November 2017

The Promotion of Adipogenesis in a Rat Model of Radiation-Induced Mammary Fat Pad Fibrosis

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Graduate Program in Surgery

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Abstract

Radiofibrosis of the breast makes satisfactory breast tissue reconstruction challenging and is associated with complications. Autologous fat grafting can improve surgical outcomes but fat retention is variable. We developed RHAMM mimetic peptides (NPI-110) that decrease tissue fibrosis and increase mammary fat pad adipogenesis by promoting the differentiation of mesenchymal progenitor cells into adipocytes. We tested the efficacy of NPI-110 in a rat model of radiation-induced mammary fat pad fibrosis. Effects of radiation and NPI-110 were quantified by visible skin changes, fat pad volume estimates using high frequency ultrasound, mRNA expression of genes involved in fibrosis (Collagen-1:Collagen-1, TGFβ1) and adipogenesis (PPARγ, adiponectin and perilipin) using qPCR, and collagen fibril deposition using picrosirius red staining. NPI-110 significantly reduced skin inflammation and radiofibrosis, the latter assessed by collagen fibril deposition, and increased mRNA expression of adipogenic markers. Results from this study will aid in creating a micro-environment that optimizes fat transplantation success.

Keywords

radiation fibrosis, radiodermatitis, adipogenesis, hyaluronan, RHAMM
Co-Authorship Statement

Chapter 8: Manuscript for “Reducing Radiofibrosis in Rodent Mammary Fat Pads”

All of the laboratory experiments outlined in this manuscript were performed in Dr. Eva Turley’s laboratory. The radiation experiments were performed under the supervision of Dr. Eugene Wong at the London Regional Cancer Program. The ultrasound measurements were performed in Dr. James Lacefield’s facilities at Robarts Research Institute.

Meredith Barr assisted with the animal experiments, immunohistochemistry for the markers of adipogenesis and fibrosis, and analysis of immunohistochemistry and ultrasound volume assessments. Cecilia Dai assisted with the animal experiments, immunohistochemistry for the markers of adipogenesis and fibrosis, and analysis of immunohistochemistry. Thomas Carlos Raissi assisted with the qPCR for the markers of adipogenesis and fibrosis. All of the aforementioned were involved with manuscript preparation.

In terms of the objectives of the current thesis outlined in Chapter 2: Carl Postenka paraffinized and embedded all of the mammary fat pad specimens used in the analysis. Caroline O’Neil and Hao Yin from the Molecular Pathology Core Facility at Robarts Research Institute assisted with immunohistochemical staining of TGFβ1 and Picrosirius Red. Matt Lowerison assisted with the ultrasound experiments on the rats.
Acknowledgements

I am thankful to have had the opportunity to be a part of the Master of Science in Surgery program. Through the stimulating and collaborative environment fostered by Dr. Turley’s lab, I have been able to connect with various scientists at different facilities with different expertise. It has certainly been a very academically stimulating period of time for me.

I am very thankful for the continued guidance and support of my supervisors, Drs. Eva Turley and Arjang Yazdani. Your commitment to and mastery of your fields is truly inspiring, and you both have been a great role models.

To the members of the Turley Lab (Conny, Jenny, Kaustuv, Katelyn, Kitty), thank you for all your help along the way – from teaching me new techniques, to helping me troubleshoot problems.

Thank you, Dr. Francisco Perera, for your expertise and early guidance regarding radiotherapy-induced skin changes. I’d also like to thank Carl Postenka for processing my tissue specimens, Caroline O’Neill and the Pickering Lab for your expertise in immunohistochemistry, and Hao Yin for your help with the polarizing microscope. I’d like to thank Matt Lowerison from the Lacefield Lab: you were integral to the ultrasound portion of my experiments. I’d like to thank Trisha Carter from Animal Facilities and all the other staff involved with the Animal Care Team for all the coordination that had to occur for the success of my animal experiments.

To my medical students Thomas Raissi, Meredith Barr, and Cecilia Dai – a special thanks for responding to my e-mails at all hours of the day. Without you all, this project would not have been possible.

Lastly, Dr. Eugene Wong, your guidance and input has been invaluable to the completion of my Masters as one of my committee members.
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<th>Description</th>
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<tbody>
<tr>
<td>ADIPOQ</td>
<td>Adiponectin</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>ASCs</td>
<td>Adipose derived stem cells</td>
</tr>
<tr>
<td>BFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer gene</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate inducer</td>
</tr>
<tr>
<td>CD34</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine)-enhancer-binding proteins</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CTCAE</td>
<td>Common Toxicity Criteria-Adverse Event</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>D&amp;F</td>
<td>Douglas &amp; Fowler</td>
</tr>
<tr>
<td>ECD</td>
<td>Doppler ultrasound examination</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EN</td>
<td>Homeobox protein engrailed</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid/hyaluronan</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HIV Tat</td>
<td>Human immunodeficiency virus trans-activator of transcription</td>
</tr>
<tr>
<td>HMWHA</td>
<td>High molecular weight HA</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methyl-xantine</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin 1α</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IORT</td>
<td>Intraoperative radiation therapy</td>
</tr>
<tr>
<td>KLF5, 15</td>
<td>Kruppel-like factors 5, 15</td>
</tr>
<tr>
<td>Krox20</td>
<td>EGR2, early growth response protein-2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LYVE1</td>
<td>Lymphatic vessel endothelial hyaluronan receptor 1</td>
</tr>
<tr>
<td>MAPK/ERK</td>
<td>Mitogen-activated protein kinase/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MEK1/ERK1,2</td>
<td>Mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega Hertz</td>
</tr>
<tr>
<td>MicroCT</td>
<td>Micro computer tomography</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
</tr>
<tr>
<td>ONS</td>
<td>Oncology Nursing Society</td>
</tr>
<tr>
<td>PLIN</td>
<td>Perilipin</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3 kinase/AKT</td>
<td>Phosphoinositide 3-kinase/protein kinase B</td>
</tr>
<tr>
<td>Pref-1</td>
<td>Preadipocyte factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>RDS</td>
<td>Radiation Dermatitis Severity</td>
</tr>
<tr>
<td>RHAMM</td>
<td>Receptor of hyaluronan mediated motility</td>
</tr>
<tr>
<td>ROS/NOS</td>
<td>Reactive oxygen species/nitrogen oxide species</td>
</tr>
<tr>
<td>RTOG</td>
<td>Radiation Therapy Oncology Group</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cells antigen-1</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Transforming growth factor 1</td>
</tr>
<tr>
<td>TLR2,4</td>
<td>Toll-like receptor 2, 4</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TRAM</td>
<td>Transverse abdominis myocutaneous</td>
</tr>
<tr>
<td>WBI</td>
<td>Whole breast irradiation</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
1 Introduction

1 in 9 Canadian women will be diagnosed with breast cancer in her lifetime, and 1 in 30 will die from it\(^1\). Approximately 45 to 50% breast cancer patients undergo curative radiotherapy\(^2-4\). Recent data have shown that post-mastectomy radiation therapy reduced the rate of loco-regional recurrence in node-positive patients from 9 to 27\(^%\)\(^5\).

Although there are newer radiotherapy techniques that reduce skin and overall toxicity, acute skin toxicity, chronic inflammation, and tissue fibrosis still occur\(^6,7\). The underlying mechanisms driving radiation-induced chronic inflammation and fibrosis are not well understood, and so there are no effective interventions to prevent or modify the course and severity of this damage\(^3,7-9\).

In the context of plastic surgery, breast reconstruction in the irradiated breast is a challenge. Fat grafting has emerged as a technique that improves contour abnormalities, reduces tissue fibrosis, scar formation, and post-mastectomy pain\(^10\). There is also early evidence that fat grafting improves scar formation following radiation and enhances quality of soft tissue envelope. However, fat take is variable, with the prevailing theory that successful grafting results from the survival and differentiation of pre-adipocyte and other adipose derived multi-potent stem cells (ASCs) in the graft\(^11\).

1.1 Radiation Damage

The use of ionizing radiation is predicated on attempting to achieve lethal effects on tumour cells while sparing normal tissue\(^12\). The majority of breast cancer patients, however, still develop radiation-induced skin toxicity and underlying tissue fibrosis\(^2\).

Radiation injury may be categorized as acute or chronic (late), with acute injury occurring within hours to weeks after radiation exposure, and chronic injury presenting months to years after radiation exposure\(^7-9\). Acute injury primarily involves skin cell death through cellular alterations and inflammation in the epidermis, starting with erythema, edema, pigment changes, and depilation\(^8\). Severe acute injury involves complete loss of epidermis, persistent edema, fibrinous
exudates and disturbance in skin barrier function\textsuperscript{8,13}. The severity of skin reactions thus range from mild erythema and dry desquamation, to moist desquamation and ulceration\textsuperscript{3,14}. Later, chronic effects include chronic inflammation, delayed ulcers, telangiectasias, atrophy, poikilodermatous changes, necrosis, fibrosis and osteoradionecrosis, and are from other as yet poorly defined mechanisms\textsuperscript{2,8}. Factors that affect the severity of reaction include dose fraction, total dose delivered, use of bolus or other beam-modifying devices, site, size of treatment field, chemotherapy and individual factors\textsuperscript{15}.

Newer technologies such as conformal radiation techniques and intensity-modulated radiation therapy (IMRT) have considerably reduced the area of skin toxicity\textsuperscript{7}. However, radiation dermatitis cannot be avoided in certain areas where the skin or superficial tissues are the target, such as breast cancer\textsuperscript{3}. As a result, approximately 74-100\% of breast cancer patients experience radiation dermatitis\textsuperscript{7,16,17}. While the majority of these changes resolve, there is also a trend towards combining chemotherapy with larger fractions of radiation. Chemotherapeutic drugs can induce radiosensitivity, which is more effective in killing tumour cells but a side effect is often severe xerosis, inflammation, skin thinning, and skin necrosis. This condition develops into a dynamically progressing fibrosis with reversible and irreversible components in approximately 35\% of patients, and results in a greater proportion of patients developing a persistent chronic inflammation and localized fat pad fibrosis\textsuperscript{4,10,18,19}. Radiation boosts to the tumour bed using electrons, external-beam megavoltage photons, or interstitial brachytherapy after external whole-breast irradiation (WBI) has been shown to reduce the risk of local recurrence following breast-conserving surgery. However, these and more novel boost methods such as intraoperative radiation therapy (IORT) have also been associated with an increased rate of severe subcutaneous fibrosis, as a higher total radiation dose is delivered\textsuperscript{17}. Although not life threatening, fibrotic breast tissue is challenging to reconstruct and contribute to variability of autologous fat grafting success in these patients.

The underlying mechanisms driving the development of these radiation-associated changes are not well understood, due in part to a lack of suitable pre-clinical models\textsuperscript{8}. As a result of this knowledge deficit, there is currently no effective intervention, by topical or systemic means, to prevent or favorably modify the course and severity of dermatitis\textsuperscript{5,7-9}. Studies and reviews have observed interventions such as “washing with mild soap”, topical corticosteroids, non-steroidal creams,
hyaluronic acid, ascorbic acid, silver leaf dressing, amifostine and oral enzymes for radiation-induced skin reactions\textsuperscript{3,8,9}. Long treatments of Pentoxifylline and tocopherol have reduced radiation-induced fibrosis, but are associated with undesired outcomes (e.g., their cessation results in a “rebound effect”) \textsuperscript{7,20}.

Overall, very few studies have demonstrated statistically significant results of the aforementioned interventions\textsuperscript{3,16}. The development of effective radiation mitigators and protectors, and the elucidation of mechanisms responsible for radiation-associated changes in skin and other normal tissues in comparison to tumours necessary for such a task are thus of interest\textsuperscript{21}. A possible genetic basis for risk has been suggested in a study that had observed clinical heterogeneity in the manifestation of fibrosis-related to radiation-induced dermatitis\textsuperscript{22}. As such, target gene therapy and stem cell therapy combined with surgical excision are new techniques and emerging areas of interest\textsuperscript{8}.

1.1.1 Pathophysiology

There are two discrete types of radiation: ionizing, which has enough energy to change the chemical composition of matter and is what radiotherapy is comprised of; and nonionizing, which has less energy, but can still excite molecules and atoms\textsuperscript{23}.

Radiotherapy interferes with normal processes of epidermal and hair matrix cells, as well as fibroblasts and cutaneous vasculature. Ionizing radiation causes various types of initial tissue injury through two major pathways. First, an oxidative stress and radiolytic hydrolysis process occurs that leads to the production of free radicals resulting in DNA damage and alterations of proteins, lipids, and carbohydrates\textsuperscript{24–26}. Inhibition of normal granulation tissue, angiogenesis, and fibrogenesis occur as a result of this process\textsuperscript{24,26}. Second, there is stimulation of the innate immune system leading to tissue inflammation processes; transcriptionally activated pro-inflammatory cytokines and growth factors result in alteration of cellular proliferation\textsuperscript{24}. For example, Interleukin 1\(\alpha\) (IL-1\(\alpha\)) is a cytokine that initiates the interaction between epithelial and mesenchymal cells, and modulates the synthesis of other pro-inflammatory mediators and proteases in surrounding fibroblasts\textsuperscript{27}. 
Ionizing radiation resulting in free radicals immediately produces damage to the epidermal basal keratinocytes, hair follicle stem cells, endothelial cells, Langerhans cells, and vascular bed through DNA damage\textsuperscript{24,25,28}. These free radicals, primarily reactive oxygen species, also cause fragmentation of extracellular matrix (ECM) proteins such as collagen, laminin, and polysaccharides\textsuperscript{29}. The signals generated from the fragmented HA components then trigger an influx of pro-inflammatory innate immune cells, and together result in aberrant stem cell function and predisposes tissues to a hyper-activation of fibrotic pathways as a compensatory mechanism\textsuperscript{8,30}. These effects may persist and progress, particularly if DNA damage is incompletely repaired. These events altogether contribute to barrier function impairment, bacterial colonization, and superinfection\textsuperscript{24}.

On a cellular level, perivascular inflammatory infiltrate around dilated blood vessels, swelling, growth arrest, and sloughing of epithelial cells is seen in acutely irradiated skin\textsuperscript{24}. Effects depend on the radiation dose, and range from clumping of nuclear chromatin, nuclear swelling, nuclear disfiguration, mitochondrial distortion, to cellular necrosis\textsuperscript{24,31,32}. The physiology behind telangiectasia is unknown, but is thought to be due to microvasculature damage due to inflammation during acute injury, and the excessive production of cytokines and growth factors such as TGFβ\textsubscript{1}, platelet-derived growth factor (PDGF) and fibroblast growth factor by macrophages or damaged endothelial cells\textsuperscript{24,26}.

1.1.2 TGFβ\textsubscript{1}

TGFβ\textsubscript{1} is a peptide and cytokine that has many functions, of which include promoting the development of chronic inflammation, radiation dermatitis, and fibrosis\textsuperscript{33}. TGFβ\textsubscript{1} activates fibroblasts to secrete extracellular matrix protein including collagen\textsuperscript{24,34–36}. Up-regulation of TGFβ\textsubscript{1} is common in the fibrotic tissues of irradiated patients and induction of its expression is a major cellular response to ionizing radiation\textsuperscript{34}. Additionally, TGFβ\textsubscript{1} and other pro-inflammatory, pro-fibrotic cytokines such as interferon, IL-1, IL-2, and tumour necrosis factor α (TNFα) inhibit adipogenesis. Collectively, the sustained presence of these cytokines creates a microenvironment that is hostile to the promotion of adipocytes. This compromises the success of tissue
reconstruction.

Fibrosis in response to growth factors such as TGFβ1 may be focal or widespread, and also contribute to tissue retraction, restriction of movement, and chronic pain that can be difficult to manage\(^3\). Although TGFβ1 is clearly involved with the development of fibrosis, its numerous effects on normal tissue function make it a poor target for therapy.

1.1.3 Effect on skin
Radiodermatitis, or radiation-induced skin reactions, have been recognized since the beginning of the 20\(^{th}\) century, and remains a significant clinical problem with no definitive evidence supporting any one intervention in its prevention or treatment\(^2,24\). While many skin changes are minor and reversible, radiation dermatitis remains a serious side effect, and may limit the duration of treatment, dose delivered, and affect the quality of life of patients\(^6\).

1.1.3.1 Clinical assessment Scales
The ability to evaluate and quantify the severity of radiation-induced skin changes is important from a clinical and research standpoint. This is especially important when evaluating potential therapeutics to reduce such skin changes. There are scales described and regularly available for the evaluation of radiation-induced dermatitis in humans and animals\(^16,23\).

In general, it has been suggested that further research is needed to develop a consensus reaction assessment tool in order to provide management options for higher levels of skin toxicity in sensitive areas\(^16\). A gold standard for clinically rating radiation skin injury does not exist, and individualized skin assessment tool uptake and use is poor\(^23\). However, there are commonly used scoring systems such as the National Institutes of Health Common Toxicity Criteria-Adverse Event (CTCAE) and the Radiation Therapy Oncology Group (RTOG) toxicity scoring system\(^3,12\). Other newer scoring scales include Douglas & Fowler (D&F), Oncology Nursing Society (ONS), and Radiation Dermatitis Severity (RDS) scales\(^8\). It has been suggested that the use of more than one scale should be considered when evaluating interventions to modulate radiation dermatitis due
to the limited evidence for support of these scales\textsuperscript{3}.

The RTOG Acute Radiation Morbidity Scoring Criteria were developed in 1985 as a complement to the Late Effects Scoring Criteria previously developed, and describes a scoring system 0-4 for various organ tissues that may be affected by radiation (Supplemental Figure 1)\textsuperscript{12}. Its original purpose was to allow an international registry to be established to facilitate joint research studies.

Within model-based literature, the Kumar score is a scale that has been developed originally for assessment of radiation injury in the hind leg of mice; it was used to provide a high level of detail to cutaneous radiation injury including erythema, dry desquamation, moist desquamation, ulceration, and full thickness injury (Supplemental Figure 2). This scale has also been used in a study by Rodgers \textit{et al}., in a guinea pig skin model to determine the dose-dependent response to soft X-ray radiation to the dermis\textsuperscript{37}. 

<table>
<thead>
<tr>
<th>Organ Tissue</th>
<th>[0]</th>
<th>[1]</th>
<th>[2]</th>
<th>[3]</th>
<th>[4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>No change over baseline</td>
<td>Follicular, faint or dull erythema/epilation/dry desquamation/decreased sweating</td>
<td>Tender or bright erythema, patchy moist desquamation/moderate edema</td>
<td>Confluent, moist desquamation other than skin folds, pitting edema</td>
<td>Ulceration, hemorrhage, necrosis</td>
</tr>
<tr>
<td>Mucous membrane</td>
<td>No change over baseline</td>
<td>Injection/may experience mild pain not requiring analgesic</td>
<td>Patchy mucositis that may produce an inflammatory serosanguinous discharge/may experience moderate pain requiring analgesia</td>
<td>Confluent fibrotic mucositis/may include severe pain requiring narcotic</td>
<td>Ulcerations, hemorrhage or necrosis</td>
</tr>
<tr>
<td>Eye</td>
<td>No change</td>
<td>Mild conjunctivitis with or without scleral injection/increased tearing</td>
<td>Moderate conjunctivitis with or without keratitis requiring steroids &amp;/or antibiotics/dry eye requiring artificial tears/iritis with photophobia</td>
<td>Severe keratitis with corneal ulceration/objective decrease in visual acuity or in visual fields/acute glaucoma/panophthalmitis</td>
<td>Loss of vision (unilateral or bilateral)</td>
</tr>
<tr>
<td>Ear</td>
<td>No change over baseline</td>
<td>Mild external otitis with erythema, pruritis, secondary to dry desquamation not requiring medication. Audiogram unchanged from baseline</td>
<td>Moderate external otitis requiring topical medication/serious otitis medius/hypacusis on testing only</td>
<td>Severe external otitis with discharge or moist desquamation/symptomatic hypacusis/tinnitus, not drug related</td>
<td>Deafness</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>No change over baseline</td>
<td>Mild mouth dryness/slightly thickened saliva/may have slightly altered taste such as metallic taste/these changes not reflected in alteration in baseline feeding behavior, such as increased use of liquids with meals</td>
<td>Moderate to complete dryness/thick sticky salivamarkedly altered taste</td>
<td>—</td>
<td>Acute salivary gland necrosis</td>
</tr>
<tr>
<td>Pharynx &amp; esophagus</td>
<td>No change over baseline</td>
<td>Mild dysphagia or odynophagia/may require topical anesthetic or non-narcotic analgesics/may require soft diet</td>
<td>Moderate dysphagia or odynophagia/may require narcotic analgesics/may require puree or liquid diet</td>
<td>Severe dysphagia or odynophagia with dehydration or weight loss &gt; 15% from pretreatment baseline/ requiring N-G feeding tube, i.v. fluids or hyperalimentation</td>
<td>Complete obstruction, ulceration, perforation, fistula</td>
</tr>
<tr>
<td>Larynx</td>
<td>No change over baseline</td>
<td>Mild or intermittent hoarseness/cough not requiring antiusis/erythemas of mucosa</td>
<td>Persistent hoarseness but able to vocalize/referral ear pain, sore throat, puffy fibrotic edema/may or mild arytenoid edema not requiring narcotic/cough requiring antiusis</td>
<td>Whispered speech, throat pain or referred ear pain requiring narcotic/confluent fibrotic exudate, marked arytenoid edema</td>
<td>Marked dyspnea, stridor or hemoptysis with tracheotomy or intubation necessary</td>
</tr>
<tr>
<td>Upper G.I.</td>
<td>No change</td>
<td>Anorexia with &lt; -5% weight loss from pretreatment baseline/nausea not requiring antiemetics/abdominal discomfort not requiring parasympatholytic drugs or analogics</td>
<td>Anorexia with &lt; -15% weight loss from pretreatment baseline/nausea &amp;/or vomiting requiring antiemetics/abdominal pain requiring analogics</td>
<td>Anorexia with &gt; 15% wt loss from pretreatment baseline or requiring N-G tube or parenteral support. Nausea &amp;/or vomiting requiring tube or parenteral support/abdominal pain, severe despite medication/hematensis or internal bleeding</td>
<td>Bean, subacute or acute obstruction, perforation, GI bleeding requiring transfusion/abdominal pain requiring tube decompression or bowel diversion</td>
</tr>
</tbody>
</table>

Supplemental Figure 1. Radiation Therapy Oncology Group (RTOG) acute radiation morbidity scoring criteria. This scale was originally developed to complement the Late Effects Scoring Criteria to report toxicity in patients enrolled in studies between physicians, RTOG staff, and the European Organization for Research and Treatment of Cancer (EORTC).
<table>
<thead>
<tr>
<th>SCORE</th>
<th>SKIN CHANGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>No effect</td>
</tr>
<tr>
<td>1.5</td>
<td>Minimal erythema, mild dry skin</td>
</tr>
<tr>
<td>2.0</td>
<td>Moderate erythema, dry skin</td>
</tr>
<tr>
<td>2.5</td>
<td>Marked erythema, dry desquamation</td>
</tr>
<tr>
<td>3.0</td>
<td>Dry desquamation, minimal dry crusting</td>
</tr>
<tr>
<td>3.5</td>
<td>Dry desquamation, dry crusting, superficial minimal scabbing</td>
</tr>
<tr>
<td>4.0</td>
<td>Patchy moist desquamation, moderate scabbing</td>
</tr>
<tr>
<td>4.5</td>
<td>Confluent moist desquamation, ulcers, large deep scabs</td>
</tr>
<tr>
<td>5.0</td>
<td>Open wound, full thickness skin loss</td>
</tr>
<tr>
<td>5.5</td>
<td>Necrosis</td>
</tr>
</tbody>
</table>

**Supplemental Figure 2. Kumar Scale**[^38]. A non-linear, semi-quantitative scale used to assess skin damage; used in assessment of acute skin reactions of radiotherapy in mice.

[^38]: Kumar Scale is a non-linear, semi-quantitative scale used to assess skin damage; used in assessment of acute skin reactions of radiotherapy in mice.
1.1.4 Animal models
Animal models have been developed to study radiation-induced changes such as dermatitis and ulcers. In general, the dosages studied in animals are comparatively higher than human doses. As a reference, in humans, current National Comprehensive Cancer Network (NCCN) guidelines recommend dosing regimens of 46-50 Gy in 23-25 fractions, or 40-42.5 Gy in 15-16 fractions. Typical boost doses, recommended in patients at higher risk for recurrence, are 10-16 Gy in 4 to 8 fractions. These boost doses are relevant to patients who undergo breast-conserving therapy (lumpectomy with whole breast irradiation), the primary breast local treatment for the majority with Stage I and II disease\textsuperscript{39}.

Ertekin \textit{et al.} has described an animal model of radiotherapy-induced dermatitis while observing the effects of zinc sulphate as a protective agent against radiotherapy; rats were given a one-time dose of 30 Gy\textsuperscript{40}. Takikawa \textit{et al.} has developed a new animal model for intractable skin ulcers in irradiated rats. Four groups of six rats each were given single doses of 10 Gy, 15 Gy, 20 Gy, and 30 Gy and observed for 24 weeks for presence or absence of epilation, depigmentation, erosion, and ulcers amidst wounding with a punch biopsy over the radiation sites at various timepoints. They found no visible changes in the skin in the 10 Gy group, depilation and depigmentation present at 2 weeks in the 15 Gy group, minor erosions at 4 weeks in the 20 Gy group, and epilation and depigmentation present at 2 weeks progressing to erosion and ulcers at 4 weeks in the 30 Gy group\textsuperscript{41}. Gu \textit{et al.} have reported a different model of radiation-induced skin ulcer in rats, at 35-55 Gy resulting in a death rate of 20\%\textsuperscript{42}.

There is currently no literature on an animal model of radiation-induced changes concerning the mammary fat pad; this will be the focus of this thesis.

1.1.5 Breast reconstruction
A prior history of radiation treatment affects the complication profile and available breast reconstructive alternatives. There is an increasing number of patients receiving post-mastectomy radiation therapy, and the timing and technique of breast reconstruction in this population is controversial\textsuperscript{43}. 
Alloplastic, or tissue expander and implant based reconstruction, is the most common method for breast reconstruction after radiotherapy. Studies evaluating such two-stage breast reconstruction in post-mastectomy radiation therapy patients with implant following tissue expander placement reveal consistently high rates of acute and chronic complications such as capsular contractures and poor aesthetic outcomes. Ascherman et al. found higher complication rates, extrusion rates, and asymmetry for irradiated breasts as compared to non-irradiated breasts in patients who underwent two-stage implant-based reconstruction. Interestingly, lower rates and severity of capsular contracture has been described in a study by Cordeiro et al., where two-stage reconstruction was completed before post-mastectomy radiation therapy. Other types of implant-based reconstruction along with an autologous flap such as transverse abdominis myocutaneous (TRAM) and latissimus dorsi myocutaneous flap are associated with capsular contracture.

Although there is consensus that autologous tissue-based reconstruction is preferable to breast implants in an irradiated operative field, post-mastectomy radiation nevertheless has adverse effects on outcomes in this population with respect to fat necrosis, vessel thrombosis, flap necrosis, fibrosis, and flap contracture. It has thus been suggested that autologous reconstruction be delayed in those who will require post-mastectomy radiation therapy.

1.2 Fat Quantification
Animal models involving transplantation of whole inguinal fat pads into mice scalp have been used to study adipogenesis, graft survival, and differential contributions of graft-derived and host-derived cells. In these cases, the ratio of harvested sample to body weight has been used to evaluate the change in sample weight. More recently, Bahrami et al. have characterized fat content in dorsal subcutaneous skin and fourth mammary fat pad, utilizing micro CT imaging, morphometry, histology, RT-PCR and ELISA analyses of adipogenic gene expression to quantify fat content. Overall, there is very limited literature on the study of adipogenesis in an animal model, and much fewer in the context of the mammary fat pad.
1.3 Fat Grafting, Adipogenesis, and Fibrogenesis

The anti-fibrotic effects of fat transplants, in their reversal of radiation-induced damage, are attributed to adipose-derived stem cells (ASCs) in the graft that produce adiponectin. Adiponectin regulates myofibroblast and fibroblast differentiation\(^56,57\).

Adiponectin is a cytokine that is secreted predominantly by adipocytes. It has anti-fibrotic, anti-inflammatory, anti-atherogenic and insulin-sensitizing effects\(^{58-60}\). Its receptors are found in adipose tissue, skeletal tissue, and liver\(^61\). It is upregulated when pre-adipocytes differentiate into mature adipocytes\(^{58,61,62}\), and depleted in lipodystrophy in animal studies\(^60\).

Mature adipocytes reduce tissue rigidity by altering the mechanical properties of connective tissue, thus sustaining the differentiation and survival of myofibroblasts. Fibrosis is characterized by excessive Collagen-I to Collagen-3 ratio\(^63\). We also know that TGF\(_{\beta1}\), a fibrogenic cytokine, inhibits adipogenesis\(^64\). Fibrogenesis is increased when there is loss of adipocyte function due to reduced expression of anti-fibrotic adipokines such as adiponectin\(^65\). Technologies that target this pathway, effectively promoting adipogenesis while reducing fibrosis in breast tissue would improve fat take and reduce side effects of radiotherapy.

The optimal properties of fat grafting mentioned above result in part from the ability of mature adipocytes to reduce tissue rigidity by altering the mechanical properties of connective tissue – thus blocking the differentiation and survival of myofibroblasts – and from anti-fibrotic functions of adipokines such as adiponectin and leptin. Therefore, development of therapies that can enhance the long-term survival of adipocyte progenitor cells would greatly benefit successful reconstruction of breast tissue in cancer patients. One microenvironmental factor that affects pre-adipocyte survival and differentiation, and fibrosis is the tissue polysaccharide, hyaluronan.

1.4 Hyaluronan

Hyaluronan (or hyaluronic acid, HA) is a carbohydrate in the glycosaminoglycan family in mammalian tissues first discovered in bovine vitrous humour of the eye in 1934\(^{66}\). It is a major extracellular matrix (ECM) component, particularly in skin, and consists of repeating polymeric
disaccharides D-glucuronic acid and N-acetyl-D-glucosamine linked by alternating glucuronidic β(1,3) and β(1,4) bonds\textsuperscript{29,67,68}. It plays an important role in tissue repair, displays antioxidant properties, and promotes cell migration and proliferation\textsuperscript{69,70}. It is also well known that when HA is fragmented it functions as a molecule that transmits signals and is involved in cell adhesion, motility, growth, and differentiation\textsuperscript{71}. Notably it is important for maintaining tissue homeostasis and regulating inflammation, fibrosis and stem cell renewal in damaged tissues\textsuperscript{29,72,73}.

In fetal skin, HA remains in its native high molecular weight form (HMWHA), and attenuates inflammation and fibroplasia\textsuperscript{74–77}. It also provides a hydrated, anti-inflammatory and cytokine rich microenvironment for various skin progenitor cells that maintains their “stemness” and protects them from ionizing radiation-induced DNA damage. In injured adult skin, HA is degraded into low molecular fragments through hyaluronidases and reactive oxygen/nitrogen species (ROS/NOS)\textsuperscript{73,78–80}. Rapid alterations in hyaluronan production and polymer size within tissues are among the earliest changes that can be detected following injury from exposure to ionizing radiation\textsuperscript{29,80–83}. These low molecular weight fragments are well characterized to stimulate inflammation, and if chronically present, result in tissue destruction from uncontrolled fibrosis\textsuperscript{84}. As well, HA promotes the trafficking and differentiation of progenitor cells in particular mesenchymal lineages\textsuperscript{73,78,85}. Notably, HMWHA supports survival, proliferation, and differentiation of pre-adipocytes, while fragmentation of HA suppresses adipocyte maturation\textsuperscript{55,86,87}.

HA fragments mediate their pro-inflammatory and pro-fibrotic effects through interactions with various cell-surface receptors. CD44 is the major constitutively expressed HA receptor of the epidermis\textsuperscript{82}. It is also required for subcutaneous adipogenesis, and is implicated in the stimulation of aggregation, proliferation, migration, and angiogenesis\textsuperscript{71,88,89}. The receptor of hyaluronan mediated motility (RHAMM) is another cell surface receptor and CD44 binding partner that is expressed during tissue inflammation and repair, and will be a focus of this thesis. Other additional HA cell surface receptors include lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), a homologue of CD44, and toll-like receptor 2 and 4 (TLR2,4)\textsuperscript{74,90}. Phosphoinositide 3-kinase/protein kinase B (PI3 kinase/AKT), and mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase 1,2 (MEK1/ERK1,2) are downstream pathways that are
activated by these receptors\(^34\). Importantly, the activation of signaling cascades through RAS-MAP (ERK) kinase regulates mesenchymal differentiation required for tissue injury repair\(^91,92\). This will be further elaborated upon below.

1.4.1 ERK1

HA receptors such as CD44, RHAMM, and TLR2,4 as mentioned detect and bind to HA fragments, collectively controlling the activation of MAP kinase signaling networks. Ultimately this plays a part in regulating the migration, proliferation, and differentiation of progenitor cells into specific mesenchymal lineages to maintain tissue homeostasis and repair tissues. It is known that fibrosis and adipogenesis originate from a common mesenchymal progenitor\(^93\), with MAP kinase and ERK1 pivotal for substantial tissue formation. ERK1 is required for the clonal expansion of mesenchymal progenitor cells into proliferating pre-adipocytes (Figure 1).

The importance of the MAP kinase pathway in adipogenesis is demonstrated by the report that ERK1-/- mice have fewer adipocytes and reduced adiposity than their wildtype (WT) counterparts due to the key requirement of ERK1 in clonal expansion of pre-adipocytes. In order to transition from pre-adipocytes into mature adipocytes, the progenitors must respond to differentiating factors present in their microenvironment that activate PPAR\(\gamma\), a master adipogenic transcription factor. ERK inactivates PPAR\(\gamma\). Connective tissue growth factor (CTGF), one of ERK’s downstream transcription targets also inhibits PPAR\(\gamma\) expression. Sustained ERK activity thus promotes fibrogenesis, while inhibition of ERK1 kinase promotes adipogenic differentiation of the expanded pre-adipocytes.
Figure 1. Model of the roles of ERK activity and RHAMM in mesenchymal progenitor cell differentiation into adipocytes. This model highlights the key role of ERK1,2 activity in the clonal expansion of pre-adipocytes and predicts that expanded pre-adipocytes express RHAMM which sustains ERK activation. Together these suppress the differentiation of pre-adipocytes to mature adipocytes. Either loss or function blocking of RHAMM and ERK activity results in the differentiation of expanded pre-adipocytes into mature adipocytes. 

*Figure from the Turley Lab, (unpublished).*
1.5 RHAMM

RHAMM (gene name HMMR) is a regulator of ERK activity. It is a multifunctional cell surface and intracellular protein, and is located on the cytoskeleton, in mitochondria, and in cell nucleus. It is not constitutively expressed in most homeostatic tissues, with the exception of some type of progenitor cells, but is transiently expressed in tissues repairing an injury. Although it is secreted, it does not contain a signal peptide for export through the golgi apparatus or endoplasmic reticulum. Thus, it resembles proteins such as basic fibroblast growth factor (BFGF), Human immunodeficiency virus trans-activator of transcription (HIV Tat) protein, heat shock proteins (HSPs), the homeobox protein engrailed (EN), and epimorphin in its ability to be unconventionally exported through the plasma membrane. The binding of extracellular hyaluronan to secreted RHAMM, which couples with CD44, plays a key role in activating signal cascades MAP kinases, ERK1,2. This occurs in response to mesenchymal growth factors and receptors such as PDGFß and PDGFR. Intracellular RHAMM is a regulator of ERK activity, and co-associated with approximately 20% of total cellular ERK1 kinase.

Activation of this CD44 signaling pathway thus promotes fibrogenesis and myofibroblast differentiation and blocks PPARγ and adipogenesis. Intracellular RHAMM performs adapter protein functions that include coupling ERK to its upstream activators. It also targets these to its downstream substrates in the nucleus. These intracellular RHAMM/ERK1,2 signaling complexes participate in expression of a subset of fibrogenic genes, some of which suppress adipogenesis. HA is the major RHAMM ligand that plays a role in controlling adipogenesis and fibrosis; it is particularly enriched in mesenchymal progenitor niches.

Unlike ERK, RHAMM does not appear to be essential for clonal expansion of pre-adipocytes, knockout of HMMR (protein product: RHAMM) has resulted in an increase in adipose tissue. In other words, if RHAMM were required for pre-adipocyte clonal expansion like ERK1 is, adipose tissue formation would be decreased when it is lost. Its expression instead directs the differentiation of progenitors towards myofibroblasts and dermal fibroblast phenotypes, and blocks differentiation of pre-adipocytes into mature adipocytes.

Unique properties of RHAMM that make it a better therapeutic target than ERK1 for controlling
fibrogenesis and adipogenesis include its restricted expression in most homeostatic cell types. It is also limited to these progenitor cells. This is predictive of a good safety profile, alongside its dual functions of myofibroblast-promotion and adipocyte-suppression.

1.5.1 RHAMM-based peptides

The low molecular weight HA fragments that promote myofibroblasts and inhibit adipogenesis bind to extracellular RHAMM and CD44. These interactions, as mentioned, activates the RAS-MAP kinase pathway and stimulates the formation of intracellular RHAMM/ERK that are required for expression of key fibrogenic genes and suppression of PPARγ. Blocking the binding of HA fragments to these receptors would thus decrease fibrogenesis and promote adipogenesis via relieving suppression of PPARγ.

RHAMM mimetic peptides have recently been developed that perform such functions. These peptides fall into two categories: those that act either by directly binding to HA fragment sizes that are typically generated in small amounts during tissue injury (Kd=10nM) or by binding directly to RHAMM to block its ability to associate with HA fragments. One of these peptides, NPI-110 has been reported to promote adipogenesis by releasing the RHAMM-mediated block on PPARγ expression. This and other adipogenic RHAMM peptides were identified by screening for an ability to promote adipogenesis in culture assays using bone marrow mesenchymal stem cells, mouse pre-adipocyte cell lines, and primary human subcutaneous pre-adipocytes. NPI-110, or 644KLKDENSQLKSEVSK was a peptide that was selected as the most effective in promoting adipocyte differentiation in bone marrow mesenchymal cells, human and mouse pre-adipocytes in culture.

NPI-110 and similar peptides appear to act specifically on RHAMM regulated pathways since they do not affect these functions in RHAMM/- cells. The limited expression of RHAMM in homeostatic tissues predicts a good safety profile, and since NPI-110 directly blocks fibrosis and promotes adipogenesis, we predicted it would be an efficacious therapy for reducing radiofibrosis and for creating a breast tissue microenvironment more favorable to the survival of grafted fat.
Our lab has shown that NPI-110 significantly blunted mRNA expression of many fibrogenic and pro-inflammatory genes expressed by fibroblasts and macrophages when cultured macrophages and mesenchymal cells stimulated with TLR-4 agonists or TGFβ1 (Figure 2). In studies in an animal model of bleomycin-induced scleroderma, which usually is associated with a TGFβ1 driven increase in fibrosis and loss of adipose tissue, NPI-110 has a reduction in mRNA expression of pro-fibrosis genes and blunted loss of adipocytes.101

ERK1, which are required for clonal expansion of mesenchymal progenitor cells into proliferating pre-adipocytes, we recall inhibits PPARγ. Blocking RHAMM, a regulator of the ERK pathway with these peptides would release inhibition of pre-adipocytes and allow it to differentiate into mature adipocytes as seen below (Figure 3).

Recall that the activation of intracellular RHAMM/ERK1,2 complexes as a result of extracellular RHAMM activation would result in the promotion of myofibroblasts, PPARγ inhibition, and adipogenesis suppression. RHAMM mimetic peptides would bind with the hyaluronan fragments, halting hyaluronan fragments from binding with the extracellular RHAMM, thus preventing the differentiation of myofibroblasts, and prevent the inactivation of PPARγ and promote adipocyte differentiation (Figure 4).
Figure 2. RHAMM peptides suppress expression of RHAMM regulated pro-fibrosis genes. Mouse macrophage and dermal fibroblast lines were stimulated with a TLR4 agonist and TGFβ1 respectively with and without the RHAMM peptide mimetic; mRNA was isolated and fibrosis PCR arrays performed. The mRNA expression of genes that were significantly (p<.05) reduced are listed in the table. Adapted from unpublished data from the Turley lab.

<table>
<thead>
<tr>
<th>GENE NAME</th>
<th>FUNCTION</th>
<th>% INHIBITION BY RHAMM PEPTIDES</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1α</td>
<td>Pro-inflammatory cytokine</td>
<td>37</td>
</tr>
<tr>
<td>IL17</td>
<td>Pro-inflammatory cytokine</td>
<td>89</td>
</tr>
<tr>
<td>CCL1, 5 &amp; 25</td>
<td>White cell chemotactic factors</td>
<td>64, 79 &amp; 64</td>
</tr>
<tr>
<td>TNFα</td>
<td>Immune regulator, promotes inflammation</td>
<td>67</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>Master fibrosis promoter</td>
<td>50</td>
</tr>
<tr>
<td>CTGF</td>
<td>TGFβ-1 target gene, promotes tissue fibrosis and blocks adipogenesis</td>
<td>82%</td>
</tr>
<tr>
<td>COL3a1</td>
<td>Contributes to collagen fibrillogenesis during tissue fibrosis</td>
<td>90%</td>
</tr>
<tr>
<td>MMP1</td>
<td>Metalloproteinase contributes to extracellular matrix remodelling during tissue fibrosis</td>
<td>90%</td>
</tr>
<tr>
<td>GREM 1</td>
<td>TGFβ-1 target that promotes tissue fibrosis and blocks adipogenesis</td>
<td>74%</td>
</tr>
</tbody>
</table>
Figure 3. The MAP kinase ERK1 is required for the clonal expansion of mesenchymal progenitor cells into proliferating pre-adipocytes but suppresses maturation into adipocyte in the presence of RHAMM. Proliferating pre-adipocytes express RHAMM, which regulates localization and transcriptional targets of ERK1 resulting in suppression of the mast adipogenic transcription factor, PPARγ to prevent maturation of pre-adipocytes into mature adipocytes. RHAMM mimetic peptides block this RHAMM function and release inhibition of PPARγ to permit adipocyte maturation. *Figure from the Turley Lab (unpublished).*
**Figure 4. Mechanistic model of RHAMM mimetic peptide inhibiting fibrogenesis and promoting adipogenesis.** A. Hyaluronan fragments bind to extracellular RHAMM expressed on adipose derived stem cells resulting in the formation of a complex with CD44 and growth factor/receptors. This activates the RAS-MAP kinase pathway and formation of intracellular RHAMM/ERK signaling complexes which promote differentiation into myofibroblasts. These ERK complexes phosphorylate and inactivate the master adipogenic transcription factor PPARγ, blocking adipogenesis. B. RHAMM mimetic peptides bind to hyaluronan fragments, which prevents differentiation of myofibroblasts and inactivation of PPARγ and thus promote adipocyte differentiation. *Figure from the Turley Lab (unpublished).*
1.6 Identifying the problem

Dermal fibrosis as a result from radiotherapy is a challenge for breast reconstruction. Fat grafting has emerged as a technique that can reverse some late radiotherapy changes, as well correct contour abnormalities. RHAMM, a receptor for HA, has been identified as a potential target for tissue fibrosis, as it is involved in the pathways that leads to increased fibrogenic and decreased adipogenic gene expression. Dr. Turley’s laboratory has developed RHAMM mimetic peptides that have been shown effectively decrease fibrogenesis and promote adipogenesis in vivo. We aimed to study the effects of this peptide, in a novel rat model of radiation-induced mammary fat pad fibrosis. We propose a novel approach for reducing radiotherapy associated changes and promoting adipogenesis with this peptide, as a potential adjunct to fat grafting in breast reconstruction outcomes following radiotherapy.
2 Thesis Objectives and Aims

The hypothesis of the study is thus that the injection of RHAMM mimetic (NPI-110) peptides decrease fibrosis and increase adipogenesis in a rat model of radiation induced mammary fat pad fibrosis. The three primary objectives of the thesis are outlined below.

**Objective 1: Create a rodent model of radiation-induced mammary fat pad fibrosis**

The creation of a rat model of radiation-induced mammary fat pad fibrosis will serve as a basis for observing the effects of the peptide in such a setting. It will also serve as a basis for future fat transplantation studies.

**Aim 1:** Following a one-time radiation dose of 26 Gy in bilateral fourth mammary fat pads of rats, perform clinical assessments via visual skin assessments with two grading scales previously described (Kumar, RTOG).

**Aim 2:** Following a one-time radiation dose of 26 Gy in bilateral fourth mammary fat pads of rats, use paraffin processed mammary fat pad specimens to quantify the amount of staining for immunohistochemistry markers of fibrosis (TGFβ1 and Picrosirius Red).

**Aim 3:** Following a one-time radiation dose of 26 Gy in bilateral fourth mammary fat pads of rats, use mammary fat pad tissues to quantify the amount of expression of fibrotic markers (TGFβ1, ratio of Collagen-1 and Collagen-3) with quantitative PCR.

**Objective 2: Examine the effect of RHAMM mimetic peptides on fibrosis in irradiated mammary fat pads**

RHAMM is implicated in the fibrotic pathway as described above. The thesis aims to examine the effect of RHAMM peptide mimetics on fibrosis in the context of a rat model of mammary fat pad fibrosis.

**Aim 1:** Using paraffin processed mammary fat pad specimens, quantify the amount of staining for immunohistochemistry markers of fibrosis (TGFβ1 and Picrosirius Red).
Aim 2: Using mammary fat pad tissues, quantify the amount of expression of fibrotic markers (TGFβ1, ratio of Collagen-1 and Collagen-3) with quantitative PCR.

Objective 3: Examine the effect of RHAMM mimetic peptides on adipogenesis in irradiated mammary fat pads

RHAMM is implicated in the adipogenesis pathway as described above. The thesis aims to examine the effect of RHAMM mimetic peptides on adipogenesis in the context of a rat model of mammary fat pad fibrosis.

Aim 1: Using high frequency ultrasound, quantify the amount of adipogenesis through measurements of mammary fat pad thickness and volume.

Aim 2: Using paraffin processed mammary fat pad specimens, quantify the amount of staining for immunohistochemistry marker of adipogenesis (adiponectin).

Aim 3: Using mammary fat pad tissues, quantify the amount of expression of adipogenic markers (PPARγ and adiponectin) with quantitative PCR.
3 Methods

All experiments were approved and compliant with the standard operating protocols of the Animal Use Subcommittee (Protocol # 2009-060) at Western University in London, Ontario, Canada.

3.1 Peptide Injection Formulation

Patented (through World Discoveries, Lawson Health Research Institute) function blocking RHAMM peptides (peptide mimetics) $^{64}$KLKDENSQKSEVSK (denoted NPI-110) were synthesized and purified to >95% purity (ProSci and gift of Dr. L. Luyt, Western University and Novare Pharmaceuticals Inc). Peptides dissolved in PBS (2 mg/ml) were sterilized by filtration through 0.22 µm filter at a concentration of 2 mg/ml. This was then mixed 1:1 with hyaluronic acid (Orthovisc, Anika Therapeutics, Bedford, MA) for a final concentration of 1 mg/ml. A vehicle mixture of 1:1 of PBS (2 mg/ml) and Orthovisc was formulated without the peptide mixed in for the control group.

3.2 Rat Experiments

Animal work performed at London Health Sciences Centre, London Regional Cancer Program, and Robarts Research Institute conformed to animal use protocol #2009-060.

3.2.1 Preliminary Experiments

Preliminary experiments involving four retired breeder female CD (Charles River) rats were used to determine clinically detectable doses of radiation from a fibrosis standpoint. Rats were irradiated at two doses using a modified procedure of Jourdan et al. to determine clinically detectable doses of radiation fibrosis. The rats were 6 to 12 months of age. Rats were caged in pairs in a temperature-controlled room with 12 hour light/dark cycle, and fed a standard rat chow diet. One rat was not irradiated and served as a control. The other three rats received a high dose of radiation in the left third mammary fat pad, and a lower dose in the left fourth mammary fat pad. The right third and fourth sets were not irradiated.
Radiation was administered to three rats with Therapax-150 (T-150) unit to achieve a focused radiation beam of 2.37 Gy/min (probe diameter: 1 cm). Animals were induced and maintained with isofluorane gas in a clear plastic chamber by nose cone during radiation. Hair over the abdomen overlying the third and fourth set of nipples bilaterally were trimmed with a razor in a 2 cm diameter area. Rats were positioned supine, with laboratory tape used to secure and position the rat in a rotated fashion so that the fat pad and overlying skin were the main target of the radiation probe. In other words, the underlying organs were spared from radiation. A high dose of 26 Gy over 11.2 minutes was given to the left third mammary fat pad. A low dose of 13 Gy over 5.6 minutes was given to the left fourth mammary fat pad.

Animals were housed in Health Sciences Animal Research Facility, Western University for 3 weeks following radiation and provided with water and standard rat diet. Animals were euthanized at 3 weeks. The mammary fat pads were dissected and fixed in 4% paraformaldehyde (pH 7.4; Sigma-Aldrich, St. Louis, Missouri) for paraffin processing for histology.

3.2.2 Main Experiment: Animals
Twenty retired breeder female CD (Charles River) rats were used for the following experiments. Rats were caged in pairs in a temperature-controlled room with 12 hour light/dark cycles, and fed a standard diet. The fourth set of mammary fat pads were chosen because of its ease of identification, relative distinction from neighbouring fat pads, and previous use in literature. There were four treatment groups with 5 rats each, with the designations outlined in Table 1:
<table>
<thead>
<tr>
<th>Group</th>
<th>Radiation</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/- (Control)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>+/-p</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>+/-r</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>+/-r/p</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 1.** Breakdown of treatment groups
3.2.2.1 Radiation
Radiation was administered in radiation treated groups using a Therapax-150 (T-150) unit to achieve a focused radiation beam of 2.37 Gy/min (probe diameter: 1 cm). Animals were induced and maintained with isofluorane by nose cone during radiation. Hair over the fourth set of nipples were trimmed with a razor in a 2 cm diameter area. Rats were positioned supine, with laboratory tape used to secure and position the rat in a rotated fashion so that the fat pad and overlying skin were the main target of the radiation probe in a manner similar to that mentioned above. Animals in the radiation group received a single dose of 26 Gy; 2.37 Gy over 11.2 minutes. Both mammary fat pads in the fourth set of nipples were irradiated in rats in the radiation-treated groups (n=10).

3.2.2.2 Peptide Injection
Animals in the -/p and r/p groups received a single injection of 100 µg peptide in vehicle formulation (1 mg/ml) as described above in the mammary fat pads of the fourth sets of nipples. Animals in the non-peptide treated group received the same volume of injection of the vehicle formulation without the peptide mixture (100 µl). This was performed under direct visualization under ultrasound on Day 0 as described below (Supplemental Figure 3).
Supplemental Figure 3. Ultrasound images of nipple pre (left) and post (right) injection of peptide or vehicle
3.2.2.3 High Frequency Ultrasound

Ultrasound experiments were carried out at Robarts Research Institute in the James Lacefield lab. Animals were induced and maintained with isoflurane by nose cone during ultrasound. Rats were placed supine on a heated platform set to 35°C. A motorized razor was used to remove hair over a 2 x 5 cm area overlying the fourth set of nipples on Day 0, followed by Q-tip® assisted application of Nair®. This was done to ensure clear images via ultrasound and only on the Day 0 to avoid further irritation of the skin. Subsequent ultrasound images taken at Day 7, 14, and 21 were augmented by motorized razor trimming of hair regrowth, and not by chemical de-epilation to reduce irritation to skin that may confound skin assessments.

A Vevo2100 ultrasound Imaging System (VisualSonics, Toronto, ON) equipped with a MS-550D transducer was used for high frequency ultrasound data. The imaging resolution was 40 µm (axial) and 80 µl (lateral). Image acquisition was transmitted at 40 MHz, with an image width of 14.08 mm, and depth of 15 mm.

A thin layer of ultrasound gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ) was applied over the transducer and 2 x 5 cm area over the nipple of interest. Care was taken to minimize the amount of bubbles in the gel so as not to interfere with quality of image acquisition. The transducer was then hooked into an upright ultrasound stand. Image acquisition was set to B mode, a two-dimensional ultrasound image display composed of bright dots, and centered on the nipple. This scanning process for 3D image acquisition utilized a step size of 0.083 mm, with a total distance of 44 mm along the length of the rat centered on the nipple.

On Day 0, after the scanning was completed, the transducer was once again centered along the nipple. A 27 gauge needle was used to inject a one-time 100 µl vehicle formulation with (-/p and r/-) or without peptide (-/- and r/-), depending on the treatment group under the fourth nipple into the mammary fat pad. The transducer was removed, and gel wiped off at this point. Photographs were then taken at this time, while supine on the heated platform, with interest in the skin condition over the fourth nipple set. Photographs were also taken in a similar fashion on proceeding days of scanning on Day 7, 14, and 21. It should be noted that only two rats per group were scanned on Day 7.
3.2.2.4 Euthanization and Dissection

After photographs were taken and ultrasound imaging performed on Day 21 under general anesthesia, rats were euthanized the same day in Western University animal facilities in a carbon dioxide (CO$_2$) chamber.

Rats were secured to a Styrofoam board with 18 gauge needles. A skin marker was used to plan the incisions, and a 15-blade used to make a longitudinal midline incision from the xyphoid process down to the pubis, and transversely to the mid-axillary line at these landmarks. Dissection was carried along an alveolar plane, deep to the level of the mammary fat pad and superficial to the parietal peritoneum. The fourth mammary fat pad was identified visually, by a deep yellow tissue with an adipose-tissue appearance. For consistency, the area of dissection was marked out by a skin marker that included the subcutaneous tissue up to the level of the xyphoid process for the cephalad border, the dorsal border of the fat pad as the medial border, the most ventral-medial border of the fat pad as the lateral border, and just proximal to where a blood vessel to the fourth mammary fat pad emerges as the caudal border of the fat pad (Supplemental Figure 4). Dissection of the subcutaneous tissue including the mammary fat pad was carried down to the plane between dermis and subcutaneous fat. Sections were subsequently weighed. An additional fat pad was taken as an intra-rat control in each rat, of a single fat pad in the 5th nipple set.

Two rats in each treatment group were planned for immunohistochemistry, and these fat pads were placed in cassettes and 4% paraformaldehyde at 4°C in anticipation of fixing and sectioning. Three rats in each treatment group were planned for qPCR experiments, and were thus placed in Eppendorf tubes, snap frozen, and kept at -80°C.
Supplemental Figure 4. Markings for dissection of 4th mammary fat pad
3.3 Effect of radiation and peptide on fibrosis

3.3.1 Immunohistochemistry – H&E, Masson’s Trichrome

Fat pads were processed for paraffin histology slides. 4 µm sections were cut using a Microm HM 200 Ergostar Microtome (GMI; Ramsey, Minnesota, USA). Sections were stained with hematoxylin and eosin (H&E) to confirm the presence of subcutaneous adipose tissue. Adipocytes were identified as large vacuoles on imaging and visual inspection as paraffin processing removed the lipid content. Sections were also stained with Masson’s Trichrome (Sigma-Aldrich, St. Louis, Missouri).

3.3.1.1 TGFβ1

Fat pads from the rats were fixed and processed for paraffin embedded histology slides. Antigen retrieval was performed using Antigen 2100 Retriever (Aptum Biologics Ltd, Southampton, UK). De-paraffinized tissue sections were incubated with Anti-TGFβ1 monoclonal antibody (1:50 dilution, Abcam ab92486) overnight at 4°C. The tissue sections were washed in PBS and then incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:500 dilution, Abcam) for 1 hour at room temperature. Colorimetric detection was then performed using nickel 3,3’-diaminobenzidine (DAB; 0.15 mg/ml in 0.03% H2O2; Sigma). Sections were counterstained with H&E. The negative control was isotype matched non-immune IgGg used in place of the anti-adiponectin antibody. From each experimental group, specimens were obtained from two rats. For each rat, both left and right mammary fat pads were analyzed.

3.3.1.2 TGFβ1 Quantification

Using Aperio© ImageScope (Aperio ePathology Solutions; http://www.aperio.com/), a total of three images per mammary fat pad specimen were taken at 10x magnification. Specimens that were too small, damaged, or ripped were excluded. The Aperio image was saved and transferred to ImageJ 1.47 software.

In ImageJ 1.47, areas that were predominately fibrosis and ductal tissue were preserved while
adipocytes were excluded from the quantification. The ductal tissue was traced by hand via Wacom Intuos 4 (Portland, OR), and the adipocytes were erased. The isolated tissue was saved as a new image.

Next, the new image was de-convoluted under the ‘H DAB’ setting, allowing ImageJ to separate the different colour channels to isolate the blue staining from hematoxylin and the positive brown staining from TGFβ1 antibody. On the brown channel, the image was adjusted at a threshold of 215 units as a conservative assessment for identifying positively stained cells, and the blue channel was adjusted at a threshold of 225 units as a conservative assessment for identifying the number of total stained pixels.

To determine the ratio of hematoxylin staining to TGFβ1 antibody staining, the ratio of pixels of the isolated brown to blue channels was taken on ImageJ 1.47. The channels were analyzed using ImageJ 1.47 and the histograms were used to obtain pixel counts for both channels. The individual pixel counts were recorded, and the ratio was calculated. A higher ratio of brown to blue staining would demonstrate greater TGFβ1 staining in those tissues, and was expressed as a percentage. Five images were analyzed per slide, with two slides per rat.

### 3.3.1.3 Picrosirius Red Staining

Paraffin processed tissue sections of skin were stained for collagen using Picrosirius Red staining Kit (Cat# 2490-250, Polysciences, Warrington, PA) with the help of the Molecular Pathology Lab at Robarts Research Institute. Again, two sections per rat (right and left fat pads) and two rats per group were stained. Under polarized light, Picrosirius Red Staining is able to comment on collagen deposition and bundling. Thus, expression of red, or denser bundling in the tissue is associated with greater fibrosis. Conversely, blue reflects a lower density of collagen bundling.

Abrio 2.2 (Cri, Woburn, MA, USA) software was used for image acquisition. Slides were examined under polarized light, and five randomized images at 40.0X magnification were taken of each slide of areas that were composed primarily of adipocytes. Five additional images at 40.0X magnification were taken in a randomized fashion of areas that were composed primarily of ductal
tissue. Image acquisition and analysis was performed by a blinded assessor. Images were saved in Grayscale and Pseudocolour format. The intensity range for the live image acquisition was set at 99.6 for each image. Analysis was carried out in Photoshop CC (Adobe, Mountain View, California, USA).

For slides containing ductal tissue and surrounding fibrosis, the ducts were isolated by manually cropping out adipocyte tissue that surrounded the ductal tissue. The total pixel count of this area was then recorded. Expression was red and blue was then isolated in the method described above. In addition, a duplicate image was further cropped to reveal only the ducts, which were exempt from collagen deposition.

Expression of extent of collagen deposition was thus examined by observing the amount of red expression, with respect to area and corresponding blue expression. The total number of red pixels multiplied by mean value (in other words, intensity), was used to provide a measure of absolute red expression. This was then compared to total pixel area when observing adipocytes.

\[ \text{Red expression in ductal tissues} = \frac{(\text{red pixels} \times \text{red mean})}{(\text{pixels of area with adipocytes} - \text{ductal area})} \]

\[ \text{Red:blue expression} = \frac{(\text{Red pixels} \times \text{red mean})}{(\text{blue pixels} \times \text{blue mean})} \]

3.3.2 qPCR

Fat pads were dissected in method above from rats and snap-frozen. Frozen mammary fat pads were thawed in Trizol (Thermo-Fisher, Waltham, MA) and homogenized on ice. Fat pads were separated at initial dissection into nipple and peripheral samples. These samples were weighed and RNA combined proportionally according to fat pad mass after extraction so they could be directly compared with whole samples.

RNA was extracted using a phenol–chloroform procedure. Homogenates in Trizol were divided into 1 mL tubes. 200 µL chloroform was added and samples were mixed and centrifuged in a tabletop centrifuge at 4°C for 15 minutes at 12,000 rpm. The top aqueous phase was transferred to a
new tube. RNA was precipitated by adding one volume of isopropanol and washed three times with cold ethanol then dried in the fume hood and re-suspended in 20 μL nuclease-free water. RNA was quantitated by spectroscopy (NanoDrop, Thermo-Fisher).

Reverse transcription was performed with SuperScript VILO cDNA Master Mix (Thermo-Fisher). Reactions consisting of 1000 ng RNA, 4 μL Master Mix, and sufficient nuclease-free water to make the reaction up to 20 μL were prepared and incubated according to the kit manual. The resulting cDNA was diluted 1:10 in nuclease-free water.

Quantitative PCR was performed with SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA) using 10 μL master mix with 1 μL of forward and 1 μL reverse primer, each at 10 ng/μL, and 8 μL of diluted cDNA per reaction. Primer sequences are available in Supplemental Table 1. qPCR was run on a Mx3000P system (Agilent Technologies, Santa Clara, CA) with 40 cycles at an annealing temperature of 60°C. Specificity of amplification was confirmed with melting curve analysis. Expression was calculated using the $2^{-\Delta\Delta Ct}$ method relative to Gapdh expression and normalized to expression in the non-irradiated, untreated samples. Please note that adipogenic markers pertaining to section 3.4 are included in Table 2 below:

3.3.3 Skin Assessments

Photographs of the rats were taken at Day 0, 7, 14, and 21 after ultrasound gel was wiped off as described above. Images of all the rats in different groups over the time points were then uploaded onto Qualtrics, an online survey software tool (Provos, Utah, USA) and randomized. A modified version of previously described skin assessment scales, Kumar and RTOG, accompanied this online survey to serve as a scoring guide (Supplemental Figure 5). The survey was then sent to a staff physician with expertise on skin changes associated with radiation damage, as well as two resident physicians in plastic surgery. The reviewers were blinded to the treatment groups and day of treatment.
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfb1</td>
<td>ATGACATGAACCGACCCCTTC</td>
<td>ACTTCCAACCCAGGTCTTTTC</td>
</tr>
<tr>
<td>Col1a1</td>
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<td>TACTCGAAGGGGAATCCATC</td>
</tr>
<tr>
<td>Col3a1</td>
<td>TGATGGGATCCATGAGGGAGA</td>
<td>GAGTCTCATGTCCTTGCTTGT</td>
</tr>
<tr>
<td>Adipoq</td>
<td>TAAGGGTGACCCAGGAGATG</td>
<td>GGAACATTGGGGGAGAGTGAC</td>
</tr>
<tr>
<td>Pparγ</td>
<td>AGAGCTGACCACATGGTTGC</td>
<td>AAGGCTTTCATGTGGCCTT</td>
</tr>
<tr>
<td>Perilipin</td>
<td>TGCAAFATTCTGACAAGG</td>
<td>GGAGCCTTCTGATCTTTTT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CTCATGACCACAGTCATGC</td>
<td>TTCAGCTCTGGGATGACCTT</td>
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</table>

Table 2. qPCR Primer Sequences for fibrotic, adipogenic markers and housekeeping gene
### A. Kumar Scale

<table>
<thead>
<tr>
<th>Score</th>
<th>Skin Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No effect</td>
</tr>
<tr>
<td>1.5</td>
<td>Minimal erythema, mild dry skin</td>
</tr>
<tr>
<td>2</td>
<td>Moderate erythema, dry skin</td>
</tr>
<tr>
<td>2.5</td>
<td>Marked erythema, dry desquamation</td>
</tr>
<tr>
<td>3</td>
<td>Dry desquamation, minimal dry crusting</td>
</tr>
<tr>
<td>3.5</td>
<td>Dry desquamation, dry crusting, superficial minimal scabbing</td>
</tr>
<tr>
<td>4</td>
<td>Patchy moist desquamation, moderate scabbing</td>
</tr>
<tr>
<td>4.5</td>
<td>Confluent moist desquamation, ulcers, large deep scabs</td>
</tr>
<tr>
<td>5</td>
<td>Open wound, full thickness skin loss</td>
</tr>
<tr>
<td>5.5</td>
<td>Necrosis</td>
</tr>
</tbody>
</table>

### B. RTOG Scale

<table>
<thead>
<tr>
<th>Score</th>
<th>Skin Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No change over baseline</td>
</tr>
<tr>
<td>1</td>
<td>Follicular, faint or dull erythema/epilation/dry desquamation/decreased sweating</td>
</tr>
<tr>
<td>2</td>
<td>Tender or bright erythema, pathy moist desquamation/moderate edema</td>
</tr>
<tr>
<td>3</td>
<td>Confluent, moist desquamation other than skin folds, pitting edema</td>
</tr>
<tr>
<td>4</td>
<td>Ulceration, hemorrhage, necrosis</td>
</tr>
</tbody>
</table>

**Supplemental Figure 5. A. Kumar scale B. RTOG scale**
3.4 Effect of peptide on adipogenesis

Immunohistochemical studies and qPCR studies for adipogenic markers were performed as previously described in section 3.3.

3.4.1 Immunohistochemistry

Fat pads were processed in same method as mentioned above for TGF\(\beta\)1. From each experimental group, specimens were obtained from two rats. For each rat, both left and right mammary fat pads were analyzed.

3.4.1.1 Adiponectin

Paraffin-embedded sections were de-paraffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed with sections in a 0.01 M aqueous sodium citrate buffer (pH 6.0) using a microwave oven, with care taken to heat the tissues to just below boiling so as not to disturb the delicate nature of the highly fatty tissues. Following this, sections were washed in 1x PBS three times for 5 minutes each and treated with 3.0% hydrogen peroxide for 10 minutes. After a further wash of 1x PBS, tissues were blocked with 3.0% BSA for 1 hour at 4°C. The tissue sections were then incubated with anti-adiponectin monoclonal antibody (dilution 1:200, Abcam, ab22554) in 3% BSA overnight at 4°C. The negative control was isotype matched non-immune IgG used in place of the anti-adiponectin antibody. Sections were washed in PBS then incubated with Goat anti-mouse secondary IgG (dilution 1:500, Dako, E3044) at 2 \(\mu\)g/ml for 2 hr at room temperature. After washing, sections were incubated in streptavidin-horseradish peroxidase (dilution 1:2000 Abcam, ab7403) for 30 min at room temperature. Staining was visualized with diaminobenzidine (DAB) according to kit instructions (DAKO Liquid DAB + Substrate Chromogen System); 35 seconds was necessary for colour development. Sections were counterstained in hematoxylin and washed with distilled water. An ascending ethanol series and xylene wash were performed before Cytoseal 60 (Thermoscientific) was applied, followed by a glass cover slip application.
3.4.1.2 Adiponectin Quantification

Images of mammary fat pads for quantification were obtained using Aperio© ImageScope (Aperio ePathology Solutions; http://www.aperio.com/). A total of five images per specimen were taken at 10.0X magnification, one in each of four quadrants and one in the center region. Each Aperio image was saved and transferred to ImageJ 1.47.

In ImageJ 1.47, adipocytes were manually excluded, leaving only the glands and surrounding stroma to be analyzed. The image of the now isolated tissue was saved. Next, the image was deconvoluted under the ‘H DAB’ setting, again similar to the process for TGFβ1. The brown deconvoluted image was set to a threshold pixel range of 165 units, to set a conservative assessment for identifying positively stained cells, and the blue channel was adjusted at a threshold of 240 units as a conservative measurement for identifying the number of total blue pixels.

The ratio of positively stained brown cells to blue cells was used to determine the strength of adiponectin staining within each image. This ratio was compared to the ratio from the image of the entire specimen, in order to determine whether there was increased adiponectin staining near the glands. Three images were analyzed per slide, with two slides per rat.

3.4.2 Volume Assessment

3.4.2.1 Weights

Weights of the rats were recorded at Day 0, 7, 14, and 21. The fourth set of mammary fat pads and additional single fat pad from 5th set of mammary fat pads were dissected from rats and weighed.

3.4.2.2 High Frequency Ultrasound

High frequency ultrasound images were analyzed using Vevo2100 ultrasound Imaging System. Each rat underwent ultrasound at Days 0, 14, and 21. As mentioned, only two of five rats in each group were analyzed on Day 7.
3.4.2.2.1 Thickness
For each fat pad, the midline was marked as the center of the nipple. Measurements of the fat pad, as well as the fat pad with the skin were taken at 2 mm proximal and 2 mm distal to the marked midline in Vevo2100 software. 2 mm was chosen because it was the closest measurement that was consistently possible to the nipple yet still avoid the mammary fat pad distortion associated with the ducts feeding the nipple at midline.

3.4.2.2.2 Volume: 3D Reconstruction
The same midline measurement was used as that used for thickness measurements in Vevo2100 software. The fat pad was contoured using the lasso tool at midline, at 5 mm proximal and at 5 mm distal to the mammary fat pad. These measurements were taken as the fat pads were consistently captured within these parameters – often further than that, the fat pad would “drop off” if the rat was smaller in size. The software was programmed to fill in the rest of the fat pad between these contour levels. The contours were checked by scrolling through the images (step size of each image is 0.083 mm) and adjusted as needed to reflect the mammary fat pads as accurate as possible.
4 Results

Our preliminary experiments radiation experiments from section 3.2.1 demonstrated hardening of the fat pad as noted by digital palpation as early as two weeks. Further hardening was noticed on palpation at 3 weeks, and animals were euthanized at that point. Masson’s trichrome (Sigma-Aldrich) staining revealed higher collagen fibril density in mammary fat pads from contralateral, non-irradiated glands of radiated animals compared to mammary fat pads isolated from non-irradiated control animals, suggestive of a systemic inflammatory response. The clinical findings provide a suitable model to test our hypothesis further.

Following these initial studies of low and high dose radiation in rats, a high dose of 26 Gy was deemed appropriate for subsequent radiation studies. Ten of twenty retired female breeder rats were irradiated with 26 Gy at the fourth mammary fat pad. Radiation changes were evaluated through clinical skin assessments and biomarker analysis via immunohistochemistry and quantitative PCR.

The irradiated rat mammary fat pads exhibited significantly higher scores on the Kumar Scale, a skin assessment scale previously used measure of cutaneous radiation injury for animals (Supplemental Figure 2)\textsuperscript{37}, amongst all days when compared to non-irradiated rats when evaluated by a clinical expert in radiation-skin changes in a blinded fashion (***\(p=0.005\); Figure 5A). When specific days were analyzed, the radiation oncologist expert observed differences in the two groups at Day 7 and 21 (equal variances not assumed; *\(p=0.0125\) and **\(p=0.0045\) respectively; Figure 5B). The clinical expert also found a difference amongst all days (**\(p=0.0015\); Figure 5C) using the Radiation Therapy Oncology Group (RTOG) toxicity scoring system. In particular, when we compared the two groups using the RTOG on day 7 alone, we found a significant difference (*\(p=0.022\); Figure 5D). These results show that the applied dose of radiation resulted in acute and clinically visible changes in the exposed skin (Supplemental Figure 5). To determine if this dose also resulted in chronic skin fibrosis typical of radiofibrosis observed in human skin, pro-fibrotic markers were measured.

An increased density of collagen fibrils is typical marker for radiofibrosis. This property was
measured by Picrosirius red staining of histology sections from tissues from day 21 that were imaged with polarized microscopy. As shown in Figure 6, radiation induced a strong increase in collagen fibril density. (*p = .025). A similar, though non-significant result was observed when collagen was detected using Masson’s trichrome staining as well (Supplemental Figure 6). There was a tendency (p=.07) towards increased expression of active TGFβ1 in immunohistochemical studies of mammary fat pad sections. TGFβ1 is typically expressed within the first week of damage, and so it is not unexpected that a significant change was not observed in this series of experiments (Figure 6).
Supplemental Figure 6. Masson’s Trichrome staining of representative images from one rat (left and right fat pads)
Figure 5. Radiation group had significantly greater clinically detectable skin changes than non-irradiated control group with the Kumar scale and RTOG skin scoring systems. Skin assessments were performed by a radiation oncologist on control and irradiated rats in all days, using the Kumar and RTOG scoring systems.

A. Clinical Expert Evaluation mean score across all days with Kumar scale (**p=.0005) B. Clinical Expert Evaluation with Kumar Scale, days separated (**p=.0045; *p=.0125) C. Clinical Expert Evaluation mean score across all days with RTOG scale (**p=.0015) D. Clinical Expert Evaluation with RTOG scale, days separated (*p=.022) Values are the Mean, analysis with 1-tailed t-tests. n = 5 rats.
Figure 6. Markers of fibrosis are increased in irradiated groups compared to non-irradiated control in immunohistochemical studies. Paraffinized sections in control and irradiated rats euthanized at Day 21 were stained for markers of fibrosis A. Representative images stained with H&E B. Representative images stained with Picrosirius Red, red indicating denser collagen bundling and networks, at 40x C. Representative images stained with Masson’s Trichrome D. Representative images stained with TGFβ1, brown is positive staining for TGFβ1, at 5x E. The percentage staining per an area that expressed red when stained for Picrosirius Red (*p=.025) F. The percentage staining per an area of fat pad at day 21 for TGFβ1 (p=.07). Values are the Mean, analysis with 1-tailed t-tests. n = 5 rats, 5 images/fat pad, two fat pads/rat.
To further probe pro-fibrotic changes in the radiated rodent skin, qPCR analyses of TGFβ1, Collagen-1, and Collagen-3 mRNA were performed. An increase in the Collagen-1 to Collagen-3 ratio is typical of scarred fibrotic tissue. Adipogenic markers were also evaluated since radiation has been observed to inhibit adipogenesis. Markers included the “master” adipogenic transcription factor, PPARγ and one of its targets the adipokine adiponectin. However, significant changes in mRNA expression was not observed in the control vs. radiated groups (Figure 7).

A goal of this study was to reduce tissue fibrosis in order to create a more favorable microenvironment to support autologous fat grafts. Thus, the effect of RHAMM mimetic peptides – (NPI-110) which have previously been shown to inhibit fibrosis and promote adipogenesis in uninjured dorsal skin and mammary fat pads – on radiofibrosis in the mammary fat pads of the irradiated rat model was next assessed. Visible skin changes were not appreciably different between the radiated and peptide treated groups when the cumulative score was assessed using the Kumar and RTOG scales (Supplemental Figures 7-9).
Figure 7. No difference in fibrotic markers were found in qPCR studies. Real time PCR was performed for markers of fibrosis in non-irradiated (control group) and irradiated rat whole mammary fat pads. A. TGFβ1 (*p=.045) B. Collagen-1:Collagen-3 (p=.118) C. PPARγ (p=.085) D. Adiponectin (p=.063). Values are the Mean, analysis with 1-tailed t-tests. n = 3 rats, 3 technical replicates/fat pad, two fat pads/rat.
Supplemental Figure 7. Representative Images of right fourth mammary fat pad rats in control and radiation group at Days 0, 7, 14, and 21.
Supplemental Figure 8. Images of right fourth mammary fat pad rats in radiation and radiation + peptide group at Day 0, 7, 14, 21 (Rat 4 and 5 of each group respectively).
Supplemental Figure 9. Peptide (NPI-110) did not reduce severity of clinically appreciable skin changes in irradiated rats. Skin assessments were performed by an expert clinician treated and non-peptide treated groups amongst irradiated rats using the Kumar Scale and RTOG scale A. Clinical Expert Evaluation mean score across all days using Kumar scale (p=.15) B. Clinical Expert Evaluation mean score across all days using RTOG scale (p=.057) C. Clinical Expert Evaluation using Kumar Score, days separated D. Clinical Expert Evaluation using RTOG score, days separated. Values are the Mean, analysis with 1-tailed t-tests. n = 5 rats.
While there was no difference in visual appearance, peptide-treated irradiated skin strongly reduced collagen fibril formation as detected by Picrosirius red staining (\( **p=0.003 \)). Masson’s trichrome staining qualitatively showed a similar effect. Active TGF\( \beta \)1 trended to a lower level in the peptide group (\( p=0.075 \)). These results show that the RHAMM mimetic peptides strongly reduces radiofibrosis as detected by collagen fibril formation (Figure 8). There was no difference found between radiation and peptide treated group as compared to the control group when Picrosirius Red staining was examined; the peptide treated radiation group was found to decrease expression of active TGF\( \beta \)1 as compared to the control group (Supplemental Figure 10). We used mammary fat pad tissues to quantify the amount of TGF\( \beta \)1, ratio of Collagen-1 and Collagen-3 via quantitative PCR in the irradiated rats. We found no difference in the expression of these markers amongst the two groups, however (Supplemental Figure 11).

In order to assess if these changes in tissue fibrosis are accompanied by altered adipogenesis, adipokine expression was analysed by qPCR. These included adiponectin (ADIPOQ), and PPAR\( \gamma \). We also analyzed the mammary fat pad for volume and thickness changes using high frequency ultrasound, but these were not significant (Supplemental Figure 12, 13).

qPCR analyses showed a significant increase in the adipokine adiponectin (Figure 5) (\( *p=0.014 \)) at Day 21 (when rats were euthanized). There was a trend towards increased expression of PPAR\( \gamma \) in the peptide treated group (\( p=0.055 \)) and a tendency towards an increase of PPAR\( \gamma \) expression (Figure 9). These results suggest that the RHAMM function blocking peptide promotes a pro-adipogenic microenvironment.

We also used paraffin processed mammary fat pad specimens to quantify the amount of staining for immunohistochemistry markers of adiponectin. We found that there was no trend towards expression of adiponectin in the peptide-treated group as compared to the non-peptide treated group amongst irradiated rat (Supplemental Figure 14) on day 21.
Figure 8. Markers of fibrosis are decreased in peptide-treated groups compared to non-peptide treated groups amongst irradiated rats in immunohistochemical studies. Paraffinized sections in peptide and non-peptide treated groups euthanized at Day 21 were stained for markers of fibrosis A. Representative samples stained with H&E B. Representative samples stained with Picosirisur Red, red indicating denser collagen bundling and networks, at 40x C. Representative samples stained with Masson’s Trichrome D. Representative samples stained with TGFβ1, brown is positive staining for TGFβ1, at 5x E. The percentage staining per an area that expressed red when stained for Picosirisur Red (*p=.003) F. The percentage staining per an area of fat pad at day 21 for TGFβ (p=.078). Values are the Mean, analysis with 1-tailed t-test. n = 5 rats, 5 images/fat pad, two fat pads/rat.
Supplemental Figure 10. Markers of fibrosis in peptide-treated groups compared to control in immunohistochemical studies. Paraffinized sections in peptide and non-peptide treated groups euthanized at Day 21 were stained for markers of fibrosis A. The percentage staining per an area that expressed red when stained for Picrosirius Red (p=.109) F. The percentage staining per an area of fat pad at day 21 for TGFβ (*p=.025). Values are the Mean, analysis with 1-tailed t-test. n = 5 rats, 5 images/fat pad, two fat pads/rat.
Supplemental Figure 11. Peptide NPI-110 did not affect markers of fibrosis in qPCR studies. Real time PCR was performed for markers of fibrosis in peptide and non-peptide treated groups in irradiated rats A. TGFβ1 (p=.074) B. Collagen-1:Collagen-3 (*p=.032). Values are the Mean, analysis with 1-tailed t-test. n = 3 rats, 3 technical replicates/fat pad, two fat pads/rat.
Supplemental Figure 12. Peptide NPI-110 did not affect volume of fat pad amongst irradiated rat in ultrasound measurements. 3D volume reconstruction was performed on fat pads on live rats in Day 0, 14, and 21. A. Between Day 0 and Day 14 (p=.64) B. Between Day 7 and Day 14 (p=.33). Values are the mean percentage difference of fat pad volume compared to Day 0, analysis with 1-tailed t-test. n = 5 rats/group, two fat pads/rat.
Supplemental Figure 13. Peptide NPI-110 did not affect thickness measurements of the mammary fat pad between the peptide and non-peptide treated group amongst irradiated rats in ultrasound measurements. Thickness measurements (mm) was performed on fat pads on live rats in Day 0, 7, and 21 under high frequency ultrasound A. Mammary fat pad thickness percentage differences between day 0 and 7 (p=.12) B. Mammary fat pad thickness percentage differences between day 0 and 21 (p=.45) Values are the mean percentage difference of thicknesses compared to Day 0, analysis with 1-tailed t-test. n = 5 rats/group, two fat pads/rat.
Figure 9. Peptide NPI-110 increased markers of adipogenesis amongst irradiated groups in qPCR studies. Real time PCR was performed for markers of adipogenesis in peptide and non-peptide treated groups in irradiated rats A. Adiponectin (*p=.015) B. PPARγ (p=.055). Values are the mean, analysis with 1-tailed t-test. n = 3 rats, 3 technical replicates/fat pad, two fat pads/rat.
Supplemental Figure 14. Peptide NPI-110 did not increase expression of adipogenic marker adiponectin in immunohistochemical study amongst irradiated rats. Paraffinized sections in peptide and non-peptide treated groups amongst irradiated rats euthanized at Day 21 were stained for adipogenic marker, adiponectin A. Representative sections of mammary fat pad stained with adiponectin; brown is positive staining for adiponectin B. qPCR results Adiponectin (p=.093). Values are the Mean, analysis with 1-tailed t-test. n = 5 rats, 5 images/fat pad, two fat pads/rat.
5 Discussion

The central hypothesis of this study is that the injection of RHAMM mimetic (NPI-110) peptides decrease fibrosis and increase adipogenesis in a rat model of radiation induced mammary fat pad fibrosis. We demonstrated that radiated rats scored significantly higher on clinical skin assessment scales, a measure of cutaneous radiation injury for animals. Pro-fibrotic markers such as active TGFβ1 and collagen density were increased in the irradiated mammary fat pads when analyzed through immunohistochemical means. RHAMM mimetic peptide NPI-110 strongly reduced active TGFβ1 and collagen fibril formation amongst irradiated rats through immunohistochemical analyses. RHAMM mimetic peptides NPI-110 also increased expression of adiponectin, an adipokine in qPCR analyses amongst irradiated rats.

**Objective 1: Create a rodent model of radiation-induced mammary fat pad fibrosis**

We describe in this study radiation-induced inflammatory (dermatitis) and fibrotic changes in mammary fat pads. There are other studies that have, using different radiation doses, observed radiodermatitis, radiation-induced lesions, and radiofibrosis in the skin of animal models. De Andrade et al. used 10 Gy, 40 Gy, and 60 Gy on dorsal excisional wounds in rats that resulted in macroscopic and microscopic injuries to rat skin with increasing doses of radiation. In wounds, visible skin changes, increased collagen fibril formation, and increased TGFβ1 expression were observed as early as five days after injury even at lower doses. Chronic changes in response to ionizing radiation have also been studied in other animal models. For example, in leg contracture models, mice exhibit progressive leg contracture and fibrosis when administered single doses of radiation between 20-80 Gy. However, to our knowledge, our study is the first report of radiation induced mammary fat pad fibrosis. From our preliminary experiments, the 26 Gy model was chosen because it demonstrated clinically palpable and observable changes. However, the 13 Gy model may also have been chosen, as more occult, lower doses of radiation changes are still associated with long term clinical sequelae.

Radiation fibrosis is a dynamic process, characterized by constant remodeling and long-term fibroblast activation. In normal wound repair, fibroblasts are transiently activated into
myofibroblasts to facilitate wound remodeling. Terminally differentiated myofibroblasts undergo apoptosis and normal tissue architecture is mostly restored. However, in fibrotic tissue, chronic myofibroblast activation is observed, which is thought to result from elevated levels of growth factors including TGFβ1, PDGFb, TNFα, bFGF’s, GM-CSF, IL-1, IL-4, CTGF106,107. In animal models of lipodystrophy and scleroderma, these pro-fibrotic cytokines reduce the survival of adipocyte progenitor cells57,106,108. Adipogenesis is also restrained by the rigid microenvironment produced by myofibroblasts’ ability to promote collagen fibrillogenesis57. Loss of adipocytes also contributes to progressive tissue fibrosis since many adipokines are anti-fibrotic. This is particularly true of adiponectin56. Our results support this connection between adipocytes and fibrosis since the RHAMM mimetic peptides reduced fibrosis concomitantly with an increase in adiponectin expression (Figure 9).

Other tools may be considered to measure clinically observable skin and fibrotic changes. One includes image processing programs that can measure amount of redness in skin as a proxy for erythematous changes. Optical coherence tomography (OCT) is a non-invasive imaging modality that provides an in vivo cross-sectional image of tissues through the use of low-coherence interferometry, and is used in clinical diagnosis in dermatology and other fields in medicine. It has the capacity to image skin collagen through its orientation, organization and reflective properties, and uses light instead of sound (as in ultrasound) to generate images109. Skin elasticity as a measure of radiation fibrosis through newer methods has been reported with the DermaLab suction cup system; it shows promise in generating reproducible measurements of radiation-induced skin fibrosis110.

**Objective 2: Examine the effect of RHAMM mimetic peptides on fibrosis in irradiated mammary fat pads**

We did not observe an increase in either the expression or activation of TGFβ1 or an altered Collagen-1 to Collagen-3 ratio that has been reported for other animal models of radiation. TGFβ1 has been detected in skin as early as 6 hours after γ-radiation111. Increased levels of TGFβ1 were
also found at 6 hours post-irradiation in other studies, which returned to basal level within 48 hours and increased after 14 days. In mouse mammary glands, one team has found that whole body irradiation induced immunoreactivity at 1 hour, and persisted for 7 days. In addition, they found that irradiation specifically generates the active (versus latent) TGFβ1, suggesting that low and moderate doses of radiation induced the activation of latent TGFβ1 in the mouse mammary gland\textsuperscript{112,113}. Martin \textit{et al.} describes two types of fibrosis: an immature, active, and inflammatory fibrotic tissue examined in most studies, versus a non-inflammatory and poorly cellularized fibrotic tissue that correspond to studies of samples 10-20 years after irradiation\textsuperscript{33}. Of note, the young fibrosis samples contained a high proliferation rate of myofibroblasts, and a high secretion of TGFβ1, whereas the older type of fibroblasts exhibited reduced proliferation and low secretion of TGFβ1\textsuperscript{114,115}.

Through previous immunohistochemical studies, collagen-1 at one day after irradiation appeared to be reduced in the stromal sheath, while collagen-3 appeared reduced in the periepithelial stromal sheath but stronger along septa. Increased abundance of collagen-3 staining was noted in both the periepithelial stroma and stroma at day 3; this new collagen-3 expression in the adipose stroma colocalized with that of new TGFβ1 expression\textsuperscript{113}.

To date, there is no approved clinical treatment for radiofibrosis, and few current options are available for restricting or reversing tissue fibrosis resulting from other forms of tissue injury. Although TGFβ1 is considered a master switch for promoting myofibroblast differentiation and survival and for reducing anti-fibrotic adipokine production, this cytokine has not been useful for clinical control of fibrosis likely because of its ubiquitous expression and multifunctional nature. Another potential target that has emerged is PPARγ, which regulates both inflammatory and fibrosis processes. In particular, the PPARγ agonist rosiglitazone has been reported to protect mice against radiation induced inflammation and reduced expression of both TGFβ1 and Collagen-1\textsuperscript{116}. Unfortunately, the use of rosiglitazone is currently restricted in humans due to trials showing increased drug related cardiovascular toxicity. One of the PPARγ target genes, adiponectin, may be a more promising approach for reducing some forms of tissue fibrosis and clinical trials are currently ongoing for non-alcoholic fatty liver disease\textsuperscript{117}. Thus far, in animal models, adiponectin
does not appear to play a role in radiation induced intestinal damage in mice\textsuperscript{118} and its anti-fibrotic effects may be restricted to specific tissue and stimuli.

We propose that blocking RHAMM function using mimetic peptides is an efficacious alternative approach to controlling tissue fibrosis resulting from radiation as it did from other forms of tissue damage\textsuperscript{101}. RHAMM mimetic peptides are predicted to be particularly useful for reducing radiofibrosis in breast tissue since they create a microenvironment that supports adipogenesis. This is predicted to both contribute to a reduction in fibrosis (e.g. through elevated production of adiponectin) and to provide a method for supporting the survival and differentiation of adipocytes in autologous fat grafts. To date, RHAMM mimetic peptides have a good safety profile in animal models likely due to the restricted expression of this protein in homeostatic tissues. RHAMM expression is an integral part of the response of tissues to stress and to disease. Its primary functions appear to be control cellular migration and mesenchymal differentiation during repair processes\textsuperscript{119–122}.

\textbf{Objective 3: Examine the effect of RHAMM mimetic peptides on adipogenesis in irradiated mammary fat pads}

Previous studies showed that the injection of RHAMM mimetic peptides result in a detectable increase in mammary fat pad size as measured by microcomputer tomography (MicroCT)\textsuperscript{55}. High frequency ultrasound is a more common imaging modality that has recently evolved as a result of more sophisticated technology and computer programs\textsuperscript{123,124}. High frequency transducers (>15 MHz) have been used to successfully study the vascularization of expansion of lesions in the skin lesions, skin diseases such as psoriasis, scleroderma, erythema nodosum, among others\textsuperscript{123}. More recently, Santolo \textit{et al.} has evaluated the use of doppler ultrasound examination (ECD) in autologous adipose tissue filling by a plastic surgeon in the setting of post-surgical, post-traumatic, and post-burn scars in patients\textsuperscript{124}. They obtained quantitative data with ultrasound examination representing various skin and subcutaneous layers in serial evaluations after lipofilling procedures, and thus a subsequent average percentage of one-year survival of autologous implanted fat was possible. Other authors have used ultrasound as a non-invasive, quick, and low-cost technique to
study fat necrosis, and subcutaneous tissue for pre-operative planning and document post-operative results in the context of lipofilling\textsuperscript{125-127}. Our study, however, was not able to detect any changes in fat volume with high frequency ultrasound. This will be elaborated upon below.

Our lab has previously injected adipogenic RHAMM mimetic peptides into dorsal rat skin and mammary fat pads, and has found increased dorsal back subcutaneous fat pad area and mammary fat pad size using micro CT imaging. Peptide-induced fat pad surface area in dorsal skin tissues was significantly larger than vehicle controls at day 21, and maintained until Day 35 with respect to fat pad retention when examined via micro-CT. Over-expression of RHAMM downregulates adipogenic transcription factors PPAR\textsubscript{\gamma}, adiponectin, and perilipin via qPCR analysis. Our lab has shown that these RHAMM mimetic peptides upregulate expression of PPAR\textsubscript{\gamma} and adiponectin\textsuperscript{55}. Consistent with this, we demonstrated an increased expression of adiponectin and a trend towards increased PPAR\textsubscript{\gamma} expression in our study. Although we did not demonstrate tissue architecture change, we provided molecular evidence for a marker change with our qPCR studies. PPAR\textsubscript{\gamma} directly regulates adiponectin expression, and is made by subcutaneous fat; it is therefore the best marker for adipogenesis that we currently have. Oil Red O uptake into fat droplets is another method used to measure adipogenesis that our lab used previously, which may be another valuable adjunct in measuring NPI-110’s effects\textsuperscript{55}.

5.1 Stem Cells and Fat Grafting
Adipose-derived stem cells from the stromal vascular fraction of fat harvest is thought to play a large role in fat take. It can be harvested with minimal morbidity, differentiated reliably down various pathways, and can be transplanted in a safe fashion with modern liposuction techniques\textsuperscript{128}. Growth hormone, insulin, glucocorticoids, and prostaglandins stimulate ASCs in adipocyte differentiation in its initial and final stages. As such, adipogenic media are often supplemented with dexamethasone, human recombinant insulin, indomethacin, and second messenger cyclic adenosine monophosphate (cAMP) inducer 3-isobutyl-1-methyl-xantine (IBMX), to activate gene expression involved in adipogenic differentiation\textsuperscript{129}. These conditions will lead ASCs to acquire intracellular lipid droplets and morphology specific to lipid-laden cells, and adipocyte marker expression of adiponectin and PPAR\textsubscript{\gamma}\textsuperscript{129}. Early stage adipogenic genes have been identified and
include \textit{Krox20}, \textit{KLF5}, \textit{C/EBP}β, \textit{C/EBP}δ. Late stage adipogenesis regulators include \textit{KLF15}, \textit{C/EBP}α, \textit{PPAR}γ, and \textit{aP2}130. Adipocyte stem cell markers include CD34 and Sca-1, and pre-adipocyte markers include Gata2 and Pref-1130. Future studies would look at the effect of our peptide on adipocyte differentiation, in looking at these factors within our tissue samples via immunohistochemistry and qPCR.

The ultimate goal would be to create a micro-environment, with adipogenic media and potential supplementation of RHAMM mimetic peptides (or RHAMM function blocking peptides) that would facilitate greater fat take and adipogenesis. It is widely understood that volume retention of the graft, or “fat take” is variable. Studies have suggested that successful grafting, or “good fat take” has been linked to the presence of progenitor cells and their ability to undergo adipogenesis11. There are numerous techniques for fat harvest and fat processing. The lipoaspirate from fat grafting contains pre-adipocytes and mature adipocytes, with the pre-adipocyte (10\% of cell population) responsible for graft survival due to their large capacity for proliferation131. Recent literature in this area emphasizes the importance of this adult stem cell population or fraction in fat grafting. It is thought that these adipose derived stem cells (ASCs), which are of mesenchymal stem cell (MSC) origin, have the capacity to differentiate into specialized cell types to have reparative effects in their home tissue132, and promote long-term volume stability, survival, and outcome predictability133,134. Lipoaspirates have shown great potential to create viable natural tissue in addition to damaged tissue10,135,136. A study has recently shown that adipose-derived stem cells from lipoaspirates led to clinical improvement in the late side effects of radiotherapy (healing of ulcers, remission of fibrosis, atrophy, and retraction10. There is thus interest in stem cell therapies, or stem cell enhanced fat grafting to promote greater volume retention of graft. In mice, adipose stromal stem cells have been shown to secrete angiogenic and antiapoptotic factors, and promote neovascularization in ischemic tissues by differentiating into endothelial cells and incorporating into blood vessels137,138.

Autologous fat transplant, also known as fat grafting, is a common technique for breast revision surgery139. It has emerged as a promising technique with a relatively low complication rate that improves contour, shape, and volume following breast reconstruction. Furthermore, experimental models and clinical research have shown that successful autologous fat grafting reduces fibrosis
from various stimuli, including radiation\textsuperscript{139}.

Fat grafting in post-mastectomy radiation patients undergoing two-stage breast reconstruction with a traditional tissue expander has shown promise in better reconstructive outcomes with the creation of new subcutaneous tissue, improved skin quality, and reduced capsular contracture rates\textsuperscript{140}. There is evidence to suggest that fat grafting may reduce morbidity associated with radiation-induced fibrosis including post-mastectomy pain, scarring, and arm movement restriction as well\textsuperscript{10}. The ability of fat grafting to reverse some of the late changes associated with radiation is promising in the clinical context; it is a clinical target for breast cancer reconstruction patients in its ability to reverse radiation-induced soft tissue damage and dermal fibrosis.

From an oncological perspective, there has traditionally been concern with the safety of fat grafting at the breast level. Most of this concerned the potential for necrosis and micro-calcification of breast tissue that would be misleading on radiographic mammography\textsuperscript{11}. In addition, the interaction of grafted adipose tissue with host cancer cells, as well as the long-term effects of fat and cell transplantation in a microenvironment of highly reproductive tissue or residual tumour cells are unknown\textsuperscript{11,141–143}. Recommendations thus exist for patient selection for autologous fat grafting to the breast: risk factors such as BRCA-1, BRCA-2, positive personal or family history of breast cancer would warrant mammography prior to surgery\textsuperscript{11,144}. Fat grafts enriched with processed adipose derived stem cells have been used in breast reconstruction following mastectomy and in non-oncologic breast augmentation without adverse outcome; however, no long term data is present\textsuperscript{145,146}. More research in long-term clinical studies in this area is needed, and it is suggested that until then, conventional fat grafting is safe in certain subgroups of patients, whereas an increase in stem cell fraction through various means cannot be recommended at present\textsuperscript{11}.

5.2 Limitations and Future Directions

One limitation of the current study is technical. A rat model was chosen as a model due to previous mammary fat pad and radiation studies. As compared to the mouse or guinea pig, the rat is larger in size and thus easier to study the mammary fat pad. Due to economic and practical administrative
reasons of a pilot study, a model that more closely resembles human skin were not selected. Our rats were retired breeders between the ages of 6-12 months, but their exact dates of birth are unknown.

Although ultrasound has been used to study lipofilling and adipose tissue in the previous studies, to our knowledge, our study was the first attempt to use high frequency ultrasound to quantify the volume of a rodent mammary fat pad. Attempts were made to set up rats in the same position for every measurement, but even so, slight movement of the rat or centering the probe on the nipple may not be identical each time and could affect the measurement. Slight angulation and rotation may have affected the field of capture as well. Efforts to bypass this were made by measuring thickness of mammary fat pad in addition to contouring the volume, whereby the irregularities around the nipple wouldn’t confound the accuracy of measurement. Other potential confounders include variation in ultrasound gel and pressure of the, potentially affecting the consistency in measurement of the fat pad secondary to differences in compression. Lastly, as with human breast volume, there exist natural variations in rat volume, and so perhaps we were unable to detect significant differences due to this with our sample size. Percentage change within rat weights, volume, and thicknesses were used to control for this, but again we saw no differences between the studied groups. We would recommend future studies repeat with a greater sample size, and have it compared to other methods of volume assessment. We believe that measuring fat volumes would thus be difficult unless there are very large increases in adipocyte differentiation, and that these imaging techniques are not robust or sensitive enough to detect small changes. Molecular markers such as PPARγ and adiponectin would thus be worthwhile adjunctive measures.

Although there were five rats per group studied, three rats per group were allocated to qPCR studies, and two rats remaining per group allocated to immunohistochemistry. This was done to preserve the homogeneity of the tissues – as the radiation probe was 1 cm in diameter, it was not possible to separate or identify the purely irradiated portion of the fat pad. Only dissecting the whole fat pad would provide a tissue sample representative of the rat, and trying to split the fat pads in half would not capture necessarily the irradiated part of the tissue. In addition, more replicates or drop PCR may be more appropriate; three technical replicates were done in this case and were likely not sufficient.
Furthermore, quantification in immunohistochemistry relies on calculating pigment density as an indicator of presence of marker of interest. There is inherent variability and uptake in the penetration of the stain, even though steps were taken to minimize the variability – ie, staining all sections in the same conditions as best as possible. Quantification methods do not capture the whole section, but rather, snapshots within the section. Although performed in a randomized fashion, it is possible that some staining was over-estimated or under-estimated because of this sampling bias; as mentioned above it is unknown where exactly on the fat pad that the irradiation beam focused on. This may affect the results as we are essentially piloting this method, and may suffer from low sensitivity.

Future studies would look at sacrificing animals within the one-week mark as TGFβ1 is an early inflammatory marker, and after 6 months as perhaps a more accurate representation of chronic radiation fibrosis. The studies above support using TGFβ1, Collagen-1 and Collagen-3 as markers of fibrosis. Additional future work with these mimetic peptides will be directed towards assessing their ability to promote pre-adipocyte survival and differentiation in fibrotic microenvironments.

Future studies of interest would involve repeated injections of peptide, as its effect have been shown to be at its peak at 14 days in our previous studies; our animals in this case would be observed for as long as 6 months.

Our goal is to create a micro-environment, with adipogenic media and potential supplementation of RHAMM mimetic peptides, that would facilitate greater fat take and adipogenesis with fat grafting. Our model had demonstrated clinically appreciable skin changes after radiation. Previous studies have described fat grafting or transplant to have an effect on skin quality. We would thus like to further assess the effects of the NPI-110 at these multiple injection times, on its own and in conjunction with fat grafting.


6 Conclusion

Radiation damaged skin as a result of radiotherapy is characterized by both acute and chronic changes, and can be replicated in an animal model. Here, a model for radiation-induced mammary fat pad has been developed, with an increase in observational skin changes, and increased collagen bundling and expression of fibrotic marker TGFβ1. RHAMM mimetic peptides (NPI-110) have been shown to promote adipogenesis and decrease fibrosis in previous animal models. Here, we show that NPI-110 increase expression of an adipogenic marker and decrease fibrotic markers in such a rat model of radiation-induced mammary fat pad fibrosis. These results support the further investigation of this approach to ultimately reduce radiofibrosis and improve breast reconstruction in breast cancer patients.
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Appendix A: Manuscript

Reducing Radiofibrosis in Rodent Mammary Fat Pads.

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Abstract

Radiofibrosis of the breast makes satisfactory breast tissue reconstruction challenging and is associated with a high risk of complications. Autologous fat grafting can improve surgical outcome but fat retention is often variable. We developed RHAMM mimetic peptides (NPI-110) that decrease chemotherapy induced fibrosis and increase subcutaneous adipogenesis by promoting the differentiation of mesenchymal progenitor cells. Here, we assessed the efficacy of these peptides in reducing radiofibrosis of rodent mammary fat pads. A model of radiation-induced mammary fatpad fibrosis was developed in retired breeder rats that exhibits acute skin inflammation and a robust increase in collagen fibril deposition. This model was used to quantify both the effect of the NPI-110 on radiofibrosis and the promotion of a microenvironment that supports adipogenesis. Acute skin changes were scored from photographs, fat pad volume estimates were quantified using high frequency ultrasound, mRNA expression of genes involved in fibrosis (Collagen-1, Collagen-3, TGFβ1) and adipogenesis (PPARγ, adiponectin and perilipin) using QPCR, and collagen fibril deposition using polarized microscopy of picrosirius red stained paraffin processed mammary fat pad tissue sections. NPI-110 significantly reduced radiation-induced skin inflammation, radiofibrosis as assessed by reduced collagen fibril deposition and increased mRNA expression of adipogenesis markers adiponectin and PPARγ. Results from this study will aid in creating a microenvironment that optimizes autologous fat transplantation success.
1. Introduction

1 in 9 Canadian women will develop breast cancer in her lifetime, and 1 in 30 will die from it. Approximately 45-50% breast cancer patients undergo curative radiotherapy\(^2\)–\(^4\). Recent data has shown that post-mastectomy radiation therapy reduced the rate of locoregional recurrence in node-positive patients from 27% to 9%\(^5\).

The use of ionizing radiation is predicated on attempting to achieve lethal effects on tumour cells while sparing normal tissue\(^1\). Newer technologies such as conformal radiation techniques and intensity-modulated radiation therapy (IMRT) have considerably reduced the area of skin toxicity\(^7\) but the higher beam intensities have increased the localized risk of fat pad fibrosis\(^2\). Although not life threatening, fibrotic breast tissue is challenging to reconstruct and contribute to variability of autologous fat grafting success in these patients.

Radiation injury may be categorized as acute or chronic (late), with acute injury occurring within hours to weeks after radiation exposure, and chronic injury presenting months to years after radiation exposure\(^7\)–\(^9\). Acute injury primarily involves skin cell death through cellular alterations and inflammation in the epidermis and begin with erythema, edema, pigment changes, and de-epilation\(^8\). Severe acute injury involves complete loss of epidermis, persistent edema, fibrinous exudates and disturbance in skin barrier function\(^8\)\(^,\)\(^13\). Later, chronic effects include but are not limited to chronic inflammation, delayed ulcers, telangiectasias, atrophy, and fibrosis\(^2\)\(^,\)\(^8\). Chemotherapeutic drugs have been discovered to induce radiosensitivity which is more effective in killing tumor cells but a side effect is often more xerosis, inflammation, thinning, and necrosis of surrounding normal skin. This condition can develop into a dynamically progressing fibrosis with reversible and irreversible components in approximately 35% of patients\(^18\),\(^19\). The underlying mechanisms driving the development of such radiation-associated damage are not well understood, due in part to a lack of suitable pre-clinical models\(^8\). As a result of this knowledge deficit, there is currently no effective intervention, by topical or systemic means, to prevent or favorably modify the course and severity of dermatitis\(^3\)\(^,\)\(^7\)–\(^9\).

TGF\(\beta\)\(^1\) is a peptide and cytokine that has many functions that include promoting development of chronic inflammation, radiation dermatitis and fibrosis\(^3\). TGF\(\beta\)\(^1\) activates fibroblasts to secrete extracellular matrix proteins including collagens\(^24\)\(^,\)\(^34\)–\(^36\)\(^,\)\(^147\). Up-regulation of TGF\(\beta\)\(^1\) is common in the fibrotic tissues of irradiated patients and induction of its expression is a major cellular response to ionizing radiation\(^3\). Additionally, TGF\(\beta\)\(^1\) and other pro-inflammatory, pro-fibrotic cytokines such as interferon, IL-1, IL-2, and tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) inhibit adipogenesis. Collectively, the sustained presence of these cytokines create a microenvironment that is hostile to the survival of mesenchymal stem cells, which compromises the success of tissue reconstruction. Fibrosis in response to growth factors such as TGF\(\beta\)\(^1\) may be focal or widespread and also contribute to tissue retraction, restriction of movement, and chronic pain that can be difficult to manage\(^3\). The development of effective radiation mitigators and protectors, and the elucidation of the mechanisms responsible for radiation-associated changes in skin and other normal tissues are therefore a necessary part of breast cancer patient management\(^21\).

A prior history of radiation treatment affects the complication profile and available breast reconstructive alternatives. There is an increasing number of patients receiving post-mastectomy
radiation therapy, and the timing and technique of breast reconstruction in this population is controversial\textsuperscript{43}. Alloplastic, or tissue expander and implant based reconstruction, is the most common method for breast reconstruction after radiotherapy. Studies evaluating such two-stage breast reconstruction in post-mastectomy radiation therapy patients with implant following tissue expander placement reveal consistently high rates of acute and chronic complications such as capsular contractures and poor aesthetic outcomes\textsuperscript{44}. Ascherman \textit{et al.} found higher complication rates, extrusion rates, and asymmetry for irradiated breasts as compared to non-irradiated breasts in patients who underwent two-stage implant-based reconstruction\textsuperscript{45}.

Fat grafting has emerged as a promising technique with a relatively low complication rate that improves contour, shape, and volume following breast reconstruction. Furthermore, experimental models and clinical research have shown that successful autologous fat grafting reduces fibrosis from various stimuli, including radiation\textsuperscript{49}. There is evidence to suggest that fat grafting may reduce morbidity associated with radiation-induced fibrosis including post-mastectomy pain, scarring, and arm movement restriction\textsuperscript{10}. These optimal properties of fat grafting result in part from the ability of mature adipocytes to reduce tissue rigidity by altering the mechanical properties of connective tissue, thus blocking the differentiation and survival of myofibroblasts and from anti-fibrotic functions of adipokines such as adiponectin and leptin. Therefore, development of therapies that can enhance the long-term survival of adipocyte progenitor cells would greatly benefit successful reconstruction of breast tissue in cancer patients. One microenvironmental factor that affects pre-adipocyte survival and differentiation is the tissue polysaccharide, hyaluronan\textsuperscript{62}.

Hyaluronan (or hyaluronic acid, HA) is a carbohydrate in the glycosaminoglycan family in mammalian tissues\textsuperscript{66}. It is a major extracellular matrix (ECM) component, particularly in skin and consists of repeating polymeric disaccharides D-glucuronic acid and N-acetyl-D-glucosamine linked by alternating glucurinidic β(1,3) and β(1,4) bonds\textsuperscript{29,67,68}. In its native high molecular weight form, it plays an important role in tissue repair, has antioxidant properties and is a key component of mesenchymal stem cell niches. When it is degraded into small fragments, which occurs in response to stimuli such as ionizing radiation, it promotes inflammation and fibrosis\textsuperscript{69,70}.

HA fragments mediate their pro-inflammatory and pro-fibrotic effects through interactions with specific cell-surface receptors. The receptor of hyaluronan mediated motility (RHAMM) is one such cell surface receptor that is only expressed during tissue inflammation and repair. The binding of extracellular hyaluronan to RHAMM, which couples with CD44, plays a key role in activating signal cascades MAP kinases, ERK1,2 controlling the cell migration that is required for initiation of inflammation and fibrosis\textsuperscript{74,93}.

RHAMM mimetic peptides have recently been developed that block the binding of RHAMM to HA fragments. These peptides fall into two categories that act either by directly binding to HA fragment sizes that are typically generated in small amounts during tissue injury (Kd=10nM) or by binding directly to RHAMM itself. One of these peptides, NPI-110 also promotes adipogenesis by releasing the RHAMM-mediated block on PPARγ expression. These peptides appear act specifically on RHAMM regulated pathways since they do not affect these functions in RHAMM-/- cells. The limited expression of RHAMM in homeostatic tissues predicts a good safety profile, and since NPI-110 directly blocks fibrosis and promotes adipogenesis, we predicted it would be
an efficacious therapy for reducing radiofibrosis and for creating a breast tissue microenvironment more favorable to the survival of grafted fat.

In order to assess these potential effects of NPI-110, a rat model of radiation-induced mammary fat pad fibrosis was developed. We show that NPI-110 significantly reduced radiofibrosis and enhanced expression of adipokines. We propose that NPI-110 is therefore a potential adjunct to fat grafting in breast reconstruction outcomes following radiotherapy.

2. Experimental

2.1 Peptide injection formulation. Patented (through World Discoveries, Lawson Health Research Institute) function blocking RHAMM peptides $^{64}$KLKDENSQKSEVSK (denoted NPI-110) were synthesized and purified to >95% purity (ProSci and gift of Dr. L. Luyt, Western University and Novare Pharmaceuticals Inc). Peptides dissolved in PBS (2mg/ml) were sterilized by filtration through 0.22µm filter at a concentration of 2mg/ml and then mixed 1:1 with hyaluronic acid (Orthovisc, Anika Therapeutics, Bedford, MA) for a final concentration of 1mg/ml. A vehicle mixture of 1:1 of PBS (2mg/ml) and Orthovisc was formulated without the peptide mixed in for the control group.

2.2 Animals Animal work performed at London Health Sciences Centre, London Regional Cancer Program, and Robarts Research Institute conformed to animal use protocol 2009-060. Twenty retired breeder female CD (Charles River) rats were used for experiments. Rats were known to be at 6-12 months of age, caged in pairs in a temperature-controlled room with 12 hour light/dark cycle, and fed a standard diet. The 4 treatment groups were the following: control (-/-), peptide injection only (-/p), radiation only (r/-), radiation and peptide injection (r/p).

Radiation. Radiation was administered in radiation treated groups using a Therapax-150 (T-150) unit to achieve a focused radiation beam of 2.37Gy/min (probe diameter: 1cm). Animals were induced and maintained with isofluorane by nose cone during radiation. Hair over the 4th set of nipples were trimmed with a razor in a 2cm diameter area. Rats were positioned supine in a rotated fashion so that the fat pad and overlying skin were the main target of the radiation probe in a manner similar to that mentioned above. Animals in the radiation group received a single dose of 26 Gy. Both mammary fat pads in the 4th set of nipples were irradiated in rats in the radiation-treated groups (n=10).

Peptide injection. Animals in the -/p and r/p groups received a single injection of 100µg peptide in vehicle formulation (1µg/ml) as described above in the mammary fat pads of the 4th sets of nipples. Animals in the non-peptide treated group received the same volume of injection of the vehicle formulation without the peptide mixture (100µl). This was performed under direct visualization under ultrasound on Day 0 as described below.

High Frequency Ultrasound. Ultrasound experiments were carried out at Robarts Research Institute in the James Lacefield lab. Animals were induced and maintained by isofluorane by nose cone during ultrasound. Rats were placed supine on a heated platform set to 35°C. A motorized razor was used to remove hair over a 2 x 5 cm area overlying the 4th set of nipples on Day 0, followed by Q-tip assisted application of Nair®. This was done to ensure clear images via
ultrasound and only on the Day 0 to avoid further irritation of the skin. Subsequent ultrasound images taken at Day 7, 14, and 21 were augmented by motorized razor trimming of hair regrowth, and not by chemical de-epilation to reduce irritation to skin that may confound skin assessments.

A Vevo2100 ultrasound Imaging System (VisualSonics, Toronto, ON) equipped with a MS-550D transducer was used for high frequency ultrasound data. The imaging resolution was 40 µm (axial) and 80 µl (lateral); image acquisition was transmitted at 40 MHz, with an image width of 14.08mm, and depth of 15mm. A thin layer of ultrasound gel (Aquasonic 100, Parker Laboratories, Fairfield, NJ) was applied over the transducer and 2 x 5 cm area over the nipple of interest. The image acquisition was set to B mode, a two-dimensional ultrasound image display composed of bright dots, and centered on the nipple. This scanning process for 3D image acquisition utilized a step size of 0.083mm, with a total distance of 44mm along the length of the rat centered on the nipple. On Day 0, after the scanning was completed, the transducer was once again centered along the nipple. A one-time 100ul vehicle formulation with (/-p and r/-) or without peptide (/- and r/-), depending on the treatment group was injected under the 4th nipple into the mammary fat pad. Photographs were then taken at this time, while supine on the heated platform, with interest in the skin condition over the 4th nipple set. Photographs were also taken at this point on subsequent days of scanning on Day 7, 14, and 21.

**Euthanization and Dissection.** After photographs were taken and ultrasound imaging performed on Day 21 under general anesthesia, rats were euthanized the same day in Western University animal facilities in a carbon dioxide (CO2) chamber. A 15-blade used to make a longitudinal midline incision from the xyphoid process down to pubis, and transversely to the mid-axillary line at these landmarks. Dissection was carried along an alveolar plane, deep to the level of the mammary fat pad and superficial to the parietal peritoneum. Dissection of the subcutaneous tissue including the fat pads were carried down to the plane between dermis and subcutaneous fat. Sections were subsequently weighed. An additional fat pad was taken as an intra-rat control in each rat, of a single fat pad in the 5th nipple set. Two rats in each treatment group were planned for immunohistochemistry, and these fat pads were placed in cassettes and 4% paraformaldehyde at 4°C in anticipation of fixing and sectioning. Three rats in each treatment group were planned for qPCR experiments, and were thus placed in Eppendorf tubes, snap frozen, and kept at -80°C.

2.3 Immunohistochemistry. Fat pads were processed for paraffin histology slides. 4µm sections were cut using a Microm HM 200 Ergostar Microtome (GMI; Ramsey, Minnesota, USA). Sections were stained with hematoxylin and eosin (H&E) to confirm the presence of subcutaneous adipose tissue. Adipocytes were identified as large vacuoles on imaging and visual inspection as paraffin processing removed the lipid content. Sections were also stained with Masson’s Trichrome (Sigma-Aldrich, St. Louis, Missouri). Antigen retrieval was performed using Antigen 2100 Retriever (Aptum Biologics Ltd, Southampton, UK). De-paraffinized tissue sections were incubated with Anti-TGFβ1 monoclonal antibody (1:50 dilution, Abcam ab92486) overnight at 4°C. The tissue sections were washed in PBS and then incubated with an anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:500 dilution, Abcam) for 1 hr at room temperature. Colorimetric detection was then performed using nickel 3,3’-diaminobenzidine
(DAB; 0.15 mg/ml in 0.03% H2O2; Sigma). Sections were counterstained with H&E. An ascending ethanol series and xylene wash were performed before Cytoseal 60 (Thermoscientific) was applied, followed by a glass cover slip application. In a similar fashion, the tissue sections were incubated with anti-adiponectin monoclonal antibody (dilution 1:200, Abcam, ab22554) and incubated with Goat anti-mouse secondary IgG (dilution 1:500, Dako, E3044) at 2 µg/ml for 2 hr at room temperature. After washing, sections were incubated in streptavidin-horseradish peroxidase (dilution 1:2000 Abcam, ab7403) for 30 min at room temperature. From each experimental group, specimens were obtained from two rats. For each rat, both left and right mammary fat pads were analyzed.

**Immunohistochemistry Quantification.** For TGFβ1, using Aperio© ImageScope (Aperio ePathology Solutions; http://www.aperio.com/), a total of five images per mammary fat pad, with two slides per rat specimen were taken at 10x magnification. The images were transferred to ImageJ 1.47 software. The ductal tissue was traced by hand via Wacom Intuos 4 (Portland, OR), and the adipocytes were erased. Images were de-convoluted under the ‘H DAB’ setting to separate the different colour channels to isolate the blue staining from hematoxylin and the positive brown staining from TGFβ1 antibody. A higher ratio of brown to blue staining would demonstrate greater TGFβ1 staining in those tissues. Quantification for adiponectin was done in a similar fashion, with a total of five images per fat pad, and two slides per fat pad, were taken at 10.0X magnification, one in each of four quadrants and one in the center region. Adipocytes were manually excluded, leaving only the glands and surrounding stroma to be analyzed. The ratio of positively stained brown cells to blue cells was used to determine the strength of adiponectin staining within each image. This ratio was compared to the ratio from the image of the entire specimen, in order to determine whether there was increased adiponectin staining near the glands.

**Picrosirius Red Staining.** Paraffin processed tissue sections of skin were stained for collagen using Picrosirius Red staining Kit (Cat# 2490-250, Polysciences, Warrington, PA) by the Pickering Lab at Robarts Research Institute. Again, two sections per rat (right and left fat pads) and two rats per group were stained. Under polarized light, Picrosirius Red Staining is able to comment on collagen deposition and bundling. Thus, expression of red, or denser bundling in the tissue is associated with greater fibrosis. Conversely, blue reflects a lower density of collagen bundling.

**Abrio 2.2 (Cri, Woburn, MA, USA) software was used for image acquisition.** Slides were examined under polarized light, and five randomized images at 40X magnification were taken of each slide of areas that were composed primarily of adipocytes. Five additional images at 40X magnification were taken in a randomized fashion of areas that were composed primarily of ductal tissue. Image acquisition and analysis was performed by a blinded assessor. Analysis was carried out in Photoshop CC (Adobe, Mountain View, California, USA). Ducts were isolated by manually cropping out adipocyte tissue that surrounded the ductal tissue. The total pixel count of this area was then recorded. Expression was red and blue was then isolated. Expression of extent of collagen deposition was thus examined by observing the amount of red expression, with respect to area and corresponding blue expression. The total number of red pixels multiplied by mean value (in other words, intensity), was used to provide a measure of absolute red expression.
2.4 qPCR analysis. Mammary gland RNA was isolated using Trizol (Thermo-Fisher, Waltham, MA) following manufacturer’s instructions and homogenized on ice. RNA was quantitated by spectroscopy (NanoDrop, Thermo-Fisher). Reverse transcription was performed with SuperScript VILO cDNA Master Mix (Thermo-Fisher). Reactions consisting of 1000ng RNA, 4µL Master Mix, and sufficient nuclease-free water to make the reaction up to 20µL were prepared and incubated according to the kit manual. The resulting cDNA was diluted 1:10 in nuclease-free water. Quantitative PCR (qPCR) was performed with SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA) using 10µL master mix with 1µL of forward and 1µL reverse primer, each at 10 ng/µL, and 8µL of diluted cDNA per reaction. qPCR was run on a Mx3000P system (Agilent Technologies, Santa Clara, CA) with 40 cycles at an annealing temperature of 60°C. Specificity of amplification was confirmed with melting curve analysis. Expression was calculated using the 2−ΔΔCt method relative to Gapdh expression and normalized to expression in the non-irradiated, untreated samples. Primer sequence: Gapdh forward: CTCATGACCACAGTCCATGC, Gapdh reverse: TTCAGCTCTGGGATGACCTT, TGFβ1 forward: ATGACATGAACCGACCCTTC, TGFβ1 reverse: ACTTCCAACCCAGGTCTCTTC, Collagen-1 forward: GAGCGGAGAGTACTGGATCG, Collagen-1 reverse: TACTCGAACGGGAATCCATC, Collagen-3 forward: TGATGGGATCCAATGGGAGA, Collagen-3 reverse: GAGTCTCATGGCCTTGCGTTT, Adiponectin forward: TAAGGGTGACCAGAGATG, Adiponectin reverse: GGAACATTGGGGACAGTGAC, Perilipin forward: TGCAAFCATTCTGACAGG, Perilipin reverse: GGAGCTCTTCTGCATCTTTTG, PPARγ forward AGAGCTGACCCAATGGTTG, PPARγ reverse: AAGGCTCTTTCATGGGCGCTG.

2.5 Skin Assessments. Photographs of the rats were taken at Day 0, 7, 14, and 21 after ultrasound gel was wiped off as described above. Images of all the rats in different groups over the time points were then uploaded onto Qualtrics, an online survey software tool (Provos, Utah, USA) and randomized. The survey was then sent to a staff physician with expertise on skin changes associated with radiation damage, as well as two resident physicians in plastic surgery. The reviewers were blinded to the treatment groups and day of treatment.

2.6 Volume assessment. Weights of the rats were recorded at Day 0, 7, 14, and 21. The 4th set of mammary fat pads and additional single fat pad from 5th set of mammary fat pads were dissected from rats and weighed. High frequency ultrasound images were analyzed using Vevo2100 ultrasound Imaging System. Each rat underwent ultrasound at Days 0, 14, and 21. As mentioned, only two of five rats in each group were analyzed on Day 7. For each fat pad, the midline was marked as the center of the nipple. Measurements of the fat pad, as well as the fat pad with the skin were taken at 2mm proximal and 2mm distal to the marked midline in Vevo2100 software. 2mm was chosen because it was the closest measurement that was consistently possible to the nipple yet avoid the mammary fat pad distortion associated at the level of midline due to the ducts feeding the nipple. 3D reconstruction. The same midline measurement was used as that used for thickness measurements in Vevo2100 software. The fat pad was contoured using the lasso tool at midline, at 5mm proximal and at 5mm distal to the mammary fat pad. These measurements were taken as the fat pads were consistently captured within these parameters – often further than that, the fat pad would “drop off” if the rat was smaller in size. The software was programmed to fill in the rest of the fat pad between these contour levels. The contours were checked by scrolling through the images (step size of each images 0.083mm) and adjusted as needed to reflect the mammary fat pads as accurate as possible.
2.7 Statistical analysis. Statistical significance between groups were assessed using 1-tailed independent sampled “t-test”. Values were considered to be significant if p < .05.

3. Results

The irradiated rats exhibited significantly higher scores on the Kumar Scale, a skin assessment scale previously used measure of cutaneous radiation injury for animals (Supp. Figure 1)\(^7\), amongst all days when compared to non-irradiated rats when evaluated by a clinical expert in radiation-skin changes in a blinded fashion (***p=.005; Figure 1). When specific days were analyzed, the radiation oncologist expert observed differences in the two groups at Day 7 and 21 (equal variances not assumed; *p=.0125 and **p=.0045 respectively). The clinical expert also found a difference (*p=.022) between the two groups at Day 7 using the Radiation Therapy Oncology Group (RTOG) toxicity scoring system, a commonly used scoring system for radiation skin reaction changes\(^12\). These results show that the applied dose of radiation resulted in acute and clinically visible changes in the exposed skin. To determine if this dose also resulted in chronic skin fibrosis typical of radiofibrosis observed in human skin, pro-fibrotic markers were measured.

An increased density of collagen fibrils is typical of radiofibrosis. This property was measured by Picosirius red staining of histology sections that were imaged with polarized microscopy. As shown in Figure 2, radiation induced a strong increase in collagen fibril density. (*p =.025). A similar result was observed when collagen was detected using Masson’s trichrome staining as well. There was a trend (p=.07) towards increased expression of active TGFβ1 in immunohistochemical studies of mammary fat pad sections. TGFβ1 is typically expressed within the first week of damage, and so it is not un-expected that a significant change was not observed in this series of experiments (Figure 2).

To further probe pro-fibrotic changes in the radiated rodent skin, qPCR analyses of TGFβ1 and Collagen 1 and collagen 3 mRNA were next evaluated. An increase in the Collagen-1 to Collagen-3 ratio is typical of scarred fibrotic tissue. Adipogenic markers were also evaluated since radiation has been observed to inhibit adipogenesis. Markers included the “master” adipogenic transcription factor, PPARγ and one of its targets the adipokine adiponectin. However, significant changes in mRNA expression was not observed in the control vs. radiated groups (Figure 3).

Since a goal of this study was to reduce tissue fibrosis in order to create a more favorable microenvironment to support autologous fat grafts, the effect of RHAMM function blocking peptides, which have previously been shown to inhibit fibrosis and promote adipogenesis\(^55,63,78\), on radiofibrosis in this rat model was next assessed. Visible Skin changes were not appreciably different between the radiated and peptide treated groups when the cumulative score was assessed using the Kumar and RTOG scales (representative images Supp. Figure 3, 4).

However, peptide treated radiated skin strongly reduced collagen fibril formation as detected by picrosirius red staining (*p=.003). Masson’s trichrome staining qualitatively showed a similar effect. Active TGFβ1 trended to a lower level in the peptide group (p=.08). These results show that the RHAMM function blocking peptide strongly reduces radiofibrosis as detected by collagen
fibril formation (Figure 4).

In order to assess if these changes in tissue fibrosis are accompanied by altered adipogenesis, adipokine expression was analysed by QPCR. These included ADIPOQ, and PPARγ. We also analyzed the mammary fat pad for volume changes using high frequency ultrasound but these were not significant (data not shown).

QPCR analyses showed a significant increase in the adipokine adiponectin (Figure 5) (*p=.015). There was a trend towards increased expression of PPARγ in the peptide treated group (p=.06) and a trend in an increase of PPARγ expression (Figure 5). These results suggest that the RHAMM function blocking peptide promotes a pro-adipogenic microenvironment.

4. Discussion

We describe in this study radiation induced inflammatory (dermatitis) and fibrotic changes in mammary fat pads. Other studies that have also observed radiodermatitis, radiation-induced lesions, and radiofibrosis in different animal models, using different radiation dosages. De Andrade et al. used 10 Gy, 40 Gy, and 60 Gy on dorsal excisional wounds in rats that resulted in macroscopic and microscopic injuries to rat skin with increasing doses of radiation. In wounds, visible skin changes, increased collagen fibril formation, and increased TGFβ1 expression observed as early as day 5 even at lower doses. Chronic changes in response to ionizing radiation have also in other animal models. For example, in leg contracture models, mice exhibit progressive leg contracture and fibrosis when administered single doses of radiation between 20-80 Gy. However, to our knowledge, our study is the first report of radiation induced mammary fat pad fibrosis.

Contrary to previous belief, radiation fibrosis is currently thought to be a dynamic process, characterized by constant remodeling and long-term fibroblast activation. In normal repair, fibroblasts are transiently activated into myofibroblasts to facilitate wound remodeling. Terminally differentiated myofibroblasts undergo apoptosis and normal tissue architecture is mostly restored. However, in fibrotic tissue, chronic myofibroblast activation is observed, which is thought to result from elevated levels of growth factors including TGFβ1, PDGF, TNFα, bFGF’s, GM-CSF, IL-1, IL-4, and CTGF. In animal models of lipodystrophy and scleroderma, these pro-fibrotic cytokines reduce the survival of adipocyte progenitor cells. Adipogenesis is also restrained by the rigid microenvironment produced by myofibroblasts ability to promote collagen fibrillogenesis. Loss of adipocytes also contributes to progressive tissue fibrosis since many adipokines are anti-fibrotic. This is particularly true of adiponectin. Our results support this connection between adipocytes and fibrosis since the RHAMM function blocking peptide reduced fibrosis concomitantly with an increase in adiponectin expression.

To date, there is no approved clinical treatment for radiofibrosis and few current options are available for restricting or reversing tissue fibrosis resulting from other forms of tissue injury. Although TGFβ1 is considered a master switch for promoting myofibroblast differentiation and survival and for reducing anti-fibrotic adipokine production, this cytokine has not been useful for clinical control of fibrosis likely because of its ubiquitous expression and multifunctional nature.
Another potential target that has emerged is PPARγ, which regulates both inflammatory and fibrosis processes. In particular, the PPARγ agonist rosiglitazone has been reported to protect mice against radiation induced inflammation and reduced expression of both TGFβ-1 and Collagen-I. However, the use of rosiglitazone is currently restricted in humans due to trials showing increased drug related cardiovascular toxicity. One of the PPARγ target genes, adiponectin, may be a more promising approach for reducing some forms of tissue fibrosis and clinical trials are currently ongoing for liver disease. However, in animal models, adiponectin did not appear to play a role in radiation induced intestinal damage in mice and its anti-fibrotic effects may be more specific to injury stimulus. We propose that blocking RHAMM function using mimetic peptides is an efficacious alternative approach to controlling tissue fibrosis resulting from radiation and also from other forms of tissue damage. RHAMM function blocking peptides are predicted to be particularly useful for reducing radiofibrosis in breast tissue since they create a microenvironment that supports adipogenesis. This is predicted to both contribute to a reduction in fibrosis (e.g. through elevated production of adiponectin) and to provide a method for supporting the survival and differentiation of adipocytes in autologous fat grafts. To date, RHAMM function blocking peptides have a good safety profile in animal models likely due to the restricted expression of this protein in homeostatic tissues. RHAMM expression is an integral part of the response of tissues to stress and to disease. Its primary functions appear to be control cellular migration and mesenchymal differentiation during repair processes. Future work with these mimetic peptides will be directed towards assessing their ability to promote pre-adipocyte survival and differentiation in fibrotic microenvironments.

We did not observe an increase in either the expression or activation of TGFβ1 or an altered Collagen-1:Collagen-3 ratio that has been reported for other animal models of radiation. TGFβ1 has been detected in skin as early as 6 hours after γ-radiation. Increased levels of TGFβ1 were also found at 6 hours post-irradiation in other studies, which returned to basal level within 48 hours and increased after 14 days. In mouse mammary glands, one team has found that whole body irradiation induced immunoreactivity at 1 hour, and persisted for 7 days. In addition, they found that irradiation specifically generates the active (versus latent) TGFβ1, suggesting that low and moderate doses of radiation induced the activation of latent TGFβ1 in the mouse mammary gland. Martin et al. describes two types of fibrosis: an immature, active, and inflammatory fibrotic tissue examined in most studies, versus a non-inflammatory and poorly cellularized fibrotic tissue that correspond to studies of samples 10-20 years after irradiation. Of note, the young fibrosis samples contained a high proliferation rate of myofibroblasts, and a high secretion of TGFβ1, whereas the older type of fibroblasts exhibited reduced proliferation and low secretion of TGFβ1. Future studies would look at sacrificing animals within the one-week mark as TGFβ1 is an early inflammatory marker, and after 6 months as perhaps a more accurate representation of chronic radiation fibrosis. The studies above support using TGFβ1, Collagen-1 and 3 as markers of fibrosis.

Previous studies showed that the injection of RHAMM function blocking peptides result in a detectable increase in mammary fat pad size as measured by micro computer tomography (MicroCT). High frequency ultrasound is a more common imaging modality that has recently evolve as a result of more sophisticated technology and computer programs. High frequency transducers (>15 MHz) have been used to successfully study the vascularization of expansion of lesions in the skin lesions, skin diseases such as psoriasis, scleroderma, erythema nodosum, among
others\textsuperscript{123}. More recently, Santolo et al. has evaluated the use of Doppler ultrasound examination (ECD) in autologous adipose tissue filling by a plastic surgeon in the setting of post-surgical, post-traumatic, and post-burn scars in patients\textsuperscript{124}. They found that they were able to have quantitative data with ultrasound examination representing various skin and subcutaneous layers in serial evaluations after lipofilling procedure, and thus subsequent average percentage of one-year survival of autologous implanted fat was possible. Other authors have used ultrasound as a non-invasive, quick, and low-cost technique to study fat necrosis, and subcutaneous tissue for pre-operative planning and document post-operative results in the context of lipofilling\textsuperscript{125–127}.

However, although ultrasound has been used to study lipofilling and adipose tissue in the previous studies, to our knowledge, our study was the first attempt to use high frequency ultrasound to quantify the volume of a rodent mammary fat pad. Attempts were made to set up rats in the same position for every measurement, but even so, slight movement of the rat or centering on nipple may not be exact each time and could affect the measurement. Slight angulation and rotation may have affected the field of capture as well. Efforts to bypass this were made by measuring thickness of mammary fat pad in addition to contouring the volume, whereby the irregularities around the nipple wouldn’t confound the accuracy of measurement. Other potential confounders include variation in ultrasound gel and pressure of the, potentially affecting the consistency in measurement of the fat pad secondary to differences in compression. Lastly, as with human breast volume, there exist natural variations in rat volume, and so perhaps we were unable to detect significant differences due to this with our sample size. Percentage change within rat weights, volume, and thicknesses were used to control for this, but again we saw no differences between the studied groups. We would recommend future studies repeat with a greater sample size, and have it compared to other methods of volume assessment.

From an oncological perspective, there has traditionally been concern with the safety of fat grafting at the breast level. Most of this concerned the potential for necrosis and micro-calcification of breast tissue that would be misleading on radiographic mammography\textsuperscript{11}. In addition, the interaction of grafted adipose tissue with host cancer cells, as well as the long-term effects of fat and cell transplantation in a microenvironment of highly reproductive tissue or residual tumour cells are unknown\textsuperscript{11,141–143}. Recommendations thus exist for patient selection for autologous fat grafting to the breast: risk factors such as BRCA-1, BRCA-2, positive personal or family history of breast cancer would warrant mammography prior to surgery\textsuperscript{11,144}. Fat grafts enriched with processed adipose derived stem cells have been used in breast reconstruction following mastectomy and in non-oncologic breast augmentation without adverse outcome, however no long term data is present\textsuperscript{145,146}. More research in long-term clinical studies in this area is needed, and it is suggested that until then, conventional fat grafting is safe in certain subgroups of patients, whereas an increase in stem cell fraction through various means cannot be recommended at present\textsuperscript{11}.

5. Conclusions

Radiation damaged skin as a result of radiotherapy is characterized by both acute and chronic changes, and can be replicated in an animal model. Here, a model for radiation-induced mammary fat pad has been developed, with an increase in observational skin changes, and increased collagen
bundling and expression of fibrotic marker TGFβ. RHAMM blocking peptides (NPI-110) have been shown to promote adipogenesis and decrease fibrosis in previous animal models. Here, we show that RHAMM blocking peptide NPI-110 increase expression of adipogenetic markers and decrease fibrotic markers in such a rat model of radiation-induced mammary fat pad fibrosis. These results support the further investigation of this approach to ultimately reduce radiofibrosis and improve breast reconstruction in breast cancer patients.

Conflicts of Interest

There are no conflicts of interest to disclose.

Acknowledgements

The authors would like to thank Carl Postenka, the Pickering Lab and Matt Lowerison for their aid in immunohistochemical and ultrasound experiments.

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Figure 1. Radiation group had significantly greater clinically detectable skin changes than non-irradiated control group with the Kumar scale and RTOG skin scoring systems. Skin assessments were performed by a radiation oncologist on control and irradiated rats in all days, using the Kumar and RTOG scoring systems A. Clinical Expert Evaluation mean score across all days with Kumar scale (***p=.0005) B. Clinical Expert Evaluation with Kumar Scale, days separated (**p=.0045; *p=.0125) C. Clinical Expert Evaluation mean score across all days with RTOG scale (**p=.0015) D. Clinical Expert Evaluation with RTOG scale, days separated (*p=.022) Values are the Mean, analysis with 1-tailed t-tests. n = 5 rats.
Figure 5. Markers of fibrosis are increased in irradiated groups compared to non-irradiated control in immunohistochemical studies. Paraffinized sections in control and irradiated rats euthanized at Day 21 were stained for markers of fibrosis. A. Representative images stained with H&E. B. Representative images stained with Picosirius Red, red indicating denser collagen bundling and networks, at 40x. C. Representative images stained with Masson’s Trichrome. D. Representative images stained with TGFβ1, brown is positive staining for TGFβ1, at 5x. E. The percentage staining per an area that expressed red when stained for Picosirius Red (*p=.025). F. The percentage staining per an area of fat pad at day 21 for TGFβ1 (p=.07). Values are the Mean, analysis with 1-tailed t-tests. *n = 5 rats, 5 images/fat pad, two fat pads/rat.
Figure 6. No difference in fibrotic markers were found in qPCR studies. Real time PCR was performed for markers of fibrosis in non-irradiated (control group) and irradiated rat whole mammary fat pads A. TGFβ1 (*p=.045) B. Collagen-1:Collagen-3 (p=.118) C. PPARγ (p=.085) D. Adiponectin (p=.063). Values are the Mean, analysis with 1-tailed t-tests. n = 3 rats, 3 technical replicates/fat pad, two fat pads/rat.
Figure 7. Markers of fibrosis are decreased in peptide-treated groups compared to non-peptide treated groups amongst irradiated rats in immunohistochemical studies. Paraffinized sections in peptide and non-peptide treated groups euthanized at Day 21 were stained for markers of fibrosis A. Representative samples stained with H&E B. Representative samples stained with Picrosirius Red, red indicating denser collagen bundling and networks, at 40x C. Representative samples stained with Masson’s Trichrome D. Representative samples stained with TGFβ1, brown is positive staining for TGFβ1, at 5x E. The percentage staining per an area that expressed red when stained for Picrosirius Red (*p=.003) F. The percentage staining per an area of fat pad at day 21 for TGFβ1 (p=.078). Values are the Mean, analysis with 1-tailed t-test. n = 5 rats, 5 images/fat pad, two fat pads/rat.
Figure 5. Peptide NPI-110 increased markers of adipogenesis amongst irradiated groups in qPCR studies. Real time PCR was performed for markers of adipogenesis in peptide and non-peptide treated groups in irradiated rats A. Adiponectin (*p=.015) B. PPAR\(\gamma\) (p=.055). Values are the mean, analysis with 1-tailed t-test. n = 3 rats, 3 technical replicates/fat pad, two fat pads/rat.
Supplemental Figure 1. Representative Images of right 4\textsuperscript{th} mammary fat pad rats in control and radiation group at Days 0, 7, 14, and 21.
**Supplemental Figure 2. A. Kumar scale B. RTOG scale**

### A. Kumar Scale

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<thead>
<tr>
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<th>Skin Changes</th>
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<tbody>
<tr>
<td>1</td>
<td>No effect</td>
</tr>
<tr>
<td>1.5</td>
<td>Minimal erythema, mild dry skin</td>
</tr>
<tr>
<td>2</td>
<td>Moderate erythema, dry skin</td>
</tr>
<tr>
<td>2.5</td>
<td>Marked erythema, dry desquamation</td>
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<tr>
<td>3</td>
<td>Dry desquamation, minimal dry crusting</td>
</tr>
<tr>
<td>3.5</td>
<td>Dry desquamation, dry crusting, superficial minimal scabbing</td>
</tr>
<tr>
<td>4</td>
<td>Patchy moist desquamation, moderate scabbing</td>
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<tr>
<td>4.5</td>
<td>Confluent moist desquamation, ulcers, large deep scabs</td>
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<td>Open wound, full thickness skin loss</td>
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### B. RTOG Scale

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<td>1</td>
<td>follicular, faint or dull erythema/epilation/dry desquamation/decreased sweating</td>
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<tr>
<td>2</td>
<td>Tender or bright erythema, pathy moist desquamation/moderate edema</td>
</tr>
<tr>
<td>3</td>
<td>Confluent, moist desquamation other than skin folds, pitting edema</td>
</tr>
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<td>4</td>
<td>Ulceration, hemorrhage, necrosis</td>
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Supplemental Figure 3. Images of right 4th mammary fat pad rats in radiation and radiation + peptide group at Day 0, 7, 14, 21 (Rat 4 and 5 of each group respectively).
Supp Figure 4: Peptide (NPI-110) did not reduce severity of clinically appreciable skin changes in irradiated rats. Skin assessments were performed by an expert clinician treated and non-peptide treated groups amongst irradiated rats using the Kumar Scale and RTOG scale A. Clinical Expert Evaluation mean score across all days using Kumar scale (p=.593) B. Clinical Expert Evaluation mean score across all days using RTOG scale (p=.231) C. Clinical Expert Evaluation using Kumar Score, days separated D. Clinical Expert Evaluation using RTOG score, days separated. Values are the Mean, analysis with 2-tailed t-tests. n = 5 rats.
Curriculum Vitae

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Publications


Truong JL, Tobe J, Fear T: Medical Humanities at the Schulich School of Medicine and Dentistry: Integrated Extracurricular Pre-clerkship Modules in Narrative Medicine, Visual Arts, and History of Medicine. Published in the Canadian Federation of Medical Students (CFMS) Annual review: Jan 2014.