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DISTINGUISHING THE ROLE OF INSULIN VS INSULINOMA CELLS AS MODULATORS OF CELL MIGRATION IN A CELL-MEDIATED MODEL OF WOUND HEALING

by

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Ronke Olabisi

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ABSTRACT OF THE THESIS

DISTINGUISHING THE ROLE OF INSULIN VS INSULINOMA CELLS AS MODULATORS OF CELL MIGRATION IN A CELL-MEDIATED MODEL OF WOUND HEALING

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Thesis Director:

Ronke Olabisi

Chronic wounds are wounds that recur or fail to heal by six weeks and cost the United States $150 million through prolonged and frequent hospital visits. Chronic wounds can be caused by poor blood circulation, compromised immune systems, and limited mobility. Current treatments include daily dressing changes that maintain moist wound environments, surgical debridement, skin grafts, and vacuum assisted wound closure. There is a need for an accelerated wound therapy that is inexpensive, nonsurgical, and reduces the patient's discomfort.

Insulin treatments have shown promise in wound healing because it is the main initiator of the mitogen-activated protein kinase – extracellular signal-regulated kinases (MAPK-ERK) pathways and the phosphoinositide 3-kinase – protein kinase B (PI3K-

Akt) pathway. This leads to the stimulation of haptotactic migration, collagen production, cell proliferation and a reduction in apoptosis. Pilot studies in the lab showed accelerated healing in a chronic wound model by using RIN-m, which are insulin secreting cells (ISCs) derived from pancreatic rat tumor cells. The results showed that wounds that were treated with ISCs closed at day 28 of the study, while the wounds treated by the controls did not close during the 35-day study. Since cancer cells also promote growth and resist apoptosis it is necessary to establish that these results stem from the insulin treatment and not the use of cancer cells. The goal of this present study is to evaluate the effects of cell lines derived from RIN-m, namely RIN-5F and RIN-14B, which secrete only insulin and somatostatin respectively.

These cells were combined with prepolymer solutions to create polyethylene glycol diacrylate (PEGDA) hydrogels. Conditioned media were used in an insulin ELISA to determine insulin concentrations. The conditioned media was also used to perform scratch assays using HaCaT keratinocytes to model an *in vitro* wound and monitor the rate of scratch closure.

The ELISA showed that the cell bioactivity and insulin release were not impeded by encapsulation, with the insulin concentrations at expected levels. RIN-5F had high levels of insulin secretion, and RIN-14B had low levels of insulin secretes. Since RIN-14B secretes somatostatin, which suppresses insulin, these results are expected. The experimental groups treated with RIN-5F conditioned media had the smallest gap area at the end of the 48 hour study than the experimental groups treated with RIN-14B conditioned media in the scratch assay. Since the RIN-5F experimental groups stimulated keratinocytes to close gaps faster than the RIN-14B experimental groups, it strongly

suggests that the results of the wound healing, both in these experiments and in previous lab work, stem from the insulin treatment and not simply the use of cancer cells.

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Chapter 1

Introduction

Chronic wounds pose a $150 million toll on the United States each year through prolonged and frequent hospital visits [1]. Chronic lower extremity ulcers affect 2.4-4.5 million people in the United States [2]. Chronic wounds are wounds that fail to close within three months [3, 4]. These wounds may take years to heal, or may never heal [5]. Key characteristics of chronic wounds include not progressing past the inflammation stage of wound healing and limited cell proliferation. Chronic wounds stem from poor blood circulation due to obstructed veins, compromised immune systems, or limited mobility. Those at risk include populations with systemic illnesses, such as diabetes, or those with repeated trauma, the immobile, the obese, and the elderly [2]. Besides the financial burden, chronic wounds affect the quality of patients' lives by causing additional financial stress due to multiple hospital visits and emotional stress due to increased pain, discomfort, loss of mobility.

Current treatments for chronic wounds include anti-inflammatory drugs, surgical debridement, vacuum assisted wound closure (VAC) and skin grafts [6, 7]. The standard treatment is anti-inflammatory drugs and compressive bandages, but these treatments require constant changing, which agitates the wound and allows for possible infections to occur which could further impair healing [8]. A more aggressive treatment is surgical debridement. Surgical debridement excises nonviable tissue and debris until healthy vascularized tissue has been reached in order to convert a chronic wound into an acute one. This is extremely painful for the patients, even under local anesthetic. Furthermore, should the wound heal, a defect remains that can impact the patient cosmetically and

functionally [9]. VAC is used as a last resort treatment. VAC involves applying negative pressure to the wound [10, 11], which improves vascularization, reduces bacteria at the wound, and increases wound contraction [12]. However, most studies involving VAC lack of standardization and therefore produce inconsistent results [13]. Autografts, or skin grafts, are considered a last-resort therapy because it comes with additional risks from surgery, further infection at the ulcer site, and graft failure [14]. There is a need for a treatment that doesn't require constant bandage changing, surgery and is not painful.

Insulin has shown promise as a treatment for chronic wounds. Insulin increases the expression of phosphoinositide 3-kinase – protein kinase B (PI3K-Akt) [15]. The Akt pathway is important for the proliferation, migration, growth and apoptosis of cells [7, 16, 17]. Pierre *et al.* conducted an experiment with six burn patients, between 15-27 years old, using a topically applied insulin treatment. The authors concluded that the insulin treatments decreased healing time by 2-3 days [18]. Lima *et al.* conducted an experiment using topical insulin cream on 22 rats. The rats were injured with a 4 x 4 mm full thickness excision wound. Complete wound healing was observed in the rats who received the treatment, but the wound in the rats that received the placebo treatment had not closed during the eight day study [19]. Rezvani *et al.* used a topical insulin treatment in a double-blind study on 45 patients, between 12-71 years old with noninfected acute and chronic extremity wounds. Patients receiving the insulin treatment experienced a 13.85 mm^2/day faster healing rate than those receiving the placebo treatment [20]. Although promising, most of these therapies require repeated, often daily, reapplication of the therapy which increases susceptibility to infection. As such, several groups have developed degradable polymers; however, their hydrolysis products have been shown to

reduce insulin bioactivity over time [19, 21]. Therefore, a therapy involving constant release of insulin through the use of live encapsulated cells contains promise as an accelerated wound therapy for chronic wounds.

Prior work from the lab has explored the effects of insulin as a wound healing therapy. Animal studies using genetically diabetic male mice (BKS.Cg-Dock7^{g}+/+Lepr^{db}/J; 10 weeks old, n=9; Jackson Laboratories, Bar Harbor, ME) were conducted with encapsulated rat insulinoma (RIN-m) cells and encapsulated glucose-stimulated insulin-releasing mouse sarcoma line (AtT-20ins) cells , and a control group receiving phosphate buffered saline (PBS). RIN-m cells provide a constant release of both insulin and somatostatin. Somatostatin is a hormone that inhibits other hormones, like insulin. AtT-20ins release insulin in a glucose-dependent manner. Experiments were conducted with both cell types entrapped in microspheres, then repeated with just RIN-m cells encapsulated in hydrogel sheets. The treatments were applied to 1 cm x 1 cm full-thickness excised wounds on dorsal skin on the mice. The results showed that the RIN-m treatment had closed 100% of wounds by day 28, AtT-20ins treatment had closed 66% of wounds by day 35, and the control treatment failed to close the wounds in the duration of the 35 day study [7]. Because insulin is a powerful wound healing agent, it was concluded that insulin was responsible for the accelerated healing. However, both cell lines were derived from carcinomas and thus it is possible these prior lab results are driven by cancer cell behavior rather than the insulin produced by the RIN-m cells. In general, cancer cells resist cell death, sustain proliferative signaling, evade growth suppressors, and enable replicative immortality [22].

This study seeks to confirm the role of insulin in the accelerated wound healing observed when insulin secreting cells (ISCs) were encapsulated and applied to *in vitro* and *in vivo* wounds. Towards that goal, the original *in vitro* experiments with sheet encapsulated ISCs were repeated with insulinoma cells that did or did not deliver insulin. RIN-5F and RIN-14B are both derived from the original study's RIN-m cell line and deliver either insulin or somatostatin, respectively. If the insulin is the cause of the accelerated growth, it is to be expected that the RIN-5F cells will close *in vitro* wounds the quickest. Conversely, if some unknown factor delivered by the cancer cells is the cause of the accelerated wound healing, it is expected that both cell lines, RIN-5F and RIN-14B, will perform similarly.

Chapter 2

Literature Review

2.1 Acute and Chronic Wounds

The skin is the body's largest organ. It is the first line of defense as it is the body's protective barrier against bacteria, dehydration and the general external environment [23, 24]. It is not atypical for the skin to suffer injuries as it is constantly exposed to the environment. Generally, wounds can be categorized as an acute wound or a chronic wound (Figure 1). Acute wounds are wounds that progress through the stages of wound healing in a timely manner. Chronic wounds are wounds that fail to fully heal within 3 months [3, 4]. Key features of acute wound healing include scab formation that prevents further infections, revascularization, keratinocyte migration to close the wound underneath the scab, and increased collagen production to rebuild the extracellular matrix. In the chronic wound, there is no scab so the wound is constantly being attacked by bacteria, blood vessels have been damaged and are not circulating blood well, there is no keratinocyte migration, there is a lack of collagen and growth factors, and there are numerous immune cells since the wound remains in the inflammation stage.

Chronic wounds include foot ulcers, pressure ulcers, and vascular ulcers [2-4]. Vascular ulcers typically occur in adults with vascular disease or diabetes, foot ulcers most commonly affect diabetics, while those with limited mobility most often suffer pressure ulcers; however, all can be triggered by additional morbidities like ageing, obesity, infection, and edema [2, 24-26]. These chronic wounds can lead to a loss of limb, function and decreased quality of life. Chronic wounds last on average for a year, and

recur in up to 70% of patients [2]. Chronic wound care costs up to 3% of healthcare budgets in developed countries, such as the United States of America [2].

Normal wound healing can be broken down into three main stages: inflammation, which lasts for 1-3 days; proliferation, which continues for 4-21 days, and remodeling, which is on the order of months [1, 19, 27]. During the inflammation stage, neutrophils migrate to the wound to remove bacteria and other foreign material. Eventually these neutrophils are replaced by macrophages, which phagocytose any remaining debris and signify the transition from inflammation to the proliferation stage of wound healing. During the proliferation stage, various growth factors and cells are recruited to the wound. This stage is characterized by angiogenesis and re-epithelization [2, 28]. For re-epithelization to occur, the body secretes chemokines, cytokines, and growth factors to recruit fibroblasts, endothelial cells and immune cells. Re-epithelialization is an importance process in wound healing, which consists of the proliferation, migration and differentiation of keratinocytes, fibroblasts, and myofibroblasts [29]. Fibroblasts differentiate from surrounding mesenchymal stem cells and migrate into the wound after the start of keratinocyte migration. This begins the reparative phase within the proliferation stage of wound healing. The arriving fibroblasts stimulate the production of collagen, which is important for the reconstruction of the extracellular matrix (ECM), and differentiate into active myofibroblasts, which are important for wound contraction [30]. The remodeling phase occurs after the wound has been closed during the proliferation stage and involves the remodeling of the resulting scar [1, 28].

Typically, chronic wounds never pass the first stage and remain in inflammation stage [2, 4, 24, 25]. There are many theories as to why chronic wounds cannot get past

this stage, including a lack of growth factors. It has been theorized that the lack of growth factors are caused by them becoming trapped in the extracellular matrix, or being degraded by proteases [1, 24].

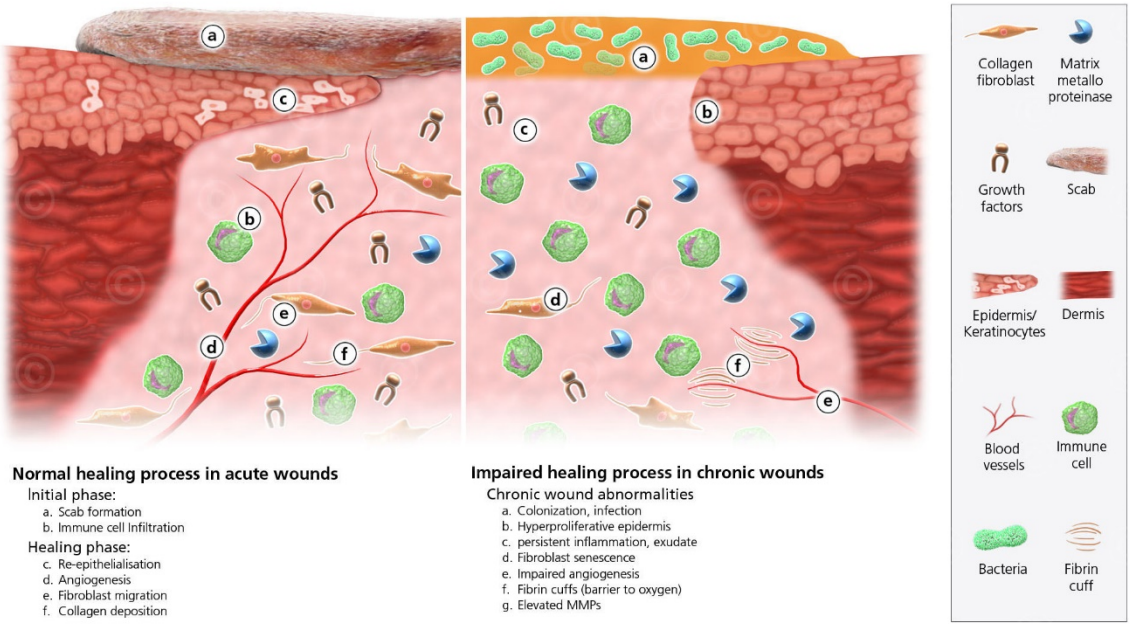

Figure 1: The Difference between an Acute (left) and Chronic (right) Wound [31]. In healthy acute wounds, wounds have begun scab formation and immune cell infiltration, continuous re-epithelialization and angiogenesis, increased fibroblast migration and collagen deposition. In chronic wounds, there continues to be infection, and bacterial colonization, the epidermis is hyperproliferative, there is persistent inflammation, continual fibroblast senescence, impaired angiogenesis, increased barriers to oxygens, and elevated matrix metalloproteinases (MMPs).

Keratinocyte migration is an important event during the proliferation stage of wound healing, it is directly involved in re-epithelialization and wound closure [27, 32-34]. In chronic wounds, keratinocyte migration is stalled. The failure of the keratinocytes to proliferate, migrate, and thus close the chronic wound exposes the wound to bacterial

infection. Uncontrolled infection can lead to amputation. In diabetic patients, 15% will develop a lower extremity ulcer and 14-32% of these patients will undergo amputation [3].

2.2 Insulin in Wound Healing

In addition to the numerous signaling molecules that play a role in wound healing, topically applied insulin has shown promise. Insulin's importance in regulating blood glucose is well known, and it is also important in wound healing. A number of cells, including keratinocytes, contain insulin receptors and although it remains unclear precisely how, insulin promotes their motility. Specifically, insulin stimulates haptotactic migration which is cell migration towards substrate-bound ECM components [35, 36]. Insulin also stimulates collagen production, which is needed to rebuild the ECM [35-37]. Insulin binds with its insulin receptor, which then activates tyrosine kinase [38]. Tyrosine kinase is responsible for activating the mitogen-activated protein kinase extracellular signal-regulated kinase (MAPK-ERK) pathways and for phosphorylating PI3K-Akt (Figure 2). MAPK-ERK pathways stimulate cell growth, proliferation, and differentiation [39], while PI3K-Akt activation prevents cell apoptosis [38, 40]. Insulin receptor-mediated PI3K-Akt activation has even proven to improve keratinocyte and vascular cell migration, which are both critical in wound healing [37, 40].

The MAPK-ERK pathway is an important wound healing pathway that mediates various events including cell proliferation, adhesion, metabolism and apoptosis [41-43]. ERKs control cell division and MAPKs regulate cellular activity like gene expression, mitosis, and cell movement [44]. In a knockout mouse study of MAPKs, activators of MAPKS, and substrates of MAPKs, the knockout mice exhibited lack of proliferation and

differentiation, resulting in embryonic lethality due to placental development defects, defective T cells, decreased apoptosis, and diminished angiogenesis [45]. Many mechanisms necessary in skin developmental embryogenesis are also necessary in re-epithelialization in cutaneous wounds, thus the lethality highlights the importance of MAPK [35]. In addition, inactivation of MAPK pathway is shown to inhibit the production of interleukin 8, which induces chemotaxis in cells, another critical process in wound healing [46].

The PI3K-Akt pathway is also an important wound healing pathway that mediates various events including proliferation, migration, growth and apoptosis of cells. The role of the PI3K-Akt pathway has also been demonstrated by its expression in the tissue of healing wounds [19].

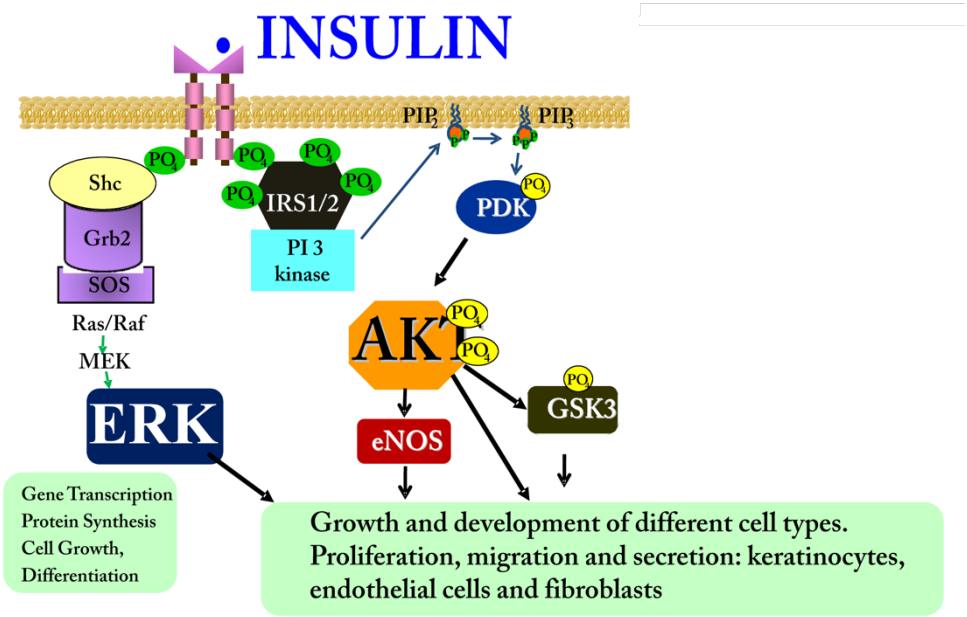

Figure 2: Insulin Receptor Binding Reactions: Modified from [19]. Insulin activates both the MAPK- ERK pathway and the PI3K-Akt pathway in cells. The ERK pathway then stimulates gene transcription, protein synthesis, cell growth, and differentiation. After activation, the PI3K-Akt pathway stimulates growth and development of many different

cells, and the proliferation and migration of various cells like keratinocytes, endothelial cells and fibroblasts.

2.3 Cancer Cells in Wound Healing

There is a possibility that the accelerated wound healing results from the RIN-m treatment from previous lab studies is caused by an unexpected artifact of using cancer cells and not from the benefits of the secreted insulin. This possibility stems from the various properties of cancer cells, such as sustained proliferative signaling, resisting cell death, and inducing angiogenesis (Figure 3) [22]. Chronic wounds are unable to enter the proliferation stage of wound healing, and cancer cells' ability to sustain proliferative signaling could jump start this stage within the wound through unknown paracrine effects. Furthermore, chronic wounds are characterized by a lack of vascularization in the wound, and cancer cells have the ability to induce angiogenesis. It has been suggested that these qualities may make cancer cells beneficial in healing chronic wounds [47, 48].

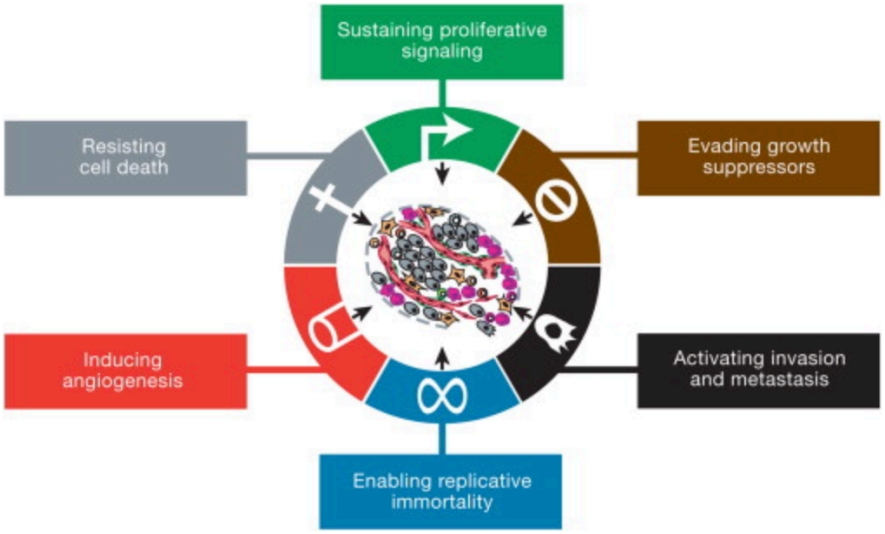

Figure 3: The Hallmarks of Cancer Cells [22]. Cancer cells sustain proliferative signaling, evade growth suppressors, activate invasion and metastasis, enable replicative immortality, induce angiogenesis, and resist cell death.

There are numerous similarities in the mechanisms cancer cells use to survive and the mechanisms to heal wounds. For example, chemokines and cytokines that are released in response to injury are similar to the ones found in invading tumors [47, 48]. The microenvironment of healing skin wounds and that of an epidermal squamous cell carcinoma are very similar. The underlying reason for the similarities is still being studied, but both secrete the same growth factors, cytokines, and MMPs. [49]. In addition, the same metabolic pathways active in wound healing are active in cancer.

The MAPK-ERK pathway is an important pathway for cell migration by its downstream activation of various growth factor receptors, including epidermal growth factor (EGF). EGF is also overexpressed in cancer, such as colorectal cancers [50]. Additionally, matrix metalloproteinase-7 (MMP7) is regulated through the MAPK-ERK pathway and is also involved in early intestinal tumorigenesis [50]. Certain members of the MAPK family are activated by cytokines and are also involved in the regulation of p53, which is a tumor suppressor [51]. Similarly, the PI3K-Akt pathway is also observed to be active in various cancers. For instance, the PI3K-Akt pathway can be activated with the activation of erbB2 tyrosine kinase receptor, which is overexpressed in various cancers, like breast cancer [28]. Additionally, a hallmark of cancer includes resisting cell death and PI3K-Akt activates cyclic AMP response element-binding protein (CREB), a transcription factor, which regulates antiapoptotic activity [52]. Thus, although the MAPK-ERK and PI3k-Akt pathways are active during wound healing, they are also

active during tumorigenesis and in other cancer behavior. And although insulin activates these pathways, cancer also does. Therefore, it is possible that an unidentified factor secreted by the insulinoma cells used is stimulating the MAPK-ERK and PI3k-Akt pathways through paracrine effects acting on the various epithelial cells in the wound environment to accelerate wound closure and the keratinocytes in the scratch assay.to accelerate gap closure.

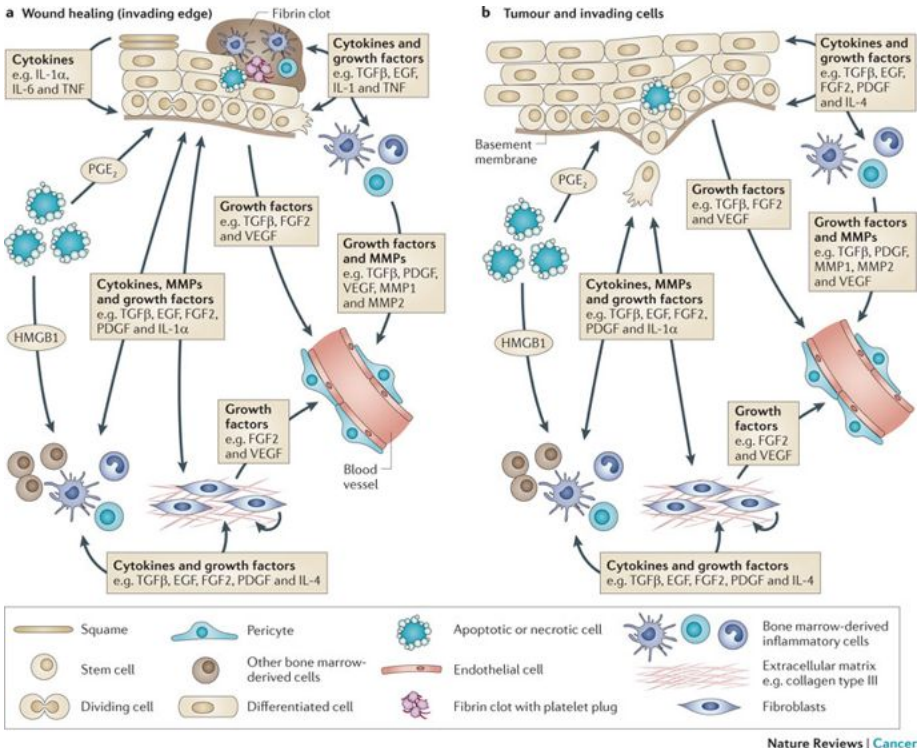

Figure 4: Comparison of Microenvironments of Healing Wounds and Invading Tumors [47]. Both healing wounds and invading tumors signal the release of cytokines, which are involved in autocrine and paracrine signaling and an increased release of various growth factors, which control cell growth, proliferation, apoptosis, and angiogenesis. These similarities could be factors influencing the accelerated would healing therapy demonstrated by prior lab work.

2.4 Cell Migration in Wounds

Cell migration is defined as the single cell or collective cell movement. Collective cell movement can be thought of as sheets of cells migrating two-dimensionally across a tissue surface. This is the mechanism in which keratinocytes migrate during both *in vivo* and *in vitro* (scratch assay) wound healing. Wound healing is a complex process that involves various growth factors, cytokines, chemokines, and microarchitectural cues from the extracellular matrix. It involves a variety of epithelial cells, including keratinocytes and skin fibroblasts. Keratinocytes proliferate, migrate, and differentiate to patch the epidermal barrier of the wound while kin fibroblasts = stimulate collagen production, as described in the section describing acute and chronic wounds [53].

Following a wound, cell migration occurs within hours of injury. Within 24 hours of injury, epidermal cells adjacent to the wound's edges detach from the basal lamina and are ready to migrate [53]. Directional influences determine the migration of the detached cells in the form of a soluble attractant, which describes chemotaxis, or a foundation-bound gradient of a matrix constituent, whether an attractant or an adhesive substrate, which describes haptotaxis, or, alternatively, a gradient of bound attractants, [30]. Wound healing *in vivo* is a complex process that involves other events such as invasion of macrophages, migration of fibroblasts, and coordination of angiogenesis[53].

Cell migration in *in vivo* wounds is composed of several processes. Although this migration is very complex, scratch assays offer a way of testing keratinocyte migration *in vitro*.

2.5 Scratch Assays as a Model of Wound Healing In Vitro

The scratch assay is a convenient, low-cost, well-developed model of *in vitro* wound healing. This model evaluates the effects of various treatments on cell migration by creating an artificial gap within a monolayer of cells, usually keratinocytes, and analyzing time lapse photos until the gap closes.

Keratinocyte migration that closes scratches in a scratch assay can be related to re-epithelization within *in vivo* wounds. *In vivo* wound healing consists of the proliferation, migration, and differentiation of keratinocytes and scratch assays examine keratinocyte proliferation and migration. [29]. Nevertheless, there are drawbacks. For instance, although the proliferation and migration of keratinocytes in a scratch assay can be related to the proliferation and migration of keratinocytes in a wound, there are certain disadvantages to using scratch assays. For example, scratch assays do not establish any chemical gradient and thus are not a good model to analyze the effects of chemotaxis on cell migration. However, scratch assays are a good model to evaluate the effects on collective cell migration since the cells are migrating as a group to fill the artificial gap created.

This thesis conducted scratch assays with conditioned media from RIN-5F and RIN-14B experimental groups. The goal was to compare the effect of insulinoma-conditioned media with and without insulin on the speed of keratinocyte migration in order to determine whether the previous wound healing results stemmed from the secreted insulin or an unknown factor from the carcinomas cells. RIN-5F and RIN-14B cells are derived directly from RIN-m cells, which were used in the original study. The scratch assay will allow the

assessment of the effect of these experimental groups on the speed of keratinocyte migration. If the insulin has no effect and there is only a paracrine effect from the use of insulinoma cells causing accelerated gap closure, the expected result is that not only will both RIN-14B and RIN-5F close the artificial gap at similar rates, but these experimental groups will also perform at a similar level as the RIN-m cells. Since all cell lines are derived from the same insulinoma cells, if cancer factors alone had an effect on the accelerated wound healing, all cell lines would be expected to perform similarly since any paracrine effect causing the accelerated wound healing would appear in all cell lines. However, if the accelerated gap closure occurs from the secreted insulin alone, the expected result is that RIN-5F cells will close the artificial gap faster than the RIN-14B cells, which do not secrete insulin. Since it is known that insulin assists wound healing, a final possibility is that both insulin and some cancer factor are combining somehow and driving wound healing. If this were the case, then RIN-14B cells would be expected to close scratch assays faster than controls, but slower than RIN-5F cells.

Chapter 3

Methods and Materials

3.1 Cell Culture

Frozen RIN-5F and RIN-14B cells were thawed and transferred into 175 cm^2 culture flasks. The flasks were incubated at 37°C in a humidified incubator with 5% CO_2. Cells were expanded in complete cell medium: Roswell Park Memorial Institute (RPMI) 1640 from ATCC, 10% fetal bovine serum, and 1% penicillin-streptomycin. The media was changed three times a week and cells were passaged when they achieved 75% confluency. After the fifth passage the RIN-5F and RIN-14B cells were encapsulated into PEDGA hydrogels at $2x10^6$ cells or $0.5x10^6$ cells per 1 cm x 1 cm x 0.4 mm hydrogel.

3.2 Hydrogel Encapsulation

A prepolymer hydrogel solution was prepared as previously described, with the exception of acetophenone.[cite] Briefly, the following were combined: 0.1 g 10 kDa PEGDA, 493 µL HEPES-buffered saline, 1 µL triethylolamine, 10 µL 1.0 mM eosin Y solution, 3.95 µL N-Vinylpyrrolidone (NVP), and cells at $0.5x10^6$ for low cell density (LCD) or $2.0x10^6$, for intermediate cell density (ICD) hydrogels. The RIN-5F and RIN-14B cells were harvested, pelleted via centrifugation and were resuspended in 50 µL of the prepolymer hydrogel solution. This cell-prepolymer solution was then pipetted into a custom made glass-Teflon mold that was composed of a 0.4 mm thick Teflon sheet sandwiched between two glass microscope slides clamped together with binder clips. A 1 cm x 1 cm section was cut from the Teflon. After injection into the mold, the solution was polymerized by white light (Figure 5) and polymerized hydrogels were removed

from molds. Six hydrogel sheets were formed, with three containing RIN-5F and three containing RIN-14B. The six sheets were placed in each well of a 6-well plate and submerged with 5 mL of complete cell culture media. Conditioned media was collected from cells in monolayer ($0.5x10^6$ cells per flask) or from cells encapsulated in hydrogels ($0.5x10^6$ or $2x10^6$ cells per hydrogel) on days 1, 7, 14, 21, 28 and stored at -20°C until analysis. Conditioned media collected from encapsulated cells and monolayers were analyzed with an insulin enzyme-linked immunosorbent assay, ELISA, Kit, (EMD Millipore Corporation) and used in scratch assays. All materials were provided with the insulin ELISA kit and procedures were followed according to the included instructions.

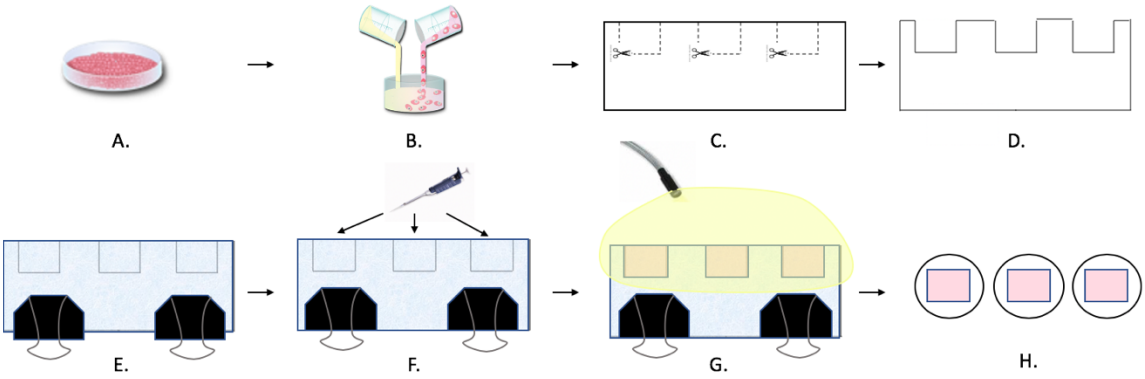

Figure 5: Schematic of Hydrogel Encapsulation. **A.** Harvest cells at $0.5x10^6$ or $2x10^6$ million cells. **B.** Combine cells and prepolymer hydrogel solution. **C.** Measure 1 cm x 1 cm squares in 0.4 mm thick Teflon sheet. **D.** Cut the squares out. **E.** Sandwich the Teflon sheet with glass slides and hold secure with binder clips. **F.** Pipette the combination of cell and prepolymer hydrogel solution into the precut space. **G.** Polymerize hydrogels with white light. **H.** Carefully remove hydrogel sheets from mold, place in a 6-well plate and immerse in complete culture media.

3.3 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a plate-based assay that uses antibodies to measure the levels of peptides, proteins, antibodies and hormones in samples. Insulin ELISAs use two antibodies to capture insulin within samples, and then a plate reader records the amount of insulin within the samples. The insulin ELISAs were conducted to verify the concentration of insulin secreted by various experimental groups. This test is important to verify the bioactivity of each experimental group.

3.4 Scratch Assay

As described in the previous chapter, scratch assays simulate a wound healing response *in vitro* and verify insulin bioactivity by stimulating the migration of keratinocytes. HaCaT keratinocyte cells were thawed and transferred into 175 cm^2 culture flasks. The flasks were incubated at 37°C in a humidified incubator with 5% CO_2. Cells were expanded with HaCaT complete culture medium: Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, and 1% penicillin-streptomycin. The media was changed three times a week and passaged when cells achieved 75% confluency. After the third passage the HaCaT cells were plated in 24-well plates.

HaCaT cells were allowed to reach 70-80% confluency within wells then the monolayers were scratched with a 10 µL pipette tip (Figure 6). Conditioned media (0.5 mL) from RIN cells and fresh complete media (0.5 mL) were added to the wells. Controls received 1 mL of HaCaT complete culture media. For positive control, 4.3 ng/mL of human insulin was added to the wells. Negative control received no additions. The wells were imaged at 0, 4, 8, 24, 48 hours and images were analyzed using NIH ImageJ. The

scratch assays were analyzed by calculating the percentage of the original gap that has remained open with the equation: $\left(\frac{new\ gap\ area}{original\ gap\ area}\right) * 100$.

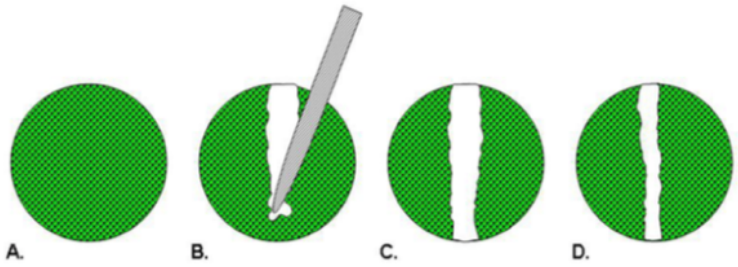

Figure 6: Depiction of the Process for the Scratch Assay. **A.** Allow keratinocytes to reach confluence. **B.** Scratch monolayer with a 10 µL pipette tip**. C.** Add the conditioned media or controls. **D.** Monitor the scratch closure over time

3.5 Statistical Analysis

All data were taken in triplicate. ELISA results are reported as mean ± standard deviation. and were compared between different treatment groups using a one-way analysis of variance (ANOVA). p-values less than 0.05 were considered statistically significant. Pairwise comparisons between groups were made using Fisher's Least Significant Difference (LSD) post-hoc test. Scratch assay results are reported as mean ± standard error and were compared between different treatment groups using a t-test with unequal variances. p-values less than 0.05 were considered statistically significant. All analysis was performed using Microsoft Excel.

Chapter 4

Results

ELISA results were analyzed to align with data from unencapsulated monolayer cells to correct a labeling error. Afterwards, data from the different days for each experiment group, RIN-5F and RIN-14B, were averaged (Figure 8).

4.1 Cell Encapsulation

Cells encapsulated at ICD were plated in a 6-well plate showed no viability at any time points. No further studies were conducted with these cells. Cells encapsulated at LCD were plated in a 6-well plate showed viability at all time points. ELISA and scratch assays were run on these sets of hydrogels.

4.2 ELISA Experiments

ELISAs were conducted to measure the levels of insulin secreted by the experimental groups and controls. The insulin concentration levels between the conditioned media collected from RIN-5F and RIN-14B monolayers were compared to RIN-m monolayer insulin concentration levels. The ELISA results were normalized by volume.

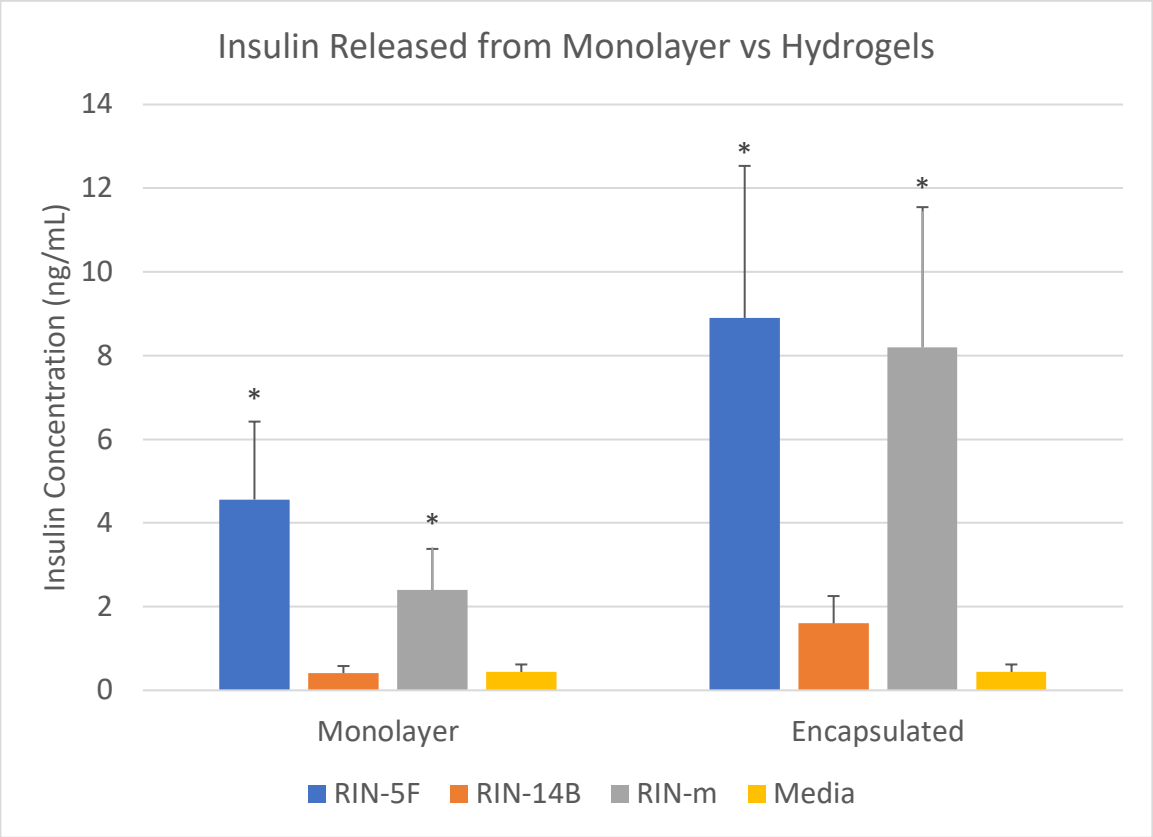

Figure 7: Difference in Insulin Concentration from Conditioned Media. The ELISA results return insulin concentration levels of the conditioned media from the treatment groups and values from day 7 monolayer and encapsulated cells are compared to insulin concentrations from previous lab data (RIN-m). Error bars show standard deviation. RIN-5F encapsulated cells released the highest amounts of insulin at 8.9± 3.633 ng/mL, RIN-14B monolayer cells released the lowest amounts of insulin at 0.413 ± 0.168 ng/mL. There was no statistical difference between insulin levels in RIN-14B monolayer vs media only controls (0.439 ± 0.156 ng/mL). Although the level was higher, there was also no statistical difference between RIN-14B encapsulated cells (1.567 ± 0.321 ng/mL) vs control. Asterisks indicate statistically significant differences ($p<0.05$) between the indicated treatment(s) and the media control.

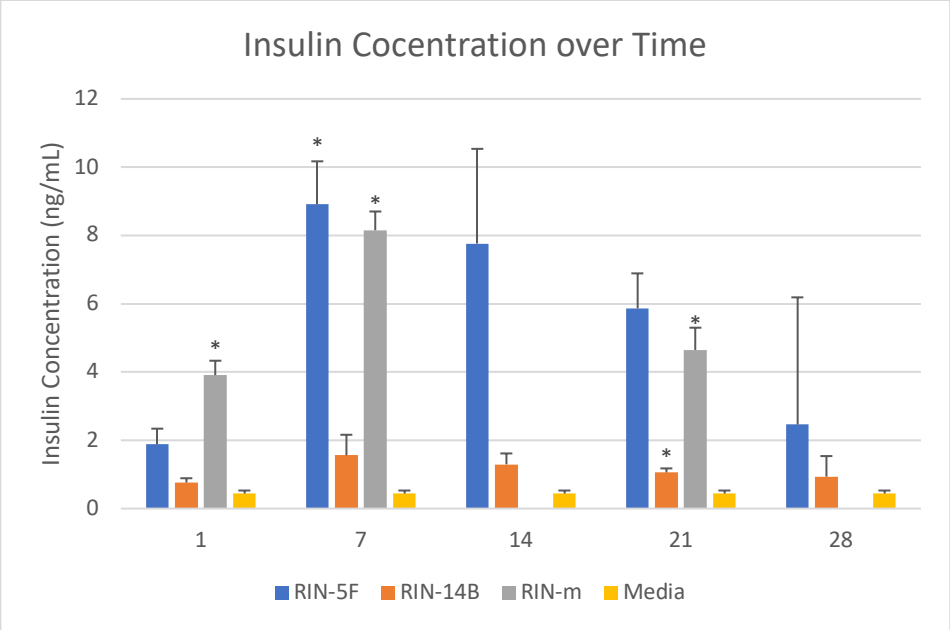

Figure 8: Difference in Insulin Concentrations with Conditioned Media from Encapsulated Cells at $0.5x10^6$ cells/gel. The insulin levels over time for the collected conditioned media. Error bars show standard error. Asterisks indicate statistically significant differences ($p<0.05$) between the indicated treatment(s) and the media controls. RIN-m data from the original studies were used for a direct comparison of the original results; this original data only included days 1, 7, and 21. The conditioned media from encapsulated RIN-5F cells from day 7 show the highest insulin concentration at 8.91 ± 1.26 ng/mL. The media show the lowest insulin concentration at 0.439 ±0.09 ng/mL.

4.3 Scratch Assays

Scratch assays were conducted to compare the effect of the conditioned media and controls from encapsulated cells on keratinocyte migration. HaCaT keratinocytes were plated in a monolayer and the artificial gap stimulates an *in vitro* wound. The scratch assay was imaged with a light microscope at various hours (Figure 9). These images

provide a time lapse in which changes in the gap areas can be measured and compared (Figures 10 - 12).

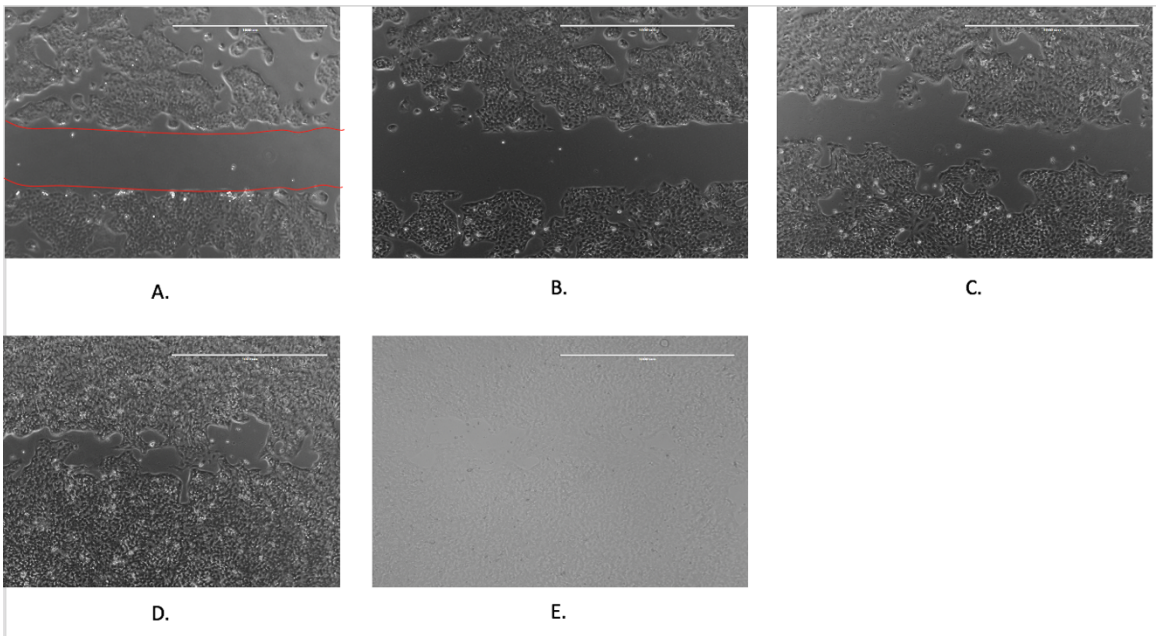

Figure 9: Representative Scratch Assay Images Over the Time.

The scale bars are 1000 μm. **A**. Artificial gap imaged at 0 hour. Red lines mark the initial scratch boundaries. **B**. Artificial gap imaged at 12 hours. The scratch has not experienced much gap closure. **C.** Artificial gap imaged at 24 hours. The original boundaries of the gap is less apparent. The gap area is smaller than B. **D.** Artificial gap imaged at 48 hours. The gap is almost non-existent. **E.** Artificial gap imaged at 72 hours. The gap is fully closed.

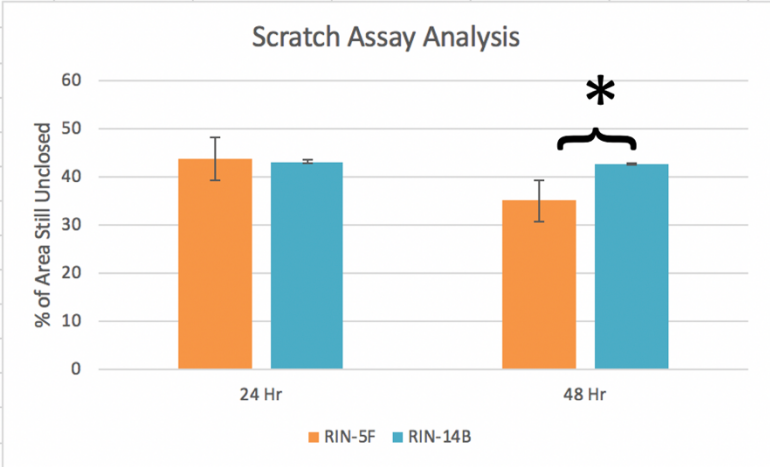

Figure 10: Scratch Assay Analysis with Conditioned Media from Monolayer.

This graph shows the percentage of area still unclosed. The scratch was conducted with conditioned media extracted from the monolayer. The assay was imaged at 0, 24, 48, and 72 hours. The scratch assay was done in triplicate, and the results were averaged. The error bars show standard deviation. The gaps for all of the experimental were closed at 72 hours. At 48 hours, we can see that the gap was smaller for the group that was treated with the conditioned media from the RIN-5F, insulin producing, monolayer. The error bars show standard error. At 24 hours 43.77% ±4.3 and 43.11±0.28 of the area was still unclosed for RIN-5F and RIN-14B respectively. At 48 hours 35% ±4.3 and 42±0.28 of the area was still unclosed for RIN-5F and RIN-14B respectively. There was no significant difference between the experimental groups.

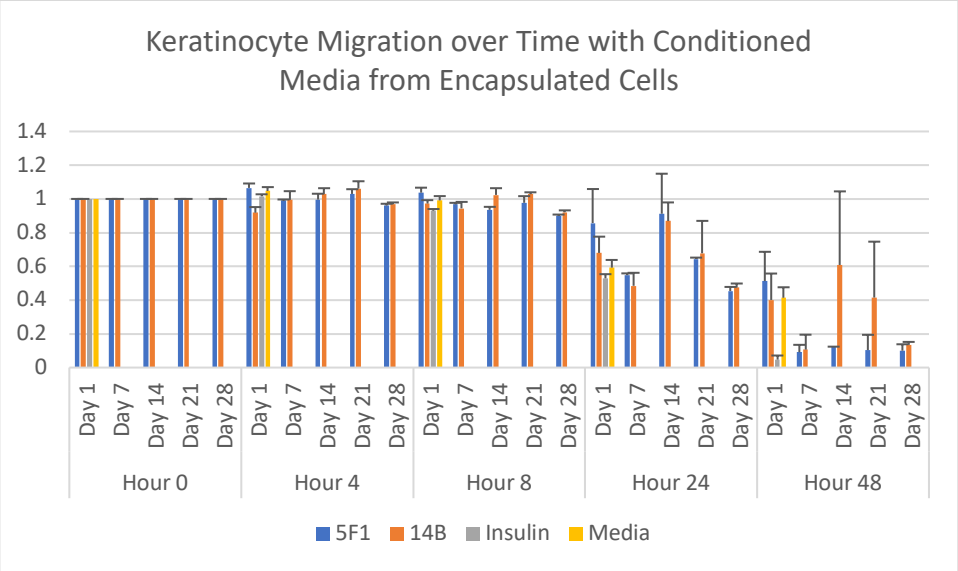

Figure 11: Scratch Assay Analysis with Conditioned Media from Encapsulated Cells. This graph shows the percentage of area still unclosed for each experimental group, which are the conditioned media collected from encapsulated cells ($0.5x10^6$ cells/gel) on various days. The scratch assay was imaged at 0,4,8,24 and 48 hours. The bars represent experimental group. The scratch assay was done in triplicate and averaged to show the following data. The error bars represent standard error.

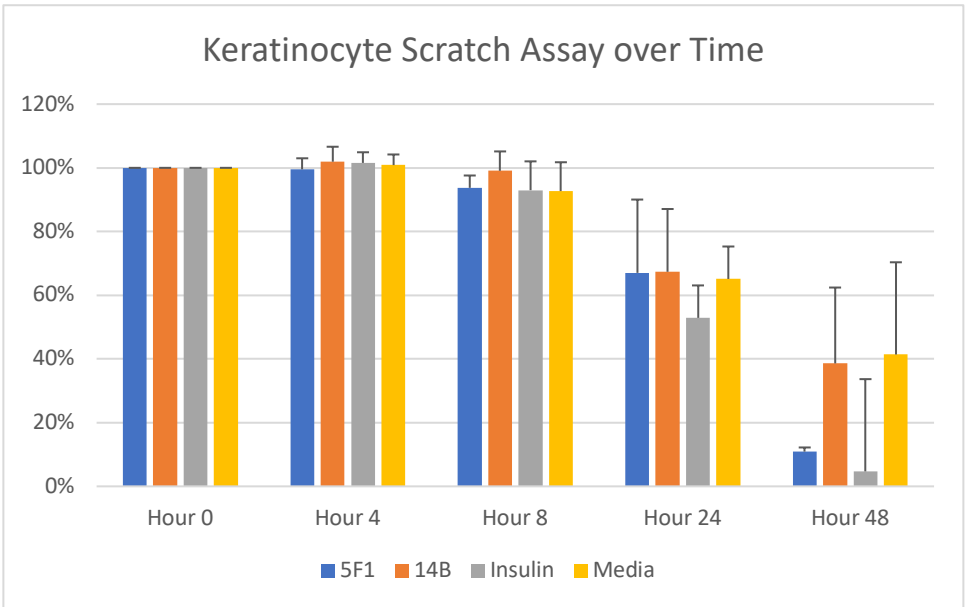

Figure 12: Scratch Assay Analysis with Results from Days Averaged at Each Time Point.

The data from the various days shown in Figure 11 was averaged if not statistically different (Day 1 was excluded as an outlier). The media experimental group were averaged from current and previous experiments. By 48 hours, the gaps treated with media from gels encapsulated with RIN-5F cells were smaller than the gaps treated with conditioned media from gels encapsulated with RIN-14B cells. Due to large variation, there was no difference between scratches in hour 48. However, the means of the RIN-5F group and the insulin only group were similar and the means for the RIN-14B and media only groups were similar. The error bars represent standard deviation. The percentage of area still unclosed at and at 48 hours is 0.109±0.013, 0.386±0.238, 0.048±0.24, 0.353±0.359 for RIN-5F, RIN-14B, insulin only positive controls and media only negative controls, respectively.

Chapter 5

Discussion

The goal of this thesis was to verify that the lab's previous accelerated wound healing results stem from the insulin treatment and not from the use of cancer cells. To prove this, experiments conducted with RIN-m cells were conducted with RIN-5F and RIN-14B cells, both insulinoma cells from pancreatic rat tumor cells derived from the same RIN-m cell line.

A total of 12 hydrogels were created. Hydrogels (n=12) were encapsulated at LCD (n=6) or ICD (n=6), with either RIN-5F (n=6 total; n=3 per seeding density) cells or RIN-14B cells (n=6 total, n=3 per seeding density). Previous lab work tested cell viability at LCD, ICD, and high cell density (HCD) of $5x10^6$ cells/hydrogel. The cells in this thesis were encapsulated at LCD and ICD to examine whether the RIN-5F would behave the similarly to RIN-m cells and if they would also produce more insulin at lower cell densities. After the cell death in the ICD hydrogels, HCD hydrogels were not evaluated. Results from this study show that cells encapsulated at LCD had the highest viability and released the most insulin, as analyzed with an ELISA. The hydrogels encapsulated at ICD showed no cell viability, which differed from previous results. These hydrogels appeared to deplete their media faster than other hydrogels and may have been starved in a way that RIN-m were not in the prior study. Potential reasons that this occurred may be due to the use of different cell lines, the RIN-m, RIN-5F, and RIN-14B cells grow at different rates and the latter two could have been too crowded in the ICD encapsulation to be viable. This result reconfirmed previous data that cells encapsulated at LCD was better for overall viability.

The media from the RIN-5F cells showed the highest level of insulin secreted when encapsulated at LCD. The media from the RIN-14B cells showed the lowest levels of insulin secreted when conditioned media was collected from the monolayer. It is unsurprising that RIN-14B insulin levels are near zero since RIN-14B is designed to secrete somatostatin but not insulin. The conditioned media collected from day 7 hydrogels encapsulated with RIN-5F cells had the highest level of insulin secretion, higher than the same cells in monolayer. This was true even when correcting for media volume and cell number. This supports the prior lab results that encapsulating the ISCs helps to stimulate insulin release. The insulin levels between RIN-5F and RIN-m were not statistically different. This is also in line with expectations since RIN-5F are derived from RIN-m cells and both cell lines secrete insulin. In addition, the insulin levels between media only and RIN-14B were not statistically different. This was also expected since RIN-14B do not produce insulin but small levels of insulin were detected and the media used was complete cell media containing serum, and there is insulin in serum [54].

In the scratch assay experiments conducted in this study, the experimental groups treated with RIN-5F conditioned media and insulin only had smaller gaps at the end of 48 hours than the experimental groups treated with RIN-14B conditioned media or media only controls. Since RIN-14B did not accelerate gap closure faster than media only, it suggests that nothing released by RIN-14B cells accelerates wound closure. Because RIN-5F and RIN-14B are both derived from insulinoma cells, if the results had stemmed only from the use of cancer cells, both of the experimental groups treated with RIN-5F conditioned media and RIN-14B conditioned media should have performed similarly and closed the gaps at similar rates. Had this occurred, it could have been due to the

insulinoma cells releasing certain pro-migration factors that were accelerating the keratinocyte migration. Since it is known that insulin accelerates wound healing, which was confirmed by our positive control, the other possibility was that insulin and cancer signaling was accelerating keratinocyte migration. If that were the case, RIN-5F should have closed gaps the fastest since these cells would have cancer signals plus insulin, followed by RIN-14B since these cells would have cancer signals alone, followed by media only, which should have nothing additional. However, the experimental groups treated with RIN-5F conditioned media consistently outperformed the experimental groups treated with RIN-14B conditioned media in closing scratches, and these did not outperform media only controls. The difference between these two lines is that RIN-5F secretes insulin and RIN-14B secretes somatostatin. Therefore, these results strongly suggest that the previous lab results stem from the insulin treatment and not the use of cancer cells.

Chapter 6

Conclusions

Wound healing is a complex process that involves various complex biological processes. When this fine-tuned process is disturbed, it could lead to chronic wounds. Chronic wounds can be caused by poor blood circulation, a compromised immune system, or limited mobility. These wounds cause patients increased pain, loss of mobility, and discomfort and create a financial burden due to multiple hospital visits. Current treatments are costly, require constant bandage changes, surgeries, or are extremely painful. Thus, there is a need for a treatment that is inexpensive, nonsurgical, reduces the possibility of infection, and does not cause the patients discomfort.

An insulin treatment could fill this need. Insulin prevents apoptosis, when tethered to a hydrogel or other substrate can stimulate haptotactic migration, promotes collagen production, and triggers cell growth, proliferation, and differentiation through the activation of the MAPK-ERK and PI3k-Akt pathways in cells. The work in this thesis established that the previous lab results that showed accelerated wound healing through the use of a therapy involving encapsulated RIN-m cells can be credited to the insulin secretion and not to the release of cancer signals.

Overall, the insulin treatment explored by this study presents great potential as a therapy for chronic wounds.

Chapter 7

Future Work

Due to lab closures stemming from the current coronavirus pandemic, control experiments were not performed as preferred. This section describes the experimental plan for remaining experiments if the work had not been interrupted.

Although the experiments were designed to exactly follow the previous protocols to allow a direct comparison, a limitation is that although the levels are very low, fetal bovine serum contains insulin [54]. Another limitation was that the media only and insulin only controls used fresh media while all other groups received 50% conditioned media, which had been depleted of some of its nutrients by the encapsulated cells. In the repeat experiments, there would be 4 additional experimental groups and 3 additional controls, all in serum free media: 1) RIN-m; 2) RIN-5F; 3) RIN-14B; 4) insulin-releasing fibroblasts 5) empty hydrogels; 6) insulin only; and 7) conditioned media only. The experimental groups would be repeated exactly as described in the methods section of this thesis, except serum would not be added to complete culture media. Fibroblasts (group 4) that were genetically modified via adenoviral transduction to release insulin would be obtained from collaborators at Baylor College of Medicine (BCM). The goal would be to have cells release insulin but not cancer signals to compare the rate of gap closure of these cells to those of RIN-5F cells.

To avoid any artifact due to experimental groups receiving 50% conditioned media and 50% fresh media while control groups receive 100% fresh media, conditioned media from keratinocytes not used in scratch assays will be combined with fresh media for controls. For group 5, empty hydrogels, or hydrogels that lack cells, would be created

and placed in 6 well plates with the same media as experimental groups. For group 6, the insulin only control, 6 mL of 50/50 keratinocyte conditioned media would be combined with 12.9 ng of commercial insulin, which gives a final concentration matching the insulin released into the media from the previous RIN-m study. The negative control media (group 7) will be composed of just the 50/50 keratinocyte conditioned media.

Expected results include the groups containing insulin (Groups 1, 2, 4, 6) closing gaps faster than all other groups. If cancer signals play no role, it would be expected that the insulin secreting fibroblasts would perform similarly to the RIN-5F, when normalized for cell number and insulin release. It would also be expected that the positive control (Group 6) would close the artificial gap quicker than groups without insulin, the RIN-14B and negative control (Groups 3 and 5).

Table: Expected experimental results for future experiments.

Experimental Group	Released Product	Expected Results
1) RIN-m	Insulin + somatostatin + cancer signals	Accelerated scratch closure
2) RIN-5F	Insulin + cancer signals	Accelerated scratch closure
3) RIN-14B	Somatostatin + cancer signals	No effect
4) Insulin-releasing fibroblasts	Insulin + ECM macromolecules	Accelerated scratch closure
5) Empty hydrogels	Nothing	No effect
6) Commercial Insulin	Insulin	Accelerated scratch closure
7) Conditioned media only	Fibroblast signals	No effect

Conditioned media from all groups would then be used to conduct a fast-activated cell-based (FACE) -Akt ELISA. A FACE Akt ELISA is used to determine the amount of activated Akt within cells that have been treated by conditioned media. The procedure would be followed as previously described [7]. Briefly, L6 myoblasts would be incubated with conditioned media from the groups in the table and lysed to determine whether experimental groups caused phosphorylation of Akt. All materials are provided with the FACE Akt ELISA Kit and the provided instructions will be followed. This would establish whether RIN-14B are in fact activating the PI3K-Akt pathway in keratinocytes via a non-insulin signaling molecule.

Future work beyond this thesis includes conducting animal studies with RIN-5F cells encapsulated at LCD in PEGDA hydrogels. *In vitro* wound models, like the scratch assay, are a good first step towards evaluating the beginnings of a wound healing therapy. However, *in vivo* studies are also necessary. This is because the body is much more complex than an *in vitro* model can capture. *In vivo* studies allow a better model to test the wound healing therapy developed. However, the mouse model has its drawbacks too because mouse skin is different than human skin. Mouse skin is covered with a thick layer of fur, has a thin epidermal layer, and panniculus carnosus muscle, a thin layer of striated muscle, under their skin [55].

Additionally, following the first insulin wound healing study, the lab co-encapsulated RIN-m cells with mesenchymal stem cells (MSCs) and demonstrated greatly improved *in vitro* and *in vivo* wound healing over RIN-m cells alone. Future work should also include co-encapsulating RIN-5F, RIN-14B, and insulin releasing fibroblasts with MSCs and comparing these results to the previous experiments conducted with the

RIN-m cells and MSCs. MSCs accelerate wound healing by secreting anti-inflammatory cytokines, antimicrobial factors, and decreasing apoptosis [26, 56-59] If RIN-14B cells co-encapsulated with MSCs do not heal wounds faster than MSC cells alone, this would further support the hypothesis that the insulin is responsible for the accelerated healing. Co-encapsulated RIN-5F cells or insulin releasing fibroblasts and MSCs may heal wounds faster than co-encapsulated RIN-m cells and MSCs. This is due to the fact that in addition to secreting insulin, RIN-m cells secrete somatostatin, which inhibits insulin. Since neither RIN-5F cells nor the insulin releasing fibroblasts have somatostatin, none of their released insulin should be inhibited. This would potentially increase the downstream effects of the insulin secreted by the entrapped cells, which may cause the wounds to heal more rapidly than the treatments with RIN-m.

This study has further confirmed insulin's role in wound healing. In addition, the thesis further demonstrates the successful use of entrapped cells to release insulin for wound healing, rather than just to achieve normoglycemia. Overall, there is great potential of having an insulin treatment for chronic wounds, and this study helps move research in the direction of fully developing such a treatment.

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