burdened with, are all reasons for the urgent need for improvements in treatment.

**Mechanobiology**

It is becoming increasingly recognized that in addition to chemical signalling, mechanical forces play equally important roles in breast cancer progression \(^\text{22,23}\). Mechanobiology is a growing field that uses engineering to affect biology and biological functions. It involves the application and manipulation of mechanical cues including physical forces, changes in extracellular matrix (ECM) mechanics and alterations in cell shape, to alter cell and tissue structure and processes \(^\text{23}\). For example, in drosophila, applying mechanical forces to the embryos modifies the embryonic shape and thereby regulates the transcriptional activities of the transcription factor Twist and subsequent gene expression, effectively controlling pattern formation during morphogenesis \(^\text{24,25}\). During Drosophila development, these mechanical cues alter other developmental processes like cell proliferation, tissue growth and organ size, and assist in determining tissue boundaries and organ-specific cell fates \(^\text{23}\). Furthermore, there is a lot of evidence showing the different
fundamental roles these physical factors play in sustaining physiological function of organs in the adult body 23.

Some mechanical/physical forces that can be manipulated are stiffness, shear stress and cellular compression. In mechanobiology, shear stress is the frictional force of fluid flow on the surface of cells, running parallel to said surface; among other roles, it plays an important part in cell determination in the systemic circuit of the cardiovascular system 23. Compressional forces often lead to an increase in physical compaction, resulting in the packing together of the cells under duress; this may sometimes be accompanied by alterations in the physical appearance of the cells 26. This last mechanical factor, compression, will be the focus of this project.

**Compression in Organ Formation**

The Ingber Lab recently experimented and found the role of mesenchymal condensation in organ development in mice. Mesenchymal condensation involves the crowding together of mesenchymal cells, such that they undergo cell differentiation in the different organs; this alters the physical nature of the cells, which experience a paired decrease in cell size and increase in cell density, which in turn modifies the mechanical and chemical signalling that controls the progression of the cells, affecting organogenesis 26. With this knowledge, an experiment studying mesenchymal condensation in mice teeth was conducted. It was shown that gradients of antagonistic forces in the dental mesenchyme resulting from two opposing chemical factors, namely Fibroblast Growth Factor 8 and Semaphorin 3f, result in a conversion of cells leading to an increase in cell compaction in the tooth. Subsequently, this rise in mesenchymal condensation kicks off a series of mechanisms necessary for tooth development, beginning with the expression of organ-specific transcription factors, including Pax9 and Msx1 27 (Figure 1).
Figure 1 Mechanochemical control of mesenchymal condensation during organ formation. Retrieved from Mammoto, Mammoto et al. (2013). (a) Alterations in tensional forces generated by the actomyosin system cause (b) changes in the extracellular matrix (ECM) structure and mechanics. This leads to the upregulation of Pax9 and other odontogenic markers in dental mesenchyme tissue.
This concept of compaction was also applied to an experiment analysing the progression of a brain tumour known as Glioblastoma Multiforme because rapidly growing tumour cells are also compacted in confined spaces. The research was aimed at determining whether cell compaction controlled tumour angiogenesis and if inhibition of said cell compaction would then lead to the inhibition of tumour angiogenesis and progression. In particular, by implanting glioblastoma cells in orthotopic mouse brain tumour models, it has been demonstrated that the drugs β-aminopropionitrile (BAPN) and D-penicillamine, which inhibit the collagen crosslinking enzyme lysyl oxidase (LOX) and modulate collagen structures, attenuate the progression of tumour growth (Figure 2). Thus, disruption in collagen structure and inhibition of tumour cell compaction decrease tumour angiogenesis, thereby hindering Glioblastoma Multiforme growth.
Figure 2 Effect of cell compaction and/or BAPN drug on brain tumour progression. (a) Heightened development of blood vessels in tumour following cell compaction. Retrieved from Mammoto et al. (2013). (b) Histology showing decrease in tumour size after BAPN treatment. Retrieved from Mammoto et al. (2013). (c) Comparison of sizes of whole control tumours and treated tumours. Retrieved from Mammoto et al. (2013). (d) Quantification of effect of BAPN on tumour size. Retrieved from Mammoto et al. (2013).
Resistance due to Cell Compaction

As previously mentioned, one problem current cancer treatment faces is resistance to the various treatments. Importantly, it has been reported that tumour cell compaction raises resistance to chemotherapy and radiotherapy in various tumour types. For example, advanced endometrial cancer regularly displays resistance to chemotherapy, hindering the apoptosis of more compact multicellular endometrial cancer structures. More compact colon cancer cells also exhibit an increased resistance to ionizing radiation.

Experimental Hypothesis

With knowledge that cancerous cells are often resistant to radiotherapy, our project sought to find the best means to efficiently inhibit tumour growth and prevent a potential rise in resistance without having to increase the power of the radiation. The goal of this project is to identify the role of cell compaction in breast cancer progression and to analyse its effects on resistance to radiation therapy, in order to improve breast cancer treatment. We hypothesized that inhibition of cell compaction should reduce resistance to radiation therapy and hence enhance the effect of radiation therapy on breast cancer tumours. If this study proves the hypothesis, the possibility of reducing radiation dosage in order to reduce some of the afore-mentioned side effects could potentially be addressed. We analysed the effects of cell compaction on the effect of irradiation on 4T1 breast cancer cells by culturing at different densities or applying compression in vitro, and by using a mouse orthotopic mammary tumour model in vivo.
Materials and Methods

Cell Culture

We chose the 4T1 breast cancer cell line because the Ingber lab has already established the methods and accumulated the data regarding breast cancer progression, mechanobiology and nanotherapy for breast cancer using this cell line. The 4T1 cells were cultured with 500ml of RPMI 1640 Medium mixed with 5 ml of Glutamax, 50 ml FBS and 1 vial of Gentamicin/Amphotericin antibiotics.

To passage the cells, the old media were aspirated from the 150mm-diametre dish and washed with 15 ml of PBS (or 10 ml in the 100mm dish). The PBS was aspirated and 2ml of Trypsin (or 1 ml in the 100mm dish) was added to detach the cells from the dish. The dish was tapped and/or placed in the incubator to aid detachment. After about 5 minutes when all the cells had been detached from the base of the dish, 10 ml of medium (or 7 ml in the 100mm) was added to the dish and pipetted up and down to detach any remaining cells. The suspension was transferred to a 50ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes to collect the cells at the bottom of the tube. The supernatant was aspirated and the cell pellet was resuspended in a volume of medium dependent on the number of the cells it contained and pipetted up and down to break apart the pellet. In order to count the number of cells/calculate the cell concentration, 10 µl of the cell suspension and 10 µl of Bio-Rad Trypan blue dye were mixed, and 10 µl of the mixture was placed on cell counter slides and inserted into the Bio-Rad Automated Cell Counter to be analysed. According to the cell concentration, a determined volume of the cell suspension was then plated onto each of the dishes and subsequently topped up with medium.
Microcontact printing system

10 g of the base Silicone Elastomer (PDMS) and 1 g of Curing Agent were mixed and then centrifuged at 14000 rpm for 5 minutes to remove visible bubbles in the reagent. Dust on the glass coverslips was air-blown off and each coverslip was set in the Spin coating machine. Approximately a drop of PDMS was added on top of the coverslip. Using the machine, the coverslips were uniformly coated with PDMS and the PDMS was later cured at 60°C for 16 h. Micro patterned stamps were coated with 500 μl of the Fibronection (FN) mixture (45.45 μg/ml in nuclease-free water) for 1 h at room temperature. The PDMS coated coverslips were cut into small squares (2 cm x 2 cm) and placed in the large dish with the coated side up. The surfaces of the PDMS coverslips were subsequently activated in a Plasma Oxidizer Machine for approximately 15 minutes. The micro patterned stamps were air-blown and dried and placed on the surface of the PDMS coated coverslips for 1 min at room temperature. After printing the patterned FN mixture, the coverslips were placed in 35 mm diameter dishes with the patterned side up. The non-specific binding site was blocked by soaking the coverslips in 2 ml of 1% Pluronic acid for at least 16 h at 4°C. The coverslips were then washed with Ca/Mg free PBS extensively and a cell suspension of 1.5 x 10^5 cells/cm^2 was applied on the dish.

Tissue compression

A mechanical compressor resting on a supporting wire mesh consisting of a base and a piston was put together with a bendable, gas-permeable PDMS elastomer membrane attached to the base such that cell pellets could be compressed meticulously. The cell pellet was placed
between the PDMS base and the piston, and a weight of 30g was placed atop the piston, applying a constant pressure of 1 kPa on the pellet for 16 hours\textsuperscript{26} (Figure 3).

![Mechanical compression](image)

**Figure 3** Mechanical compressor designed for tissue compaction. Retrieved from Mammoto et al. (2013).

Irradiation of 4T1 cells

The 4T1 cells were radiated at 8 Gy in the Gammacell 40 Exactor Low Dose-Rate Research Irradiator. After 4 hours and/or 24 hours, cells were characterized using immunocytochemical or molecular biological (qRT-PCR) analysis (below in detail).

Immunocytochemistry

The irradiated and control (unirradiated) cells were fixed with 4% PFA 4 hours and/or 24 hours post-radiation, in order to detect DNA damage by radiation.
Visualizing DNA fragmentation

A chromatin-associated binding protein, p53BP1, has been shown to play a major role in DNA damage response \(^{30}\). When cells are hit with ionizing radiation, double strand breaks (DSBs) develop in their DNA. After irradiation, some proteins are recruited to these DSBs resulting in the formation of subnuclear structure termed foci \(^{31}\). One of these proteins is p53BP1, which, originally confined in the nuclei in normal cells, relocates to these discrete foci structures in response to radiotherapy \(^{30}\). For example, DNA fragmentation was clearly detected by staining Hela cells (human uterine cancer cells) with p53BP1 antibody (Figure 4).

![Figure 4](image)

**Figure 4** Effect of increasing radiation strength on DNA fragmentation detected by staining with p53BP1 in Hela cells. Adapted from Rappold, Iwabuchi et al. (2001).

As such, we used p53BP1 to detect DNA damage by irradiation in 4T1 cells. After aspirating the media we washed cells with PBS one time and fixed the cells with 4% PFA for 20 minutes, after which they are aspirated and then re-washed with PBS. The coverslips were treated for 5 minutes with 0.3% Trx (TritonX-100), a detergent used to permeabilize the cell membrane, and then re-
washed. Afterwards, they were covered in 10% FCS/PBS, a process called ‘blocking’, and then kept for about an hour at room temperature or till the next day at 4°C.

After blocking, the cells were stained with the primary antibody detecting p53BP1 by mixing with blocking buffer (10% FCS/PBS) in a 1 µl: 100 µl ratio, and 100 µl of the antibody solution was added onto each coverslip, coating their entire surfaces. To avoid them drying out, the coverslips were covered with parafilm and then left for about an hour at room temperature, after which they were washed three times over with PBS. The secondary antibody (488 rabbit green) was mixed with blocking buffer in the same ratio as the primary antibody, and the mixture was added onto the coverslips, which were then covered with parafilm. The dishes were covered with aluminium foil to keep out the light and left at room temperature for 45 minutes. Again, the coverslips were washed with PBS three times. Slides were obtained and a drop of Vector Shield Mounting Medium was placed on each to ensure the coverslips stuck to the slides. Each coverslip was placed face down on a separate slide and stored in a slide tray at 4°C. Confocal Imaging was performed using the Leica SP confocal microscope.

**Immunohistochemistry**

Histology was performed on the tumours in order to compare the radiated and control samples in both compressed and uncompressed tumours. The radiated and control compressed and uncompressed tumour samples were transferred to 4% PFA to fix the tumour tissue and stored at 4°C overnight. Afterwards, the PFA was suctioned and replaced with 30% sucrose buffer in order to stabilize the fixed tissue overnight. The next day, the sucrose was sucked up and the tumours were covered with OCT compound in order to get them to the right consistency. After a
few minutes of waiting to ensure that there were no bubbles, the molds were filled with OCT and the tumours were transferred to them and aligned vertically for optimal cross-sectioning, a process referred to as Embedding. The molds were placed on dry ice to freeze and kept in the -80°C freezer until they were used.

Molecular biological assay

To collect RNA from cells, we used the RNeasy mini kit (QIAGEN). A mixture of RLT and BME (2-mercaptoethanol) was made in the ratio 1 ml to 10 µl of BME. 70% EtOH was prepared with DEPC water, and RW washing buffer and RPE buffer were set out for the experiment. Filter tips were utilized during this experiment. When collecting RNA from a 6-well plate, 350 µl of RLT with BME was added to each sample; the wells were scraped to ensure all cells were collected, before the mixtures were pipetted up and down and transferred to a 1.5 ml tube. Alternatively, in collecting RNA from microcontact printing patterns, the cells were initially obtained from the patterns with Trypsin and after collecting the cell pellets, 350 µl of RLT with BME was added to each sample and the mixtures were transferred to 1.5 ml tubes. Sterile 1 ml syringes with 22G needles were used to homogenize the samples, drawing and injecting out the samples 7 to 10 times each. 350 µl of 70% EtOH was added to each sample, pipetted up and down about 4 times and applied to the RNeasy spin column, after which the samples were centrifuged at 10000 rpm for 30 seconds and their flow-through discarded. 700 µl of RW buffer was added to each column, which were subsequently centrifuged again at 10000 rpm for 30 seconds to wash the membrane and the flow-through discarded. 500 µl of RPE buffer was added to the columns, which were centrifuged yet again at 10000 rpm for 30 seconds. After the flow-through was discarded, another 500 µl of RPE buffer was added and to dry them completely, the
columns were span for 2 minutes at the same speed. Afterwards, the spin columns were placed in new 1.5 ml collection tubes and 45 µl of Nuclease-free water was added to each and subsequently, the columns were span at 10000 rpm for 1 minute. The flow-through was then stored in the -20°C fridge.

In order to analyse specific genes, qRT-PCR was performed. After measuring the RNA concentrations, about 10 µg of each sample was mixed with 4 µl of Reaction Mixture, 1 µl of Reverse Transcriptase Enzyme and 4 µl of water (to reach a total of 10 µl) in order to convert RNA to cDNA. After the reverse transcriptase process was completed, some water, the Forward and Reverse Primers of the gene to be amplified, and the Master Mix, which is used as a buffer for running PCR, were pipetted into the plate, with all volumes summing up to about 23 µl. 2 µl of the different cDNAs were then pipetted into different wells. Cyclophilin, a housekeeping gene, was used to normalise the results. The plate was sealed and span at 1000 rpm for 10 seconds, before being run in the Bio-Rad real-time PCR machine.

Biochemical assay

About 1 ml of conditioning media was collected from each dish, centrifuged at 4°C at 10000 rpm for 5 minutes and the supernatant was collected in new 1.5 ml tubes and stored in the -80°C fridge.

In order to detect and quantify the PDGF-bb present in the samples, ELISA tests specific to this antigen were performed according to the protocol provided by R&D systems. A pre-coated 96 well microplate containing a capture antibody for PDGF-bb was provided in the kit. 50 µl of
Assay Diluent was pipetted into each well, followed by an equal volume of the provided Standard, Control or our sample, and these were mixed by tapping for 1 minute. The microplate was covered with an adhesive strip and left to sit at room temperature for 2 hours, while the monoclonal antibody bound to any PDGF-bb present. Afterwards, the wells were washed with 400 $\mu$l of Wash Buffer 5 times, and 100 $\mu$l of Mouse/Rat PDGF-bb Conjugate, an enzyme-linked antibody, was subsequently added to each well. Again, the microplate was covered with adhesive and incubated at room temperature for 2 hours. The wells were washed as before to get rid of any enzyme-linked antibodies that remained unbounded. 100 $\mu$l of Substrate Solution was pipetted into each well and covered with aluminium foil to protect from any light radiation. After 30 minutes at room temperature, 100 $\mu$l of Stop Solution was added to each well, which turned the product yellow after mixing. The well plate was inserted into the Bio-Rad xMark microplate spectrophotometer, which was set to 540 nm or 570 nm for this procedure. Colour intensity, which is proportionate to the amount of PDGF-bb present that had been bound in the first step, was measured by the plate reader and values were analysed using the standard curve\textsuperscript{32}.

\textit{In vivo} animal study

4T1 cells were harvested from the dishes, resuspended in PBS, and the mixture was injected using a 23-gauge needle into the fat pads surrounding the inguinal mammary glands of the mice. Once the tumours reached about 50 mm\textsuperscript{3} in volume, we treated the mice with irradiation.

In order to irradiate solely the injected region of the mice, the mice were positioned in a lead block such that the inguinal region was exposed to radiation and the rest of the body was
shielded from radiation (Figure 5). The mice were anesthetized with ketamine/xyladine and then radiated in the Gammasell 40 Exactor Low Dose-Rate Research Irradiator at 5 Gy at a rate of 1.03 Gy/min for slightly less than 4.85 minutes. The mice were then returned to their cages. To treat the mice with BAPN, a new supply of water containing BAPN at 3 mg/kg of mouse body weight was inserted into the cage.

Figure 5 Setup for Irradiation of Mice. The mouse in the lead block was positioned such that most of its body was shielded from the irradiation.

Tumour size was monitored every other day. 15 days later, the tumours were harvested and the samples were prepared for mRNA extraction, immunohistochemistry, and protein analysis as described above.

Statistical analysis

The unpaired T-test after ANOVA was utilized to analyse statistical significance using GraphPad Prism V5.0. The $p$ values and the error bars (SEM) were derived from the data.
Results

Radiation induces DNA fragmentation in 4T1 breast cancer cells

Our initial experiment aimed at determining if radiation causes DNA fragmentation in 4T1 breast cancer cells. The cells were irradiated (8 Gy), stained for p53BP1, and detect DNA fragmentation using confocal microscopy. We found an increase in the spottiness of p53BP1 4 hours after irradiation of the cells as compared to the control untreated cells, suggesting that radiation causes DNA fragmentation in 4T1 cells. However, 24 hours post-radiation, there was a significant decrease in this spottiness implying some sort of cell recovery had occurred; yet, the amount of fragmented cells observed in the irradiated sample was still significantly higher than that of the control (Figure 6a,b).

Platelet Derived Growth Factor (PDGF), one of the major angiogenic factors, has been shown to restrict DNA fragmentation of serum-deprived v-Haras-transformed cells \(^{33}\) by decreasing the rate of DNA fragmentation \(^{34}\). Consequently, we next examined whether PDGF-b mediates radiation-induced DNA fragmentation in the 4T1 cell-line. The ELISA test on the conditioned media collected from the irradiated 4T1 cells revealed that there was a decrease in PDGF-b expression compared to the control 4 hours after irradiation, while after 24 hours, PDGF-b expression increased by 25% compared to the control non-irradiated cells; this suggests that PDGF-b mediates radiation-induced DNA fragmentation, resulting in a decrease in spottiness of p53BP1 expression (Figure 6c).
Figure 6 Effect of radiation on 4T1 cells. (a) Increase in DNA fragmentation with radiation 4 hours post-radiation and a subsequent decrease in fragmentation 24 hours post-radiation. (b) Quantification of increase in fragmentation with radiation 4 hours-post radiation and subsequent decrease in fragmentation 24 hours post-radiation. (c) Graph showing decrease in PDGF expression with radiation 4 hours post-radiation followed by a rise in PDGF expression after 24 hours with cell recovery in the Conditioning Media.

*P < 0.05. Error bars represent SEM.
Effect of irradiation and a PDGF-b inhibitor on DNA fragmentation in 4T1 cells

In order to confirm whether PDGF-b mediates radiation-induced DNA fragmentation in 4T1 cells, we treated 4T1 breast cancer cells with a PDGF-b inhibitor in conjunction with the radiation. 24 hours after radiation, although the PDGF-b inhibitor did not have effects on DNA fragmentation in the non-irradiated cells, it increased the number of the cells with p53BP1 fragmentation as compared to the purely irradiated-sample (Figure 7).

Figure 7 Effect of radiation and a PDGF inhibitor on 4T1 cells. (a) Greater DNA fragmentation 24 hours after irradiation when radiation is used in conjunction with the PDGF-inhibitor as compared to control. (b) Quantification of greater fragmentation with PDGF-inhibitor 24 hours after radiation. *P < 0.05. Error bars represent SEM.
The effect of cell densities on DNA fragmentation induced by irradiation

We then examined whether 4T1 cells plated at different densities respond differently to radiation. We plated 4T1 breast cancer cells at low-density (0.2 x 10^5 cells/cm^2) and high-density (2.4 x 10^5 cells/cm^2). DNA fragmentation 24 hours after radiation detected by p53BP1 staining was significantly inhibited when 4T1 cells were plated at high density compared to when the cells were plated at a low density (Figure 8a). Given that angiogenic factor expression is controlled by cell compaction \(^2\) and that irradiation decreases PDGF-b expression, we set out to examine whether PDGF-b expression mediates compaction-dependent radiation-induced DNA fragmentation. Compared to their corresponding unirradiated samples, PDGF-b mRNA expression 24 hours after irradiation was 40% lower in the 4T1 cells plated at a low density, while it was 1.8 times higher in the cells plated at high-density, suggesting that the increase in cell density induces PDGF-b expression 24 hours after radiation (Figure 8b). This provides support for our previous conclusion, showing increases in PDGF-b expression with a decrease in DNA fragmentation in the cells plated at a high-density, and decreases in PDGF-b expression with an increase in DNA fragmentation in the cells plated at a low-density.
Figure 8 Effect of radiation on different densities of 4T1 cells. (a) Images showing an increase in DNA fragmentation in the low density sample and a decrease in DNA fragmentation in the high density sample as compared to their controls, 24 hours post-radiation. (b) Graph showing different responses of PDGF expression to radiation 24 hours post-radiation due to difference in cell densities. The 4T1 cells at low density experienced a decrease in PDGF expression, whereas the 4T1 cells at high density experienced an increase in PDGF expression. *P < 0.05. Error bars represent SEM.
The effect of tissue compression on PDGF-b expression after irradiation

To further confirm this relationship between compaction and radioresistance, we compressed the breast tumour tissues collected from orthotopically implanted 4T1 cells into the mouse mammary fat-pad for 5 days, using our unique compressing system for 16 hours (Figure 3). We compressed the extracted tumour tissues harvested from mice, and subsequently irradiated them. H&E staining of the compressed sample exhibited a 1.25 times higher cell density than our uncompressed sample (Figure 9a, b). We then examined whether the effects of radiation are different between compressed and uncompressed tissues. The ELISA test on these tumour tissue samples revealed that the compressed uniradiated tissue exhibited about 30% higher protein expression levels of PDGF-b than the control. Consistent with the results from the cells plated on high or low density, irradiation decreased PDGF-b expression in the uncompressed tumour tissue, while PDGF-b expression was about 1.23 times higher with radiation in the compressed sample (Figure 9c). These results suggest that cell compaction increases PDGF-b expression, and subsequently attenuates radiation-induced DNA fragmentation.
Figure 9 Effect of compression on radiation response. (a) H&E staining image showing effects of compression on cell compaction (b) Quantification of the effect of compression on cell compaction. (c) Graph from an ELISA test showing the combined effects of compression and radiation on PDGF expression. Error bars represent SEM.
Since cell compaction increases PDGF-b expression and inhibits radiation-induced DNA fragmentation, we next examined whether inhibition of cell compaction restores the effects of irradiation. To explore this possibility, we treated breast cancer tumours harvested from mice with BAPN, which inhibits collagen crosslinking enzyme Lysyl Oxidase and suppresses tumour cell compaction, and then compressed the tumour tissue as described above. BAPN treatment decreased the expression of PDGF-b induced by irradiation (Figure 9), further confirming the role of tumour cell compaction in the effects of irradiation in breast cancer.

**Figure 10 Effect of radiation, compression and BAPN on PDGF expression.** ELISA test showing the decrease in PDGF-b expression and radioresistance in the presence of BAPN. Error bars represent SEM.
The effect of BAPN and irradiation on tumour growth in mouse tumour model *in vivo*

To examine whether tumour cell compaction also affects the effects of irradiation in a mouse breast cancer model *in vivo*, we performed mammary fat-pad injections of 4T1 breast cancer cells into BalbC mice, irradiated the mice at a power of 5 Gy when the tumours reached the size of 50 mm$^3$, and treated the mice with BAPN (3mg/kg in drinking water) for 14 days. To analyse tumour progression, we recorded and compared tumour sizes in each group. When we averaged volumes of the mouse tumours (length x breadth x height) over this period, tumours that had been irradiated and/or treated with BAPN were significantly smaller compared to control untreated tumours (Figure 11). Based on the *in vitro* results, we expected a greater effect on tumour size in the mice treated with both BAPN and irradiation, however we did not observe any significant signs of the synergy. Since we started BAPN treatment after irradiation and BAPN may take more time to remodel tumour ECM structures, we are planning to pre-treat mice with BAPN before irradiation and examine the effects on breast cancer growth as a next experiment.
Figure 11 Combined effect of BAPN and irradiation on tumour progression. Line graph showing progression of tumours upon different treatments and showing lack of expected synergistic effects upon combination of BAPN and irradiation.
**Discussion**

Recently, it has been realised that cancer progression can be manipulated, not solely by affecting the gene or using chemical signalling, but also by applying and altering mechanical cues within the tumour microenvironment. In our experiment, we focused on extending this knowledge of mechanobiology to breast cancer progression in order to improve tumour response to radiation therapy. Our *in vitro* experiment began with determining that radiation results in DNA fragmentation by decreasing PDGF-b expression in 4T1 breast cancer cells. After doing so, we experimented with changing cell density and compressing tumour tissues by way of mechanical compression, concluding that tumour cell compaction decreases DNA fragmentation by increasing PDGF-b expression, and induces a rise in resistance to radiation. In order to improve cellular response to radiotherapy, we treated the breast tumour tissues with BAPN to decrease collagen-crosslinking. We were able to counteract the effects of cell compaction with the BAPN treatment, decreasing PDGF-b expression in the tumour tissue. With the knowledge that BAPN was successful in hindering PDGF-b expression *in vitro*, we decided to experiment directly with mice *in vivo*. We found that irradiation resulted in a decrease in the rate of tumour growth. We also found that BAPN-treated mice (without radiation) exhibited a decrease in tumour progression. However, we didn’t observe the synergistic decrease in tumour growth we had expected would occur after combining the two treatments.

Our results showed us that our current conditions were not producing synergistic effects in mice *in vivo*; the combined effect of BAPN and irradiation at a power of 5 Gy was not that much different from their individual effects, contrary to what we had expected. In order to amplify their collective effect relative to their discrete effects, we are planning on altering our experiment
in several ways. We plan on using a higher concentration of BAPN in treatment and pairing this with irradiation. We also plan on repeating the experiment with the addition of another factor, a PDGF inhibitor, with the hopes that manipulating these factors—a higher concentration of BAPN, the PDGF inhibitor and irradiation—would further improve the effects of irradiation. Another element we would like to experiment with is the timing of our initial BAPN treatment. In prior experiments we treated the mice with BAPN after irradiation. However, it takes some time for the BAPN to modulate the ECM structure. As such, we plan on modifying this by treating the mice with BAPN before irradiation to analyse how timing would affect tumour progression.

Breast tumours also progress by the expression of many other angiogenic factors and growth factors apart from PDGF-b. In upcoming experiments, we wish to observe how various approaches we performed in this study affect the expression of other growth or angiogenic factors, including VEGF. Our initial step would involve utilizing radiation to inhibit these growth factors, much like we did with PDGF-b. Prior research has demonstrated that radiation might actually enhance VEGF expression in Glioblastoma Multiforme (GBM)\textsuperscript{35}. Radiation has also been shown to induce Mitogen Activated Protein Kinase (MAPK) signalling, which, in addition to phosphorylation, works to prevent the fragmentation of cells by radiation and to up-regulate expression of VEGF and other growth factors\textsuperscript{36}. By manipulating our dosage of radiation, we could potentially attempt to block VEGF expression to decrease tumour angiogenesis and improve treatment\textsuperscript{37}. Our experiment would thus involve testing the effect of radiation on these other growth factors in 4T1 breast cancer cells and seeing how we could
optimize radiation such that we can achieve a decrease in expression of multiple growth factors at once.

Our experiments have shown that compression leads to a greater expression of PDGF-b and subsequently, a weaker tumour response to radiotherapy. We would like to further investigate this compression-induced rise in resistance by examining the effect of compression on some of the other growth factors partaking in tumour growth. Experiments have shown a potential link between compression and an increase in VEGF expression in GBM\textsuperscript{38}. We plan to construct an experiment to test if these results translate to our 4T1 breast cancer cells, and if so, hopefully work to inhibit this compression-induced tumour growth.

It has been known that oxygen-deficient or hypoxic environments result in many problems, which manifest in the recurrence of tumours. Hypoxia has been shown to counteract the cytotoxic effects of irradiation by causing an inhibition of the chemical ionization of intracellular macromolecules and water, \textsuperscript{39} which aids in DNA fragmentation. Hypoxia also stimulates activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1)\textsuperscript{39}, which is critical for tumour progression, regulating important pathways in this process. Thus, with knowledge from prior experimentation of the effect of compression on radiation response, we aim to analyse how a combination of compression and radiation could potentially affect either the tumour microenvironment directly, hopefully resulting in a change from hypoxia to normoxia, or indirectly, by altering HIF 1 gene expression to revert tumour progression.
With all these approaches, we hope to compile more concise and coherent ideas of the effects of compression on radiation response. We wish to improve cellular response to radiation therapy and reduce resistance to the therapy by altering compression to change cell compaction in order to decrease expression of multiple angiogenic/growth factors, not limited to PDGF-b. This reduction in radioresistance would then open up the possibility of further decreasing radiation dosage, such that any possible side effects resulting from the irradiation could be minimized considerably.
References


