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Metabolic reprogramming in wound healing

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Master's Degree Project in Medical Research, 30 credits, Spring 2021
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Abstract

Background: Chronic leg ulcers are a major medical problem that accounts for 2-4 % of the healthcare budget in western countries. While wound healing is a highly dynamic and energy-requiring process, the role of metabolism in chronic wound healing remains largely unexplored.

Aim: To determine if metabolic changes occurring during wound healing might influence cell migration or proliferation, two critical processes for wound healing. To investigate the molecular mechanism underlying the metabolic changes in wound healing.

Methods: Human fibroblast and keratinocyte cells were treated with different glucose concentrations along with rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) to assess the role of glycolysis and mitochondrial respiration in wound healing *in vitro*. Additionally, fibroblasts and keratinocytes were treated with the fatty acid oxidation inhibitor etomoxir to investigate the role of lipid metabolism in wound healing. Firstly, the minimum inhibitory concentrations of these inhibitors and enhancers were determined using a seahorse XF24 extracellular flux analyzer. Cell viable concentrations of all pharmacological metabolic drugs for treatments were determined using live-cell viable reagent presto blue. To understand if any of the metabolic perturbation affects wound healing, we performed scratch assays on dermal keratinocytes and fibroblasts. Additionally, fibroblast cells were treated with Wnt ligand inhibitor WNTC59 to assess effects on cellular respiration and migration.

Results: Seahorse titrations revealed that acute injections of 8 mM or higher of glucose significantly increased the extracellular consumption rate in fibroblast and keratinocyte cells, while acute treatment with etomoxir significantly reduced the oxygen consumption rate of fibroblast cells at 20 and 40 μM in the presence of 200 μM palmitate. Interestingly, scratch wound healing assays revealed that glucose treatment together with mitochondrial inhibitors rotenone and antimycin A significantly improved migration of fibroblast cells ($p < 0.0001$) when compared to controls. However, keratinocyte migration and proliferation were not dependent on increased glycolysis in *in vitro* wound healing assays. Etomoxir mediated inhibition of fatty acid oxidation had no significant impact on dermal fibroblast migration and proliferation. Treatment of fibroblasts with Wnt signaling inhibitor improved the mitochondrial respiration and migration of fibroblasts.

Conclusion: Our key findings indicate that fibroblast migration in wound healing is dependent on enhanced glycolysis. Furthermore, Wnt signaling might play a role in the metabolic reprogramming of chronic wounds.

Keywords*: Wound healing, chronic wounds, glycolysis, fatty acid oxidation, metabolism, cell migration, Wnt signaling*

Popular scientific summary

Metabolism: The key to chronic wound healing?

Every living organism needs energy to maintain structure, grow and survive, including cells. Cells require a constant supply of energy in order to undergo the biological processes they need to survive. Energy is primarily created through metabolic pathways, these pathways are defined by a set of chemical reactions that breaks down molecules to create more energy. Metabolism is a very dynamic process and therefore energy demands change as cells undergo processes like wound healing. Wound healing is a very complex process as it consists of many different phases that need to work together for successful wound closure. However wounds can fail to heal, and when that happens they can develop into chronic wounds. Chronic wounds are a major socioeconomic healthcare problem that affects around 2-4% of the population globally. Furthermore, chronic wounds have a negative impact on the patient's life as they reduce their quality of life and often cause depression.

Just like me and you need food to fuel our bodies with energy, cells do too. Cells need to be fed with carbohydrates, fats, and proteins that are broken down into smaller molecules like glucose. These small molecules can enter the cell and be further processed in metabolic pathways such as glycolysis or fats that undergo fatty acid oxidation to make energy. Our group has found that these metabolic pathways are altered in chronic wounds compared to normal healthy skin, exhibiting increased activity of both glycolysis and fatty acid metabolism. As we observed this metabolic shift occurring in chronic wounds it made us wonder how these changes affect cell migration and proliferation. This also made us wonder about what the molecular mechanism behind these changes was.

Our group therefore studied how migration and proliferation, two very important processes of wound healing, are affected when fibroblast and keratinocyte cells undergo this metabolic shift. To do this, we used different drugs like glucose to enhance metabolism and inhibitors to stop parts of the cell's metabolism. To first make sure, our enhancers and inhibitors worked properly we monitored how the cell's oxygen consumption changed as they used oxygen to produce energy as well as how much acidified the environment around the cells became when they underwent glycolysis to produce energy. We then used a live camera to monitor how the cells migrated and proliferated in response to these drug changes after we created a wound. We also treated cells with a Wnt signaling inhibitor to investigate if it might be a contributing factor in metabolic reprogramming.

We found that fibroblast cells are dependant on glucose for their ability to migrate and close a wound. When fibroblast cells are pushed into using more glucose for their energy production we saw that the more glucose we provided the cells the better were their migration ability. However, when we inhibited fatty acid metabolism it did not affect fibroblasts migration and proliferation. These new insights with metabolic modulators can help us in taking steps towards finding new and better treatment options for patients suffering from debilitating chronic wounds.

Introduction

As the most extensive organ system in our bodies, the skin, is very complex and acts as our primary line of defense with regenerative abilities. The two main layers of the skin is the epidermis and dermis (Fig. 1). The epidermis is the most outer layer and consists mainly of keratinocytes. The epidermis protects us against external factors such as microbial infections, ultraviolet radiation, and chemical damage. Underneath the epidermis lays the dermis which is comprised of dense collagen fibers providing elasticity and protection against mechanical forces (1–3). Loss of skin integrity will begin the wound healing process. Wound healing is a complex process consisting of multiple overlapping phases defined as; hemostasis, inflammation, proliferation and remodeling (2). Chronic wounds are classified as wounds that do not succeed to progress through the normal stages of wound healing in a proper and timely manner and take more than more than 12 weeks to heal (4, 5). These wounds usually have a prolonged inflammatory and proliferation phase. Chronic wounds are a global issue as they affect around 1-2% of the population with an increased prevalence (4%) in people over 64 years of age (6, 7). These wounds are a significant healthcare burden, costing the healthcare system billions of dollars yearly and more importantly leads to a substantial reduction in the quality of life of patients (8). One of the most common types of chronic wounds is venous leg ulcers (VLU) as they make up 70-80% of all ulcers. VLU has a high re-occurrence rate (50-70% at 6 months) as a result of limited or failed treatment options. Thus a better understanding of the pathophysiology is needed in order to develop new and better treatment options (8).

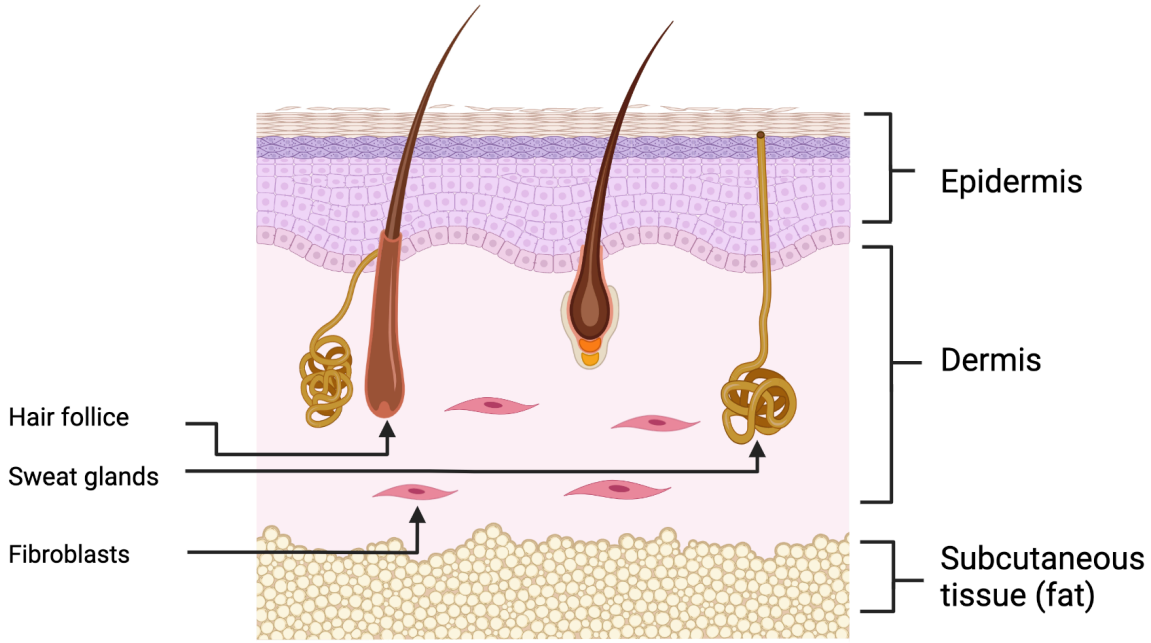

Figure 1. Simplified image of the skin anatomy. The skin consists of three layers, the outermost layer being the epidermis followed by the dermis, and lastly the subcutaneous tissue. Image created with BioRender.com

The wound healing process

Hemostasis occurs immediately after injury and is the first stage of the healing process. This phase is defined by vascular constriction, platelet aggregation, and fibrin clot formation to stop

the bleeding and recruit inflammatory cells to the wound site (Fig. 2) (9). Upon dermal injury, the endothelial monolayer in vessels is disrupted causing vascular smooth muscles to reflexively contract (10). The vascular ruption exposes platelets in the blood to the vascular subendothelial matrix which allows G-protein receptors on their surface to bind to extracellular matrix (ECM) proteins such as collagen and fibronectin released from the vessels creating a fibrin meshed blood clot (2, 11). Aggregated platelets then degranulate and secrete α-granules which release essential growth factors like platelet-derived growth factors (PDGF), epidermal growth factors (EGF), and transformative growth factor-β (TGF-β). Additionally, platelet secreted α-granules express P-selectin on their surface which recruits neutrophils, monocytes, and lymphocytes and gives rise to an inflammatory response (11, 12).

Neutrophils secrete mediators like reactive oxygen species (ROS) and cytokines that recruit macrophages together with promoting fibroblast and keratinocyte proliferation (13). A late inflammatory response occurs 2-3 days after injury when monocytes that become macrophages infiltrate the wound site. Similar to neutrophils, macrophages aid in phagocytosing bacteria and debris as well as secrete important cytokines such as tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6). Anti-inflammatory macrophages are important in initiating the next phase of healing, proliferation, by secreting growth factors such as PDGF (13). During the inflammatory phase, fibroblast cells start to migrate to the wound bed and secrete metalloproteinases (MMPs) which degrade ECM proteins (14). Simultaneously fibroblast cells produce new ECM rich in collagen III, hyaluronic acid, and fibronectin (15).

Proliferation focuses on wound closure through re-epithelization, establishing a competent blood flow by angiogenesis and ECM formation. Keratinocyte proliferation and migration is the basis of re-epithelization. Approximately one day after injury, keratinocytes around the wound perimeter are activated and start to migrate due to loss of contact inhibition and tension at adhesion structures (desmosomes and hemidesmosomes) (16). Activated keratinocytes are also responsible for alerting fibroblasts, epithelial cells, and lymphocytes by sending out paracrine signals. Furthermore, they release autocrine signals to target neighboring keratinocytes which is important for the mobilization action of surrounding cell types in tissue repair. In chronic wounds, keratinocytes are dysfunctional as they continuously proliferate at the wound edge instead of migrating as a result of c-MYC overexpression (3). During days 1-3 of skin injury, fibroblasts in the surrounding tissue start to proliferate in response to extracellular signals such as TNF- α, TGF-β, PDGF, and FGF (17, 18). Activated fibroblasts migrate into the wound site and transform the provisional ECM to a more collagen-rich ECM by producing collagen III, glycosaminoglycan, and proteoglycans (9, 17, 19, 20).

Remodeling is the final phase of the healing process where the skin regains its tensile strength. As the wound is closed and covered by a monolayer of keratinocytes, type III collagen is degraded by fibroblast cells and replaced by collagen type I which has much higher tensile strength and restores the tissue (21). Wound healing is a very energy-requiring process involving multiple cellular and biosynthetic processes that require amino acids, adenosine triphosphate (ATP), and other precursor molecules to repair injured skin (22).

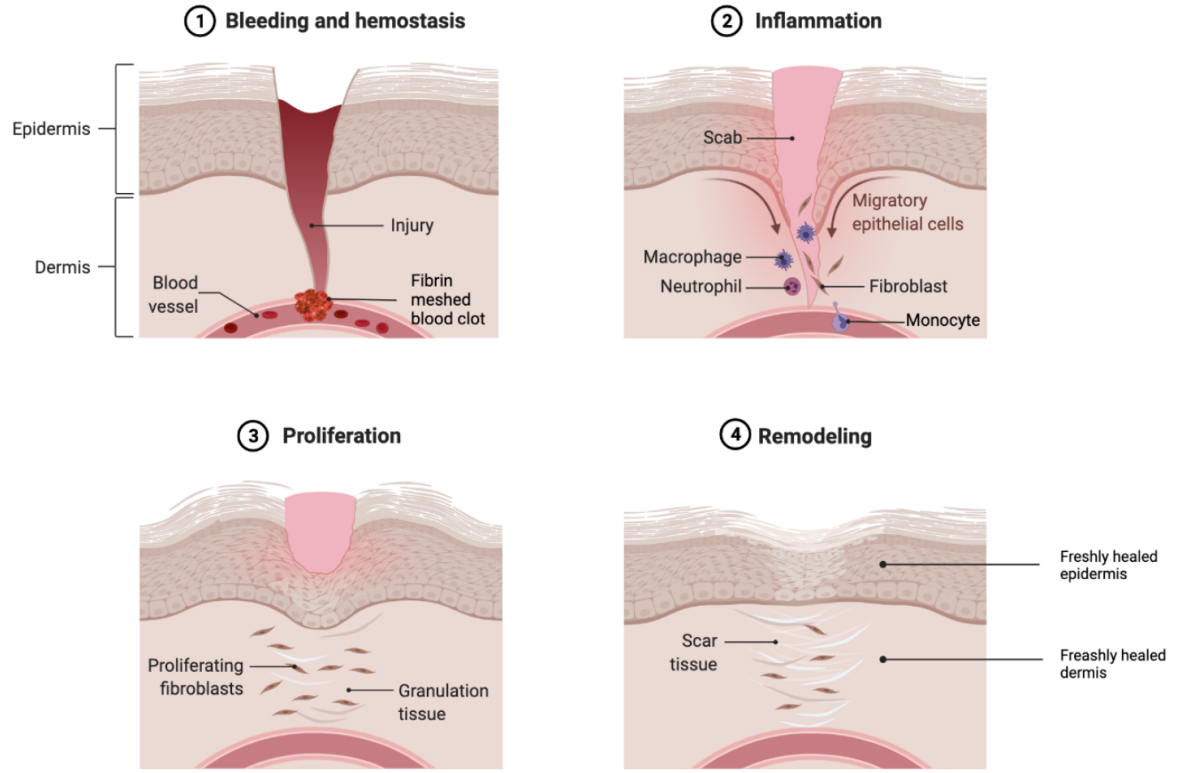

Figure 2. Simplified image of the wound healing process. The wound healing process consists of four overlapping phases, hemostasis, inflammation, proliferation, and remodeling. (1) Hemostasis occurs directly after injury. During this phase, the blood vessels constrict and blood platelets aggregate to form a blood clot. (2) During inflammation neutrophils and macrophages infiltrate the wounds and aid in phagocytosis of debris and bacteria as well as release cytokines. Additionally, fibroblasts migrate to the wound site to stimulate the production of new ECM. (3) During the proliferative stage Kerationcytes migrate to close the wound, new blood supplies occur through angiogenesis, and fibroblast starts to proliferate and transform the provisional ECM to a more collagen-rich matrix. (4) Remodeling is the final phase of wound healing. During this phase a monolayer of keratinocytes covers the wound, collagen III is broken degraded by fibroblast cells, and replaced by collagen I restoring the skin's tensile strength. *Adapted from "Wound Healing", by BioRender.com 2021. Retrieved from* https://app.biorender.com/biorender-templates

Metabolism and wound healing

The mitochondrion is an important organelle in regards to energy production in cells. Inside of the mitochondria is where the majority of adenosine triphosphate (ATP) production occurs; in other words, this is where energy production transpires (23). Carbohydrates, fats, and proteins are sources of energy in the body as they are broken down into smaller molecules used inside of the cells. Carbohydrates, for example, can be converted into simple sugars such as glucose. Glucose is then converted into two pyruvate molecules in the cytosol via glycolysis (24). In aerobic conditions, pyruvate is transported to the mitochondria where it is converted to acetyl-CoA which enters the tricarboxylic acid (TCA) cycle to produce ATP, NADH, and $FADH^{+}$ (25). Pyruvate that is not converted into acetyl-CoA and fed into the TCA cycle is anaerobically metabolized and converted into lactate before being transported out of the cell (26). Furthermore, fatty acids and proteins can also undergo enzymatic reactions to produce ATP,

NADH and $FADH^+$ in the TCA cycle. Free fatty acids are transferred to the mitochondrion inner membrane by carnitine acetyltransferase 1 and 2 (CPT1 and CPT2). Inside the mitochondrial matrix, the free fatty acids undergo β-oxidation and are converted into acetyl-CoA which enters the TCA cycle and undergoes the same processes as mentioned earlier (27). Additional ATP is produced as $FADH^+$ and NADH produced in the TCA cycle transfers electrons to the electron transport chain (ETC) in a process called oxidative phosphorylation (OXPHOS). The ETC is located on the inner mitochondrial membrane and consists of respiratory chain complexes I-V (28). OXPHOS is the reduction of electrons via this chain of proteins creating a proton gradient which increases the acidity in the intermembrane space making the inside of the membrane positively charged and the outside negatively charged. This charge difference makes it possible for protons to be transported out of ATP synthase, creating ATP (28).

Cutaneous wounds mostly depend on carbohydrate metabolism via the glycolytic pentose phosphate pathways to support wound healing processes like angiogenesis and collagen disposition in the proliferative stage (29). Carbohydrate metabolism is also important for fibroblast production and movement (30). Analysis of wound fluid and ulcer biopsies on VLU identified metabolites lactate and glutamate, amongst others, to be upregulated. This is in accordance with previous studies done by our group where metabolomics experiments in wounded tissues were performed. Several key metabolic metabolites related to glycolysis, fatty acid metabolism and amino acid metabolism, namely glutamine, were found to be upregulated in chronic wounds compared to healthy normal skin. Hence, chronic wounds seem to undergo metabolic reprogramming to meet new energy demands.

Wnt signaling in wound healing

The Wnt signaling pathway is an evolutionarily conserved pathway that plays a major role in tissue homeostasis, cell growth, cell differentiation and proliferation that are also characteristics of skin regeneration (31). The Wnt family consists of 19 Wnt ligands that can activate several different signaling pathways classified as either canonical or non-canonical depending on their β-catenin dependence (32, 33). Intracellular Wnt signaling is branched into three main pathways, the β-catenin pathway (canonical pathway), the planar cell polarity pathway (non-canonical pathway) and the Wnt/Ca^{2+} pathway (non-canonical pathway). The β-catenin dependent pathway regulates cytoplasmic β-catenin levels (Fig. 3). β-catenin accumulates in the cytoplasm and translocates to the nucleus where it is involved in the activation of downstream genes such as c-MYC, cyclin-D and Axin2 by binding to the TCF/LEF (T-cell factor/lymphoid enhancer factor) transcription factors. When Wnt ligands bind to the transmembrane receptor, intracellular protein Dishevelled (Dvl) is recruited and will bind to the receptor intracellularly. Dvl then inactivates the β-catenin destruction complex consisting of four proteins: Axin, Adenomatous polyposis (APC), Glycogen synthase kinase 3 (GSK3) and Casein kinase 1 (CK1) (34). In the absence of canonical Wnt ligands (e.g. Wnt3a), binding to Frizzled (FZD) and LRP-5/6 co-receptor the cytosolic β-catenin is phosphorylated and ubiquitinated by the β-catenin destruction complex.

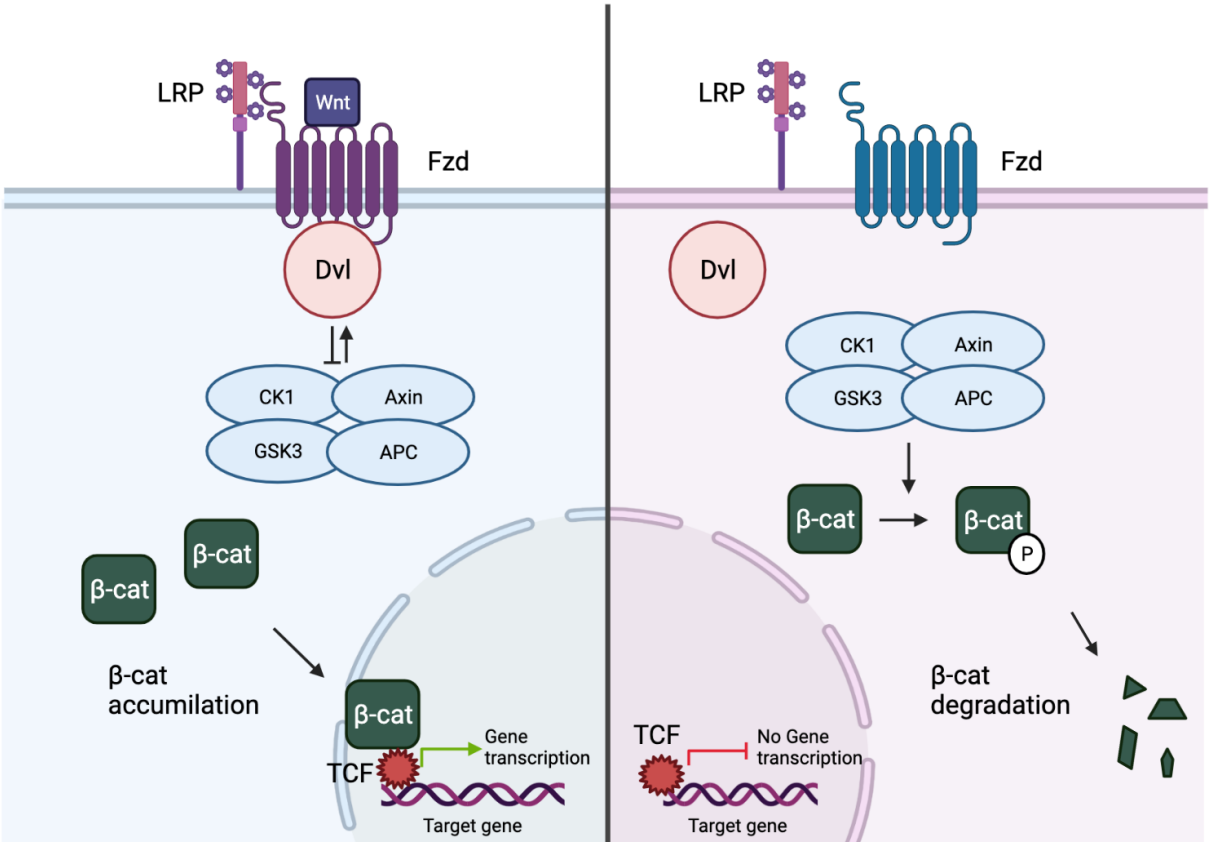

Figure 3. Simplified overview of the Canonical β-catenin (β-cat) dependent pathway. Wnt ligands bind to the Fzd receptor, causing the activation and recruitment of Dvl. The activation of Dvl will recruit and inhibit the protein complex consisting of Axin, CK1, GSK3, and APC. The inhibition of the protein complex results in the accumulation of β-catenin in the cytosol which will translocate to the nucleus and activate transcription of target genes. In the absence of Wnt ligands binding to the Fzd receptor, the protein complex will phosphorylate and ubiquitinate cytosolic β-catenin. *Image created with BioRender.com*

The non-canonical Wnt pathways are less understood but also rely on the binding of Wnt ligands (e.g. Wnta5a) to the frizzled receptor (FZD) receptor. There are multiple non-canonical Wnt signaling pathways, the more known being the planar cell polarity pathway (PCP) and Wnt/Ca^{+} pathway. The activation of the PCP pathway recruits small GTPase proteins resulting in changes in cell polarity and migration. The Wnt/Ca2+ pathway increases intracellular calcium levels. This occurs as G-proteins are activated and calcium is released which activates secondary messengers such as protein kinase C and calmodulin-dependent kinase II which modulate cell migration and differentiation (35).

Previous studies have described the involvement of β-catenin and its activator c-MYC in chronic wounds. Miller et al. described c-MYC to be an activator of lactate dehydrogenase (LDHA) and other glucose metabolism genes. In the same study, they also described c-MYC involvement in mitochondrial biogenesis by increasing mitochondrial production of acetyl-CoA (36). Studies by Stojadinovic et al. have described how c-MYC is active at the epithelial edge of chronic ulcers and how when wounds are treated with topical wound-healing inhibitors it induces its expression in the nucleus. Additionally, they describe β-catenin stabilization to be

an inhibitor of keratinocyte migration, not only as a c-MYC activator but also by blocking epithelial growth factor (EGF) stimulated migration (37).

Taken all this together, it suggests that Wnt signaling might play a role in the metabolic reprogramming that occurs in chronic wounds; however, this is not clear and needs to be further investigated.

It is well known that wound healing increases the energy demands of the skin. Studies performed by our group has identified several metabolic pathways, particularly carbohydrate, lipid, and amino acid metabolism to be altered in chronic wound healing. Targeting wound metabolism could provide a new treatment strategy for chronic wounds. A better understanding of how metabolic changes in chronic wounds influence cell proliferation and migration as well as the signaling pathway responsible for these changes is essential for improving treatment and developing new therapeutic options.

Aim(s)

- To investigate the relevance of cell metabolic adaptation in wound healing *in vitro* by using enhancers and inhibitors treating different metabolic pathways.

- To investigate the signaling pathways involved in metabolic changes during the process of dermal wound healing.

Materials and Methods

Cell lines and cultures

Human Epidermal Keratinocytes (HEKa) (Gibco; Thermo fisher scientific) were cultured in serum-free EpiLife medium supplemented with 1% Human Keratinocyte Growth Supplement (HKGS) and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone; Thermo fisher scientific). Human breast Fibroblast cells (patient cells) were cultured in DMEM medium (Gibco; Thermo fisher scientific) supplemented with 0.9% glucose, 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Fungizone; Thermo fisher scientific), 10% Fetal Bovine Serum (FBS) (Gibco; Thermo fisher scientific). Cells were sub-cultured at approximately 90 % confluency. To harvest the cells, they were washed in Dulbecco's Phosphate Buffer Saline (PBS; Thermo fisher scientific), treated with 2 mL Trypsin/EDTA (Gibco; Thermo fisher scientific), and incubated at 37°C in 5% CO_2 for 5 minutes to detach cells from the flask. Trypsin was neutralized by adding 5 mL DMEM to fibroblast cells and 4 mL Defined Trypsin Inhibitor (DTI) (1:2 ratio) to keratinocytes. Cells were centrifuged for 5 minutes at 200 x g. The supernatant was discarded and the cell pellet was re-suspended in 5 mL of appropriate medium. Cells were seeded as detailed below depending on the experiment and incubated overnight.

WNTC59 treatment

Cells were treated with 50, 100, 250, 500 and 1000 nM WNTC59 **(**Tocris Bioscience**)** diluted from a stock concentration of 10 µM. Following treatment, cells were incubated with WNTC59 for 48h at 37°C in 5% CO_2 before experiments were conducted.

RNA extraction

6 x 10^4 fibroblast cells/1 mL were seeded in 12 well plates and 4 x 10^4 HEKa cells/500 µL in 24 well plates per well. Cells were treated with 100 and 500 nM of WNTC59 as detailed above. After incubation cells were washed twice in 500 µL cold PBS and lysed by adding 500 µL Trizol to each well. Cells were incubated for 5 minutes at room temperature before being transferred to 1.5 µL RNase free Eppendorf tubes. 100 µL chloroform was added to each tube before they were manually shaken and incubated at room temperature for 3 minutes. Cells were centrifuged at 12 000 x g for 15 minutes at +4°C. 1.5 µL glycogen was added to new RNAse free Eppendorf tubes. The aqueous upper layer was transferred to the new Eppendorf tubes with 1.5 µL glycogen. 250 µL isopropanol was added to the upper aqueous layer, tubes were then incubated for 10 minutes. Tubes were centrifuged at 12,000 x g for 10 minutes. The surfactant was discarded and 500 µL of 75% ethanol was added to the RNA pellet. The RNA pellet was soft vortexed for 5 seconds and centrifuged at 7500 x g for 5 minutes. Ethanol was removed and the pellet was airdried. 15 µL RNA storage solution was used to dissolve the pellet. RNA concentration was determined by measuring absorbance at 260 nm using a nanodrop machine.

RT-PCR

RNA samples were diluted to a final concentration of 500 ng/µL with RNAse free water in a 96 well plate. Primer mix was then added according to Table 1. Primers and RNA were annealed for 5 minutes at 70°C (Table 3). The 96 well plate was placed on ice and RT Mix was added according to Table 2. RNA was reverse transcribed according to Table 4.

Table 1. RT Primer Mix

RT Primer Mix	1 rxn
Oligo dT	0.6 µL
Random hexamer	0.1 µL

Table 2. RT Mix

RT Mix	1 rxn
5x Buffer	4 µL
Ribolock	1 µL
dNTPs	2 µL
RevertAid MulV	1 µL

Table 3. Step 1 cDNA conversion

Step	Temperature	Time
1 Hold	70°C	5 min
1 Hold	4°C	∞

Table 4. Step 2 cDNA conversion

Step	Temperature	Time
1 Hold	25°C	10 min
3 Holds	42°C	60 min
	70°C	10 min
	4°C	∞

qRT-PPCR

Reagents and primers were added to Ependorftubes to make SYBR™ Green PCR master mix according to Table 5 and Table 6. 1.7 µL cDNA and 8.3 uL SYBR™ Green PCR master mix were added to a 384 well plate (Thermo fisher scientific). qRT-PCR was run on Quantflex system 6/7 according to Table 7.

Table 5. SYBR™ Green PCR master mix

PCR reaction PCR	1 rxn
Primer forward 20 µM	0.1 µL
Primer reverse 20 µM	0.1 µL
SYBR Mix	5 µL
Water	3.1 µL

Table 6. Primer list

Primer	Forward strand	Reverse strand
Wnt5a	5′-AGGGCTCCTACGAGAGTGCT-3′	5′-GACACCCCATGGCACTTG-3′
Wnt3a	5′-CCTGCACTCCATCCAGCTACA-3′	5′-GACACCCCATGGCACTTG-3′
β-catenin	5′-TCTGAGGACAAGCCACAAGATTACA-3′	5′-TGGGCACCAATATCAAGTCCAA-3′
AXIN II	5′-GAGTGGACTTGTGCCGACTTCA-3′	5′-GGTGGCTGGTGCAAAGACATAG-3′
CCNDI	5′-CATCTACACCGACAACTCCATC-3′	5′-TCTGGCATTTTGGAGAGGAA-3′
C-MYC	5′-CGTCCTGGGAAGGGAGAT-3′	5′-CGCTGCTATGGGCAAAGT-3′
LDHA	5′-TTGGTCCAGCGTAACGTGAAC-3′	5′-CCAGGATGTGTAGCCTTTGAG-3′
COL I	5′-GATTCCCTGGACCTAAAGGTGC-3′	5′-AGCCTCTCCATCTTTGCCAGCA-3′
BACTIN	5′-CACCATTGGCAATGAGCGGTTC-3’	5′-AGGTCTTTGCGGATGTCCACGT-3′
HKII	5′-GAGTTTGACCTGGATGTGGTTGC-3’	5′-GAGTTTGACCTGGATGTGGTTGC-3′
MT-ND2	5′-GGGTCATGATGGCAGGAGTAAT-3′	5′-CCACCTCTAGCCTAGCCGTTTA-3′
FLG	5′- AAAGAGCTGAAGGAACTTCTGG-3′	5′- AACCATATCTGGGTCATCTGG-3′
K1	5′- TGAGCTGAATCGTGTGATCC-3′	5′- CCAGGTCATTCAGCTTGTTC-3′
K10	5′- GCAAATTGAGAGCCTGACTG-3′	5′- CAGTGGACACATTTCGAAGG-3′

Table 7. qRT-PCR Quantflex system protocol

Time	Temperature	Cycles
2 min	50°C	1
10 min	95°C	1
15 sec	95°C	40
1 min	60°C	

Seahorse bioenergetic assay

Oxygen consumption rate (OCAR) and extracellular acidification rate (ECAR) were measured using Seahorse XFe24 extracellular flux assays. 3 x 10^4 (HEKa) and 2,2 x 10^4 (Fibroblast) cells were seeded to each well in a Seahorse XFe24 cell culture microplate (Agilent) and incubated overnight at 37°C in 5% CO_2. Cells were treated with WNTC59 as described above to assess the effects of Wnt inhibition on cellular bioenergetics. To determine acute responses to pharmacological inhibitors and enhancers cells were injected with 5-40 μM of the fatty acid inhibitor etomoxir (Sigma-Aldrich) and 8-20 μM glucose (Thermo fisher scientific). Prior to etomoxir injections, BSA-palmitate 200 μM (Cayman chemical; 5mM) and BSA-control (Cayman chemical; 5 mM) (i.e. 6:1 molar ratio to BSA) was added to the assigned wells. A XFe24 sensor cartridge was hydrated with 1 mL Seahorse calibrant (Agilent) at least 6 hours before the experiment was performed. Cells were starved in Seahorse XF basal medium supplemented with 2 mM L-glutamine, 10 mM glucose, 1 mM Sodium Pyruvate (WNTC59 experiments), or 2 mM L-glutamine, 3 mM glucose, 1 mM Sodium Pyruvate (acute response experiments) for 45 minutes to 1h. Mitostress test drugs were prepared and loaded into the senor cartilage. Oligomycin (10 μM; Sigma-Aldrich) was loaded into injection port A, FCCP a mitochondrial oxidative phosphorylation uncoupler (10 μM), was loaded into injection port B and 5 μM Rotenone (Merck) + Antimycin A (Sigma-Aldrich) each was loaded into injection port C. A bicinchoninic acid assay (BCA) assay was performed as described below to determine protein concentration and normalize data. Data were analyzed using Wave Software (Agilent).

Scratch assay

2 x 10^4 cells/well were seeded in an Incucyte® Imagelock 96-well plate (Essen BioScience). When reaching ~80-90 % confluency cells were treated with different metabolic inhibitors and enhancers as described above and incubated for 24h. To inhibit mitochondrial respiration glucose-treated cells were also treated with 75 nM rotenone, or 75 nM rotenone together with 500 nM antimycin A. On the day of the experiment, 1.2 μL of mitomycin C (Sigma-Aldrich) was added to the five columns, the cells were then incubated at 37°C for 2h. Scratches were created using an Incucyte® WoundMaker Tool (Essen BioScience). Scratches were observed to confirm unification, cells were then washed with 100 μL of PBS. Cell treatment and fresh media as the control was then added to appropriate wells. Cell plates were incubated in IncuCyte ZOOM™ (Essen BioScience) and live-cell migration was imaged until columns reached approximately 90% relative wound density. Images were analyzed with the IncuCyte software (Essen BioScience).

BCA protein assay
Cells were lysed using 50 µl RIPA lysis and Extraction Buffer (Thermo fisher scientific). Protein concentration was measured with Pierce Microplate BCA Protein Assay Kit (Thermo fisher scientific) according to the manufacturer's instruction.

Human *ex vivo* wound model
Normal full-thickness human skin samples were obtained from abdominal reduction surgeries of healthy young donors (Nordiska Kliniken, Stockholm). 6 mm biopsies with 2 mm punches were taken from the skin and placed in 12 well plates used for *ex vivo* wound healing. Biopsies were treated topically and trans-dermally with 500 nM and 10 µM of WNTC59 respectively, or vehicle (H_2O) dissolved in 30% pluronic F-127 gel (Sigma-Aldrich) which was applied on the wounds immediately following injury and thereafter, every other day. For transdermal treatment, WNTC59 and vehicle were dissolved in medium. Wound samples were collected for histological analysis on days 0, 3, 5, and 7. *Ex vivo* slides were analyzed using a Light microscope and ImageJ software (38).

Cell viability
20 x 10^4 cells/100 µL were seeded to each well of a Corning® 96 Well Black Polystyrene Microplate. Cells were treated with different concentrations of drugs for 24-48 h. Cell viability test was performed using PrestoBlue™ (Invitrogen) Cell Viability Reagent according to manufacturer's protocol.

Statistical Analysis
Statistical Analysis and graphical representations were performed using GraphPad Prism and Microsoft excel. The statistical test used was two-way ANOVA with Dunnett's multiple comparisons test and t-test. A p-value < 0.05 was considered significant and will from now on and throughout the paper be referred to with (*).

Results

Glucose increases ECAR and does not affect cell viability in keratinocytes and fibroblasts

To determine viable doses, cells were treated with glucose to enhance glycolysis, or etomoxir to inhibit fatty acid oxidation (FAO) for 24h. Neither glucose nor etomoxir was toxic for the cells as cell viability was at 100-90% for both cell types (Fig. 4).

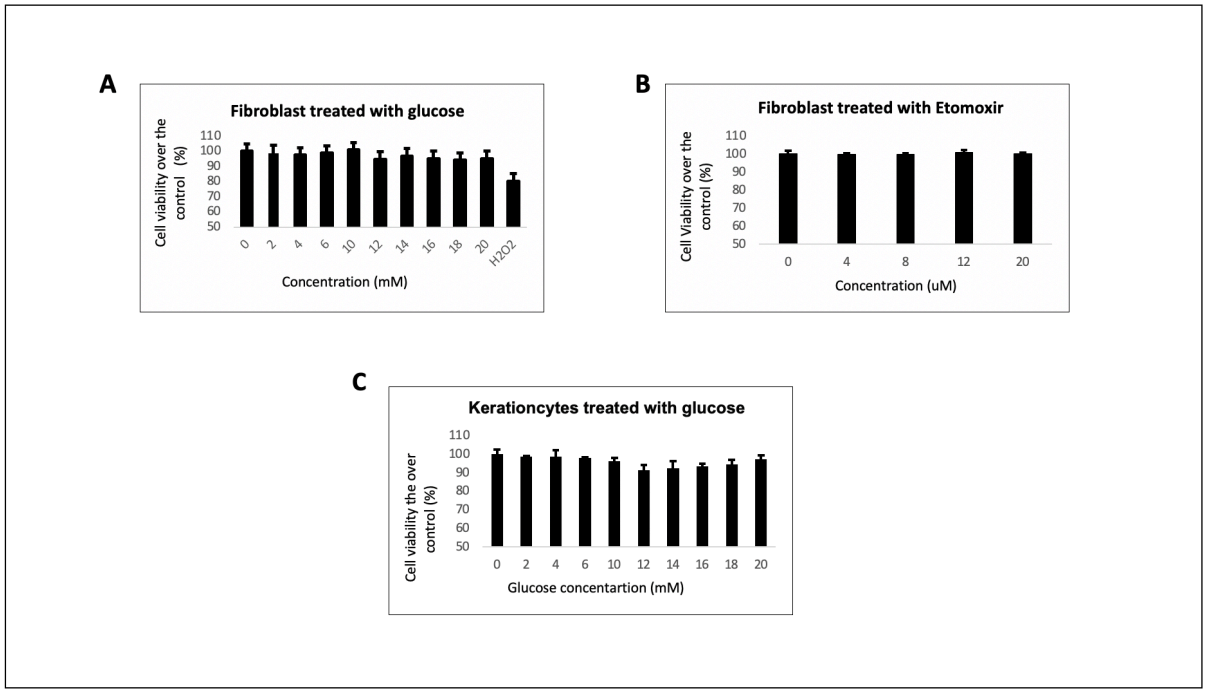

Figure 4. Glucose and CPT1 inhibitor does not affect cell viability in fibroblast and keratinocyte cells. Cells were treated for 24 hours and cytotoxicity was detected using PrestoBlue® **A.** Fibroblast cells were treated with different concentrations of glucose, no significant toxicity was detected. **B.** Fibroblast cells treated with different concentrations of etomoxir, no significant toxicity was detected.**C.** Kerationcytes treated with glucose, no significant toxicity was detected.

To assess the effects of these two drugs on cellular respiration we performed seahorse XFe24 assays. Seahorse XF24e assays, in real-time, measure oxygen consumption rates (OCAR), as cells respire to produce ATP, and extracellular acidification rates (ECAR), as cells produce lactate while undergoing glycolysis to produce ATP (41). Glucose treatment in both fibroblasts and keratinocytes significantly increased ECAR at approximately 8 mM and higher (Fig. 5A-B). Etomoxir significantly decreased OCAR at 20 uM and 40 uM as seen in Fig. 5C.

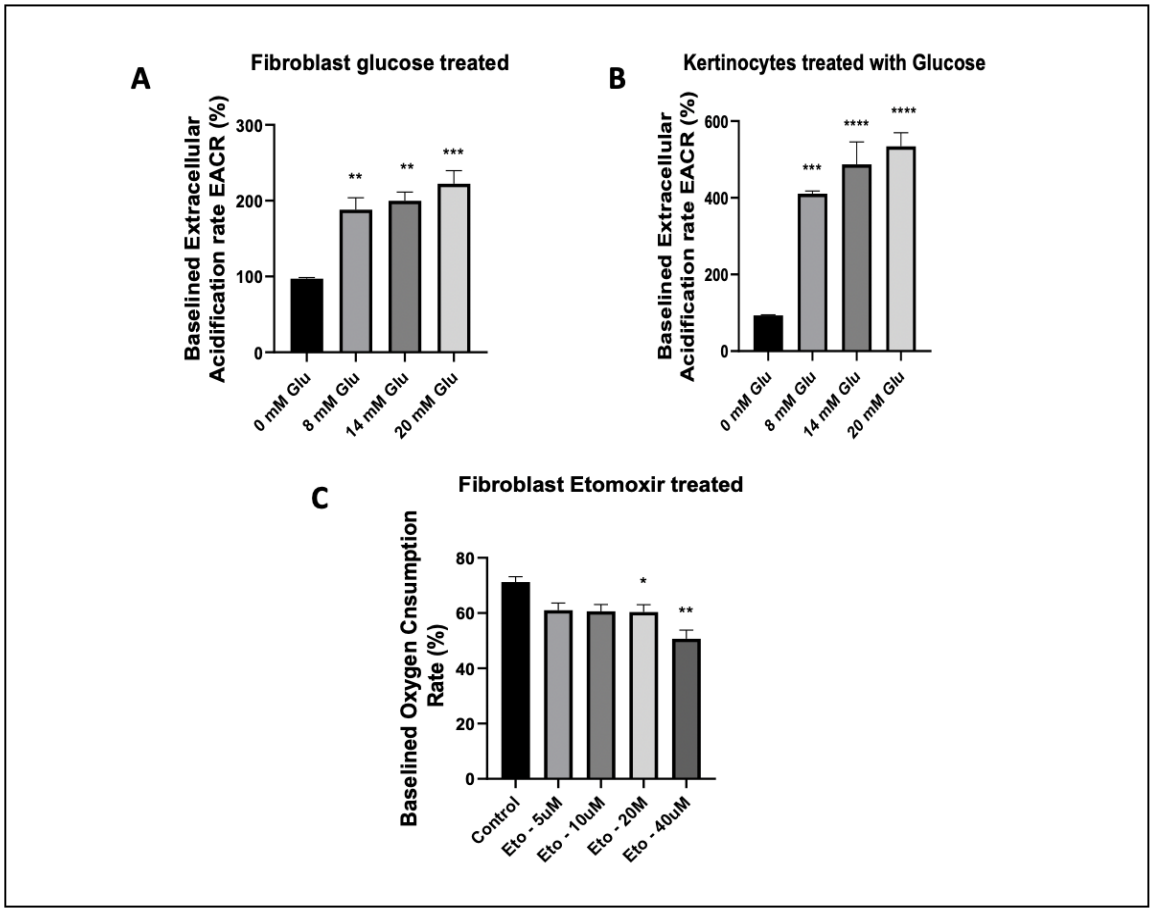

Figure 5. Glucose increases ECAR in fibroblast and keratinocytes. Etomoxir decreases OCAR in fibroblast cells. A., B. Extracellular acidification rates (ECAR) in response to acute injections of glucose measured by Seahorse Analyzer. **C.** Oxygen consumption rate (OCAR) in response to acute etomoxir injection in fibroblast cells measured by Seahorse Analyzer. N = 3, analyzed using two-way ANOVA. ****p< 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

Glucose enhances cell migration in fibroblasts

As our results showed that glucose and etomoxir affected cells OCAR and ECAR values, we wanted to assess how these metabolic perturbations impact wound healing by observing migration and proliferation using Incucyte scratch assays. Cells were treated with different concentrations of the metabolic mediators which were selected based on cell viability and seahorse assay results. To assess proliferation cells were also treated with or without the cell proliferation inhibitor mitomycin C (MMC). The fatty acid oxidation inhibitor etomoxir did not affect cell migration or proliferation in fibroblast cells when compared to the control, indicating that fibroblast migration and proliferation is not dependent on fatty acid oxidation (Fig. 6A). Cell migration results from glucose-treated cells showed that migration was improved in treated fibroblast cells compared to the control. Cells were unable to migrate when glucose was absent from the culture medium both with and without MMC as seen in Fig. 6B. Fibroblast cells needed relatively high glucose levels, compared to normal levels for their full migratory

capacity (42). On the contrary keratinocyte cells migrated well in low concentrations of glucose both with and without MMC.

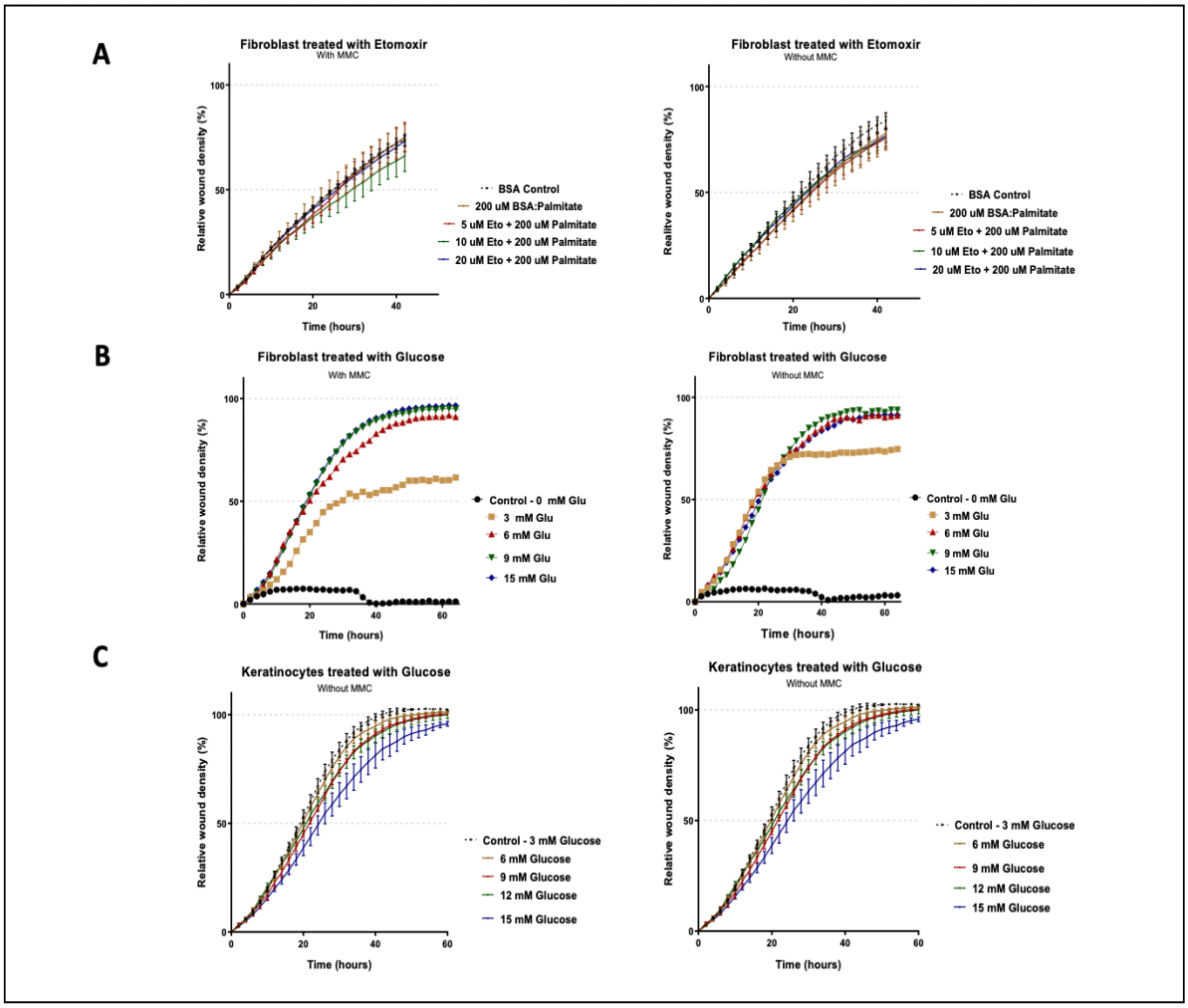

Figure 6. Glucose enhancement improves cell migration in fibroblast cells while keratinocytes migrate well in low glucose conditions. Fatty acid inhibition does not affect cell migration in fibroblasts cells. A. Scratch wound assay of fibroblast cells treated with different concentrations of etomoxir and 200 uM of BSA: Palmitate, with and without mitomycin C (MMC) for 40 hours. There was no significant difference in cell migration between the different treated groups. **B.** Scratch wound assay of fibroblast cells treated with different concentrations of glucose, with and without MMC for 60 hours. **C.** Scratch wound assay of keratinocytes treated with different concentrations of glucose, with and without MMC for 60 hours. N = 3, analyzed with two-way ANOVA.

Following the entry into cells through transporter GLUT1, glucose is metabolized via glycolysis and converted to pyruvate. Pyruvate can aerobically be metabolized via the TCA cycle and OXPHOS in the mitochondria to produce ATP. In addition to this, pyruvate can anaerobically be converted into lactate and ATP, thus glucose feeds both mitochondrial respiration and glycolysis. Based on our preliminary results, glucose seemed to be important for fibroblast cell migration and proliferation. To investigate how cells behave when parts of the ETC is inhibited, therefore being more dependent on glycolysis, rotenone was used. Rotenone is a complex I

inhibitor of the ETC and previous data from the group showed that 75 nM of rotenone decreased OCAR by 50%, therefore this concentration was chosen for further scratch assay experiments. Scratch assay results of fibroblasts treated with high glucose and 75 nM rotenone, similarly to previous glucose data, showed that fibroblast cells migrate significantly better with glucose compared to the control and 3 mM glucose-treated cells, despite parts of the ETC being inhibited. Results of keratinocyte cells treated with glucose and rotenone were also similar to previous glucose data and showed a trend in increased cell migration when treated with glucose. However, keratinocytes seem to be less dependent on glucose than fibroblasts for cell migration (Fig. 7).

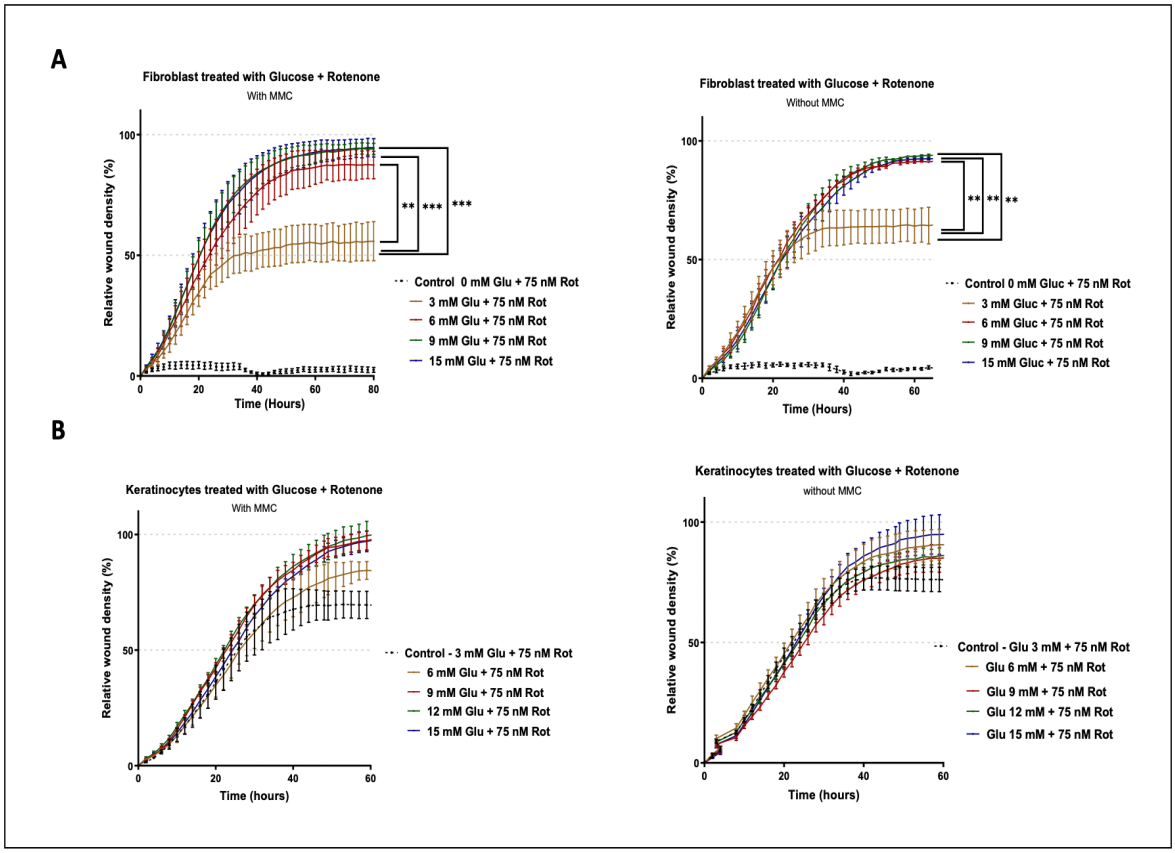

Figure 7. Enhancement of glycolysis together with ETC inhibition improves wound healing in fibroblasts. **A.** Scratch wound assay of fibroblasts treated with 75 nM of complex I inhibitor rotenone and different concentrations of glucose, with and without MMC for 60 hours. Cell migration was improved with glucose treatment while cells were unable to migrate without glucose. **B.** Scratch wound assay of keratinocytes treated with 75 nM of rotenone different concentrations of glucose, with and without MMC. There was no significant change in wound cell migration between different treatments. N = 3, analyzed with two-way ANOVA. ****p< 0. 0001, ***p < 0.001, **p < 0.01, *p < 0.05.

Glycolysis is more important than OXPHOS for fibroblast migration

Partially inhibiting mitochondrial respiration did not affect fibroblast or keratinocyte cell migration. This made us wonder how migration and proliferation would be affected if we

pushed cells further towards relying on glycolysis for their energy production. To investigate this, antimycin A (complex III inhibitor) was used in combination with rotenone to completely inhibit OXPHOS. To determine the cell viable dose of antimycin A in combination with rotenone a cell viability assay using PrestoBlue was run with 75 nM rotenone and concentrations of antimycin A ranging from 0-2000 nM incubated for 24h. Results as seen in Fig. 8 showed that none of the combined concentrations were toxic to cells as cell viability was 100-90%. We were interested in assessing how the combination of antimycin A and rotenone would further decrease OCAR in comparison to rotenone alone, for this reason, we ran a seahorse assay. 75 nM rotenone and 500 nM antimycin A significantly decreased OCAR by 20% compared to the control (75 nM rotenone alone), therefore this concentration was chosen for further experiments. Combining 500 nM antimycin A and 75 nM rotenone and significantly inhibiting both complex I and complex III of the ETC and consequently forcing cells to become more glycolytic, improved wound healing in fibroblast cells compared to our previous data. Cells needed a lower concentration of glucose for maximal migration. Lower concentrations of glucose such as 3 mM where cells only reached 50-60% relative wound density, increased to 60-75% relative wound density (Fig. 9). These results showed that enhancing the glycolytic pathway significantly increases fibroblast migration compared to when both glycolysis and OXPHOS are used. In contrast to fibroblast cells, there was no significant change in keratinocyte cell migration when treated with both rotenone and antimycin A. Results show that keratinocyte cells migrate well in low glucose conditions and OXPHOS inhibition, indicating that keratinocyte migration is independent of both glycolysis and mitochondrial metabolism.

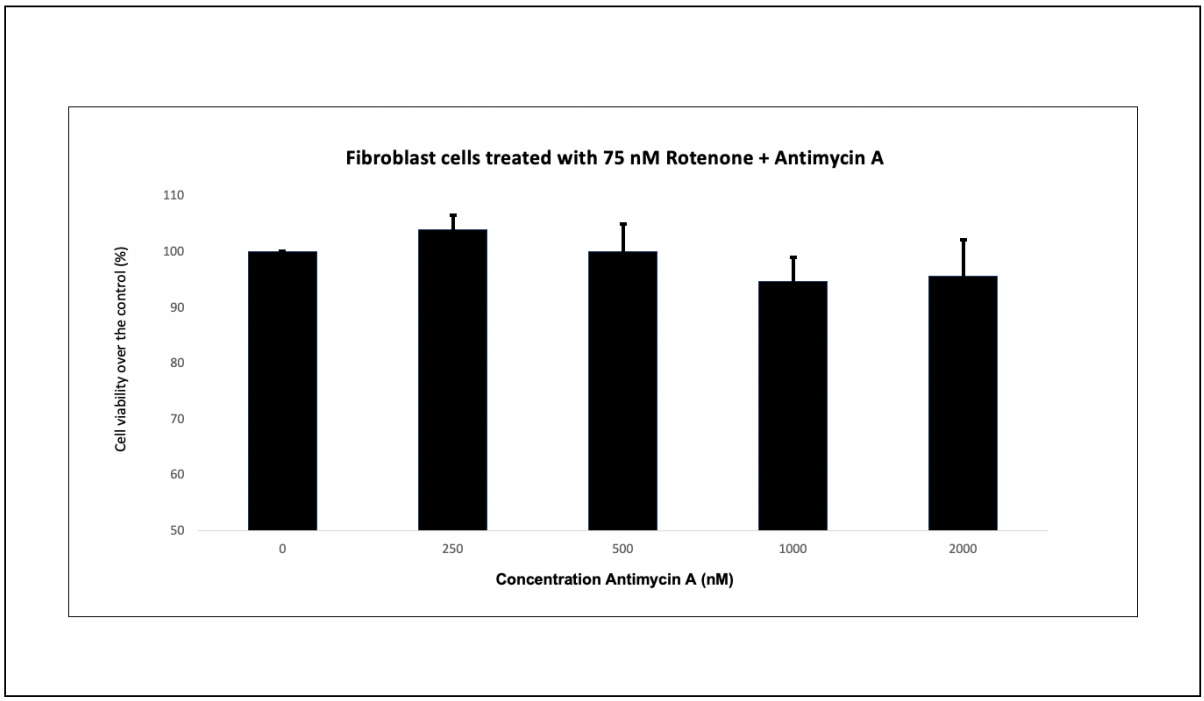

Figure 8. Combining rotenone and antimycin A does not affect cell viability in fibroblast and keratinocyte cells. Cells were treated for 24 hours and cytotoxicity was detected using PrestoBlue®. Fibroblast cells were treated with 75 nM rotenone and different concentrations of antimycin A, no significant toxicity was detected.

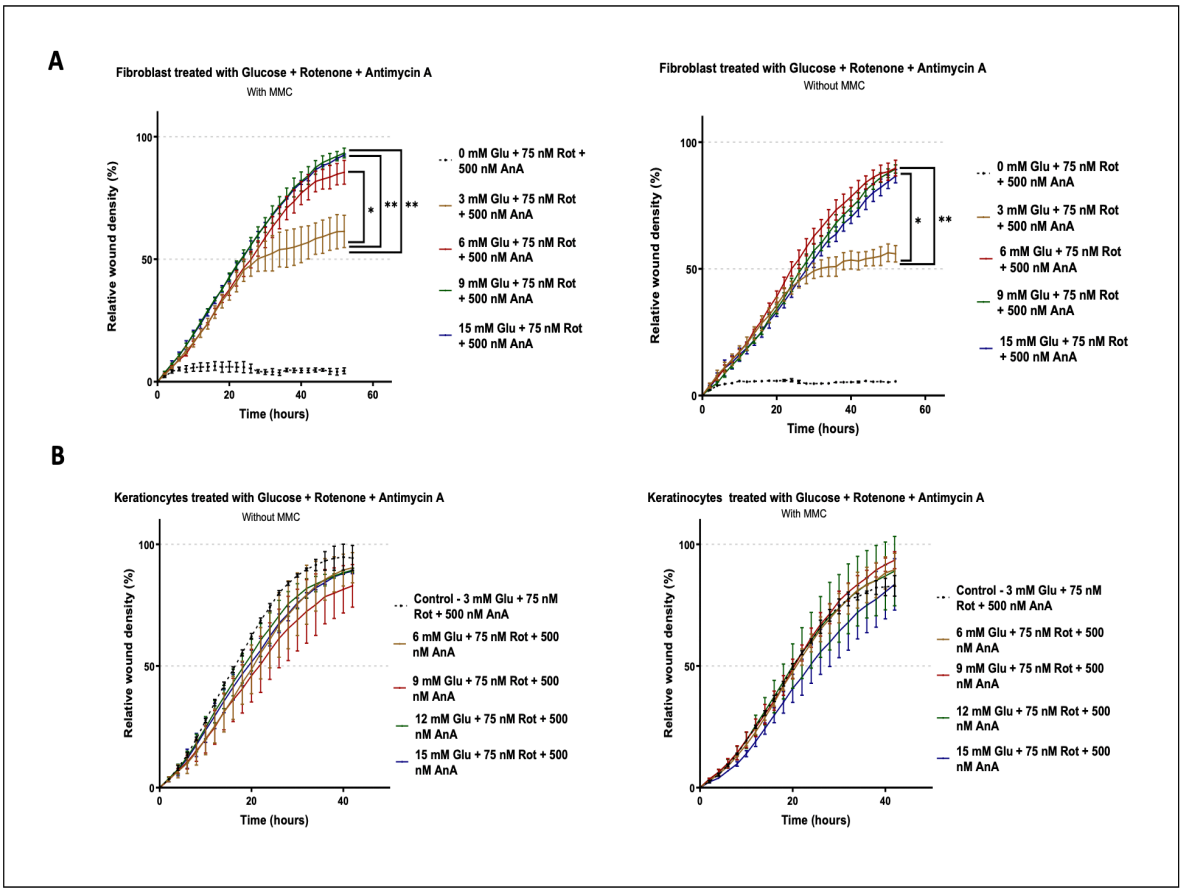

Figure 9. Pushing cells towards glycolysis by complete ETC inhibition improves fibroblast wound healing. A. Scratch wound assay of fibroblast cells treated with 75 nM rotenone, 500 nM antimycin A and different concentrations of glucose, with and without MMC for 55 hours. Forcing fibroblast cells to depend on glycolysis improves cell migration. **B.** Scratch wound assay of keratinocyte cells treated with 75 nM rotenone, 500 nM antimycin A and different concentrations of glucose, with and without MMC for 40 hours. There was no significant change in wound cell migration between different treatments. N = 3, analyzed with two-way ANOVA. ****p< 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

Wnt signaling: Regulator of wound healing reprogramming?

WNTC59 significantly downregulates keratinocyte markers K1 and K10

nCounter nanostring data from an unpublished study previously performed by our group profiling the gene expression of normal skin, acute and chronic wounds revealed that the Wnt signaling pathway is significantly altered in chronic wounds in comparison to healthy skin. Wnt signalings involvement in both migration and proliferation has been described in previous oncogenic studies, along with its role in metabolic homeostasis (43, 44). To investigate if Wnt signaling is involved in the metabolic reprogramming that occurs during chronic wound healing we performed seahorse assays and topically treated skin biopsied with exogenous Wnt inhibitor WNTC59.

To assess cell viable doses of the Wnt ligand inhibitor we conducted a cell viability assay. Fibroblast and Kerationcyte cells were treated with the chemical Porcupine O-acyltransferase

(PORCN) enzyme inhibitor WNTC59 for 48 hours. All concentrations were viable as the cell viability was 90-100% in keratinocytes and 80-100% in fibroblast cells as seen in Fig. 10. SYBR™ GREEN qRT-PCR was performed to validate the downregulation of Wnt ligands and downstream targets. A trend in the downregulation of non-canonical Wnt ligands (wnt5a) and downstream target axin (axin II) was observed in both cell types. Keratinocyte WNTC59 treated cells also showed a trend in the downregulation of mitochondrial transport (MT-ND2) and the keratinocyte differentiation marker K1. Interestingly, as seen in Fig. 11 K10, the basal keratinocyte differentiation marker is significantly downregulated on the mRNA level following WNTC59 treatment. Although showing a trend in the downregulation of Wnt ligands and downstream targets, a better validation of Wnt inhibition is needed.

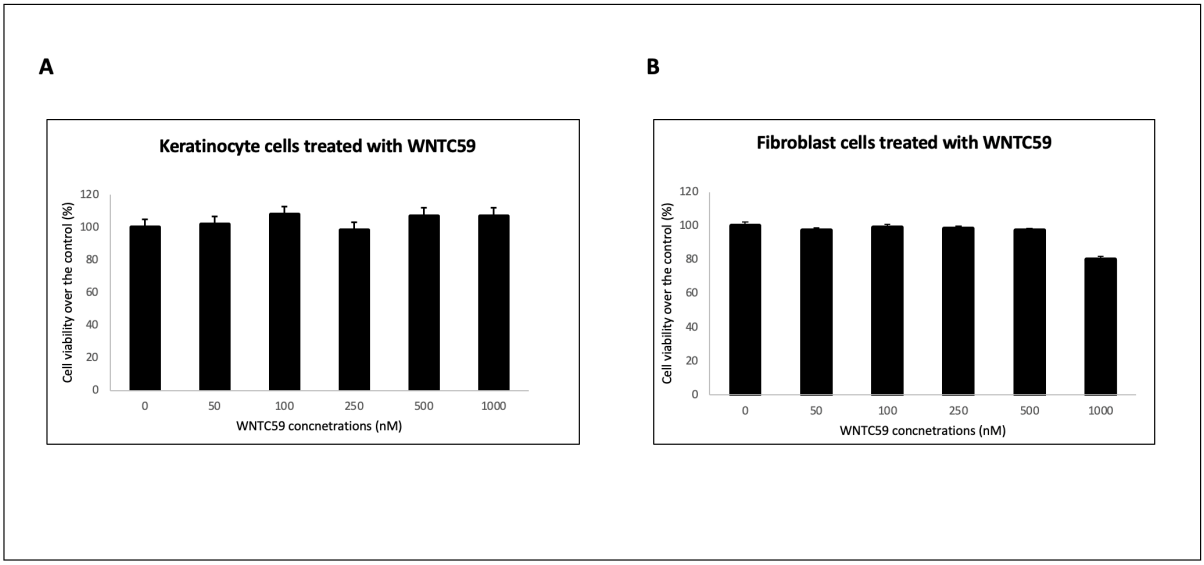

Figure 10. WNTC59 does not affect the cell viability of fibroblast and keratinocyte cells. Cells were treated for 24 hours and cytotoxicity was detected using PrestoBlue® **A.** Keratinocyte cells were treated with different concentrations of WNTC59, no significant toxicity was detected. **B.** Fibroblasts treated with different concentrations of WNTC59, no significant toxicity was detected.

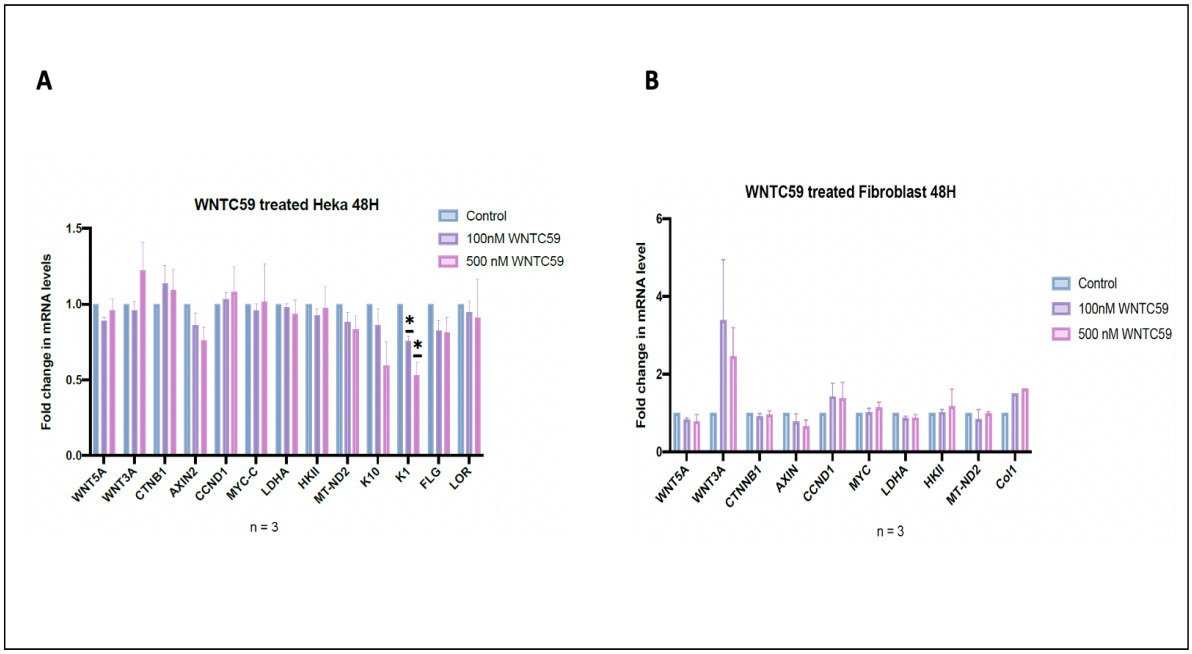

Figure 11. WNTC59 chemical inhibitor downregulates keratinocyte differentiation marker K1. A. qRT-PCR of keratinocytes treated with 100 nM and 500 nM of Wnt ligand chemical inhibitor WNTC59. There is a trend in the downregulations of downstream Wnt signaling targets and significant downregulation of differentiation marker K1; *p<0.05. **B.** qRT-PCR of fibroblasts treated with 100 nM and 500 nM of Wnt ligand chemical inhibitor WNTC59. There is a trend in the downregulations of downstream Wnt signaling targets. N = 3, analyzed with Microsoft excel, *p < 0.05.

WNTC59 treatment increases ATP production and OCAR in fibroblasts

We wanted to investigate if Wnt inhibition had any effect on cell respiration and migration, therefore we conducted mitochondrial stress test (Mito stress test) seahorse assays along with cell migration assays. The mitochondrial stress test measures mitochondrial function in real-time and reports the cells OCAR and ECAR as mitochondrial modulators are injected via built-in injection ports. Oligomycin, an ATP synthase inhibitor, is injected first. This is followed by Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) injection which is a mitochondrial membrane uncoupler allowing electrons to flow freely through the ETC. Lastly, a combination of rotenone and antimycin A is injected, these two drugs inhibit complex I and commplex III of the ETC respectively as described earlier. We performed the Mito stress test on cells treated and incubated with 100, 250, and 1000 nM of WNTC59 for 48 hours. The results revealed that inhibiting Wnt increased the OCAR and ATP production in fibroblasts treated with 100 and 500 nM of WNTC59 as seen in Fig. 12. In contrast, keratinocyte cells showed no change in OCAR or ATP production. This points toward increased energy production when Wnt signaling is inhibited in fibroblast cells.

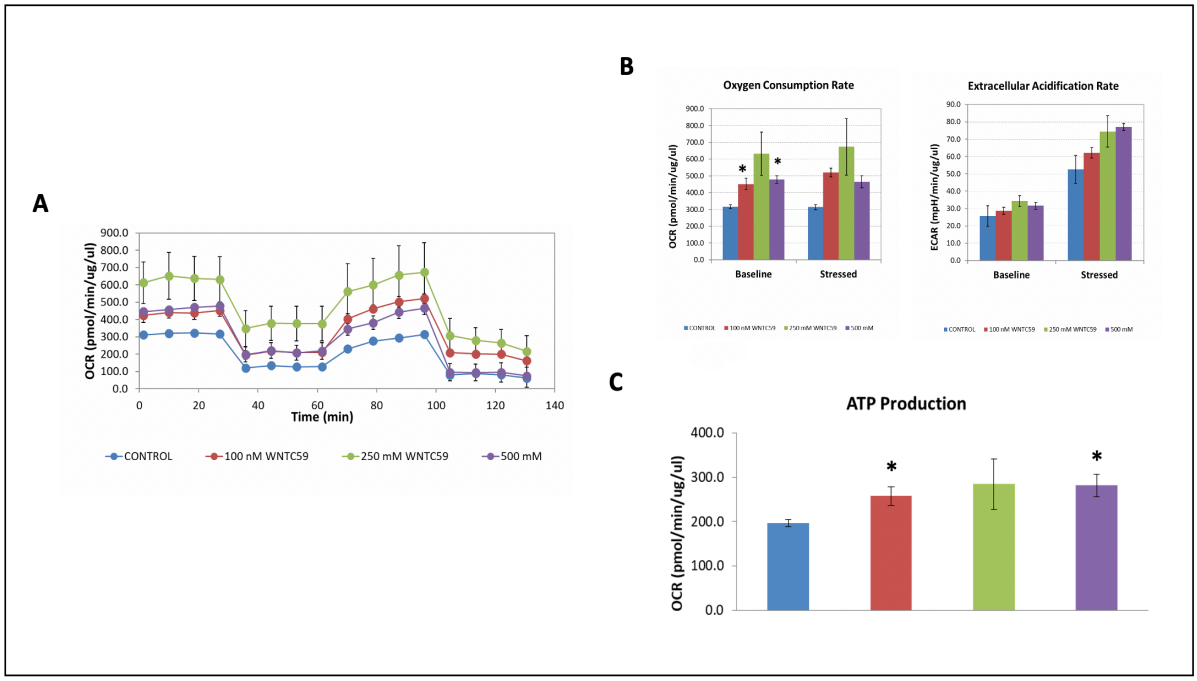

Figure 12. WNTC59 treatment makes fibroblast cells more energetic. A. Mitochondrial stress test of fibroblasts pre-incubated with WNTC59 for 48 hours. Cells were injected with oligomycin, FCCP and rotenone together with antimycin A. **B.** Oxygen consumption rate (OCAR) and extracellular acidification rate (ECAR) of fibroblasts treated with WNTC59 for 48 hours. OCAR increased significantly with 100 nM and 500 nM WNTC59 treatment. **C.** ATP production of fibroblasts treated with WNTC59 for 48 hours. 100 nm and 500 nM WNTC59 treatment increased ATP production of cells making them more energetic. Data analyzed with Wave Software. *$p < 0.05$.

ATP production is important for cell migration as more energetic cells migrate better. Due to this, we wanted to investigate how increased oxygen consumption and ATP levels affect cell migration and proliferation. Therefore we conducted a cell migration assay with 50-500 nM WNTC59 treatments. Results revealed that WNTC59 improved wound healing in fibroblast cells in a dose-dependent manner with the highest dose of 500 nM being significant ($p < 0.05$) but had no effect on proliferation (Fig. 13). In keratinocytes, WNTC59 treatment did not affect cell migration or proliferation (Fig. 14).

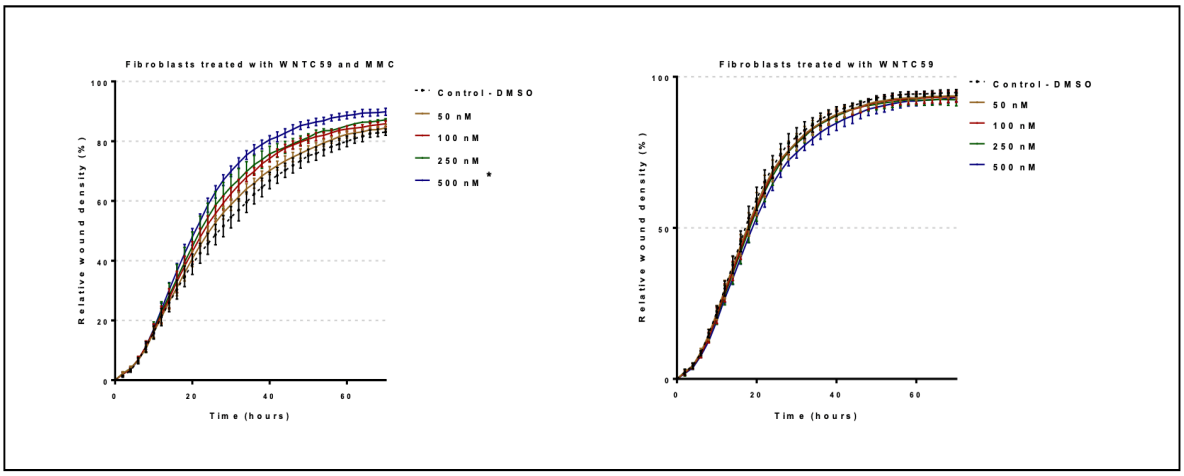

Figure 13. WNTC59 improves cell migration in a dose-dependent manner. A. Scratch wound assay of fibroblast cells treated with chemical Wnt inhibitor WNTC59 for 65 hours. WNTC59 improves wound healing in a dose-dependent manner. N = 3, analyzed with two-way ANOVA. ****p< 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

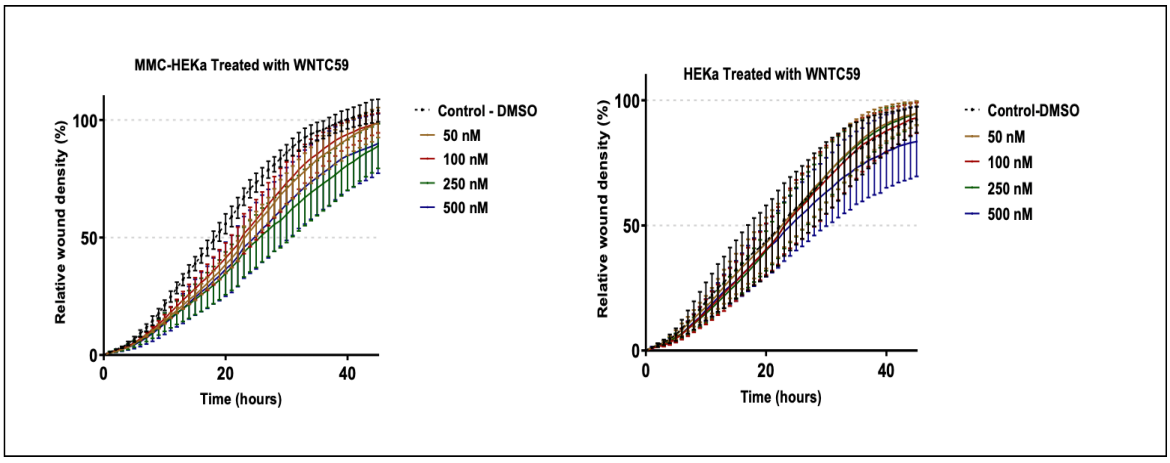

Figure 14. Keratinocyte WNTC59 treatment does not affect wound healing. A. Scratch wound assay of keratinocytes treated with chemical Wnt inhibitor WNTC59 for 40 hours. Treated cells showed no significant change in cell migration. N = 3, analyzed with two-way ANOVA.

Ex vivo WNTC59 treatment

Our findings thus far showed that Wnt ligand inhibition improves wound healing in fibroblast cells *in vitro*. This made us interested in investigating its effects on re-epithelization and wound healing in human skin. As mentioned earlier, re-epithelisation is part of the proliferation phase of the wound healing process where keratinocytes migrate, proliferate and differentiate to close the wounded area (45). To do this, we performed *ex vivo* experiments on skin biopsies collected from healthy individuals. Topically treated biopsies showed that wounds treated with 500 nM WNTC59 have a longer epithelial growth on days 1, 3, and 5 (Fig. 15A). Similarly, in biopsies treated with 10 µM WNTC59 in the medium, the epidermal growth was larger on days 1, 5, and 7 (Fig. 15B). This suggests that both topical and transdermal treated biopsies aid re-epithelization and thus wound healing in full-thickness skin wound models.

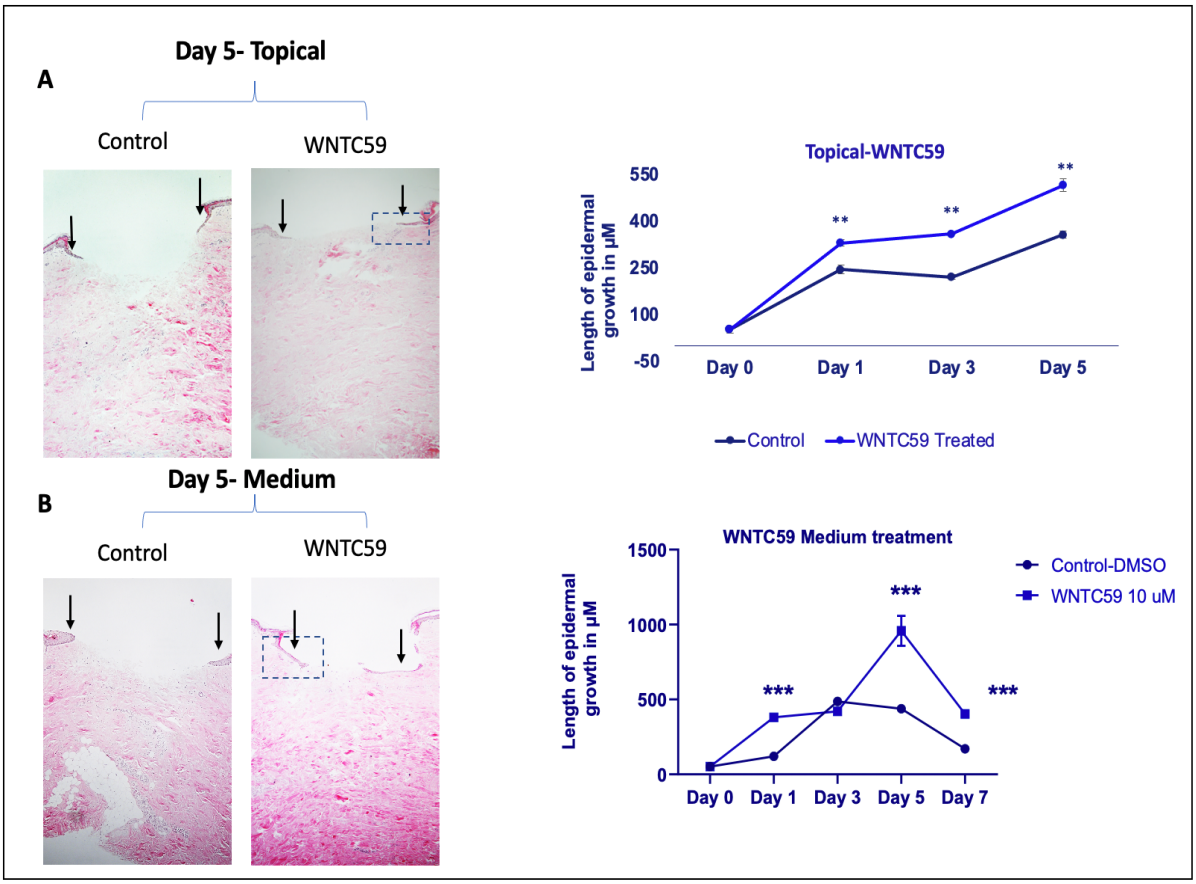

Figure 15. Topical and transdermal application of WNTC59 on skin biopsies improves re-epithelization. A. Topical treatment of skin biopsies with WNTC59 from day 0 to day 5. Re-epithelization is improved in treated skin biopsies compared to untreated control. **B**. Transdermally treated skin biopsies with WNTC59 from day 0 to day 7. Re-epithelization is improved on days 1, 5, and 7 in treated biopsies compared to control. Data analyzed with ImageJ.

Discussion

Chronic leg ulcers pose an extensive socioeconomic burden on the healthcare system. More importantly, it causes a significant reduction in the quality of life of affected patients (8). Tissue damage can result in cells undergoing metabolic adaption due to low oxygen availability. Previous studies have reported metabolic changes in chronic wounds (46), findings from a metabolomics study performed by our group showed key metabolic pathways carbohydrate, lipid, amino acid metabolism, and TCA cycle metabolites to be upregulated in chronic wounds compared to normal healthy skin. Not many studies have reported how these metabolic changes influence important processes of wound healing, migration and proliferation. Our study suggests that fibroblast cells mostly depend on glycolysis for cell migration and proliferation while keratinocyte migration is independent of glycolysis during wound healing. Furthermore, we also show that fatty acid metabolism in contrast to glycolysis does not improve cell migration in fibroblast cells.

Here, we examine how keratinocytes and fibroblast cells migrate in response to metabolic alterations in the chronic wound milieu. It has been described that proliferating cells and wounded skin primarily depend on glycolysis (47). Glycolysis role in cell migration has also previously been studied, Heiss et al. reported how increased glycolysis improves cell migration which is in accordance with other studies where they have described how the inhibition of glycolysis decreases cell migration (48, 49). However, it has not been reported in studies thus far how inhibition of mitochondrial respiration together with increased glycolysis in fibroblast and keratinocyte, thus pushing cells towards glycolysis, impacts migration and proliferation.
To investigate if metabolic changes occurring during wound healing influence cell migration and proliferation, we conducted *in vitro* cell migration assays with fibroblast and keratinocytes. Our data points towards that the metabolic shift occurring in chronic wounds increases glycolytic activity and improves cell migration. We report for the first time, a study comparing cell migration of fibroblast cells in medium lacking glucose and high glucose conditions. Our results show that fibroblast cells are unable to migrate in culture medium without glucose while a concentration of 15 mM glucose improves migration compared to non and normal glucose conditions. These results lead us to think that glucose might play an essential role in fibroblast cell migration as well as proliferation. The experiment should however be repeated for confirmation and statistical significance as the experiments were only performed once. Interestingly, these findings complement recent studies performed by our group, where the inhibition of glycolysis using 2-deoxyglucose decreased cell migration, as well as other previous published literature where studies on various model systems have described the inhibition of glucose to reduce migration (50). Additionally, we found that inhibiting the ETC had little effect on fibroblast and keratinocyte migration. We used rotenone and antimycin A to fully inhibit ETC forcing the cells to depend on glycolysis for energy production but still leaving the TCA cycle functioning. This data suggest that an increase in glucose metabolism is beneficial for fibroblast cells as they are more dependent on glycolysis for their cell migration than mitochondrial respiration. One possible explanation for the shift towards glycolysis is the resulting acidification of the microenvironment due to increased lactate production which has been linked to increased MMPs. MMPs, as mentioned earlier, are important in the mediation of matrix degradation and stimulate migration (51). Although OXPHOS is more energy-efficient than glycolysis, glycolysis generates important metabolic intermediates and enzymes required for cell growth and proliferation (52).

It is well known that re-epithelization is a very important phase in wound healing regulated by keratinocyte migration and proliferation. As described earlier glucose is transported into the cell via a glucose transporter called GLUT1. GLUT1 is highly expressed in proliferating keratinocytes and is also upregulated during wound healing (53). Upregulated GLUT1 expression is linked to increased glycolytic activity. Li and colleagues amongst many have shown in an *in vitro* cell migration study on keratinocytes that high glucose conditions (25 mM) decrease cell migration compared to normal glucose conditions (5.5 mM) (54). Moreover, few studies have reported how low glucose concentrations affect migration and proliferation *in vitro*. We found that keratinocytes migrate well in low glucose (~3 mM) conditions, there was no significant change between the different treated concentrations. Murine experiments performed by Zhang and colleges showed that keratinocytes from *K14 GLUT1* mice showed a significant reduction in cell migration compared to cells from wild-type mice (55). Our data which showed that keratinocyte cell migration and proliferation were independent of glycolysis, is thus in accordance with previous studies that describe both increased and decreased glycolytic activity to be disadvantageous for these processes in keratinocytes. This suggests that keratinocyte cells might use alternative substrates, from amino acid or fatty acid metabolism, as energy fuel (53). Due to limitations with the medium, we did not include controls without any glucose in our experiments. This makes it difficult to differentiate if keratinocytes are insensitive to glucose concerning cell migration and migrate well regardless of low glucose (i.e not dependent on glucose for migration) or sensitive, where only low concentrations are enough for full migratory capacity (i.e very low concentrations of glucose saturates glycolysis). Preferably more experiments should be performed in lower or no glucose conditions to assess this.

Data from previous experiments show that fatty acid metabolism is upregulated in chronic wounds compared to normal healthy skin, however, this is largely unexplored in the context of wound healing. Fats are one of the major metabolic pathways used for energy production and proliferation as it supports membrane growth and integrity. Therefore it would be logical that its upregulation and or inhibition would trigger changes in wound healing and possibly cell migration and proliferation. Long-chain fatty acids rely on the rate-limiting enzyme CPT1 to be shuttled into the mitochondrial matrix as they cannot pass through freely, thus inhibiting CPT1 also inhibits mitochondrial oxidation (56). A previous study on CPT1 overexpression in MDA-MB231 cancer cell lines showed a decrease in cell migration suggesting fatty acid metabolism involvement in wound healing (57). Etomoxir is a specific CPT1 inhibitor that is clinically approved and has been used in phase I clinical trials for the treatment of heart failure and diabetes. Etomoxir mediated FAO inhibition has been linked to decreased proliferation in myeloma and other tumor cells (58). Our data demonstrate that there is no significant change in cell migration or proliferation in FAO inhibited dermal fibroblast cells. This might be due to the fibroblast cell dependence on glycolysis as both glycolysis and free fatty acids (FFA) feed the TCA cycle via Acetyl-CoA because the culture medium contains glucose. Additionally, we treated cells with 200 μM FFA to emulate the chronic wound environment which has relatively high concentrations of FFA. This increase in FFA might have been too high for the drug to act on. Therefore removing BSA-Palmitate and conducting the experiments in more normal FFA conditions may be a better option for the future. Furthermore, something to take into consideration is the off-target effect of etomoxir seen in concentrations greater than 5 μM, it

has been reported in studies that complex I of the electron transport chain is inhibited with higher concentrations of etomoxir which should be addressed.

Wnt signaling has been described as a contributing factor in tumor metabolism - similar to chronic wounds, tumor cells also undergo metabolic reprogramming. Current research indicates that Wnt signaling is involved in cancer reprogramming through the regulation of downstream targets such as c-MYC, transcription factors TCF/LEF, and the Akt-mTOR pathway. As transcription factor TCF/LEF binds to the promoter in the nucleus it upregulates the expression of GLUT1, LDH and c-MYC which in turn increases glycolysis (59). Our results show that Wnt signaling increased cellular oxygen consumption rate and ATP synthesis which points towards increased mitochondrial respiration and the cells being more energetic when Wnt signaling is inhibited. Opposing our results, the previous literature has described canonical Wnt inhibition to decrease mitochondrial metabolism in adipocytes and another study by Yoon et al. shows how wnt3a treatment, whose mode of action is mainly through the canonical pathway, in mouse myoblast cells increased mitochondrial OXPHOS (60). A study by Pate et al. where they examined Wnt signaling in colon cancer metabolism revealed that inhibiting Wnt signaling reduced lactate levels and increased ATP production and oxidative phosphorylation similar to our study (43). However, to assess if Wnt inhibition has an effect on glycolysis a glucose stress test needs to be performed. A glucose stress test assesses the glycolytic capacity of cells by measuring the extracellular acidification rate in the surrounding culture medium.

Bastakoty et al. have reported that topical use of small-molecule Wnt inhibitors similar to WNTC59 promotes the closure of ear punch wounds. The study investigated how Wnt signaling affects wound healing by topically treating wounds with three different inhibitors of the canonical and non-canonical pathway with the mode of action via axin and CK1-α. All three inhibitors enhanced regeneration and promoted wound closure (61). Concurring with this our data on topical treatment of skin biopsies with Wnt inhibitor showed improved healing compared to controls in both topical treatments straight into the wound and transdermal treatment with WNTC59 in the culture medium. Taking this together Wnt signaling could be a possible factor attributing to the metabolic reprogramming in chronic wounds and aid wound healing, but the cellular mechanism needs to be further explored. Although results are promising, a limitation to our study is that better validation of Wnt signaling downregulation is needed as we only saw a trend in the down-regulation of Wnt signaling gene-expression on the mRNA level. This should preferably be done through techniques as western blot which shows the expression on a more relevant protein level.

Conclusion

It can be concluded that fibroblast cells are dependant on glycolysis for their cell migration, while keratinocytes migrate well in low glucose conditions seemingly more dependent on mitochondrial respiration. Wnt signaling may play a role in the reprogramming of fibroblast cells as Wnt inhibition increases mitochondrial activity. This suggests the metabolic shift towards glycolysis in fibroblast cells in chronic wounds is beneficial. These findings deepen our understanding of metabolism in chronic wounds and suggest potential future therapeutic options.

Acknowledgments

I want to thank Dr.Wikström as well as Dr.Bachar-Wikström for giving me the opportunity to do my thesis project in their lab, for their guidance and for allowing me to work so independently which helped me grow tremendously as a young researcher.

A huge thank you to Mansi Manschanda who trained me, helped me plan, guided and counseled me all the way from Gothenburg. Thank you for being my mentor and dear friend in the lab. You have helped me so much helping me in ways I cannot put into words.

To Naricha, Ritu, and everyone at CMM, I want to thank you for helping me navigate through the lab, answering all my questions and supporting me throughout my project.

And finally, I want to thank my friends and family who have been my support system and cheered me on throughout this semester.

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