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Mechanism of Metformin Action in Dermal Fibroblasts

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Mechanism of Metformin Action in Dermal Fibroblasts

by

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Abstract

Burn injury is a significant problem that affects approximately half a million people in the U.S. annually. One of the major complications emerging from burn injury is hyperglycemia, which can last for weeks following the initial trauma. The primary cause of hyperglycemia is dysregulated AMPK signaling and mitochondrial complex activity, which regulates both glucose uptake and cellular energy status. Current therapeutic methods to counter hyperglycemia include tight euglycemic control and/or intensive insulin treatment, which are associated with increases in hypoglycemia and mortality, but also accelerate wound healing rates, potentially decreasing scarring. Alternatives to insulin therapy include biguanide drugs such as metformin. Metformin downregulates gluconeogenesis, and reduces blood glucose levels by activating glucose transport via

GLUT4 in muscle cells. Importantly metformin has been shown to decrease mitochondrial respiratory complex I activity in hepatocytes. However its effects in dermal fibroblasts are unknown. Furthermore, up to 90 percent of burn patients develop hypertrophic scars due to dysregulated collagen secretion, which can lead to functional impairment due to excessive collagen deposition associated with hypertrophic scarring.

In this study the hypothesis is that in dermal fibroblasts metformin will alter mitochondrial oxidative phosphorylation and alter cAMP and AMPK signaling activity, resulting in increased wound healing and improved mitochondrial function. Our experimental data shows that metformin induced AMPK phosphorylation, reduced mTOR phosphorylation, and downregulated cAMP accumulation in dermal fibroblasts. Fibroblast proliferation and migration were downregulated in hyperglycemic conditions with metformin exposure. Expression of mitochondrial biogenesis genes were altered by metformin treatment. Significantly, metformin specifically upregulated oxygen flux activity in fibroblasts, which was independent of AMPK activity. Metformin also altered expression of epithelial-mesenchymal transition (EMT) genes and markers. The primary EMT pathway regulated by metformin was SMAD3, which was decreased by metformin treatment resulting in decreased collagen I gene expression. Metformin also reduced total collagen secretion in low glucose conditions. These results indicate that metformin alters metabolism in fibroblasts through mitochondrial respiratory chain independently of AMPK. This will allow a better understanding of the effects of metformin on fibroblasts leading to new therapeutic options to prevent burn injury complications for wound healing in burn patients.

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List of Abbreviations

UTMB: University of Texas Medical Branch

GSBS: Graduate School of Biomedical Science

TDC: Thesis and Dissertation Coordinator

ACC: acetyl coA carboxylase

AMPK: 5' adenosine monophosphate-activated protein kinase

ANOVA: analysis of variance

ATCC: American Type Culture Collection

BCA: bicinchoninic acid

BMP2: bone morphogenic protein 2

BSA: bovine serum albumin

cAMP: cyclic AMP

CREB: cAMP-response element binding protein

CBP: CREB binding protein

coQ: coenzyme Q2

DG: 2-deoxyglucose

2-DGP: 2-deoxyglucose 6 phosphate

DMEM: Dulbecco's Modified Eagle Media

ECL: enhanced chemoluminesence

ER: endoplasmic reticulum

EMT: epithelial-mesenchymal transition

eNOS: endogenous nitric oxide synthase

EPC: epithelial progenitor cells

ETC: electron transport chain

$FADH_2$: flavin adenine dinucleotide

FBS: fetal bovine serum

FOXO1: forkhead box protein O1

FSP-1: fibroblast specific protein 1

GAPDH: glyceraldehyde 3 phosphate dehydrogenase

HG: high glucose (4.5 g/L or 25 mM)

HDL: high density lipoprotein

HSD3B2: 3β-hydroxysteroid dehydrogenase type 2

HTS: hypertrophic scar cells from burn area

IBMX: 3-isobutyl-1-methylxanthine

IGF: insulin growth factor

IL: interleukin

IRS: insulin receptor substrate

JNK: c-jun terminal kinase

KRGB: Krebs ringer bicarbonate buffer

kDa: kilodaltons

LG: low glucose (1 g/L or 5 mM)

LKB1: liver kinase protein B1

MET: mesenchymal-epithelial transition

Mfn: mitofusion

miRNA: micro RNA

mTOR: mammalian target of rapamycin

mTORC: mammalian target of rapamycin

MATE1: multidrug and toxin extrusion transport 1

MMP: matrix metalloprotease

MTT: 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

MEF2A: myocyte enhancer factor 2A

NADH: nicotinamide adenine dinucleotide

NBS: non-burn skin

OCT: organic cation transporter

PI3K: phosphoinositide-3 kinase

PBS: phosphate buffered saline

PEPCK: phosphoenolpyruvate kinase

PPAR: peroxisome proliferator-activated receptor

PGC1α: PPAR gamma co-activator 1α

PKA: protein kinase A

PKC: protein kinase C

PI: propidium iodide

PMSF: phenylmethanesulfonyl fluoride

PTEN: phosphatase and tensin homolog

PVDF: polyvinylidene fluoride

Rheb: Ras homologue enriched in brain

rhGH: recombinant human growth factor

ROS: reactive oxygen species

RT PCR: reverse transcriptase polymerase chain reaction

SOX: superoxide dismutase

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate gel electrophoresis,

SEM: standard error of means

SIRT: sirtuin

SLC2A: solute carrier 2A

SMA: smooth muscle actin

SNP: single nucleotide polymorphism

SREBP-1: sterol regulatory element binding protein 1c

STAT3: signal transducer and activator of transcription

TBSA: total body surface area

TGF: transforming growth factor

TNFα: tumor necrosis factor α

TORC: transcription co-activator

TSC2: tuberous sclerosis complex 2

TWIST: twist family bHLH transcription factor

UCP: uncoupling protein

Chapter 1: Introduction

Burn Injury Pathology

Large burn injuries induce systemic alterations in metabolism, inflammation, and immune function that significantly impact morbidity and mortality. Each year in the United States approximately 2 million people suffer burn injuries with 500,000 burn patients admitted for treatment (1). Severely burned patients with burns over 30% of their total body surface area (TBSA) experience hypermetabolic and inflammatory responses that are associated with infection, metabolic syndrome, stress, and muscle catabolism (2-5). Among the most pervasive effects is hyperglycemia, which has been linked to insulin resistance, muscle catabolism, and multiple organ failure (5, 6). Furthermore, hyperglycemia in burn patients is associated with graft rejection, infection, and mortality (3, 7). Burn injury is frequently associated with metabolic syndrome; the clinical manifestation of dysregulation of metabolic pathways involved in regulating blood glucose levels and fat balance. Patients with metabolic syndrome frequently exhibit altered blood pressure, high fasting glucose concentrations, and low high density lipoprotein (HDL) cholesterol levels (8). Pathways such as insulin receptor signaling and gluconeogenesis regulate glucose transport and fatty acid synthesis/oxidation affected by metabolic syndrome by mediators such as 5' adenosine monophosphate-activated protein kinase (AMPK) and phosphoinositide-3 kinase (PI3K) (1, 9-11). Many of these signaling pathways are modulated by burn injury, with effects that can last long after the initial trauma (3, 12-14).

A number of therapies have been utilized to modulate burn injury associated hypermetabolism, with varying levels of success (4). Insulin therapy is the most commonly used due to beneficial outcomes including blood glucose clearance, improved muscle synthesis and reduced infections, and increased type IV collagen in early wounds (15, 16). However insulin can induce hypoglycemia, and is not associated with significant improvements in survival rates (17). Recombinant human growth hormone (rhGH) has been linked to improved insulin growth factor (IGF-1) activity and less scarring 12 months post burn injury, but also increased hyperglycemia (9, 16). Fenofibrate, a peroxisome proliferator antagonist receptor α (PPARα) agonist, improves mitochondrial function, reduces hepatic steatosis, and prevents coronary artery disease in both burned and diabetic patients (18-20). However fenofibrate does not reduce post-burn endoplasmic reticulum stress in hepatic tissue (21, 22). Therefore it is of paramount importance to implement therapies to effectively combat aspects of the hypermetabolic response inducing fewer side effects. Metformin is one such drug undergoing clinical trials that has the potential to improve aspects of burn induced hypermetabolism such as mitochondrial function, glucose clearance, and muscle wasting while avoiding many of the side effects associated with other therapies. Some of the effects of metformin use in burn patients and non-burned patients suffering from metabolic syndrome are listed in **Table 1** (23-29).

	Burn patient	Non-burn patient with metabolic syndrome
Reduce blood glucose	Yes	Yes
Improve arterial blood flow	Yes	Yes
Improve muscle fractional synthetic rates	Yes	No
Increase AMPK activity in muscle, fat, liver	Yes	Yes
Reduce hepatic gluconeogenesis	Yes	Yes
Reduce adipose fatty acid synthesis	No	Yes
Induce risk of lactic acidosis	Yes	No
Reduce serum inflammatory cytokines	Yes	Yes
Improve mitochondrial oxygen utilization	Yes	Yes
Reduce risk of infection	Yes	No
Reduce wound collagen deposition	Yes	No
Synergistic action with other drugs	No	Yes

Table 1: Effects of metformin in burned and non-burned patients experiencing metabolic dysfunction.

Burn injury upregulates gluconeogenesis and increases circulating inflammatory cytokines that can alter insulin signaling (30). Burn injury is also associated with elevation of catecholamines, glucagon, and cortisol, stimulating increased hepatic glucose production, and upregulating inflammatory pathways via non-canonical NF-κB signaling (7, 31). Another consequence of burn associated hyperglycemia is muscle protein degradation due to catabolic rates exceeding synthetic rates (4). Current therapies to reduce hyperglycemia in burn patients include tight euglycemic control and conventional or intensive insulin therapy, or other drugs used to control hyperglycemia in diabetic patients. Although insulin therapy reduces hyperglycemia in some patients (30), there is evidence that these strategies actually put some patients at increased risk for hypoglycemic episodes. For example, insulin therapy can be less effective in young children, elderly patients, and diabetics, and the risks of hypoglycemia sometimes outweigh the risks associated with hyperglycemia (6). Our group has shown that in

severely burned children receiving insulin therapy, 20% of patients develop mild hypoglycemia, with another 20% experience severe hypoglycemia (12). Insulin administration was also associated with a more hypoglycemic episodes, greater resting energy expenditure, and higher mortality rates than observed in patients not receiving insulin treatment (32). One of the primary challenges of burn therapy is to attenuate the hyperglycemic response commonly associated with burn injury.

Metformin and metabolism

Metformin belongs to the biguanide class of drugs which inhibit hepatic glucose production, and upregulates circulating glucose uptake by enhancing insulin sensitivity in a cell selective manner (33). Derived from the French lilac *Galega offifinalis*, metformin has been one of the most widely used treatments to manage type 2 diabetes in Europe for 50 years, and in the US since 1995 (33, 34). Administration of metformin increases insulin sensitivity in muscle and liver, decreases hepatic glucose production, and increases insulin receptor tyrosine kinase activity (34-36). Metformin action is cell type and tissue specific. In skeletal muscle, metformin increases mitochondrial oxidation and uptake of glucose and fatty acids (37). In adipose tissue, metformin reduces fatty acid synthesis and lipolysis, while in liver metformin reduces lipid synthesis and gluconeogenesis (37). Metformin reduces insulin secretion by deactivating beta cells in the pancreas (37). Fatty acid oxidation is reduced by metformin by suppression of *Sreb-1c* expression to inhibit steatosis induced by a high-fat diet (38). The most well-known action attributed to metformin is the stimulation insulin sensitivity through upregulation of canonical AMPK and Akt signaling in muscle cells via atypical protein kinase C (PKC) signaling, leading to GLUT4 membrane translocation for glucose uptake (34, 36,

39). Metformin downregulates glucose production in human hepatocytes primarily through upregulation of the alpha subunit and associated inhibition of mitochondrial complex 1 (27). Metformin also has effect on other signaling pathways in various cell and tissue types. In 3T3-L1 preadipocyte cells, metformin reduced phosphatase and tensin homolog (PTEN) expression and Akt phosphorylation in an AMPK dependent fashion while stimulating the phosphorylation of J-n terminal kinase (JNK) and mammalian target of rapamycin (mTOR). Loss of PTEN expression restored insulin induced Akt phosphorylation and upregulated JNK and mTOR (35). Metformin administration to diabetic mice inhibited gluconeogenesis in cardiac muscle by downregulating gluconeogenic genes such as hexokinase and phosphofructokinase (40). Biguanide treatment of primary hepatocytes inhibited glucagon signaling by accumulation of AMP and inhibition of cAMP, which led to lower gluconeogenesis via reduced protein kinase A (PKA) signaling (41). Although the functions of metformin vary considerably among different cell types, a common pathway inhibited in almost all experiments has been mitochondrial respiratory complex I (33). Metformin decreased intracellular concentrations of succinate, fumarate, and malate, mimicking the effects of the complex I inhibitor rotenone (42, 43). Interestingly, the androgen inhibiting effects of metformin in adrenal cells were due solely to complex I inhibition, independent of AMPK activity, mostly by downregulation of 3β-hydroxysteroid dehydrogenase type 2 (HSD3B2), which was also downregulated by the complex I inhibitor rotenone (42), suggesting that metformin can exert its activity through a variety of pathways in many cell types.

Metformin has also been used to treat microvasculature complications by its effects on endogenous nitric oxide synthase (eNOS), which are common in burned and diabetic patients (33, 36, 44). It has been shown that mice with streptozotocin-induced diabetes treated with 250 mg/kg metformin for 14 days experienced increased blood glucose clearance, wound healing, and capillary formation following punch biopsy injury. Circulating endothelial progenitor cells (EPCs) were downregulated in diabetic mice, but increased following metformin treatment (44). Metformin treatment also increased AMPK and eNOS activity in murine EPCs. This increase was abrogated by the AMPK α-subunit inhibitor Compound C, indicating that metformin improves wound healing in diabetic state conditions through an AMPK dependent mechanism (44). Metformin also inhibits cancer stem cell proliferation by allowing expression of miRNAs that are typically lost in cancer (45). Although the functions of metformin vary considerably among different cell types, a common effect of metformin administration is inhibition of mitochondrial respiratory complex I (33). Complex I, or NADH ubiquitone oxidoreductase, is comprised of 45 subunits located in the inner mitochondrial membrane and is the first of five complexes that phosphorylates ADP by coupling electron flow and proton transfer out of the inner membrane (46). Complex I oxidizes nicotinamide adenine dinucleotide (NADH) to generate two electrons that are passed on to ubiquinone to continue the electron transport chain (46). Metformin mimics the effects of the complex I inhibitor rotenone by decreasing intracellular concentrations of succinate, fumarate, and malate (42, 43). Interestingly, the androgen inhibiting effects of metformin in adrenal cells were due solely to complex I inhibition independent of AMPK activity (42), suggesting that metformin can exert its

Figure 1

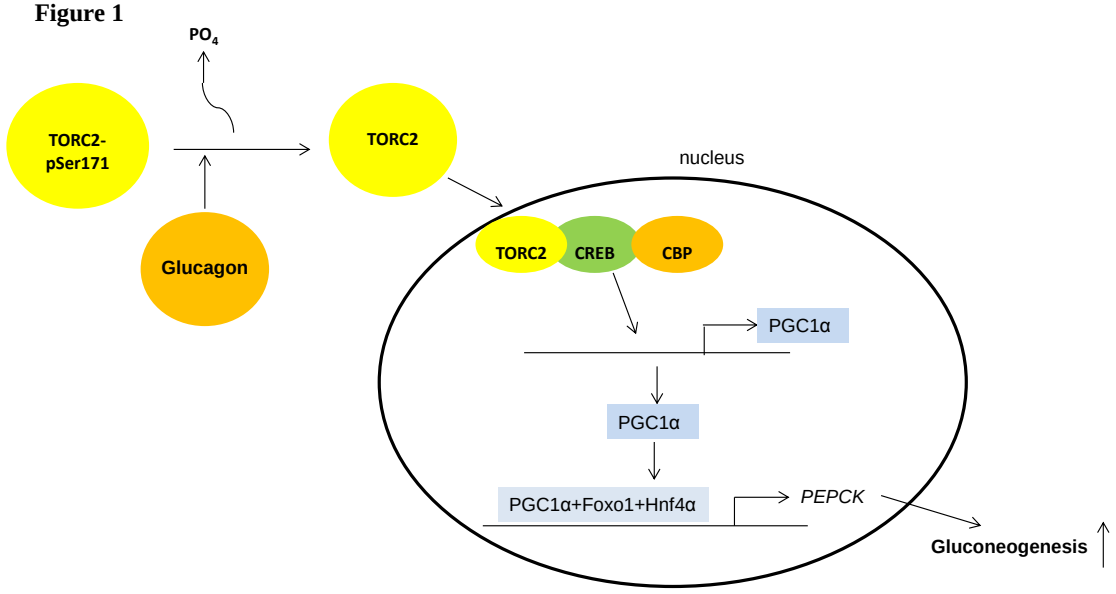


Figure 1: Activation of PEPCK by glucagon. Glucagon dephospohorylates TORC2, which translocates to the nucleus and forms a complex with CREB and CBP, activating PGC1α gene transcription. PGC1α, FOXO1, and Hnf4α bind to the PEPCK promoter to activate the gene and stimulate gluconeogenesis.

activity through a variety of pathways dependent on cell type. The clinical impacts of these actions of metformin are not understood.

Regulation of hyperglycemia by metformin

A key feature in metabolic syndrome is elevated hepatic gluconeogenesis and failure of plasma glucose clearance through dysregulation of genes regulating glucose utlilization, along with increased muscle catabolism (3, 47). Gluconeogenesis is controlled by the rate limiting enzyme phosphoenolpyruvate kinase (PEPCK), which has been shown to be elevated in diabetic individuals (48). As shown in **Figure 1**, under fasting conditions hormones such as glucagon activate cAMP-response element binding protein (CREB) regulated transcription coactivator 2 (TORC2), which leads to formation of TORC2-CREB-CREB binding protein (CBP) complex that induces PEPCK's coactivator PGC1α (48). Sirtuin 1 (SIRT1) is a multifunctional regulator of gluconeogenesis which

deacetylates TORC2 and activates PGC1α. Metformin inhibits gluconeogenesis through induction of SIRT1 and induction of the PGC1α inhibitor GCN5, thus inhibiting glucagon mediated gluconeogenesis (48). Cellular GLUT4 mediated glucose transport is regulated by a signaling cascade that begins with insulin binding to its receptor, stimulating autophosphorylation of the IRS family that recruits downstream effectors such as PI3K, Akt and PKC (36). Although metformin has been well documented to improve insulin sensitivity in many cases, metformin's role in glucose uptake is less understood. Metformin increased activation of Akt and PKC through IRS-1 and 2 activation (36, 39) resulting in increased GLUT4 activity. Others have shown that metformin mainly acts to reduce gluconeogenesis via downregulation of gluconeogenic genes and their upstream regulators such as *PEPCK* and CREB binding protein (CBP) (49).

The effects of metformin on AMPK signaling

AMPK, a heterotrimer comprised of a catalytic α subunit and 2 regulatory (β and γ) subunits that is often activated by liver kinase protein B1 (LKB1), is a critical regulator of energy homeostasis that is activated when cellular energy stores are low (34, 35). AMPK also regulates cell proliferation, fatty acid metabolism, mitochondrial synthesis, and blood flow (39, 44). Some of the many pathways regulated by AMPK are illustrated in **Figure 2**. Downregulation of AMPK occurs in diabetic states following severe burn injury (1, 50). AMPK upregulates ATP producing pathways such as fatty acid oxidation while inhibiting ATP consuming

Figure 2

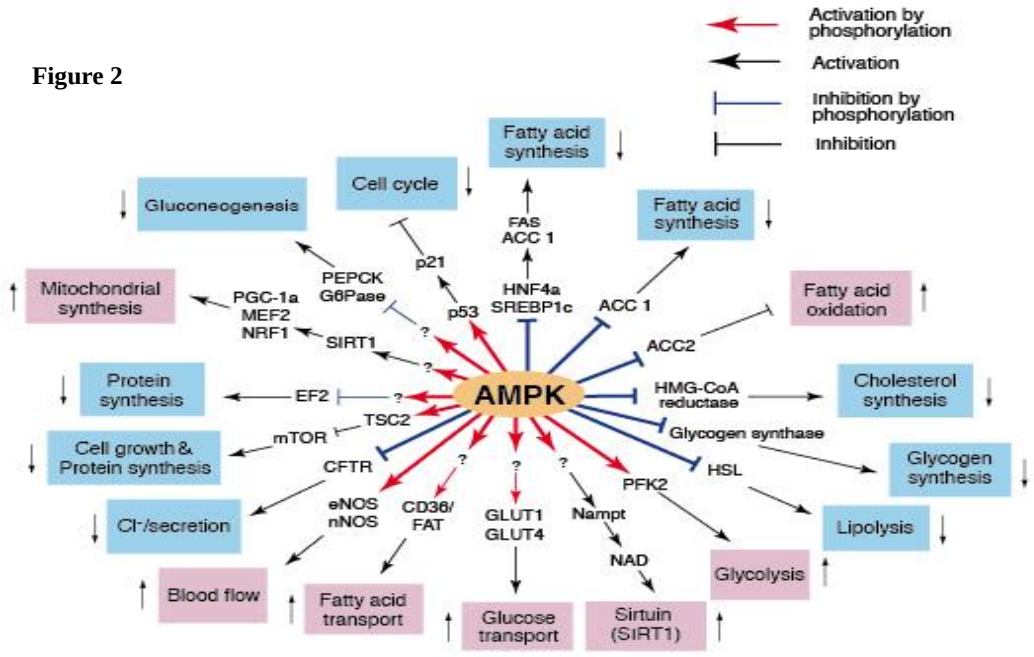


Figure 2: AMPK regulation of cell signaling pathways. From http://ruo.mbl.co.jp.

pathways such as fatty acid and cholesterol synthesis (27, 34, 35, 51). Downstream effectors include activation of GLUT4 by PI3K and Akt signaling, and inhibition of cell proliferation via downregulation of mTOR1 (39, 50, 52). Both the mitochondrial and non-mitochondrial AMPK associated pathways are affected by metformin (**Figure 3**). Interestingly, the AMPK activating effects of metformin appear to be dependent upon its ability to bind to mitochondrial copper. As shown by Logie *et al.*, treatment of H4IIE liver cells with trein, which sequesters intracellular copper, resulted in π electron delocalization and abrogation of AMPK activity (53). Many studies have elucidated the effects of metformin on AMPK regulation to treat hyperglycemic and diabetic states in a variety of cell types. This is demonstrated in the distinct effects of AMPK in adipocytes and muscle. AMPK induces downregulation of PTEN in adipocytes while stimulating both insulin induced Akt phospohrylation (35) and PKC activation in C2C12 skeletal

muscle cells (34). Other studies performed in primary mouse skeletal muscle cells confirm activation of AMPK by metformin, associated with inhibition of atypical and typical PKC (54). These changes resulted significantly increased glucose uptake, indicating that metformin may act via PKC rather than AMPK (39). Following stressors such as burn injury cyclic AMP (cAMP) levels are elevated, and acts to promote

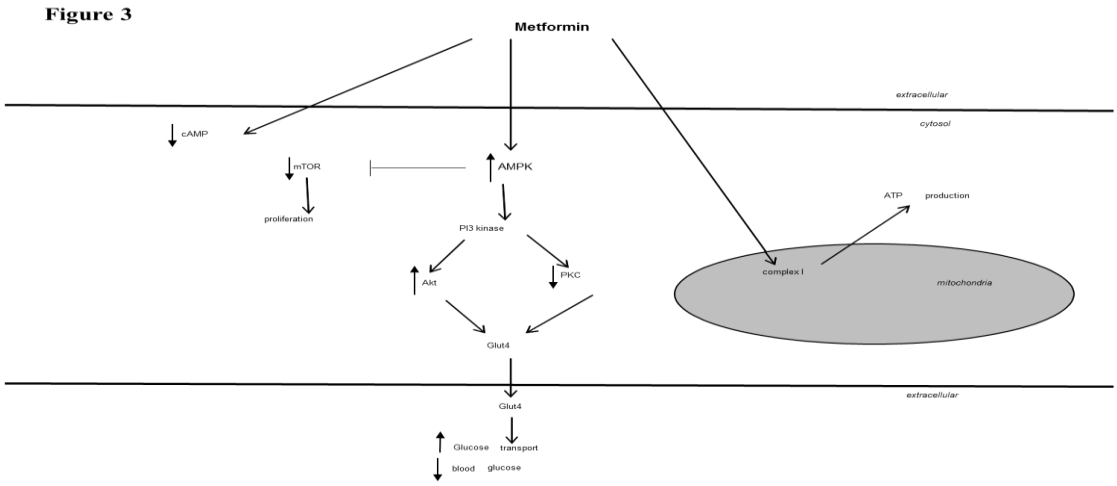


Figure 3: Model of the effects of metformin on AMPK and mitochondrial activity. Metformin upregulates AMPK activity, which inhibits mTOR and cell proliferation. Akt activity is upregulated, which leads to increased GLUT4 activation to promote blood glucose clearance. Metformin also alters mitochondrial complex I activity depending on cell type, which can alter ATP production.

hyperglycemia and insulin resistance (41, 55-57). Metformin blocks gluconeogenesis through inhibition of cAMP signaling by phosphorylating CBP, preventing CBP association with CREB and TORC2 (49), and abrogating adenyl cyclase activation by glucagon, resulting in sequestration of cAMP in cells (41). It appears that metformin may not act solely on AMPK; instead stimulating downstream targets via typical and atypical PKCs, and the anti-gluconeogenic effects are regulated by cAMP inhibition (58).

Glucose transport mechanisms

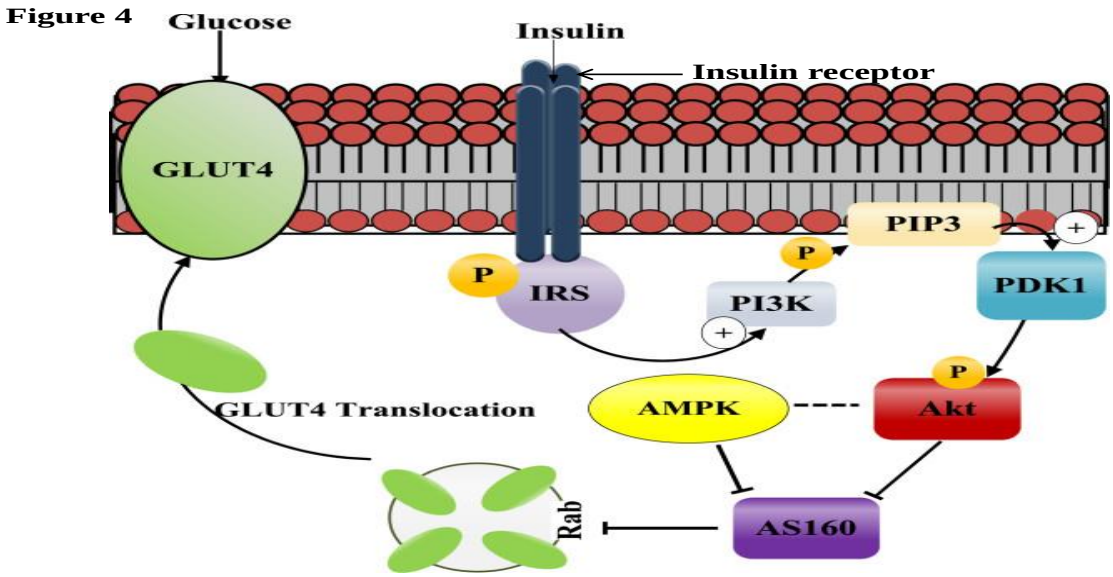


Figure 4: Insulin stimulated GLUT4 signaling pathway. Image from Thorn et al. *EJNMMI Res*;3(1):48, 2013.

All cell types utilize glucose as a major energy source, making the import of glucose into cells a priority in order to begin the glycolysis process. Most cells utilize facilitative diffusion using the GLUT (also known as solute carrier 2A: SLC2A) family of transporters. Fourteen GLUT transporters have been identified in human cell types so far, with GLUT1 and 4 being the most physiologically relevant (59). GLUT1 is found in most cell types, including erythrocytes, muscle, fat, brain, and liver (60). GLUT2 is expressed in liver and pancreatic β cells, GLUT3 in neural cells and fetal skeletal muscle, GLUT5 in intestinal epithelial cells, and GLUT7 in liver (59, 60). GLUT4 is of particular interest in diabetic syndrome, since it is the only insulin responsive glucose transporters. GLUT4 was first identified by James *et al*. at high levels in insulin responsive tissues; mainly adipose, skeletal and cardiac muscle tissue (61). As shown in **Figure 4** insulin biding to its receptor stimulates phosphorylation of insulin receptor substrate (IRS),

which phosphorylates phosphioinositide-3-kinase (PI3K) and subsequently Akt to inhibit Akt substrate of 160 kilodaltons (AS160), which phosphorylates GLUT4 inhibitor Rab, causing Rab to dissociate and allow GLUT4 to translocate to the cell membrane where it binds glucose for membrane translocation (34, 62). AMPK also plays a role in GLUT4 transcription by downregulating its repressor histone deacetylase (HDAC) 5, reducing HDAC5 association with the GLUT4 promoter (50). GLUT4 translocation is known to be dysregulated in diabetic states due to failure of the insulin signaling pathway (63). Interestingly metformin has been associated with improved GLUT4 function in muscle cells. L6 muscle cells treated with metformin displayed a dose dependent increase in GLUT4 membrane translocation and media glucose clearance, along with increased SIRT1 activity (64). Metformin had no effects on ACC activity, suggesting that fatty acid metabolism in muscle cells in not affected by increased AMPK activity. This effect was dependent on the function of the AMPKα1 subunit; mutation of AMPKα1 but not AMPKα2 resulted in less metformin induced glucose uptake (64), suggesting that AMPKα1 is the catalytic subunit. These effects are not limited to muscle cells. In the endometria of hyperinsulinemic polycystic ovary syndrome patients with lower AMPKα phosphorylation metformin treatment not only increased AMPK activity, but also increased expression of myocyte enhancer factor 2A (MEF2A), another regulator of GLUT4 translocation (65). Despite the extensive literature detailing the functions of GLUT4 in response to insulin and metformin, particularly in muscle and liver, the effects of metformin on glucose transport in dermal fibroblasts remain unknown.

Regulation of mitochondrial respiration and biogenesis by biguanides

The mitochondrial electron transport chain (ETC) is comprised of 5 subunits that utilize the electron carriers NADH and flavin adenine dinucleotide ($FADH_2$) to ultimately drive ATP production via a proton gradient (**Figure 5**). Complex I oxidizes NADH and reduces coenzyme Q (CoQ). Succinate is oxidized by complex II, which also reduces CoQ. Complex III oxidizes CoQ and reduces cytochrome c that donates an electron to complex IV, reducing oxygen to water. Complex V couples proton flow from the outer to the inner membrane with an

Figure 5

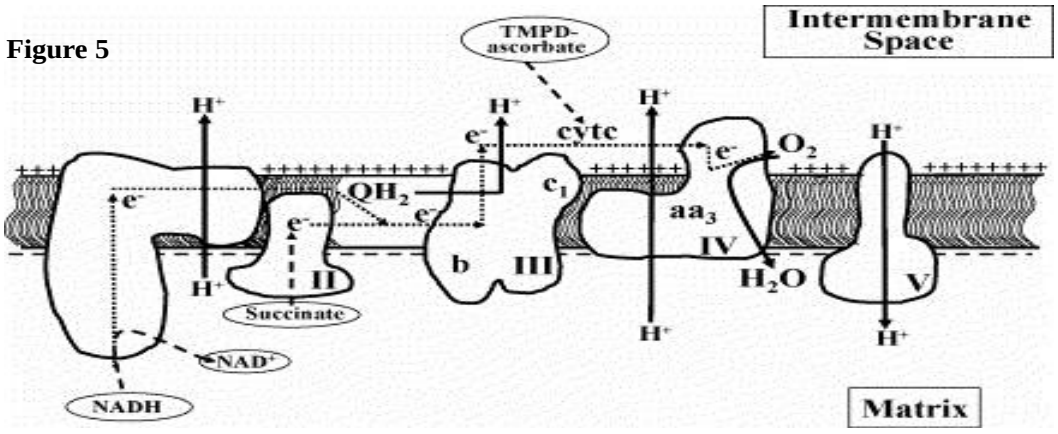


Figure 5: Electron transport chain in inner mitochondrial membrane. From Lesnefsky and Hoppel. *Ageing Research Reviews*: 5(4):402-33, 2006.

electrochemical gradient to drive the complex to phosphorylate ADP. The activity of complex V is regulated by ADP levels; in low energy states the rate of ATP hydrolysis is reduced, resulting in less available ADP to be phosphorylated by complex V (66). Respiration uncoupling occurs in pathologic states due to damage to the inner membrane or to complex V, resulting in loss of ADP regulation, impaired energy production, and increased mitochondrial permeability (66). Burn injury results in increased total body oxygen consumption, particularly in skeletal muscle, along with increased uncoupling of

respiration and ATP production (67-69). Metformin has been used to counteract hypermetabolic induced mitochondrial dysfunction by ainhibiting NADH oxidation and complex I, particularly in skeletal muscle (69, 70) By inhibiting complex I, and possibly downregulating SIRT3 expression, metformin decreases cellular energy status (27, 42, 43, 58, 71). This in turn leads to AMPK dependent decreases in mitochondrial respiration (72). These effects are tissue specific. Metformin may not affect skeletal muscle complex I activity in diabetic individuals (73), and AMPK might not be necessary for complex I activity, as AMPK knockdown mice displayed increased oxidative phosphorylation with metformin treatment over 2 weeks (74). Although most of the data thus far has linked metformin with inhibition of mitochondrial respiration, metformin administration for 2 weeks in AMPK knockout mice resulted in increased complex I activity (74), suggesting that metformin activity on cell respiration is dependent upon both cell type and length of metformin treatment. It is also possible that studies performed on isolated mitochondria yield different results when compared to mitochondria in intact tissues (75, 76). One study by Vytla and Ochs on intact L6 muscle cells showed that doses of metformin from 10-20 mM increased free ADP and AMP without affecting ATP production, along with increased cell survival measured by MTT assay (75), suggesting that metformin dosage and cell intactness can lead to AMPK and ATP independent effects on metabolic activity. It appears that metformin inhibits mitochondrial biogenesis through downregulation of transcription factors such as CREB and co-activators PGC-1α (77, 78), showing that metformin has profound effects on many aspects of mitochondrial activity.

Metformin transport by organic cation transporters

The organic cation transporters (OCTs) are members of the SLC22A family of soluble carriers that are the primary transporters of metformin across cell membranes. Humans express 3 isoforms. OCT1 and OCT3 are highly expressed in liver, while OCT2 is predominantly expressed in kidney (79, 80). The OCTs are crucial for metformin clearance by the liver and kidneys. Metformin transport by OCTs is significantly inhibited by proton pump inhibitors such as rabeprazole and omeprazole, which are pharmacological inhibitors used to promote renal clearance of metformin to prevent lactic acidosis and in the treatment of gastroesophageal reflux disease (79). The OCTs are also necessary for the glucose regulating actions of metformin. Deletion of OCT1 in mouse hepatocytes resulted in less AMPK and ACC activation and higher plasma glucose levels in mice fed on high fat diet (81). As metformin is not metabolized by the liver, failure to clear metformin can have detrimental side effects such as lactic acidosis and kidney dysfunction (82, 83). Several single nucleotide polymorphisms (SNPs) in OCT1 have been linked to the inability to clear metformin due to renal failure as well as metformin therapy resistance. An association of SNP at rs628031 and 8 base pair insertion in the OCT1 gene have correlates with gastrointestinal side effects (80). This SNP also downregulates metformin clearance by inhibiting multidrug and toxin extrusion transport 1 (MATE1), which enables metformin excretion into the urine (84). OCT1 amino acid substitutions (R61C, S401G, G465R, and deletion at M420) are associated with failure of metformin uptake and increased renal clearance (80, 81). Mutations of the OCTs can also increase the weight loss associated with metformin in diabetic patients. Carriers of

the MATE1 allele rs2289669 A displayed lower body mass index than those with the GG genotype (84). Although OCT2 is the primary renal metformin transporter, no effects of SNPs within OCT2/3 variants were not clinically meaningful in a small study of 103 Caucasian patients (80). OCT1 polymorphisms rs12208357, rs34059508, rs628031, and rs72552763, and OCT2 polymorphism rs316019 have been associated with complications of metformin therapy such as lactic acidosis or renal failure (80, 84). Although uncommon (0.00005 percent of users), metformin associated lactic acidosis has severe effects in patients, with a fatality rate up to 50 percent (82). One case study by Riesenman *et al.* of a type II diabetic patient admitted with a 12% total body surface burn area injury was administered 1000 mg of oral metformin twice daily; after 3 days the patient was found unresponsive with highly elevated of arterial lactate, serum creatine, and alanine aminotransferase. Intensive tracheal intubation and intravenous fluid administration was required to stabilize the patient (82). Genetic screening of patient samples by next generation sequencing to identify at-risk patients prior to administering metformin treatment will improve clinical outcomes and avoid complications arising from transporter mutations.

Metformin and burn injuries

A hallmark of burn injury is dysregulated glucose metabolism that mimics a diabetic like state. Following burn injury, fasting blood glucose levels can rise to over 180 mg/dl, and remain elevated up to 3 years post-injury (3, 32, 85). Insulin therapy and tight euglycemic control are the current standards of care for controlling burn induced hyperglycemia to maintain blood glucose levels between 80 and 160 mg/dl. However

insulin therapy is not always the best clinical intervention in burn patients. Insulin treatment can be ineffective, even detrimental in patients with pre-existing diabetes or severe burn injuries over 40% of total body surface area (3, 32). In a recent study of children with burns over 30% of total body surface area receiving either insulin treatment or no insulin Finnerty *et al*. found that patients treated with insulin had greater muscle strength and lower blood glucose concentrations, but also higher mortality rates and elevated inflammatory cytokines such as TNFα, CCL4a, and IL-1β (32). Additionally, older children required more insulin to maintain a blood glucose concentration between 80 and 110 mg/dl (32). Hypoglycemic episodes were significantly more common in patients treated with insulin than those who received no insulin (43% insulin, 8% non-insulin) (32). Furthermore, insulin has been shown to have no effects on aspartate and alanine aminotranserafses or blood urea nitrogen levels, which are increased following burn injury and associated with decreased clinical prognosis (85). Our group and others have explored additional glucose controlling strategies used in conjunction with insulin to improve clinical outcomes. These studies are promising due to the ability to administer these medications safely in outpatient settings.

Metformin had shown promise as a therapy to reduce hypoglycemic incidents associated with insulin treatment in burned patients. Preclinical models of burn injury showed that subcutaneous injections of 120 mg/kg metformin elevated blood lactate concentrations, increased clearance of [5-3H] glucose, and reduced liver glycogen content, suggesting that metformin promotes cellular glucose utilization (86). The few clinical studies of metformin administration to severely burned patients demonstrated the efficacy of this

therapy. Gore *et al.* demonstrated that 850 mg metformin administered every 8 hours for 7 days along with insulin infusion improved muscle fractional synthetic rate and blood flow, while decreasing arterial glucose concentration. Energy expenditure measured by muscle ATP concentration was not affected (87). This suggests that metformin works synergistically with insulin to prevent muscle catabolism by reducing hyperglycemia. Metformin achieves similar outcomes when compared to conventional therapy, but with fewer detrimental effects. Jeschke *et al*. randomized 44 severely burned adult patients to insulin or metformin and measured blood glucose, stress markers, and lipid profiles. Metformin significantly reduced blood glucose concentrations during oral glucose tolerance tests compared with insulin treatment (25). Significant reduction of serum fatty acid levels and expression of inflammatory cytokines IL-1β and MCP-1 were found with metformin, suggesting that metformin blocks burn injury induced lipolysis and inflammation (25). Furthermore, only 6 percent of patients undergoing metformin treatment had hypoglycemic incidents, compared with 15 percent for insulin treated patients (25). The effects of glucose clearance may be due to increased glucose uptake along with decreased glucose production. Metformin reduces hepatic gluconeogenesis through phosphorylation of CREB binding protein, SIRT1 induction, and inhibition of mitochondrial glycerophosphate dehydrogenase (48, 49, 88, 89). A study of patients with burns over 60% of the total body surface area showed that 8 days of metformin treatment (850 mg) increased glucose uptake and oxidation, along with increasing serum lactate concentrations (90). This same research group demonstrated no significant glucose oxidation in patients with smaller burn injuries affecting 20% of total body surface area,

suggesting that larger burns may derive more benefit from metformin administration in promoting glucose clearance and oxidation (87).

The site of metformin action might also be important in determining patient response. Metformin is mostly absorbed by the jejunum and duodenum where it acts to stimulate release of enteroendocrine L-cell hormones such as glucagon like peptide 1(GLP-1) and peptide YY (PPY) via AMPK activation (91), before it enters the bloodstream to reach other target tissues. Buse *et al*. demonstrated that administration of metformin formulated to release in the lower bowel (metformin DR) at doses of 600-1000 mg demonstrated superior glucose uptake. Metformin DR reduced plasma glucose concentrations 50% less than instant release metformin (metformin XR), increased GLP-1 secretion, and did not cause lactic acidosis (92). This same group also showed that although administration of metformin DR and oral metformin treatment resulted in similar glucose clearing activity, along with comparable GLP-1 and PYY levels, metformin DR treated patients experienced fewer gastrointestinal effects such as vomiting and diarrhea (91). This suggests that directing the site of metformin release may result in greater clinical efficacy. It has not yet been determined if site directed metformin release will improve clinical outcomes in burn patients. Further work is needed in order to determine if metformin alters mitochondrial complex activity in human trials, the mechanisms regulating muscle anabolism, and the dependence of glucose clearance on AMPK.

<u>EMT and fibroblasts in wound healing</u>

Epithelial-mesenchymal transition (EMT) is the process by which cells lose their adhesiveness and become motile. The converse of EMT is MET (mesenchymal-epithelial transition), which results in cells reverting to an epithelial phenotype (93, 94). Both processes are found in many physiological functions, such as wound healing, cancer, development, and stem cell programming. The key events in EMT and MET are loss or formation of cell junctions, rearrangement of cell polarity, extracellular matrix (ECM) alteration, cytoskeletal structure and cell shape alteration, and changes in epithelial gene expression (94). There are 3 types of EMT: type I is involved in development, type II in wound healing, and type III in cancer (95). During EMT the cells lose or gain epithelial and mesenchymal markers such as the cadherins and fibronectin, along with changes in extracellular influences, transcription factors, microRNAs, and epigenetic modifications; these changes are fluid and dynamic (93). Some of these changes are shown in **Figure 6**.

Figure 6

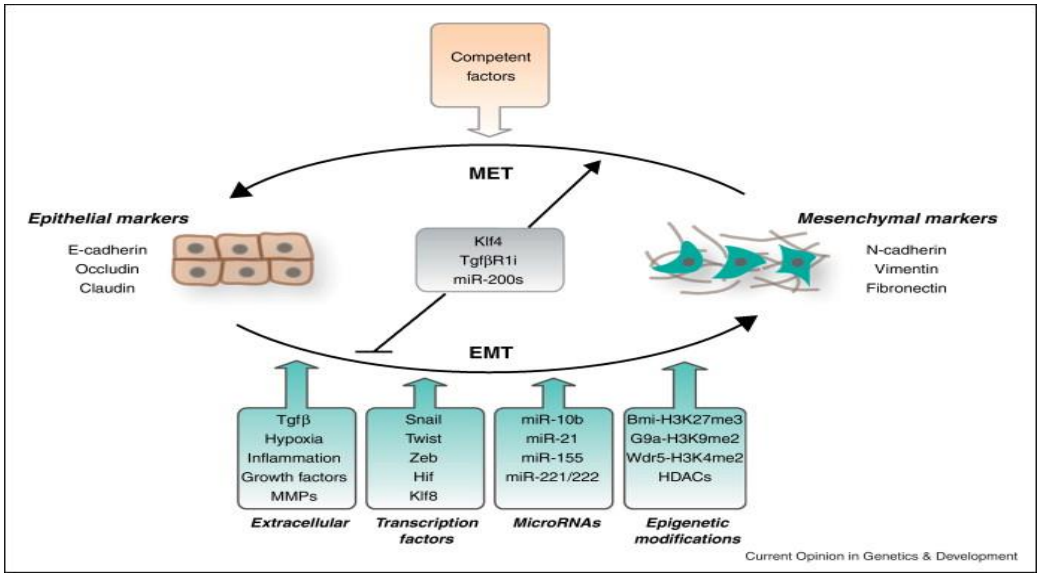


Figure 6: Cellular alterations involved in EMT and MET. From Esteban et al., *Curr Opin Genet Dev* 22: 2012.

Transforming growth factor β (TGFβ) is one of the main extracellular factors that induces

EMT. TGFβ is elevated during stress events such as oxidative damage and injury, and alters many of the signaling pathways involved in EMT. TGFβ binds to the TGFβ receptor (TGFβR), and affects downstream signaling in canonical and noncanonical pathways. The canonical pathway involves phosphorylation of SMAD2 and 3, which can activate and translocate SMAD4 to the nucleus in order to promote cellular excretion of collagen types, particularly collagen I and III (95, 96). The noncanonical pathway involves activation of pathways such as Akt, Wnt, β-catenin, and ERK, which promotes expression of EMT transcription factors such as Snail, twist family bHLH transcription factor 1 (TWIST), and Slug (95). Activation of both the TGFβ canonical and noncanonical pathways lead to increased expression of ECM remodeling matrix metalloproteinases (MMPs), loss of E-cadherin expression, increased N-cadherin, increased α-smooth muscle actin (SMA) expression, elevated vimentin expression, and increased fibronectin and collagen gene expression and secretion (95, 97, 98). During burn injury, TGFβ is frequently upregulated, which leads to increases in immunosuppression, hypertrophic scarring, and excessive collagen secretion due to upregulation of SMAD activity (96, 99). Extracellular matrix remodeling is marked by decreased collagenase activity and cell apoptosis of mesenchymal cells, with increases in collagen secretion, fibroblast proliferation, and TGFβ expression (100). Of the 28 collagen types found in the body, types I, II, and III make up 90 percent of total collagen (101). Types I and III the most important in wound healing (101, 102). Collagen type III is secreted in the initial phase of injury, but is quickly degraded and replaced by collagen type I, which forms most of the collagen found in hypertrophic scars (101). Collagen V is a minor collagen in wound healing that associates with collagen I (103) .

Downregulation of several microRNAs (mIR-185 and mIR-186) inhibit TGFβ-induced EMT and collagen V expression in fibroblasts derived from idiopathic pulmonary fibrosis patients (103). The SMAD pathway is one of the main regulators of collagen secretion in fibroblasts. It has been shown that Smad ubiquitination regulatory factor 2 (Smurf2), an ubiquitin ligase for SMADs, is upregulated in hypertrophic scar fibroblasts from burned children and was further induced by TGFβ1 treatment (99). Inhibition of Smurf2 by siRNA resulted in lower TGFβ levels and reduced collagen I secretion (99), indicating that the SMAD pathway is a critical regulator of hypertrophic scar induced inflammatory signaling and collagen production. SMAD3 is of particular interest, as SMAD3 inhibition by siRNA has been shown to reduce procollagen I and III expression in fibroblasts, and mice lacking SMAD3 have displayed lower inflammation and accelerated wound healing (104, 105). Targeting SMAD3 might be a mechanism to reduce hypertrophic scarring in burn patients and reducing fibrosis due to excessive collagen deposition.

Figure 7

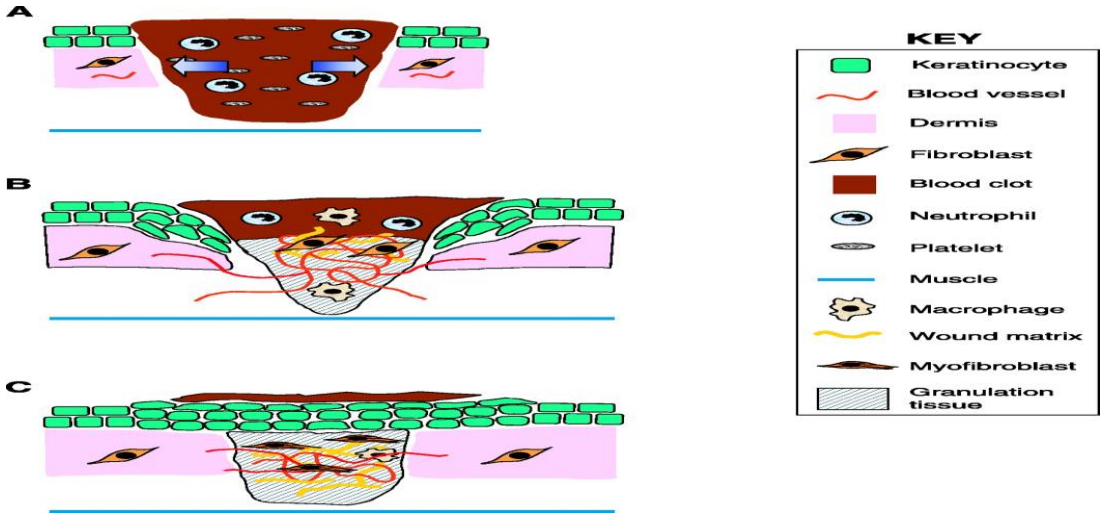


Figure 7: Stages of wound healing. A: inflammatory phase, B: remodeling phase, C: proliferative phase. From Werner and Grose. *Physiol Rev* 83: 2003.

As shown in **Figure 7** wound healing consists of three phases: A) the inflammatory phase, which is marked by increased expression of inflammatory cytokines which encourage macrophage and neutrophil infiltration to the injury site to combat infection; B) the proliferative phase in which fibroblasts and keratinocytes infiltrate the injury site in an EMT regulated manner and begin to proliferate, C) the remodeling phase, where fibroblasts and keratinocytes differentiate and secrete collagen to close and remodel the injury site, resulting in granulation tissue and scar formation in place of the original tissue architecture (106, 107). Fibroblasts and myofibroblasts help to rapidly repair the skin following injury, but the resulting repair lacks the integrity of normal connective tissue. Dysfunctional wound healing often results in dysregulation from EMT and collagen production, which can lead to formation of pathologic scars (hypertrophic scars and keloids), and limb dysfunction due to contracture (108). Fibroblasts are the most common cell type in connective tissue, and are one of the main cell types involved in wound healing. Fibrosis can occur in many tissues undergoing remodeling and

inflammation, including heart, liver, kidney, muscle, and skin. Epithelial cells can stimulate fibroblast migration through EMT, and fibroblasts can become epithelial cells by undergoing MET (109). Fibroblasts exhibit much plasticity depending on their extracellular environment and paracrine factors. Fibroblasts cultured with media from pro-inflammatory macrophages displayed had MMP and matrix degrading protein activity, while fibroblasts cultured with media from noninflammatory macrophages had higher proliferation rates (110). Subsequent treatment of fibroblasts subjected to inflammatory factors with noninflammatory macrophage media reversed the matrix degrading phenotype, indicating that fibroblast activity is highly adaptable depending on their culture conditions (110). Fibroblasts are distinguished by specific markers including CD90, fibronectin, and TE-7, which can be used to distinguish fibroblasts from myofibroblasts and monocytes (111, 112). One of the primary markers linked with fibroblast EMT is FSP1, which was originally identified in kidney fibroblasts and is elevated following inflammation (113). During inflammation elevated levels of TNFα can induce FSP1, MMP, and vimentin in hypertrophic scarring through induction of bone morphogenic protein 2 (BMP2), which has been observed in hypertrophic scar skin fibroblasts (114). FSP1 also appears to show tissue specific expression and phenotype in hypertrophic scarring. A study using liver fibroblasts showed that while FSP1 was induced by injury and cancer, and FSP1 positive fibroblasts did not display fibroblast markers such as SMA, FSP1 was not expressed in hypertrophic scar, and increased collagen I expression was not linked with elevated FSP1(115), which is a contradiction to other studies linking increased fibrosis with increased collagen gene expression associated with hypertrophic scarring (100, 116). Burn patients with keloids and

hypertrophic scars display increased circulating TGFβ, interleukins (IL), and TNFα, along with increased TGFβ receptor induced SMAD2 and 3 phosphorylation, leading to increased collagen deposition (96). It is unknown how biguanide therapy will alter EMT in burn patient derived skin fibroblasts, and the mechanisms regulating collagen production in these cells.

Metformin and EMT

In addition to its metabolic regulating activity through AMPK signaling, metformin is also regulates EMT in epithelial cells, and has even been used as an adjunct cancer therapy due to its effects on cell proliferation regulation (71). As shown in **Figure 8** the primary mechanism by which metformin inhibits cell proliferation is through attenuation of the AMPK-mTOR pathway. Metformin has a direct effect on mTOR by preventing formation of the mTOR complex (mTORC), and also impedes complex formation by activating AMPK to activate tuberous sclerosis complex 2 (TSC2) and subsequent inhibition of Ras homologue enriched in brain (Rheb), which forms part of the mTORC (52). Furthermore, metformin promotes insulin sensitivity, increasing Akt and PI3K activity

Figure 8

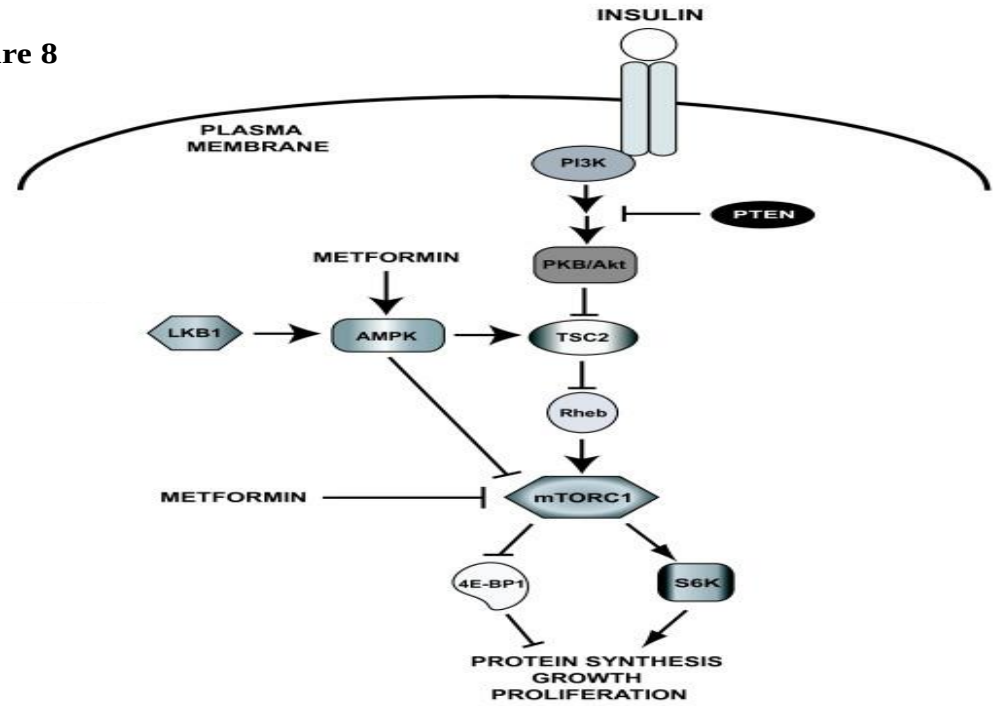


Figure 8: Metformin blocks cell proliferation by attenuating mTOR complex formation. From Dowling et al. *BMC Med*; 9(33), 2011.

to further inhibit mTORC (35, 52). Increased IGF has been linked with increased proliferation in endometrial cancer cells, and high doses of metformin (1 to 2 mM) induced apoptosis in these cells, which acted synergistically in combination with an IGF-1 receptor inhibitor (117). The ability of metformin to inhibit cell proliferation through reduction of IGF-1 receptor downregulation was AMPK dependent, as the AMPK inhibitor Compound C reversed the effects of metformin in endometrial cancer cells (117). The result was lower cell proliferation and protein synthesis, which can target many cell types. In esophageal cancer cells, mTOR activity and cell proliferation were decreased by metformin in a dose dependent manner (118). Metformin-induced inhibition of IGF-1 receptor also prevents uterine serous carcinoma cells from proliferating and migrating in a p53 independent manner; cell growth was disabled in both wild type and p53 mutant cells (119). This indicates that metformin can modulate

cell growth independent of common cell cycle checkpoints. Tumor growth was reduced nude mice injected with esophageal cancer cells displayed less tumor growth when treated with 200 μg/ml metformin daily compared to untreated control mice (118). Metformin also inhibited mTOR in endometrial cancer cells through an AMPK dependent mechanism, prevented phosphorylation of the cell cycle regulator p70S6 kinase, and increased expression of progesterone receptor, which is downregulated in many cancer types (120). This suggests that metformin-induced AMPK activity results in inhibition of tumor growth and increased survival. In prostate cancer cells, metformin increases expression of microRNAs that inhibit cancer stem cell formation by acting on targets such as CD44 and EpCAM (121). One of the main microRNAs affected by metformin is mIR30a, which inhibits superoxide dismutase (SOX) 4, and is downregulated in prostate cancer. SOX4 is activated by TGFβ and is upregulated in prostate cancer. Treatment of prostate cancer cells with 5 mM metformin for 48 hours resulted in upregulation of mIR30a and the RNASE endonuclease DICER (a key regulator of microRNA biogenesis), decreased SOX4 expression, and reduced cell migration and invasiveness (45). Metformin also transcriptionally inhibits key EMT regulating genes associated with fibrosis such as Snail, Slug, and TWIST; all of which are targets of TGFβ-induced EMT (122). The ability of metformin to induce cancer cell death might be linked to glucose status; in high glucose conditions metformin only caused cancer cell cycle arrest but glucose withdrawal along with metformin resulted in increased apoptosis and decrease in HER2 expression (123). A hallmark of cancer cells is the ability to switch from oxidative metabolism to glycolysis by downregulation of regulators such as AMPK; promoting oxidative respiration and reducing hyperglycemia

might be beneficial for the prevention of EMT in cancer conductive extracellular environments (123, 124). Although metformin generally promotes cellular glucose uptake through AMPK upregulation, the mechanism by which this is accomplished in cancer cells might be different. Metformin treatment reduced tumor (18)F-2-fluoro-2-deoxy-D-glucose uptake in mice fed a high fat diet but not in mice fed a normal diet, indicating that diet and environmental glucose status can alter metformin activity (43). One study showed that in tobacco smoke induced lung cancer metformin treatment resulted in a 70 percent decrease in tumor incidence but only small increases in AMPK activity, suggesting that metformin can act on many EMT inducing pathways (125). Cancer and burn patients with hypertrophic scars have increased inflammation and reactive oxygen species (ROS) levels; metformin reduces ROS levels in mouse embryonic fibroblasts and protects against Ras induced DNA damage by inhibiting ERK signaling, resulting in improved survival in mice subjected to ROS induction induced by paraquat injection. (126). The protective effect against DNA damage was specific to stimulation of ERK signaling, as metformin did not protect against direct DNA damage induced by hydrogen peroxide (126). One of the main pathways involved in EMT associated fibrosis is upregulated SMAD3 and collagen deposition. Loss of SMAD3 results in faster wound healing and reduced fibroblast collagen synthesis following burn injury (104, 105). In cardiac fibrosis, metformin is associated with potential improved outcome. Mice subjected to thoracic aortic constriction followed by injection with 200 mg/kg metformin daily for 6 weeks demonstrated improved cardiac diastolic function (127). In cultured cardiac fibroblasts from these mice metformin decreased SMAD3 activity and nuclear translocation, and reduced collagen, suggesting that metformin can

inhibit TGFβ receptor-SMAD3-collagen signaling (127). No changes in blood glucose or insulin concentrations were observed between metformin and saline injected mice following aortic constriction (127). It is unknown how metformin will affect skin fibroblast EMT activity in conjunction with changes in metabolic activity, although it is likely through a SMAD dependent mechanism that may or may not be associated with AMPK signaling. It is also unknown if burn patient derived fibroblasts will demonstrate EMT, and if metformin will alter the EMT phenotype by modulating common EMT markers and transcription factors. These questions will be addressed in aim 2 of this study.

Study goals and aims

In this study I find that metformin alters fibroblast AMPK signaling, cell proliferation, and mitochondrial activity. I also deduce the roles of metformin in regulating fibroblast EMT and collagen through altering SMAD3 expression. I present my findings in the following specific aims:

1. Identify effects of metformin on fibroblast AMPK, mitochondrial, and proliferative activity.
2. Determine how metformin alters EMT and collagen secretion by SMAD activity.

The data from these two aims will delineate the effects of metformin on both metabolic and EMT pathways altered by burn injury to determine whether wound healing or fibrosis are impacted by metformin treatment.

Chapter 2: Metformin Alters Fibroblast Complex I Activity Independently of AMPK in Dermal Fibroblasts Cultured Under Hyperglycemic Conditions

Background

Burn injury is a significant medical problem in the United States, leading to significant patient morbidity and mortality (1). Patients with burns over 30% of their total body surface area (TBSA) exhibit inflammatory and hypermetabolic responses that are associated with hyperglycemia, infection, muscle catabolism, and organ failure (2-5, 128). Hyperglycemia is the result of increased gluconeogenesis along with decreased glucose utilization, frequently occurring in the first few days following burn injury (3, 128). Severely burned patients who are hyperglycemic have a higher risk of infection, decreased graft acceptance, reduced wound healing, and increased mortality (3, 7). Post burn elevation of catecholamines, glucagon, and cortisol stimulate hepatic glucose production and upregulate inflammatory cytokine production, resulting in altered insulin receptor signaling (31). Muscle catabolism also results from defective insulin signaling; muscle protein degradation occurs when muscle protein breakdown rates exceed synthetic rates (4). Furthermore, burn injury impairs mitochondrial activity in skeletal muscle, further impacting muscle function (129). In patients with type 2 diabetes, reduced expression of PGC-1α, Mfn2, and ATP synthase occurred in muscle without changes in mitochondrial proliferation (78), indicating that dysregulated glucose metabolism alters mitochondrial oxidative phosphorylation. Since elevated glucose levels negatively impact outcomes following severe burn injury, interventions to reduce hyperglycemia in these patients are of paramount importance. Current therapies to reduce hyperglycemia include standard insulin therapy or tight euglycemic control to maintain glucose levels between 60 and 110 mg/dL (32, 130). Although these strategies can be

effective in countering hyperglycemia in severely burned patients, the incidence of hypoglycemic episodes emphasizes the need for better glucose controlling agents (30). Our group has shown that in severely burned children randomized to intense insulin therapy, 20% of patients develop mild hypoglycemia, with another 20% experiencing severe hypoglycemia. Reduction of blood glucose to less than 180 mg/dl with insulin administration resulted in increased muscle strength, resting energy expenditure, and bone mineral content (32). Therefore it is of paramount importance to find glucose controlling strategies in addition to insulin. Current strategies under investigation for controlling post-burn hyperglycemia include metformin (25); which is commonly used by diabetic patients under the name Glucophage.

Metformin has been one of the most widely used treatments for managing type 2 diabetes in Europe for the last 50 years. Following metformin administration insulin sensitivity in muscle and liver are increased, tyrosine kinase activity increased, and hepatic glucose production is decreased (34-36). Metformin counters burn-induced hyperglycemia by improving insulin sensitivity and increasing muscle glucose utilization by boosting muscle protein fractional synthetic rate (26, 28). The main action of metformin is induction of AMPK activity to affect downstream mTOR and Akt (34, 35). PKCα, which increased expression in fibroblasts leads to decreased cell tight junction formation and resistance to apoptosis, is also affected by AMPK (131, 132).

Diabetic neuropathic rats treated with 100 or 500 mg/kg metformin for up to 8 weeks displayed increases in ATP and acetyl CoA in muscle and liver. Additionally, genes shown to be decreased by diabetic neuropathy induced stress were upregulated by metformin treatment (133). Metformin is primarily transported into cells by the OCTs

(79, 82, 134). Metformin affects metabolism in liver, adipose, and muscle, and also alters pancreatic insulin secretion (37). Metformin has also been used to treat microvasculature complications in burned patients by upregulating eNOS activity (33, 36). Although the functions of metformin vary considerably among different cell types, a common pathway inhibited in almost all published studies has been mitochondrial respiratory complex I (33). Metformin decreased intracellular concentrations of succinate, fumarate, and malate, mimicking the effects of the complex I inhibitor rotenone (42, 43). Interestingly, the androgen inhibiting effects of metformin in adrenal cells were due solely to complex I inhibition, independent of AMPK activity (42), suggesting that metformin can act through a variety of pathways in many cell types. Although metformin therapy has been associated with improvements in blood glucose clearance (22, 30, 87), its roles in cell proliferation and fibroblast metabolism are unknown.

In this study we determine the effects of metformin on dermal fibroblasts derived from scar and unburned skin samples from burned patients. These cells were mostly cultured under hyperglycemic conditions. We analyzed AMPK activity and related signaling and mitochondrial oxidative phosphorylation. The effects of metformin on cell proliferation, cell migration, and mitochondrial proliferation were also determined.

Materials and methods

Cell culture: Primary dermal fibroblasts were derived from excised scar areas (hypertrophic scar: HTS) or biopsies of unburned areas (non-burn skin: NBS) obtained from burned patients enrolled in clinical trials using protocols previously described (135).

Briefly, skin sections were sliced into small sections in a 6 well plate, dried, covered with coverslips, then grown in growth media for 2 weeks before coverslips were removed and fibroblasts expanded for future use. Non-transformed primary dermal derived fibroblasts from neonatal non-burned donors (PCS) and HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's Modified Eagle Media (DMEM) containing 15% fetal bovine serum (FBS), 1% antibiotic/antimyotic (10,000 I.U/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B), and 25 mM glucose (high glucose) in 5% CO_2. Metformin was purchased from Sigma. HepG2 cells were grown in DMEM with 10% FBS and 1% antibiotic/antimyotic. All cells used in experiments were under passage 15. Unless indicated otherwise, cells were treated with 5 mM metformin for 24 hours prior to harvest for experiments. For AMPK inhibition experiments, cells were treated ± 5 µM Compound C (Millipore) for 24 hours prior to metformin administration.

Western blotting: Cells were grown to confluence, then lysed in buffer containing 5 mM EDTA, 750 mM NaCl, 250 mM Tris, 5% Triton X-100, 150 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1% phosphatase inhibitor. Lysates were incubated on ice for 20 minutes, then centrifuged at 13,000 x g for 20 minutes. Protein concentration was determined using the bicinchoninic acid (BCA) method (Thermo Scientific, Waltham, MA). Next 40 µg lysate was mixed with 2x sodium dodecyl sulfate (SDS) and separated on 4-20% Tris-Glycine gels (Invitrogen, Grand Island, NY) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, blocked with 5% bovine serum albumin

(BSA) in 1% Tris buffered saline + 0.5% Tween 20 (TBS-T), and then incubated with primary antibodies at 1:1000 dilution in 5% BSA in TBS-T overnight at 4°C. Blots were washed in TBS-T, then incubated with anti-rabbit or mouse horseradish peroxidase secondary antibody at 1:4000 dilution at room temperature for 1 hour. Blots were visualized using enhanced chemoluminesence (ECL). Antibodies for 5' adenosine AMPK , phospho AMPK T172, Akt, phospho Akt S79, mTOR, phospho mTOR S2448, acetyl CoA carboxylase (ACC), phospho ACC S79,protein kinase Cα (PKC) and phospho PKCα T638/641 and glyceraldehyde3 phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Danvers, MA). Quantification was performed using ImageJ software (available at http://imagej.nih.gov/ij/) and values normalized to the loading control.

Primer	Forward 5'-3'	Reverse 5'-3'
α-smooth muscle actin	GACGAAGCACAGAGCAAAAGAG	TGGTGATGATGCCATGTTCTATCG
β-actin	GAGACCTTCAACACCCCAGCC	GGAGAGCATAGCCCTCGTAG
Collagen type 1	CGACCGCTTCACCTACAG	TTTTGTATTCAATCACTGTCTTGC
Collagen type 3A	GGGAACAACTTGATGGTGCT	CCTCCTTCAACAGCTTCCTG
GLUT1	AACTCTTCAGCCAGGGTCCAC	CACAGTGAAGATGATGAAGAC
GLUT4	CTTCATCATTGGCATGGGTTT	AGGACCGCAAATAGAAGGAAAGA
E-cadherin	GAAGGTGACAGAGCCTCTGGAT	GATCGGTTACCGTGATCAAAATC
Mfn2	CCCCCTTGTCTTTATGCTGATGTT	TTTTGGGAGAGGTGTTGCTTATTTC
N-cadherin	CAGTATCCGGTCCGATCTGC	GTCCTGCTCACCACCACTAC
OCT1	TCTTCCATCGTCACTGAGTTCAAC	AGAAGCCCGCATTCAAACAG
OCT2	GATGGCAGCAAGACCAAAAGT	ACTCCACTGGCTGTAGACCTAGGT
OCT3	CCCTGTGGTCTCTGACCCATTA	CATTCTTGATGGAGCTGTCATGAG
Porin	ACGTGGACTGAAGCTGACCT	CCAACCTCTGTCCCGTCATT
PGC-1α	TGCCCTGGATTGACATGA	TTTGTCAGGCTGGGGGTAGG
Slug	TGTTGCAGTGAGGGCAAGAA	GACCCTGGTTGCTTCAAGGA
SMAD2	GTTCCTGCCTTTGCTGAGAC	TCTCTTTGCCAGGAATGCTT
SMAD3	TGCTGGTGACTGGATAGCAG	CTCCTTGGAAGGTGCTGAAG
Snail	ACCACTATGCCGCGCTCTT	GGTCGTAGGGCTGCTGGAA
TGF-β1 receptor	CGTTACAGTGTTTCTGCGACCT	AGACGAAGCACACTGGTCCAGC
TWIST	GAGTCCGCAGTCTTACGAGG	CTGCCCGTCTGGGAATCACT

Table 2: Primer sequences used in RT-PCR.

Quantitative real time PCR: Cellular mRNA was isolated using RNeasy Plus mini kit (Qiagen, Valencia, CA) and cDNA produced using a cDNA synthesis kit (Bio-Rad. Hercules, CA). Primer sequences for real time PCR are listed in **Table 2**. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Reactions were performed on the Applied Biosystems StepOne Plus™ Real Time PCR System using a cycle of 95°C for 30 seconds, then 40 cycles of 95°C for 10 seconds, and 55°C for 30 seconds.

cAMP assay: Intracellular cAMP was analyzed using a Catchpoint cAMP fluorescent assay kit (Molecular Devices, Sunnyvale, CA) following the manufacturer's protocol. Cells were plated at a density of $2.5x10^4$ cells per well in 96 well plates, treated with 5 mM metformin for 24 hours, and then washed with Krebs-Ringer Bicarbonate buffer (KRGB). Cells were stimulated with 0.75 mM 3-isobutyl-1-methylxanthine (IBMX), then

washed with KRGB and lysed with supplied lysis buffer. Anti-rabbit cAMP antibody and HRP-cAMP were added to wells, then incubated at room temperature for 2 hours. The reaction was stopped using Stoplight Red solution with 0.0034% H_2O_2 and results read at the excitation/emission wavelengths of 530/590 nm.

Cell proliferation and migration assays: Real time cell proliferation was determined using the xCELLigence system (Roche, Indianapolis, IN). Fibroblasts were plated at a density of 2.5 $x10^4$ cells per well in E-plates with or without 5 mM metformin in DMEM containing 15% FBS and analyzed over 80 hours. Cell migration was measured after incubating cells in DMEM containing 2% FBS overnight, and then plated at a density of $4x10^4$ cells per well with or without 5 mM metformin in the upper chambers of the CIM plates. Lower wells contained DMEM with 15% FBS with or without 5 mM metformin, and migration was determined by measuring electrical impedance over 30 hours. For determination of cell survival with Compound C treatment the protocol for the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay kit (ATCC, Manassas, VA) was followed with minor variations. Cells were plated at a density of 1500 cells per well in a 96 well plate and allowed to adhere overnight, then were treated with or without 0 to 10 μM of Compound C for 16 hours. Cells were then treated with 10 μl of MTT assay reagent and incubated at 37ºC for 2 hours before being washed with 100 μl detergent reagent and incubated in the dark at room temperature for 2 hours. Spectrophotometry values were then measured at 570 nm, and were plotted after normalization to the media, while drug treatments were compared to untreated cells.

Scratch migration assays: Scratch assays were performed using the Tissue Culture Wounder (TCW). The TCW is a new patent pending (USSN 14/337,875, Apparatuses and Methods for Forming Wounds in Cell Layers) device designed to hold a 200 µl pipet tip at a precise angle and apply uniform tip pressure ensuring reproducible scratch-wounds. Briefly, 12 well plates were coated with 50 µg/ml collagen and cells plated at $8x10^4$ cells per well. After 24 hours the media was changed to DMEM with 2% FBS with or without 5mM metformin for an additional 24 hours. Scratch-wounding was then performed and photographs taken 0, 4, and 8 hours afterward using a Nikon Diaphot 300 microscope equipped with a 4x lens. Area of the scratch-wounds were quantified using the Tscratch program (http://www.cselab.ethz.ch/index.php?&option=com_content&view=article&id=363), and percent wound closure determined by the following formula: ([time 0 area-time 4 or 8 h area]/time 0 area) x100 (136).

Glucose uptake assay: Glucose uptake was determined by measuring the conversion rate of 2-deoxyglucose to 2-deoxyglucose 6 phosphate using the Glucose Uptake Assay kit (Abcam) following the manufacturer's protocol. Cells were plated in 96 well plates at a density of 1500 cells per well for 2 days, and then serum starved overnight. The next day fibroblasts were treated with Krebs-Phosphate-Ringer-Hepes buffer + 2% bovine serum albumin for 40 minutes, then washed and treated with 2-deoxyglucose (with or without 5 mM metformin) for 20 minutes, then lysed. D-deoxyglucose uptake was measured by measuring samples at 412 nM and the formula 2-DG uptake = Sa/Sv where Sa=2-DG6P amount (pmol) calculated from the standard curve and Sv=sample volume per well.

Mitochondrial proliferation: Mitochondrial proliferation was measured using MitoTracker Green ™ (Molecular Probes). Cells were treated with 75 nM MitoTracker Green for 45 minutes, then washed with PBS and suspended at a concentration of $6.67x10^5$ cells per ml in PBS. Cell viability was measured using 3 μg/μl propidium iodide (PI) and MitoTracker intensity measured by flow cytometry at excitation/emission wavelengths of 490/516 nm.

Mitochondrial respiration measurement: Oxygen consumption in pmol O_2/(seconds $x10^6$ cells) was measured using Oxygraph 2k (Oroboros Instruments, Innsbruck, Austria). Fibroblasts were treated with or without 5 mM metformin for 24 hours, then suspended at a concentration of $2x10^6$ cells per ml in growth media. Samples were loaded into the Oxygraph 2k and total oxygen consumption measured. Oxidative phosphorylation was measured by adding 1.5 mM octanoyl carnitine, 5 mM pyruvate, 2 mM malate, 10 mM glutamate, 5 mM ADP, and 10 mM succinate. Complex I activity was measured by inhibiting other complexes using 5 μM oligomycin, 12 μM antimycin A, 5 μM malonic acid, and 5 μM sodium azide (**Figure 9**).

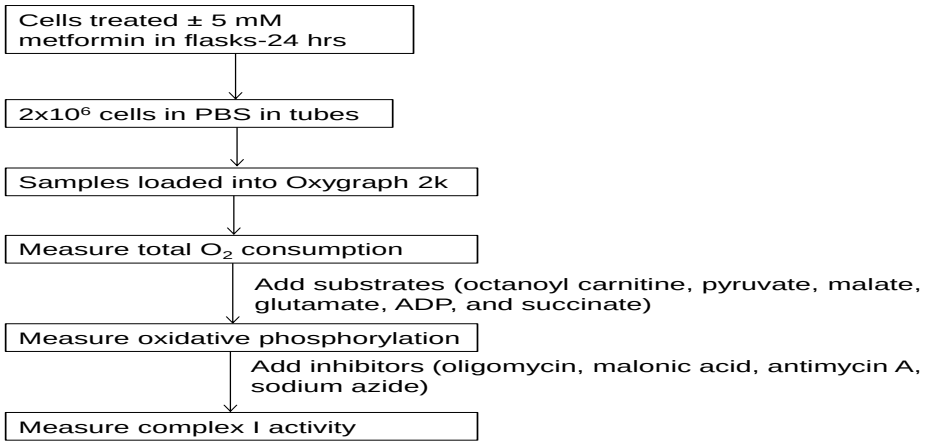


Figure 9: Method for determining mitochondrial oxygen consumption, oxidative phosphorylation, and complex I activity using Oxygraph 2k.

Statistical analysis: Statistical analysis was conducted using paired Students t-test between controls and treated samples following a Kolmogorov-Smirnov test for normality. For analysis across multiple groups, a one way analysis of variance (ANOVA) was performed, followed by a Tukey's post hoc correction when appropriate. A p value of less than 0.05 was considered significant.

Results

Metformin affects fibroblast AMPK signaling: Several concentrations (1, 5, 10 mM) of metformin were tested to determine which doses had effects on AMPK activity. We found that 5 and 10 mM metformin treatment over 24 hours had significantly elevated effects (p =0.01 and 0.03) on raising AMPK phosphorylation in HTS fibroblasts (**Figure 10**).

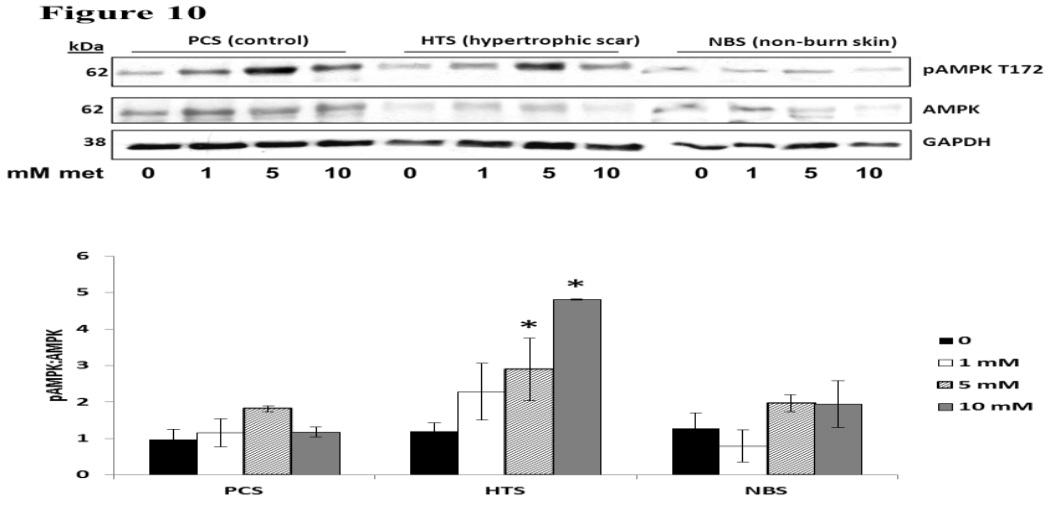
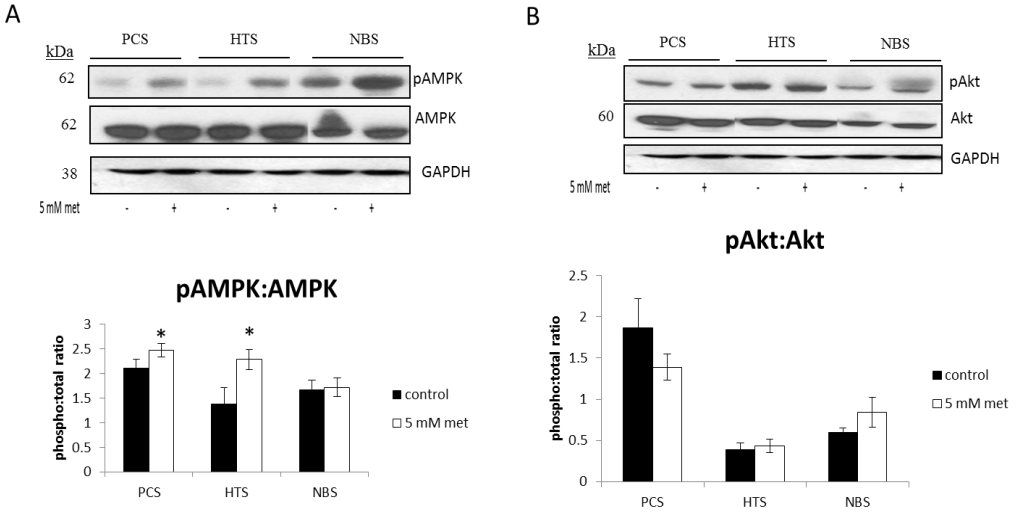


Figure 10: Determination of optimal metformin doses on fibroblast AMPK activity. Fibroblasts were treated with 0,1, 5, and 10 mM metformin for 24 hours and 40 µg of cell lysates were Western blotted for AMPK, pAMPK T172, and GAPDH. Graph compares ratio of phosphorylated to total AMPK; results are from 3 independent experiments ± SEM. *: $p<0.05$ compared to untreated controls analyzed by Student's t-test.

For all subsequent experiments 5 mM of metformin for 24 hours was used to treat the cells, unless stated otherwise. The signaling pathways affected by AMPK are shown in **Figure 2**. Since metformin has previously been shown to enhance glucose uptake by upregulation of AMPK signaling in muscle and hepatocytes (27, 35, 137), we examined the effects of metformin on dermal fibroblast AMPK expression. Phosphorylation of AMPK was upregulated by 5 mM metformin in both PCS (p=0.01) and HTS fibroblasts (p=0.04) (**Figure 11A**). No changes in Akt activity were observed with metformin treatment (**Figure 11B)**. Metformin downregulated mTOR activation in HTS and NBS fibroblasts cultured in high glucose conditions (p= 0.02 for HTS, p=0.04 for NBS) (**Figure 11C**). No significant changes in ACC phosphorylation were observed (**Figure 11D**). PKCα was increased by metformin only in NBS fibroblasts (p=0.02)

Figure 11



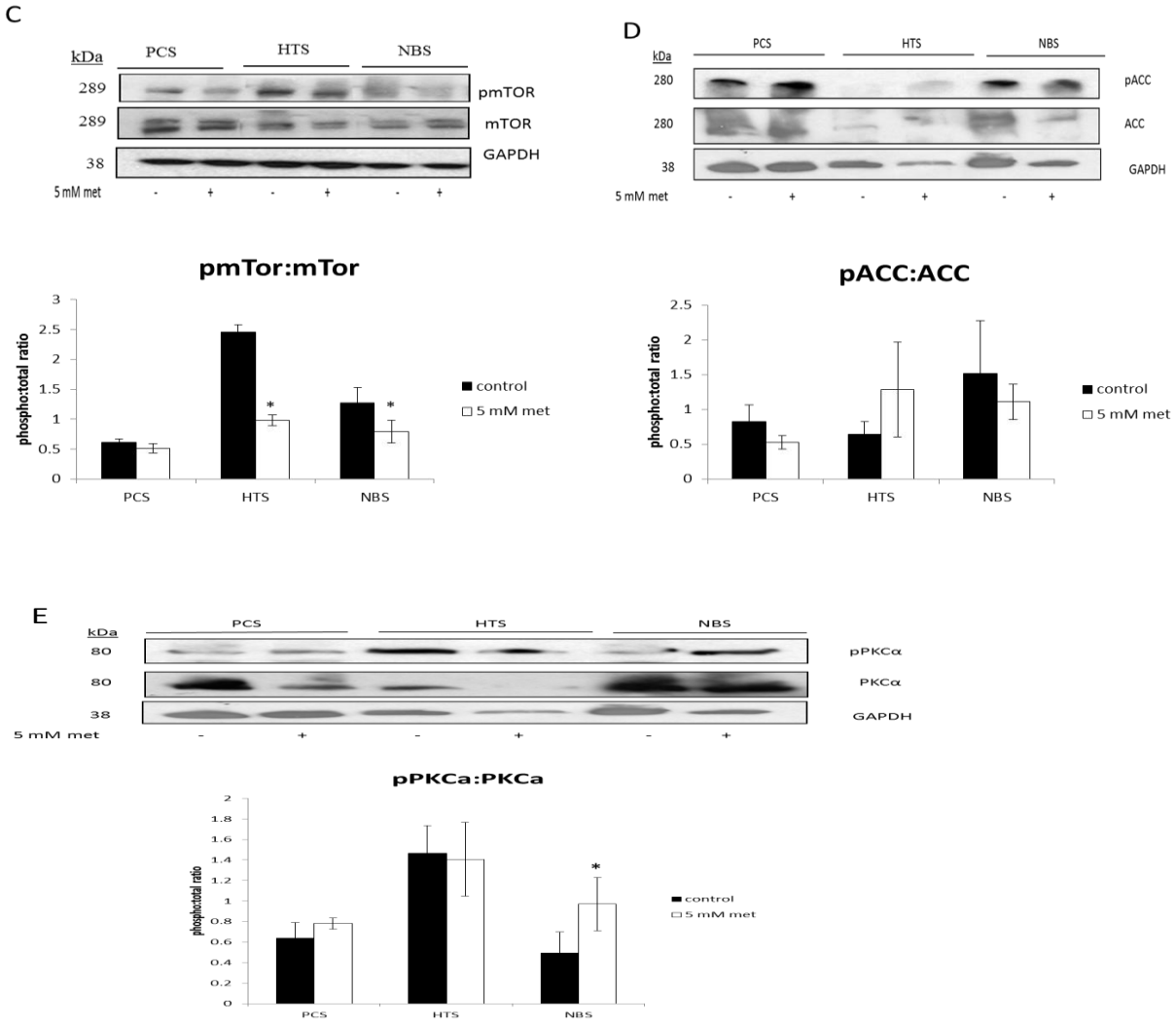


Figure 11: Effects of metformin on AMPK associated signaling: Effects of 5 mM metformin on phosphorylation of A.)AMPK, B.) Akt, C.) mTor D.) ACC and E). PKCα. Fibroblasts were grown in media containing high glucose (25 mM) ± 5 mM metformin for 24 hours. Results are combined for 5 independent experiments ± SEM. *: $p<0.05$ compared to untreated controls.

Figure 12

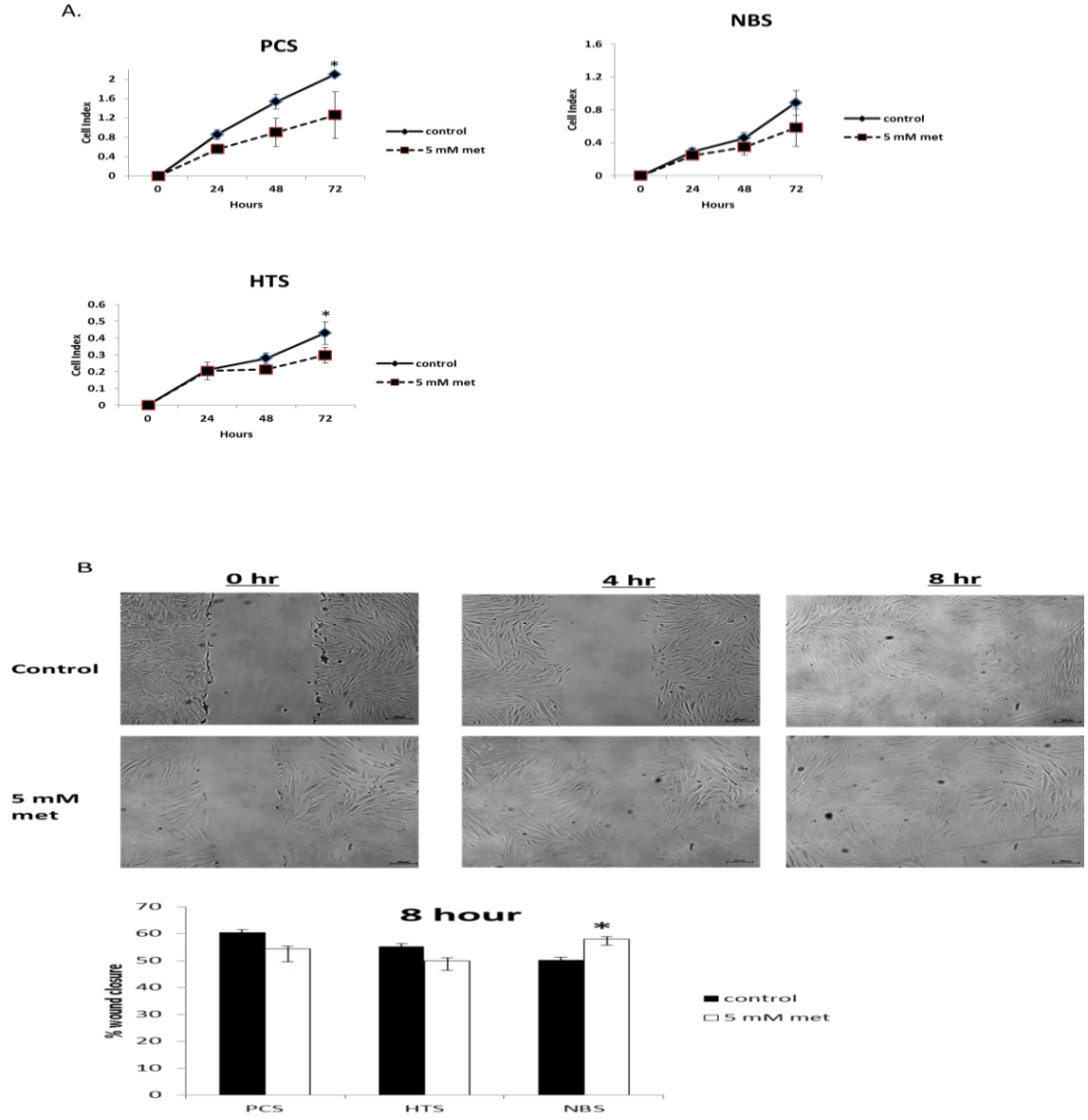


Figure 12: Metformin alters real time fibroblast proliferation and wound healing. A). Effects of metformin on fibroblast proliferation determined by xCELLigence . *: $p<0.05$ compared to untreated. N=3 ±SEM. B). Effects of metformin on wound healing. Cell migration was observed over 8 hours and percent closure determined by comparing to the initial time point. Results are from 3 independent experiments ± SEM. *: $p<0.05$ compared to untreated control.

(**Figure 11E**).These results indicate that metformin increases AMPK signaling in fibroblasts, and might reduce cell proliferation through mTOR inhibition.

Metformin impedes cell proliferation: Given our finding that metformin inhibited mTOR under high glucose conditions (**Figure 11C**), we next studied the effects of metformin on cell proliferation. Fibroblasts were treated cells with or without 5 mM metformin and monitored proliferation using the xCELLigence system. Significant inhibition of proliferation with metformin treatment was observed over 72 hours in PCS and HTS fibroblasts (PCS p=0.03, HTS p =0.05). HTS fibroblasts also displayed lower proliferation index values than non-burn and control lines (**Figure 12A**). Scratch assays were used to measure cell migration and wound closure over 8 hours (**Figure 12B**). At 8 hours metformin increased wound healing in NBS cells in high glucose conditions (p =0.03). This suggests that metformin decreases cell proliferation in hyperglycemic conditions, and may augment wound healing.

Metformin lowers cAMP accumulation but does not affect fibroblast glucose uptake: Since metformin has been implicated in the reduction of gluconeogenesis through inhibition of cAMP (41), we quantified intracellular cAMP concentrations following 24 hours of treatment with 5 mM metformin. Metformin significantly decreased cAMP levels in PCS (p=0.02) and NBS fibroblasts (p=0.04) (**Figure 13A)**. Since we observed both increased AMPK activity and decreased cAMP, fibroblast glucose uptake was determined by measuring 2-deoxyglucose conversion to 2-deoxyglucose 6 phosphate. No significant differences in glucose uptake in any fibroblast lines treated with metformin, although glucose uptake was reduced in fibroblasts from burn patients (**Figure 13B)**.

Figure 13

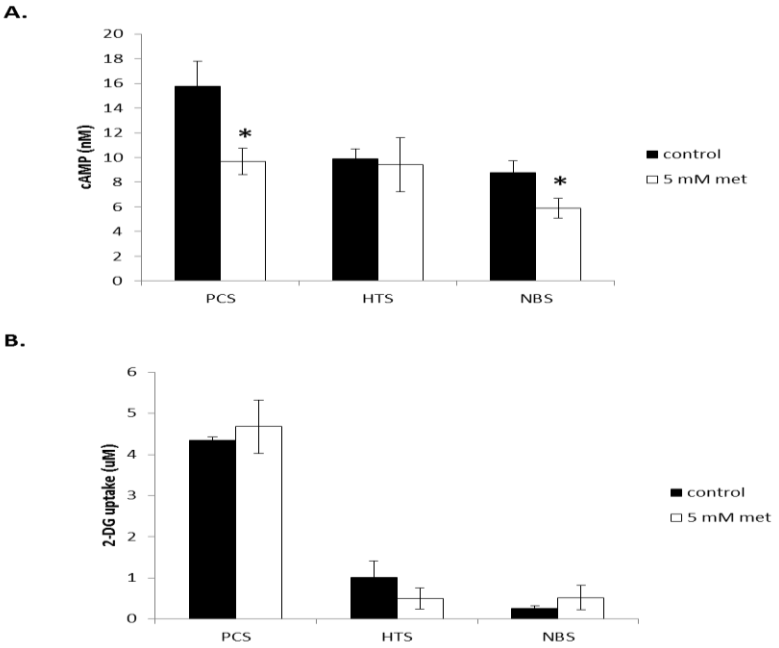


Figure 13: Metformin affects fibroblast cAMP accumulation but not glucose uptake. A). Fibroblast intracellular cAMP activity in following treatment with 5 mM metformin determined. Results are from 3 independent experiments ± SEM. *: $p<0.05$ compared to untreated controls. B). Fibroblast glucose uptake measured by 2-deoxyglucose conversion. Results are from 3 independent experiments ± SEM.

Regulation of mitochondrial oxidative phosphorylation by metformin is dependent on cell type: We next determined the effects of metformin on mitochondrial oxygen utilization. HepG2 hepatocytes were used as a control. Oxygen consumption was significantly

Figure 14

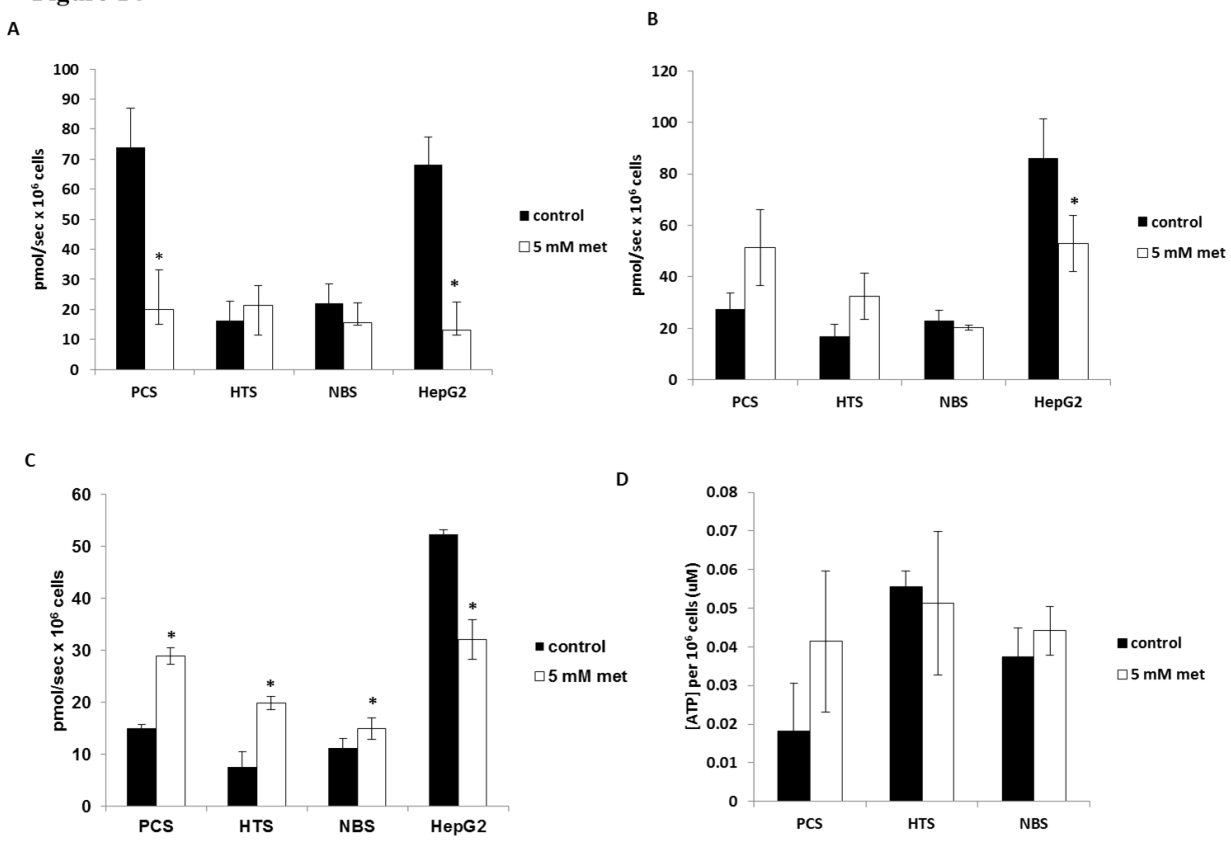


Figure 14: Metformin alters fibroblast mitochondrial activity. Measurement of A). Total oxygen consumption, B). oxidative phosphorylation, and C). Mitochondrial complex inhibitors in using Oxygraph 2k (Oroboros Instruments). Results are from 3 independent experiments ± SEM. *: $p<0.05$, compared to untreated controls. D). Intracellular ATP content. Results are from 3 independent experiments ± SEM.

reduced in HepG2 cells (p=0.004), as was oxidative phosphorylation (p=0.03). Oxygen consumption was decreased by metformin only in PCS fibroblasts (p=0.04) (**Figure 14A and B)**. Inhibition of mitochondrial complexes II-V resulted in upregulation oxygen utilization in PCS (p=0.02), HTS (p=0.03) and NBS (p=0.009) dermal fibroblasts under high conditions following metformin treatment **(Figure 14C)**. A significant decrease in complex I activity was observed in HepG2 cells (p =0.007). Existing data suggests that metformin lowers cellular energy status by reducing ATP production through complex I inhibition in hepatocytes (27), but might raise ATP production in muscle (75). To determine the effects of metformin on fibroblast ATP production we obtained lysates

from cells treated with or without 5 mM metformin and analyzed ATP content. Metformin did not significantly alter ATP production under high glucose conditions (**Figure 14D**), indicating that the altered mitochondrial respiration observed in metformin treated fibroblasts did not correlate with increased ATP production.

Figure 15

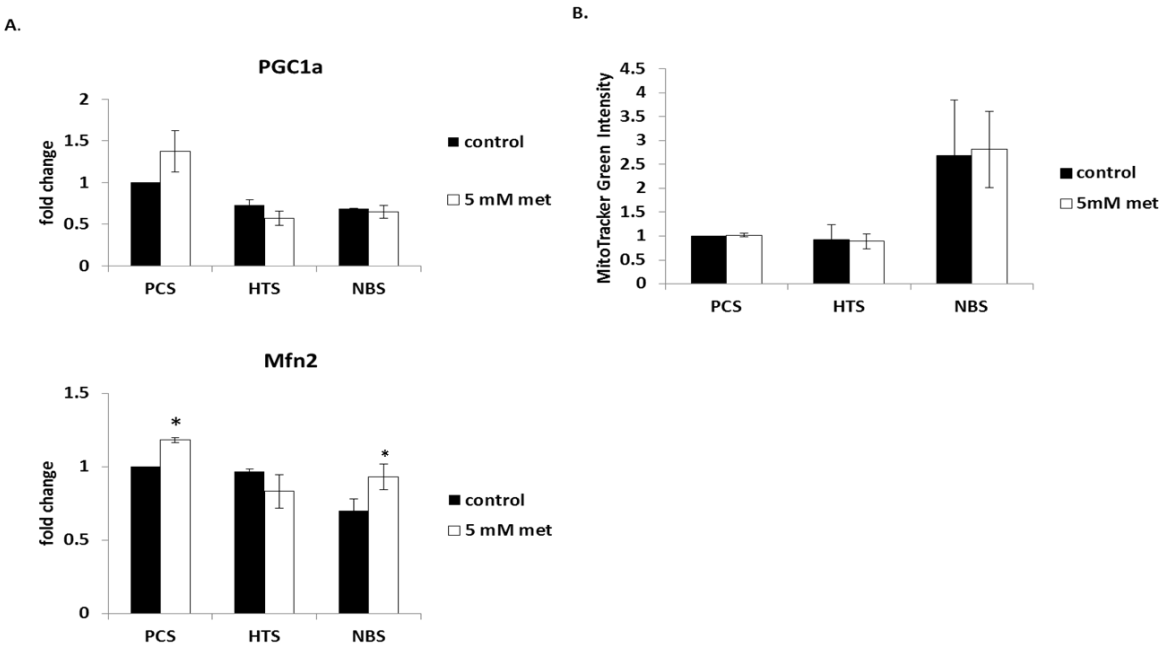


Figure 15: Effects of metformin on mitochondrial proliferation. A). Expression of PGC-1α, and Mfn2 mitochondrial biogenesis genes measured by quantitative real time PCR. *: p<005 comparing metformin treatment to controls, N=3 ± SEM. B). Total mitochondrial proliferation measured by MitoTracker Green™. Results are from 3 independent experiments ± SEM.

Effects of metformin on mitochondrial biogenesis: Since burn injury has been linked with mitochondrial dysfunction (2), we measured several genes related to mitochondrial function and insulin utilization (78, 138-140) following metformin treatment. Expression of the transcripts for the primary transcription factor PGC-1α were not affected by metformin under hyperglycemic conditions, but Mfn2, which regulates mitochondrial fusion, was increased in PCS (p=0.04) and NBS (p=0.02) fibroblasts (**Figure 15A).** To determine whether the effects observed in mitochondrial oxidative

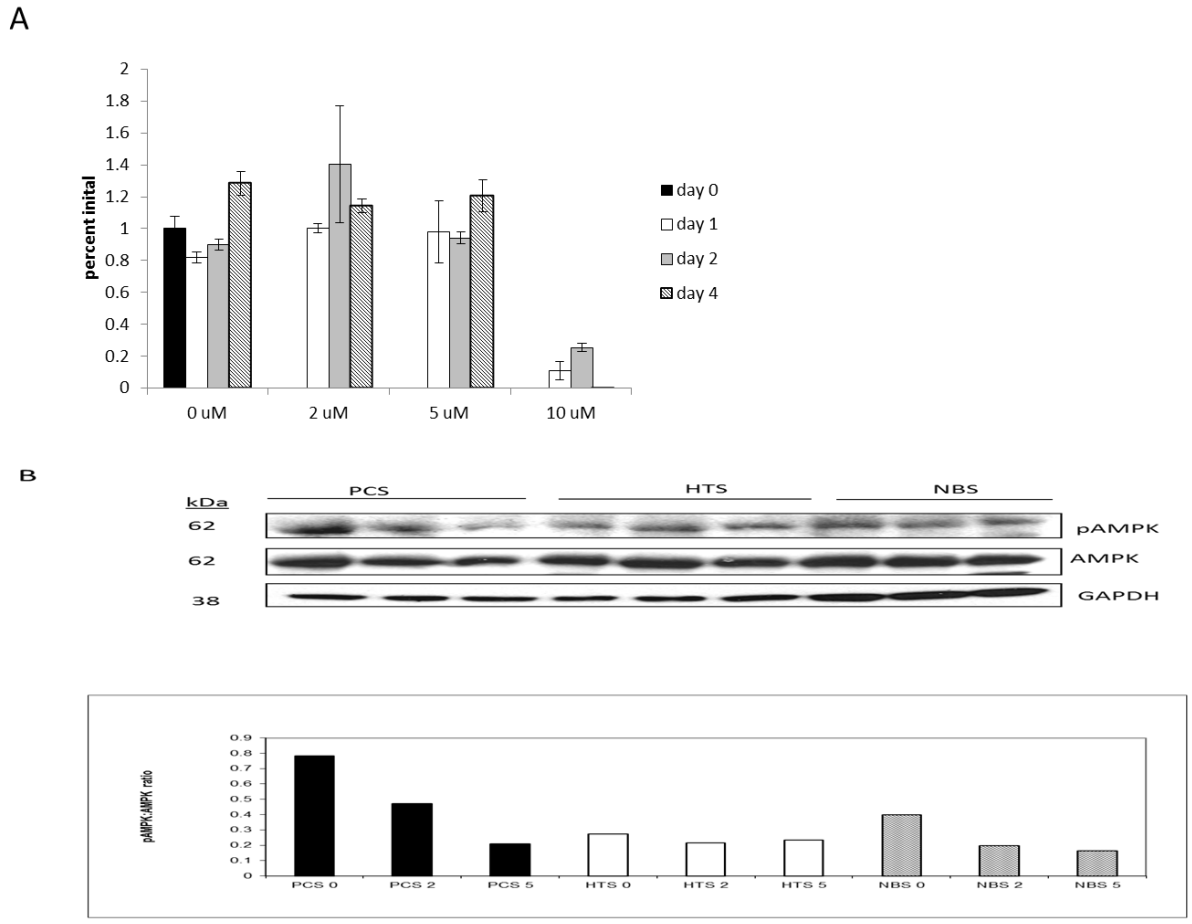
phosphorylation were due to increases in mitochondrial biogenesis, total cellular mitochondria was assessed by treating fibroblasts with 75 nM MitoTracker Green™ following 5 mM metformin treatment **(Figure 15B)**. Total mitochondrial numbers did not change with or without metformin treatment. These results suggest that while metformin does not alter total mitochondrial production in fibroblasts, it can affect transcription of mitochondrial biogenesis genes.

Metformin acts independently of AMPK. In order to determine the effects of AMPK inhibition on mitochondrial complex activity, we treated HTS fibroblasts with 0, 2, 5, and 10 μM of the AMPKα subunit inhibitor Compound C over a four day time course and determined cell viability using MTT. Lower doses of Compound C were not detrimental to cell viability, but 10 μM was fatal at all time points (**Figure 16A**). Phosphorylation of AMPK was reduced following treatment of 5 μM Compound C for 24 hours in dermal fibroblasts (**Figure 16B**).

To determine if the effects of metformin on fibroblast mitochondrial activity are dependent on AMPK activity fibroblasts were treated with 5 μM of AMPK inhibitor Compound C 24 hours prior to metformin treatment (**Figure 16C**). We observed that Compound C alone, or combined with metformin increased oxygen flux in PCS fibroblasts under high glucose ($p = 0.007$ and 0.014). Under high glucose conditions Compound C alone and with metformin increased oxygen flux in burn patient derived fibroblasts ($p = 0.037$ in HTS, $p= 0.01$ in NBS). These results indicate that both metformin and AMPK activity regulate complex I activity, and the effects of metformin

are independent of AMPK activation.

Figure 16



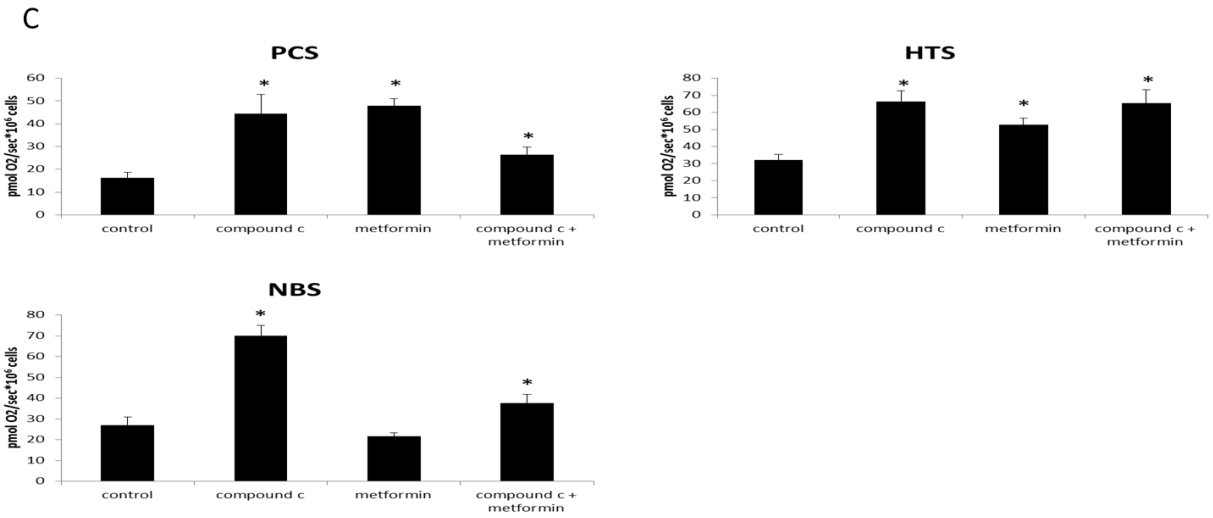


Figure 16: Metformin can act independent of AMPK. A). HTS survival with Compound C treatment. HTS fibroblasts were treated with 0-10 μM Compound C for up to 4 days. Cell survival was determined using MTT. N=3 ± SEM. B). Compound C knockdown of AMPK phosphorylation. Fibroblasts were treated with indicated doses of Compound C for 24 hours. C). Effects AMPK inhibition on oxygen flux. Fibroblasts were treated ± 5 μM of AMPK inhibitor Compound C for 24 hours, then ± 5 mM metformin for 24 hours before analysis using Oxygraph 2k. Results are from 4 independent experiments. * :$p < 0.05$ using one way ANOVA.

Effects of metformin on OCT expression. To determine if metformin had any effect on the OCTs, quantitative RT-PCR was performed on PCS, HTS, and NBS following 24 hours of metformin treatment under high glucose conditions. No significant changes were observed in the expression of OCT 1, 2, or 3, indicating

that metformin does not change expression levels of its transporters in fibroblasts, and that the effects on glucose metabolism are not due to increases in metformin transport

proteins (**Figure 17)**.

Figure 17

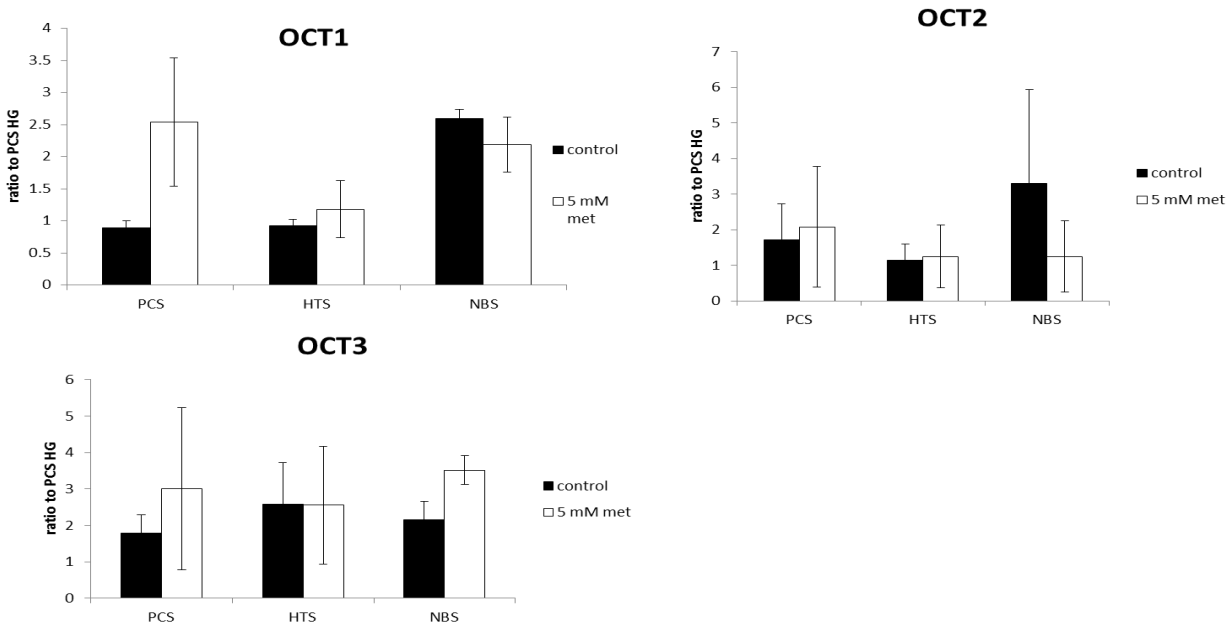


Figure 17: Effects of metformin on organic cation transporters. Expression levels of fibroblast OCT 1, 2, and 3 mRNA transcripts were measured by quantitative RT-PCR following 24 hours of metformin treatment in 25 mM glucose. Ratios are expressed relative to PCS control. Results are from 3 independent experiments ± SEM.

Discussion

We demonstrate that metformin upregulates AMPK activity and inhibits proliferation of dermal fibroblasts by reducing mTOR phosphorylation. Our data confirm that metformin activity is dependent on cell type. Unlike in hepatocytes, fibroblast oxygen utilization is upregulated by metformin. Burn injury is associated with increases in circulating glucose and muscle degradation (30, 87). Biguanides such as metformin have been used to reduce hyperglycemia by increasing AMPK and Akt activation, which also reduces muscle catabolism and cardiac fibrosis through the AMPK-PTEN pathway (35, 40, 133, 137). We demonstrate that metformin increases AMPK activity while decreasing mTOR

in dermal fibroblasts from burned patients. This suggests that metformin plays a role in both cell proliferation and metabolic regulation. We show that metformin decreases cell proliferation in high glucose conditions, while also having an effect on fibroblast wound healing. Other reports have indicated that metformin exerts maximum effects on mitochondrial activity and cancer cell growth inhibition in conjunction with other glucose controlling strategies such as insulin therapy (124, 141). Hyperglycemia also causes increased reactive oxygen species (ROS), which lead to diabetic neuropathy. In rats metformin administration results in decreased transcription of inflammatory cytokines TNFα and IL-6 while increasing ROS scavenger transcription (133). Our results indicate that metformin upregulates Mfn2, suggesting beneficial effects of metformin on mitochondrial fusion and stress reduction. We also did not observe effects of metformin on OCT gene expression, suggesting that the presence of metformin does not result in increases in transporter expression or activity.

One key finding that is contrary to existing reports is that metformin treatment increased oxygen flux in the presence of mitochondrial complex inhibitors in fibroblasts. This difference might be due to differences in measuring techniques. Most other studies utilized XF24 Seahorse Bioanalyzer, or indirect measurements using NADH oxidation (70, 142, 143). We directly observed oxygen flux using Oxygraph 2k. It has also been shown that that angiotensin I receptor antagonist losartan increased complex I, and that 10 mM metformin was required to inhibit complex I in cardiomyocytes (144), so it appears that only supraphysiological doses of metformin inhibit complex I in all *in vitro* studies, and this inhibition is cell type dependent. A study of fibroblasts treated with the coenzyme Q analog idebenone increased complex I activity but not mitochondrial

respiration (145), indicating that complex I activity is not always linked with increased energy production. Administration of metformin treatment for two weeks increased complex I activity in both the presence and absence of metformin in AMPK knockout mice (74), suggesting that metformin activity is dependent upon both cell type and duration of metformin treatment. These findings correlate with our *in vitro* data suggesting that both AMPK inhibition using Compound C or metformin increase mitochondrial complex I activity. Co-administration of both Compound C and metformin also increased complex I activity. Metformin appears to act on the mitochondria both through AMPK and another unidentified mechanism. Previous studies performed on isolated mitochondria result in lower energy expenditure. One study by Vytla and Ochs on intact L6 muscle cells showed that 10-20 mM metformin did not alter ATP production, but rather increased free ADP and AMP, and gave rise to more viable cells (75), suggesting that metformin dosage and cell intactness can lead to AMPK independent effects on ATP status. Additionally, low doses of metformin have no effects on complex I activity in skeletal muscle from diabetic individuals (73). Metformin is believed to exert inhibitory effects on complex I by altering mitochondrial membrane permeability by metformin becoming embedded in the mitochondrial membrane (76, 146). It is likely that clinical doses of metformin (< 1 mM) do not significantly alter mitochondrial membrane permeability to affect complex I activity. Skin tissue samples have less mitochondrial activity and ATP synthesis than fat or skeletal muscle (147, 148), so it is likely that mitochondria in dermal fibroblasts are utilizing less oxygen than more metabolically active cells.

In conclusion we show that metformin increases fibroblast AMPK signaling and mitochondrial complex I, that metformin reduces cell proliferation under hyperglycemic conditions by reducing mTOR activation, and that metformin-induced changes in mitochondrial fusion protein gene expression. We also demonstrate that metformin affects mitochondrial complex activity via AMPK dependent and independent mechanisms. These results establish that metformin activity is dependent on cell type, alters AMPK and other signaling pathways, and could lead to improved therapies to alter dermal fibroblast proliferation to affect wound healing in burned patients.

Chapter 3: Metformin Reduces Fibrosis in Post-Burn Hypetrophy by Targeting SMAD3

Background

One of the physiological changes associated with burn injury is dysregulated EMT: the process by which endothelial cells lose adherence and become motile (149). During EMT there is a downregulation of proteins involved in cell junctions, particularly E-cadherin, and the affected cells acquire a mesenchymal phenotype characterized by increased N-cadherin expression (**Figure 18**) (94).

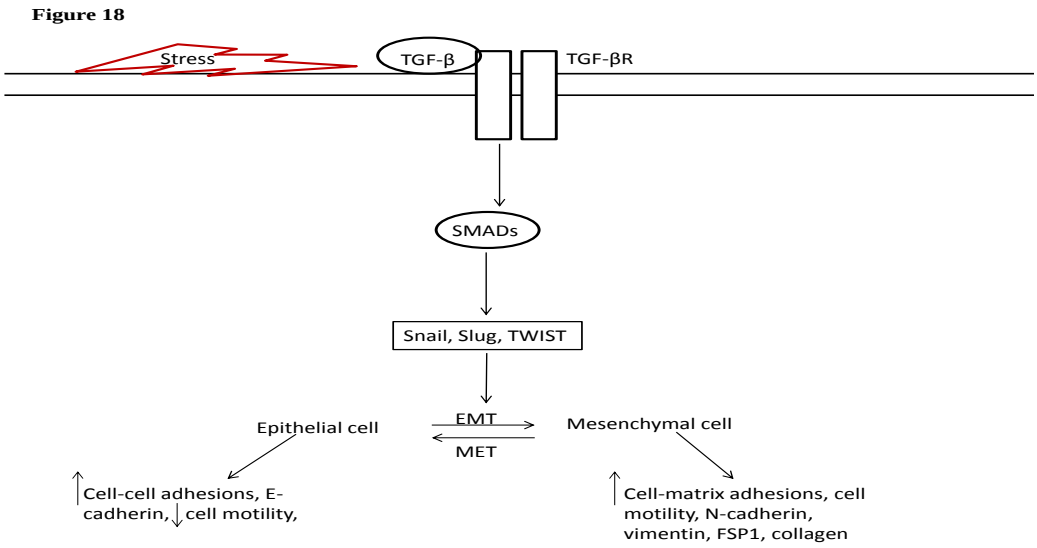


Figure 18: Stress induces SMAD induced EMT-MET pathway, altering gene expression.

This change in phenotype is further characterized by changes in gene expression, such as the downregulation of Snail and its downstream mediator Slug, upregulation of TWIST, and alterations in collagen gene expression (94). Furthermore, it has been demonstrated that the EMT phenotype is exacerbated by increases in inflammation, particularly by elevation of inflammatory cytokines such as tumor necrosis factor α (TNF-α) and tumor growth factor β (TGF-β) (96, 114). EMT is a dynamic and reversible process; mesenchymal cells can revert back to a non-motile epithelial state by gaining

expression of markers such as E-cadherin and occludin (93). MET can also be induced by transcriptional inhibition of EMT inducing genes such as Snail and TWIST, and activation of microRNAs such as miR-21 and miR-30a that repress SOX4, and signal transducer and activation of transcription 3 (STAT3) activation by TGFβ (45, 150). Wound regeneration and repair is comprised of three main stages: inflammation, proliferation, and remodeling (106); the inflammatory phase is marked by massive increases in pro-inflammatory cytokine release to attract keratinocytes and myofibroblasts in order to promote fibroblast differentiation in the wound area (149). Fibroblasts secrete collagen to promote wound closure; however collagen deposition can be a double edged sword in that excess deposition of collagen results in tissue that lacks the flexibility of normal connective tissue. Excessive collagen deposition can lead to contracture and permanent flexion at the wound site when it occurs across a joint (108). Collagen 1 is the major collagen type present in wounds, with collagen 3 prominent in early stages (101). The ratio of collagen 1 and 3 changes in hypertrophic scarring compared to non-injured skin (102, 151). TGFβ treatment increases SMAD activity and downstream collagen deposition by fibroblasts (97, 152). Fibroblasts share many characteristics of mesenchymal stem cells, including expressing some of the same markers such as integrins and CD90, and differentiating into many cell types under the proper culture conditions (109). A hallmark of post-burn wound healing is increased dermal TGF-β secretion, which can lead to dysregulated EMT and collagen secretion if unchecked (98). It is desirable to promote rapid wound healing without an overly fibrotic response in burn patients in order to avoid pathologic scarring and subsequent functional impairment caused by excessive collagen deposition (153). The SMAD signaling

pathway, which is regulated by the TGFβ receptor, is one of the major pathways regulating collagen secretion (101). It has been shown that siRNA inhibition of SMAD3 reduces collagen 1 production by fibroblasts (104), and SMAD3 knockout mice experience less inflammation and improved wound healing(105).

Metformin is a biguanide drug that is commonly used for treatment of diabetes. It affects glucose metabolism by upregulating AMPK and altering mitochondrial complex activity in liver and muscle cells (72, 154). Metformin reduces cancer phenotype in several cell types. High concentrations (≥ 1 mM) of metformin have been linked to increased apoptosis in endometrial cancer cells by inhibiting growth signaling induced by IGF-1 receptor signaling (117) and to reduced cell proliferation by downregulation of mTOR (120). Metformin induced activation of AMPK led to reduction of TGF-β and IL-6 induced EMT in renal fibroblasts, tubular endothelial cells, and lung cancer cells (127, 155-157). However the effects of metformin in skin derived fibroblasts from burn patients remain undetermined.

Due to the role of fibroblast proliferation and apoptosis, and inflammation in the development of post-burn hypertrophic scars, we hypothesized that metformin will alter the EMT phenotype in dermal fibroblasts by altering expression of genes involved in collagen production and cell motility. We first established the presence of increased EMT markers in hypertrophic scar biopsies, and showed that metformin reduces FSP1 in these tissues. We further demonstrate reduction in EMT genes, especially SMAD3, collagen 1, and SNAIL pathway genes. Finally, the effects of metformin on total collagen secretion in cultured fibroblasts under high and low glucose conditions were determined. The results of these experiments will allow a better understanding of the mechanisms

regulating EMT and collagen secretion in fibrotic burn scars. Additionally, our results lay the groundwork for the development of better therapeutic options to avoid excessive collagen deposition and pathological fibrosis in following burn injury.

Materials and methods

Cell culture: Primary dermal fibroblasts were derived from biopsies of hypertrophic scar (HTS) or unburned areas (NBS) obtained from three burned patients enrolled in clinical trials using protocols previously described (135). Fibroblasts were cultured using methods described on page 50. For TGF-β treatment, fibroblasts were cultured in media containing 0.5% FBS overnight, then treated in growth media with or without 10 ng/ml TGF-β (PeproTech, Rocky Hills, NJ) and 5 mM metformin for 48 hours. TGF-β was suspended in PBS with 1% BSA and 0.1 N hydrochloric acid at a concentration of 10 μg/ml, and stored in 20 μl aliquots at -80°C until use.

Histological analysis of patient hypertrophic scar samples: Pediatric burn patients between 0 – 18 years old with burns covering 20% or greater of total body surface area were enrolled in this study approved by the University of Texas Medical Branch Institutional Review Board. Patients enrolled in metformin clinical trials received 250-500 mg metformin daily to maintain blood glucose concentrations between 140 and 180 mg/dl. Biopsies were obtained from hypertrophic scar (HTS) and bordering non-burned skin (NBS) areas during revision procedures. For histological studies, HTS and NBS biopsies were obtained up to 24 months post-burn, washed in PBS, fixed in 10% neutral-

buffered formalin for 48 hours and dehydrated in 70% ethanol prior to processing. Following processing, tissues were paraffin-embedded and sections cut to 4 μM. For immunofluorescence studies, sections were cleared in xylene followed by rehydration to purified water. For heat-induced antigen target retrieval, sections were boiled for 5 minutes in 1X Dako Antigen Retrieval (Dako, Carpinteria, CA) then washed in PBS. Slides were blocked with 5% horse serum for 1 hour, then treated with primary antibody at 4°C overnight. Primary antibodies and dilutions used were vimentin (1:100, Abcam, Cambridge, MA), FSP1 (1:75, Abcam, Cambridge), E-cadherin (1:100, Abcam, Cambridge, MA), N-cadherin (1:100, Abcam, Cambridge, MA). Slides were treated with secondary antibodies rabbit anti-mouse 568 and donkey anti-rabbit 488 (Cell Signaling, Danvers, MA) at room temperature for 1 hour at a 1:350 dilution. Slides were then treated with 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, St. Louis, MO) at a 1:5000 dilution for 15 minutes, then cover slips were mounted and slides stored at 4°C until visualization. Images were captured on an Olympus BX41 microscope at 20x magnification. Images were analyzed using ImageJ (http://imagej.nih.gov/ij/) and corrected total cell fluorescence (CTCF) was calculated by the equation: CTCF= media of integrated density-(media of area of selected image X mean fluorescence of background). Tissues stained with secondary antibody only were used as negative controls. To confirm staining specificity, the primary antibodies were mixed with blocking peptides for N-cadherin, E-cadherin (10.4 μg/ml and 25 μg/ml; ECM Biosciences, Versailles, KY) or vimentin (26 μg/ml; Biovision,Miliptas, CA) incubated at 4°C overnight, then stained as described above.

Quantitative RT-PCR analysis: qRT-PCR was performed using the methods previously described on pages 51-52. Primer sequences are listed in **Table 2**.

siRNA induced knockdown of SMAD3: SMAD3 knockdown was achieved by Silencer® Pre-designed siRNA for SMAD3 (cat # AM16708), or scrambled control (cat # AM 4611) (Thermo Scientific, Waltham, MA). Fibroblasts were plated in 6 well plates at $4x10^4$ cells per well and grown to 80% confluency. Fifty pmol of siRNA was put into 250 µl Opti-MEM (Thermo Scientific, Waltham, MA), and held at room temperature for 5 minutes. The siRNA mix was then combined with 250 µl Opti-MEM containing 10 µl Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) for 20 minutes at room temperature. The Lipofectamine-siRNA mixture was then added to fibroblasts in Opti-MEM media and incubated at 37°C for 7 hours before changing to growth media ± 5 mM metformin. Twenty-four hours later RNA was extracted as described previously. Sequences for SMAD3 siRNA were sense 5-GGACAGCGGGAAAAAUCGAtt-3' and antisense 5'-UCGAUUUUUCCCGCUGUCCtg-3.

Collagen assay: Total collagen secretion was determined using the Sircol ™ Soluble Collagen Assay (Accurate Chemical and Scientific, Westbury, NY). Fibroblasts were plated in 12 well plates at a density of $5x10^4$ cells per well, grown for 24 hours, and then treated with 5 mM metformin for an additional 24 hours. Media was then removed, and collagen was obtained by washing each well with 1 ml of ice-cold 0.5 M acetic acid. Hydroxyproline concentrations were determined using the manufacturer's protocol and results read on a spectrophotometer at 555 nM.

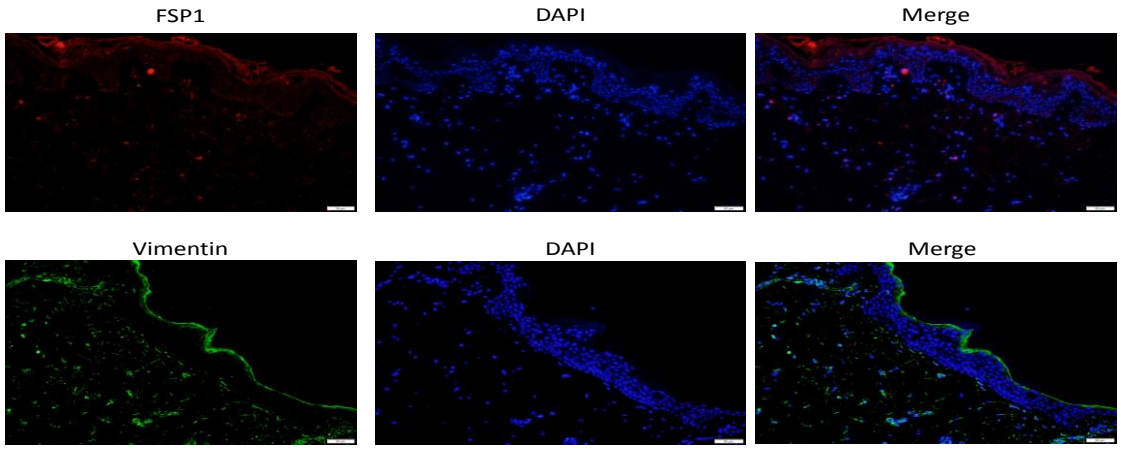
Statistical analysis: Statistical analysiswere performed as detailed on page 56.

Results

EMT occurs in post-burn patient hypertrophic scar tissues: Expression of vimentin, FSP1, E-cadherin, and N-cadherin were detected in biopsied scar tissues (**Figure 19A)**. Antibody specificity was demonstrated by incubating primary antibodies with blocking peptides (**Figure 19B**) or secondary antibody alone (**Figure 19C)**. Comparison of non-burn skin biopsies taken from burn patients 6 months post-burn and hypertrophic scar and taken from 8 patients at 6, 9, and 12 months post-burn injury

Figure 19

A



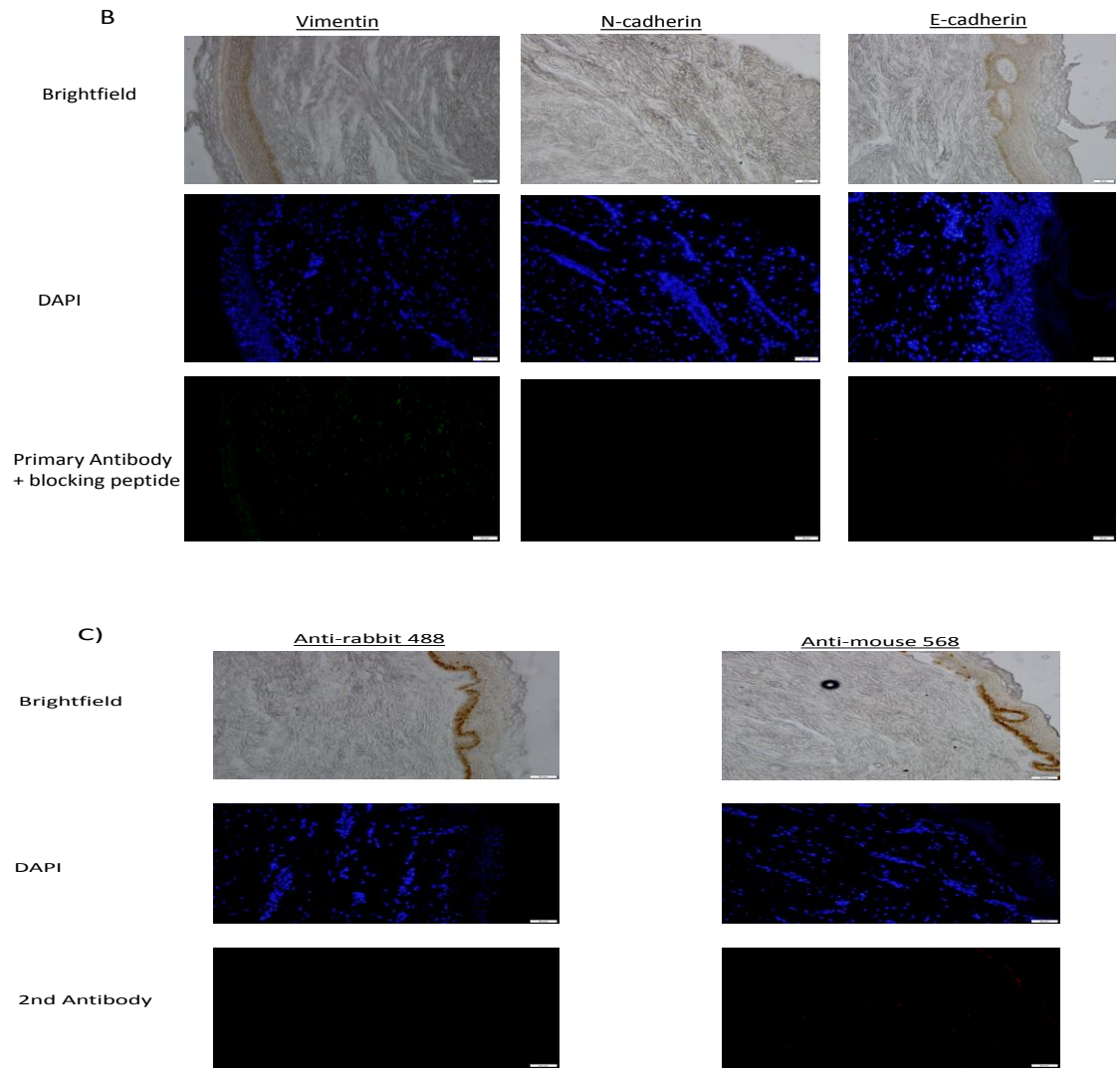
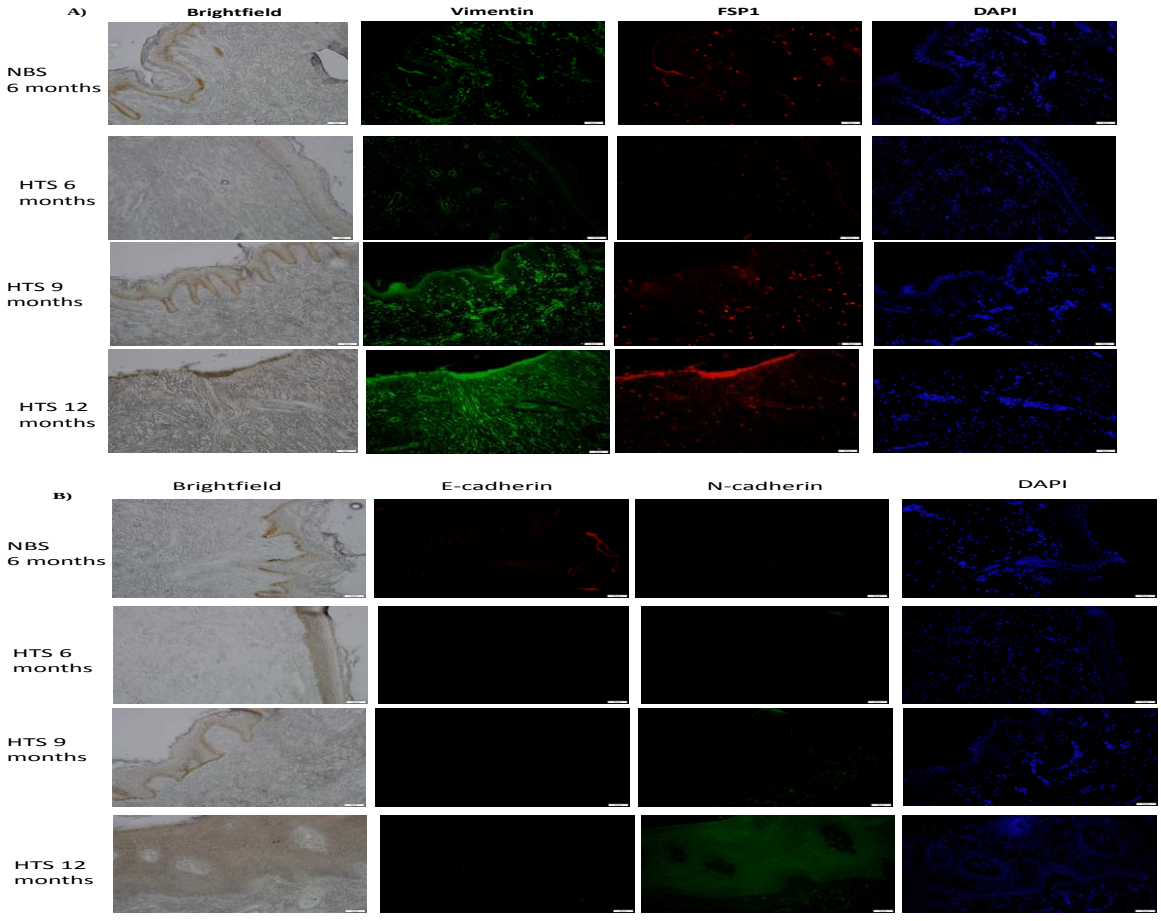


Figure 19: EMT occurs in post-burn hypertrophic scars. A). Hypertrophic scars biopsies were stained with antibodies to vimentin, FSP1, E-cadherin, and N-cadherin. B). Antibody specificity was confirmed by incubating primary antibodies with blocking peptides for vimentin, N-cadherin, and E-cadherin prior to incubation with the sections. Sections were treated with blocking peptides for 24 hours along with primary antibodies. C). Staining with secondary antibodies only.

(**Figure 20)** and observed a time dependent increase in vimentin at 9 and 12 months (p=0.017 and 0.014) and FSP1 at 12 months (p=0.04). These results indicate that EMT progression is occurring in post-burn hypertrophic scar, and that these alterations are time dependent following the burn injury.

Metformin reduces EMT markers in hypertrophic scar: The effect of metformin on the EMT phenotype in post-burn hypertrophic scars was elucidated by quantifying the expression of vimentin, FSP1, E-cadherin, and N-cadherin in biopsies obtained from burn patients randomized to receive metformin one year post-burn (**Figure 21)**. While vimentin and E-cadherin expression were not affected in metformin treated patients, expression of FSP1 was significantly reduced (p=0.026) in patients receiving metformin. N-cadherin reduction was not significant (p=0.055). This data suggests that metformin

Figure 20



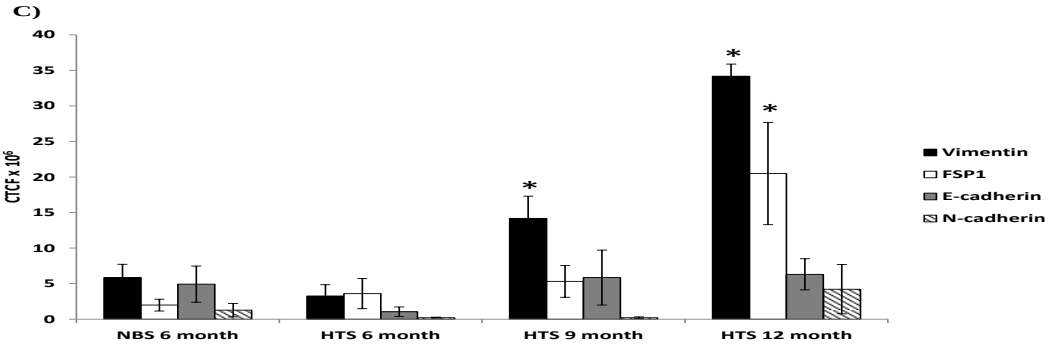
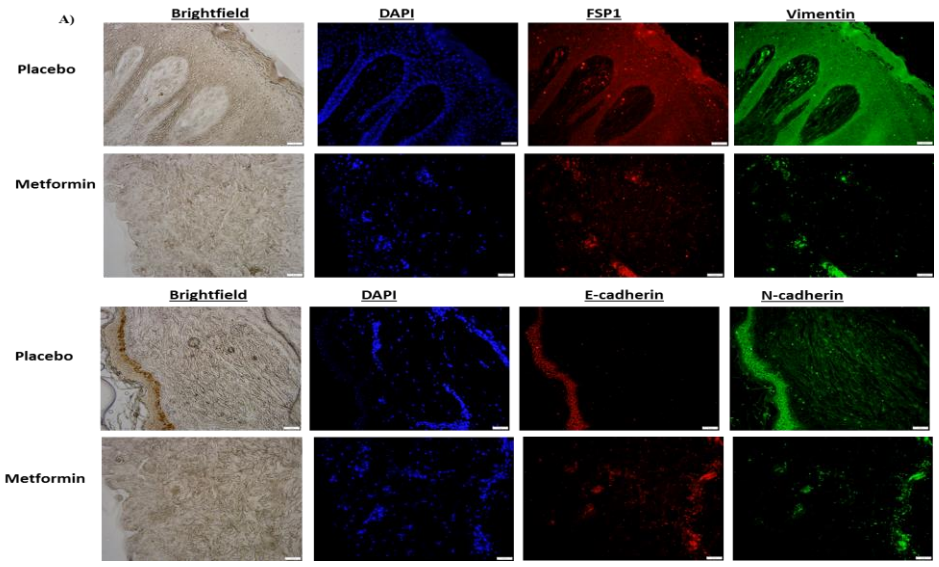


Figure 20: EMT increases in burn patient hypertrophic scars over time. Non-burned skin and hypertrophic scar samples taken from patients 6, 9, and 12 months post-burn injury were stained for A) vimentin and FSP1, or B) E-cadherin and N-cadherin. C). Quantification of EMT marker images determined by CTCF. N=8 per group, error bars indicate ± SEM, *: $p < 0.05$ compared to NBS.

Figure 21



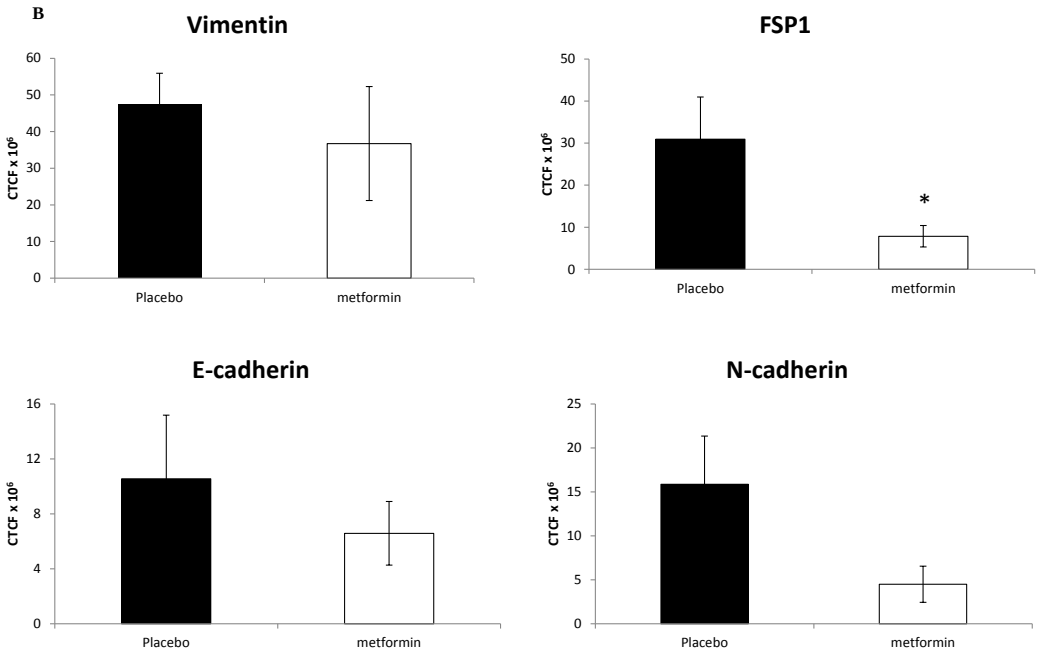


Figure 21: Metformin decreases EMT in burn patient hypertrophic scar. A) Hypertrophic scar samples from burn patients taken up to 26 months post-burn were analyzed for vimentin, FSP1, and E and N-cadherin. B) Quantification of EMT markers determined by CTCF. N=9 per group, error bars indicate ± SEM, *: $p < 0.05$ compared to placebo.

may play a role in reducing the EMT phenotype by reducing expression of FSP1, a major determinant of fibroblast differentiation, inflammation, and collagen regulation during wound healing (115, 158).

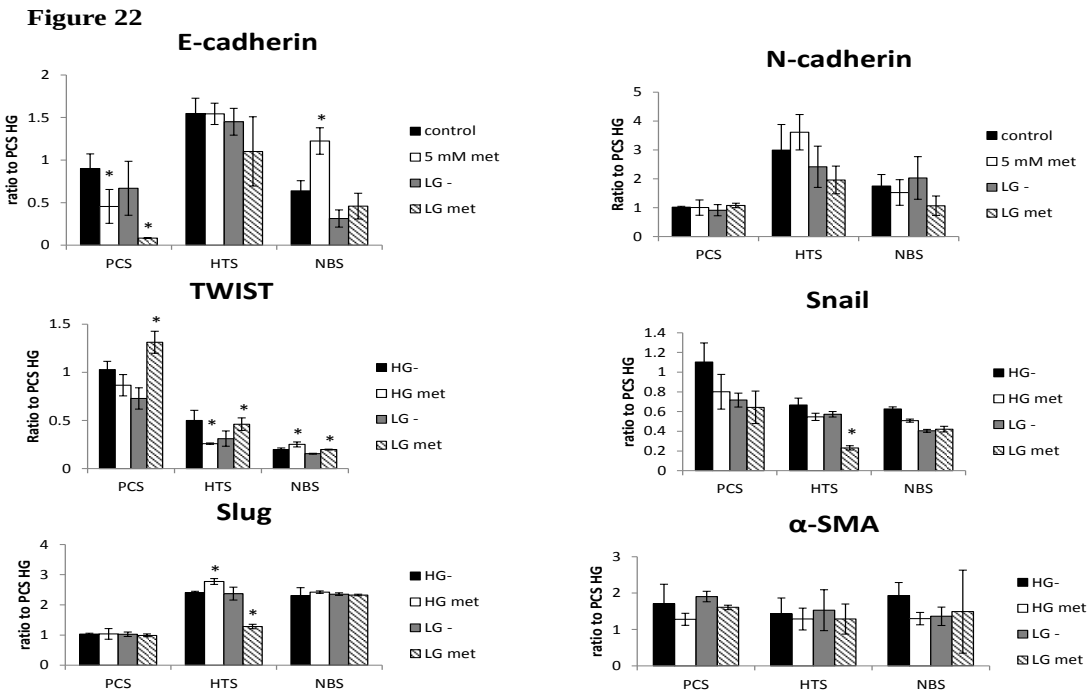


Figure 22: Effects of metformin on EMT pathway genes. Fibroblasts were treated with or without 5 mM of metformin for 24 hours in high or low glucose conditions, then mRNA was collected and quantitative RT-PCR performed for Snail, Slug, TWIST, and E and N-cadherin. Results are expressed as means ± SEM, N=3. *: $p < 0.05$ compared to untreated. HG: high glucose, LG: low glucose, PCS: neonatal fibroblasts, HTS: hypertrophic scar, NBS: non-burn skin.

Metformin treatment alters Snail pathway signaling: Snail is a key regulator of the EMT phenotype that is linked with increased cell motility (94). We determined the effects of metformin on the components of Snail signaling pathway (**Figure 22)**. This included the cadherins which regulate fibroblast adherence. Metformin treatment significantly downregulated Snail and Slug gene expression in HTS fibroblasts (p=0.01 and 0.03) grown in low glucose conditions (5 mM). TWIST was upregulated in PCS and HTS in low glucose conditions (p= 0.01 and 0.007). E-cadherin was downregulated in PCS fibroblasts, but upregulated in NBS under high glucose conditions (p=0.04 for both). No differences were observed in N-cadherin expression. These results indicate that metformin affects TWIST expression regardless of fibroblast source, but the effects on other cell adherence gene expression may be fibroblast source specific.

Figure 23

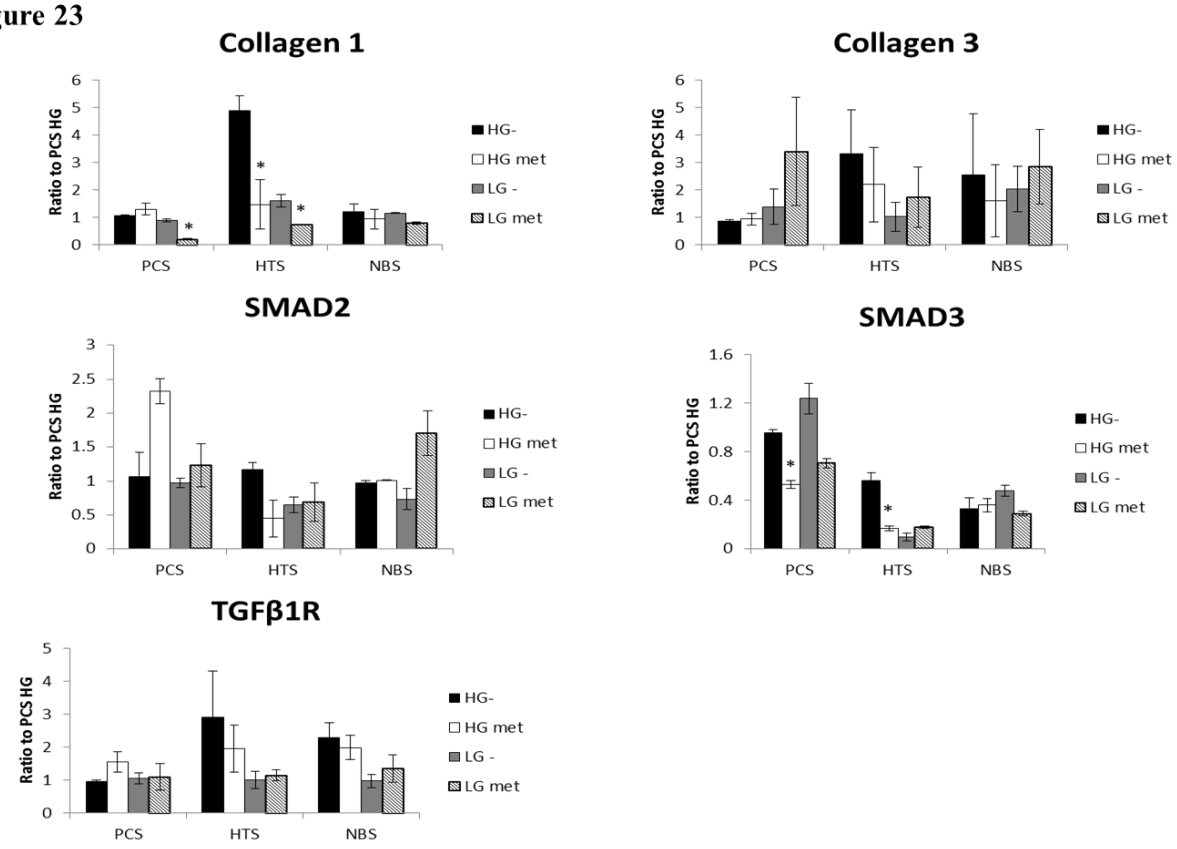


Figure 23: Effects of metformin on the SMAD signaling pathway. Fibroblasts were treated with or without 5 mM of metformin for 24 hours in high glucose conditions, then mRNA was collected and quantitative RT-PCR performed for SMAD2/3, collagen type 1and 3, and TGF-β1 receptor. Results are expressed as means ± SEM, N=3. *: $p < 0.05$ compared to untreated.

Effects of metformin on collagen expression and SMADs: Since SMAD2 and 3 are downstream effectors of TGF-β receptor mediated signaling, which is increased in post-burn scar fibroblasts and modulates collagen deposition (96, 99) we sought to determine whether metformin affected SMAD and collagen gene expression in high or low glucose conditions (**Figure 23)**. SMAD2 was unaffected by metformin in all fibroblast lines. SMAD3 was significantly downregulated by 5 mM metformin in PCS and HTS in high glucose (p=0.01 and 0.02). Downstream collagen 1 was downregulated in PCS in low glucose (p=0.003) and HTS as well (p=0.03 for HG and LG). Collagen 1 was also more highly expressed in HTS than PCS or NBS. Collagen 3 was not altered in any cell type.

Table 3

	PCS		HTS		NBS	
	HG	LG	HG	LG	HG	LG
A-SMA	-	-	-	-	-	-
Collagen 1	-	↓	↓	↓	-	-
Collagen 3	-	-	-	-	-	-
TGFβR1	-	-	-	-	-	-
SMAD2	-	-	-	-	-	-
SMAD3	↓	-	↓	-	-	-
Snail	-	-	-	↓	-	-
Slug	-	-	↑	↓	-	-
TWIST	-	↑	↓	↑	↑	↑
E-cadherin	↓	↓	-	-	↑	-
N-cadherin	-	-	-	-	-	-

Table 3: Effects of metformin on EMT genes in high and low glucose conditions.

TGF-β1 receptor expression was not significantly reduced. This suggests that metformin affects collagen 1 gene expression levels by inhibiting SMAD3 activity. The effects of metformin on EMT gene expression are summarized in **Table 3**.

Figure 24

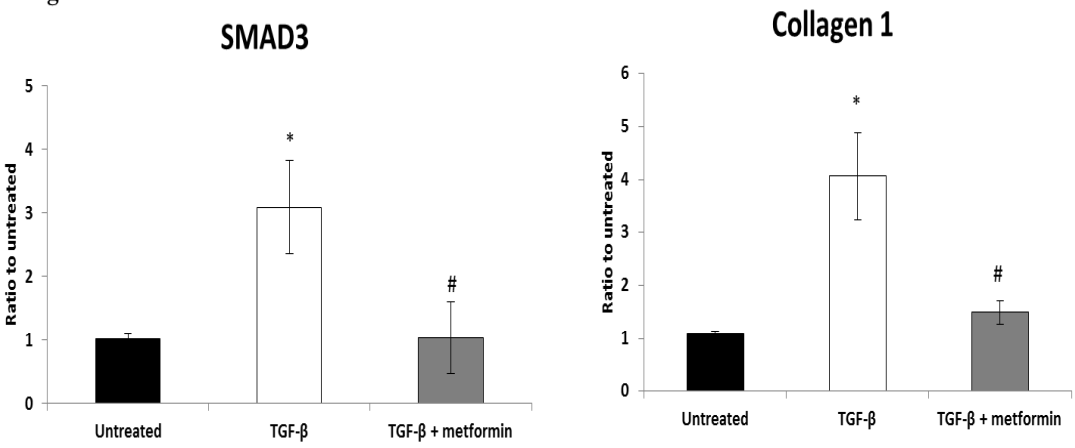


Figure 24: Metformin reverses TGF-β induced SMAD3 signaling. Hypertrophic scar fibroblasts were treated with 10 ng/ml TGF-β for 48 hours ± 5 mM metformin. SMAD3 and collagen 1 gene expression were determined by quantitative RT-PCR. N= 3 ± SEM, *: $p < 0.05$ from untreated, #: $p < 0.05$ from TGF-β.

Metformin reverses TGF-β induced SMAD3 activity: To determine whether metformin can reverse the effects of TGFβ on SMAD3 induced collagen production we treated hypertrophic scar fibroblasts with 10 ng/ml TGF-β in the presence or absence of 5 mM

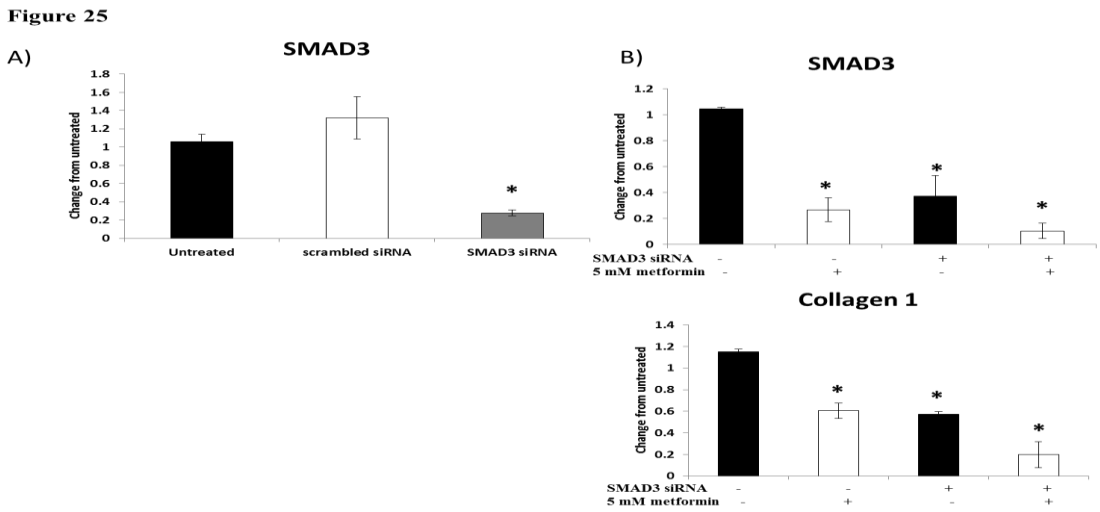


Figure 25: Modulation of collagen type 1 by SMAD3 knockdown. A. Levels of SMAD3 RNA following 24 hours of transfection with SMAD3 siRNA or scrambled control siRNA. B. Effects of metformin and SMAD3 knockdown on collagen 1 expression. HTS fibroblasts were transfected with SMAD3 siRNA or scrambled control for 7 hours, then treated with or without 5 mM metformin for 24 hours. N=3 ± SEM. *: $p<0.05$ compared to control.

metformin for 48 hours (**Figure 24**). We found that TGF-β significantly increased SMAD3 and collagen 1 gene expression in hypertrophic scar fibroblasts (p=0.04 and 0.02). Treatment with 5 mM metformin significantly reduced both SMAD3 and collagen 1 expression (p=0.02 and 0.002). This indicates that metformin is effective in reducing TGFβ induced SMAD3 activation and subsequent collagen 1 expression in burn patient derived hypertrophic scar fibroblasts.

SMAD3 inhibition and metformin reduce collagen 1 expression: To determine whether the effects of metformin on collagen 1 were specific to the SMAD3 signaling pathway we transfected cells with SMAD3 siRNA or scrambled control, and found efficient SMAD3 knockdown (p =0.005) following 24 hours of transfection (**Figure 25A)**. Following 24 hours of 5 mM metformin treatment we observed that treatment with siRNA or metformin reduced SMAD3 gene expression (p=0.013 and 0.050), and greatly reduced by

both treatments (p=0.006). Collagen 1 gene expression was reduced by siRNA or metformin (p =0.022 and 0.019), while combined SMAD3 knockdown and metformin significantly reduced collagen 1 gene expression under high glucose conditions (p=0.017) (**Figure 25B**). This indicates that metformin acts on SMAD3 to reduce collagen 1 gene expression in hyperglycemic conditions, and is augmented by SMAD3 knockdown.

Figure 26

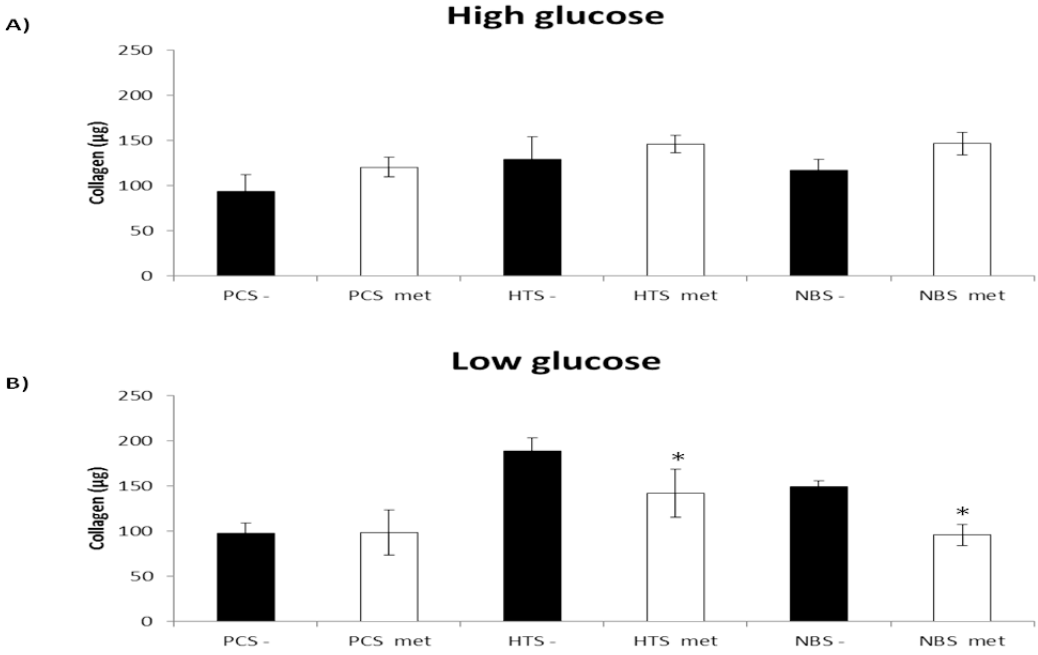


Figure 26: Metformin alteration of fibroblast total collagen secretion. Fibroblasts grown in 12 well plates were treated with or without 5 mM metformin for 24 hours, then total collagen was gathered and analyzed for hydroxyproline expression. Fibroblasts were grown in high (25 mM) glucose (A) or low (5 mM) glucose (B) during treatment. Results are expressed as means ± SEM, N=3. *: $p < 0.05$ compared to untreated.

Effects of metformin on collagen secretion: To determine the effects of metformin on soluble collagen secretion, extracellular collagen content was measured using hydroxyproline expression. Total collagen secretion was determined using Sircol™ Soluble collagen assay (Accurate Chemical and Scientific). As shown in **Figure 26A**

metformin had no effect on fibroblast collagen secretion in high glucose (25 mM), but collagen secretion in HTS and NBS cells was significantly decreased by 5 mM metformin in low glucose conditions ($p = 0.04$ and 0.01, **Figure 26B**). This suggests that while metformin decreases fibroblast collagen secretion the effect is also dependent on glucose concentration.

Discussion

The primary findings of this study are that EMT in post-burn hypertrophic scar is increased over time, possibly due to increased inflammation in the extracellular environment, that metformin downregulates FSP1 in HTS, metformin alters Snail signaling, downregulates SMAD3 and downstream collagen 1 gene expression, and metformin affects total cellular collagen secretion in low glucose conditions. The TGF-β-SMAD signaling pathway is a major contributor to collagen secretion following burn injury, and it is modulated by inflammation that often accompanies injuries (98, 159). Dermal fibroblasts derived from burn patient skin biopsies respond differently than neonatal derived controls under a variety of treatment conditions. We have identified that metformin regulates SMAD3 in a negative manner, which suggests that metformin inhibits downstream collagen type 1 gene expression. Collagen I is highly expressed during both EMT and fibrosis (116), and metformin may be a strategy to consider for downregulating the expression of collagen. This correlates with previous data that metformin inhibits cell proliferation through inhibition of mTOR, and decreases collagen synthesis via SMAD downregulation (104, 127). Metformin lowers collagen type 1 gene

expression in burn patient derived fibroblasts, which suggests that metformin might have therapeutic effects in preventing hypertrophic scarring by reducing collagen secretion or fibrosis.

One of the main findings of this study is that metformin lowers FSP1 and N-cadherin expression in post-burn hypertrophic scars**.** FSP1 drives fibroblast differentiation and promotes collagen secretion in many pathologic states, such as renal fibrosis and inflammation (115, 158). E-cadherin and N-cadherin were not affected, which is likely due to epithelial hypertrophy in the tissue compensating for local EMT and E-cadherin loss (160). Our data suggests that metformin reduces the EMT phenotype in HTS, preventing fibroblast differentiation and subsequent collagen production. Since EMT phenotype appears to progress with time following burn injury, it may be beneficial to begin metformin treatment immediately following burn injury to prevent both metabolic dysfunction and EMT progression. We also demonstrated that metformin is effective in reducing TGF-β induced SMAD3 and collagen 1 gene expression, suggesting that metformin can be effective in reducing stress induced collagen expression by attenuating SMAD3 pathway signaling. An interesting finding is that metformin only decreased fibroblast collagen secretion in low glucose conditions. This suggests that the effects of metformin on collagen secretion are dependent on extracellular glucose levels.

The wound microenvironment is highly dynamic, comprised of myofibroblasts and high levels of inflammatory cytokines such as TGFβ. It has been shown that pediatric burn wounds are characterized by increased presence of immature fibroblasts (161), which are induced to differentiate in response to stress. Our data suggests that metformin might be detrimental to collagen deposition by preventing fibroblast differentiation by

downregulation of FSP1. It is possible that AMPK activation by metformin is responsible for reducing fibroblast proliferation by inhibition of mTOR, and collagen secretion is regulated by SMAD3 activity, making it a possible therapeutic option for preventing limb paralysis. Other studies have linked AMPK with decreases in TGF-β induced fibrosis in renal and cardiac disease (127, 157), so it appears that AMPK acts on both metabolic and inflammatory pathways in wound healing.

In conclusion we have shown that metformin is a regulator of EMT in burn patient derived fibroblasts, and can have clinical use as an inhibitor of collagen gene expression. Further studies will determine the role of metformin on *in vivo* models to determine its effects on whole body EMT and wound healing.

Chapter 4: Conclusions and Significance

A major finding of this study is that metformin increased fibroblast AMPK while decreasing mTOR phoshorylation. This is consistent with other studies that link metformin with increased AMPK and lower cell proliferation due to decreased mTOR (43, 118, 120, 121). I also demonstrate that metformin decreases real time fibroblast proliferation and migration due to decreased mTOR. Metformin has been used as an adjunct cancer therapy and improves clinical outcomes when combined with glucose withdrawal, mostly through inhibiting cancer cell proliferation, inflammatory cytokines such as IL-6 and TNFα, along with growth promoting IGF-1 activity (51, 117, 122-126). Although it is uncommon, some studies have linked an increase in liver and throat cancers in only female burn patients, which typically occur 5-20 years after the initial injury (162, 163). We demonstrate that metformin reduces FSP-1 expression in burn patient HTS samples, so metformin administration might prevent long term cancer risk in some burn patients, likely due to its known effects on reducing oxidative stress induced DNA damage and inflammation (126, 133). While metformin might not be useful in reducing cancer associated EMT, it has profound effects on wound healing induced EMT. Vimentin and FSP-1 expression are increased over time following burn injury, so it is imperative to impede EMT progression in long term burn injury therapy. In addition to reducing FSP-1 expression in burn patient tissues, we show that metformin reduces E-cadherin and Slug pathway gene expression in fibroblasts,

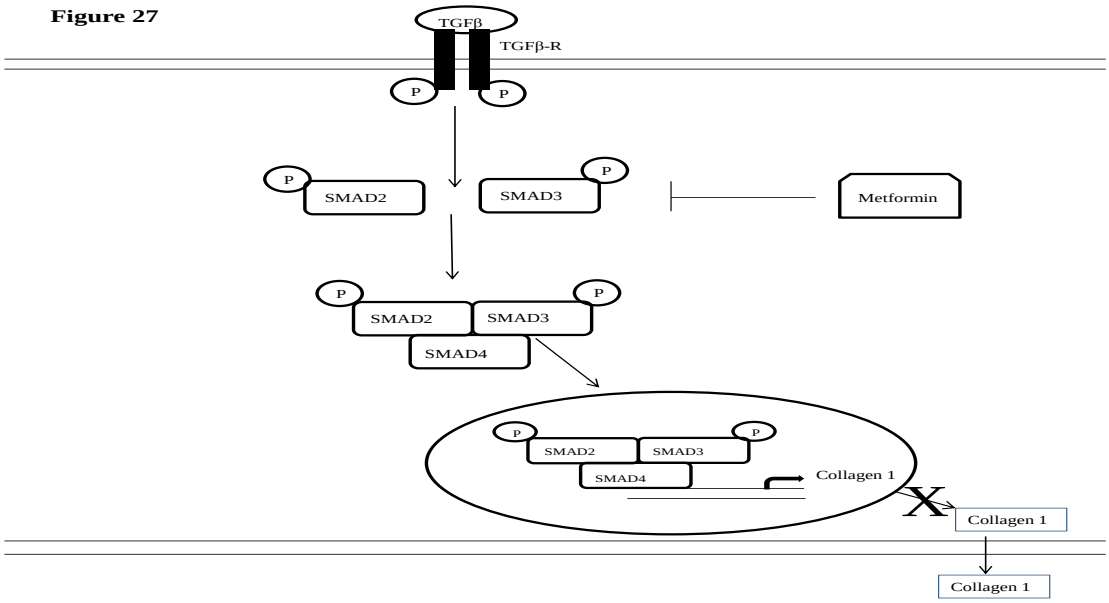


Figure 27: Inhibition of SMAD3 induced collagen 1 secretion by metformin. TGFβ binds to its receptor and promotes phosphorylation of SMAD 2 and 3, which form a complex with SMAD4 and translocate to the nucleus to promote collagen gene transcription. Metformin downregulates gene expression of SMAD3, preventing subsequent collagen 1 transcription and secretion.

decreases SMAD3 and collagen 1 gene expression, and decreases intracellular cAMP. cAMP dependent kinase has been linked with hypoxia induced EMT and invasiveness in lung cancer cells (164), and cAMP upregulates TGFβ receptor expression in breast cancer (165). The effects of metformin on reducing available cAMP likely inhibit fibroblast migration. Further experiments are needed to determine if hypoxia alters EMT gene expression, and if metformin can alter cell proliferation and migration in hypoxic environments.

The SMAD pathway is the major inducer of collagen secretion from fibroblasts (**Figure 27**). SMAD 2 and 3 induced by TGFβ, form a complex with SMAD4, and then translocate to the nucleus where it promotes collagen gene expression (166). Our results show that metformin downregulates SMAD3 and downstream collagen 1 gene expression in HTS fibroblasts. Metformin also reduced TGF-β induced collagen expression by

reducing SMAD3 . This suggests that metformin can be used to prevent excessive collagen type 1 gene expression in burn patents by selectively inhibiting SMAD3. We also found that metformin only reduced total collagen secretion in low glucose conditions, indicating that extracellular glucose status is an important regulator of collagen secretion. Further studies are needed to determine if metformin works best in synergy with other glucose controlling strategies such as insulin to control both hyperglycemia and dysregualted collagen secretion in burned patients.

Metformin increased AMPK activity and decreased mTOR, resulting in in lower cell proliferation, but not increases in 2-deoxy glucose uptake, suggesting that the AMPK increases following metformin treatment act on cell survival and proliferation pathways rather than glucose transport in fibroblasts. OCT gene expression was not altered by metformin, confirming that the effects of metformin are due to modulations in cellular metabolic activity, not increased transporter expression. Another important finding is that metformin increased mitochondrial complex I in burn patient derived fibroblasts but not HepG2 hepatocytes. Mitochondrial dysfunction is a common feature of both insulin resistance and burn injury, prompting increases in catecholamine release that prompt conversion of white adipose tissue to more metabolically active brown adipose and increases in resting energy expenditure (167, 168). In muscle burn injury leads to increased uncoupling protein 1 (UCP1) that results in uncoupling of citric acid cycle flux to ATP production in muscle directly affected by burn injury, which causes decreased ATP production, reduced state 2,3, and 4 respiratory function that can last up to 2 years post injury, and increased apoptosis and caspase expression (67, 68). Interestingly burn injury does not lead to lower mitochondrial numbers in muscle cells, as burns over 30%

of total body surface area lead to increased mitochondrial mass and hypermetabolism measured by increased resting energy expenditure (67, 167). Burn injury leads to an increased number of mitochondria with more energy expending activity but less effective oxygen utilization, leading to increased thermogenesis in muscle and adipose tissue (68). To date all studies looking at post burn injury mitochondrial function have been in muscle and adipose tissue; this is the first study using burn patient derived fibroblasts. Although most studies have linked metformin with reductions in mitochondrial complex I, we observed increased in oxygen utilization with metformin treatment. Metformin significantly increased oxygen flux, and slightly increased oxidative phosphorylation and reduced total oxygen consumption. Oxygen consumption was lower in non-burned patient derived PCS fibroblasts that HTS or NBS, indicating that burn injury impairs fibroblast oxygen utilization as in muscle and adipose tissue. This effect was not due to changes in mitochondrial numbers, but Mfn2, a major regulator of mitochondrial fusion (169) was increased by metformin treatment. ATP production was also slightly increased. It can be concluded that metformin treatment results in more efficient utilization of available oxygen by fibroblast mitochondria without affecting cellular mitochondrial mass. Another interesting observation is that both metformin and AMPK inhibition by Compound C increased oxygen flux. This agrees with previous studies that metformin activity is not dependent on its effects on AMPK (74), and AMPK inhibition can be beneficial for promoting improved mitochondrial function, possibly through less dependence on glycolysis to drive ATP production. There was no synergy of AMPK inhibition and metformin treatment, so metformin appears to be directly affecting

mitochondria by affecting membrane permeability and associated proton flow, along with impeding caspase dependent apoptosis (76).

Figure 28

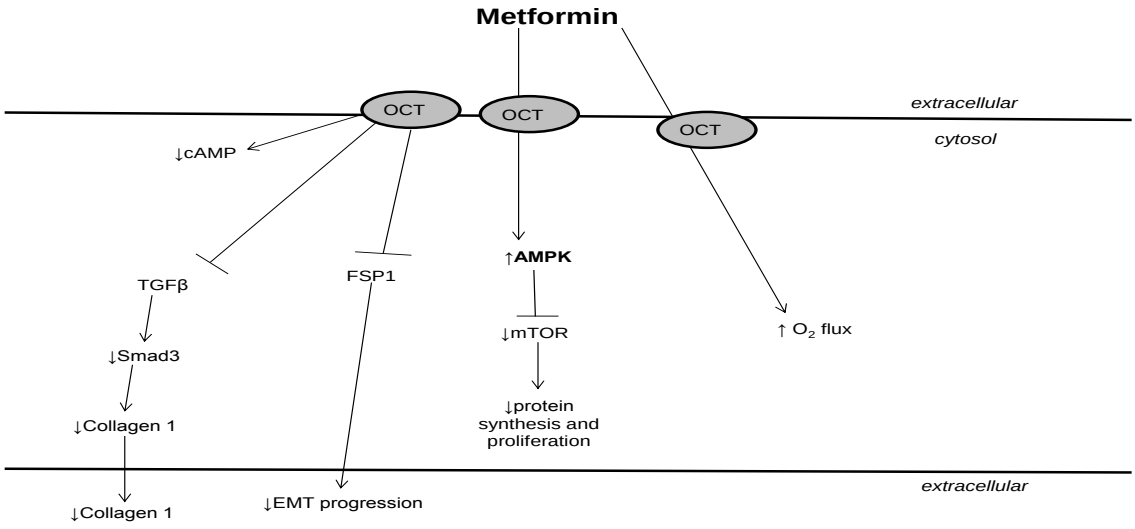


Figure 28: Cellular pathways affected by metformin in fibroblasts. Metformin is transported by OCTs and reduces intracellular cAMP, FSP1,and SMAD3 activity. Metformin increased AMPK while decreasing mTOR-regualted cellular proliferation. Metformin also acts to increase oxygen flux independently of AMPK.

The main pathways affected by metformin treatment in burn patient derived fibroblasts are shown in **Figure 28**. Following OCT-mediated transport metformin acts to reduce cAMP accumulation, FSP1 expression, and SMAD3 transcription, leading to decreased collagen 1 gene expression and secretion. Metformin acts on AMPK to reduce mTOR-regulated cell proliferation and migration, but does not consistently affect glucose transporter activity in fibroblasts. Metformin also acts on mitochondria independently of AMPK to increase complex I activity and ATP production. These results demonstrate that metformin has many functions on fibroblast metabolic and EMT activity that are distinct from other cell types.

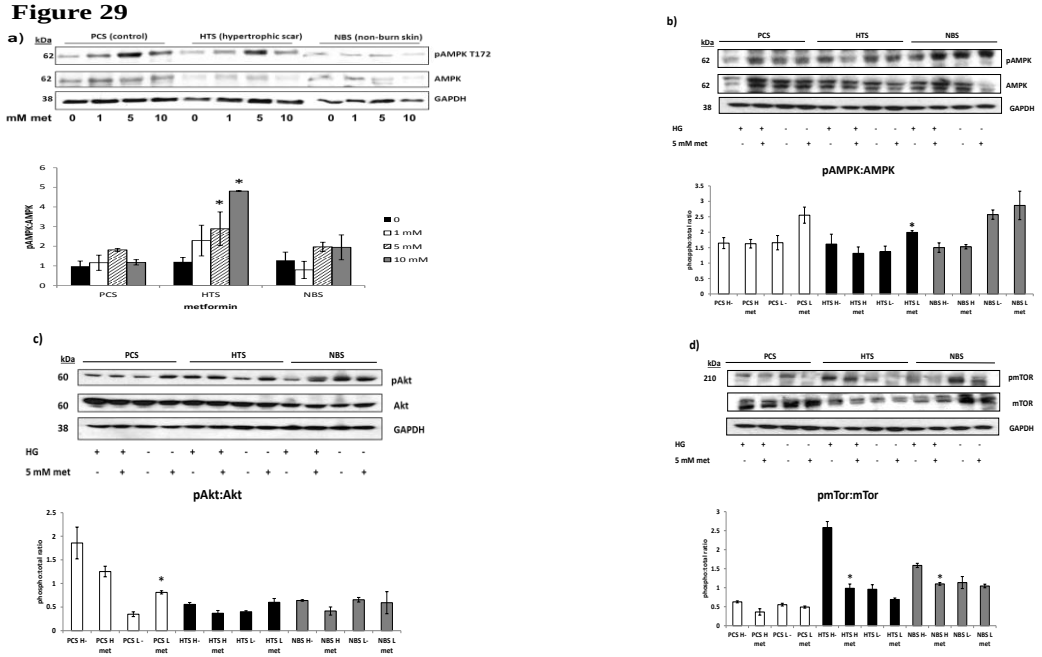
Although our results have shed much insight into the mechanisms regulating metformin action in burn patient derived fibroblasts, many questions remain about how the *in vitro* data will lead to improved burn patient therapies. Future studies will need to determine if metformin treatment will improve wound healing in rodent burn injury models by measuring wound closure and collagen content. These studies should also include analysis of burned and non-burned skin samples to determine their mitochondrial oxidative activity, and if the complex 1 inducing effects of metformin observed in isolated fibroblasts also occur in intact skin samples. In order to determine if full body AMPK inhibition affects metformin activity future studies should utilize AMPK knockout mice to compare the effects of metformin on mTOR expression, glucose utilization, and mitochondrial activity with wild type. It will be necessary to obtain muscle, fat, and skin tissue samples from patients enrolled in long term metformin trials to compare AMPK activity, EMT marker expression, and mitochondrial content. These results will confirm the tissue specific effects of metformin and how burn injury affects them compared to normal individuals. It will also be necessary to determine if metformin has different effects in pediatric patients compared to adults, which has been shown in several other therapies such as insulin (32, 147).

In conclusion we have shown that metformin is a potential therapeutic option to increase AMPK activity, promote more efficient mitochondrial oxygen utilization and complex 1 activity under hypermetabolic conditions, and reduce wound induced EMT and collagen deposition by reducing SMAD3 in fibroblasts. Future experiments will determine the *in vivo* effects of metformin

Appendix A: Comparison of Metformin Effects on Metabolic Activity in High and Low Glucose Conditions

Metformin affects fibroblast AMPK signaling: Since metformin has previously been shown to enhance glucose uptake by upregulation of AMPK signaling in muscle and hepatocytes (27, 35, 137), we examined the effects of metformin on fibroblast AMPK activity. Metformin increased AMPK activity in fibroblasts, particularly HTS (**Figure 29A**). We chose to use 5 mM metformin for subsequent studies. Phosphorylation of AMPK was upregulated by 5 mM metformin in HTS fibroblasts in low glucose conditions (**Figure 29B**). Increased Akt activity was observed in PCS fibroblasts treated

Figure 29



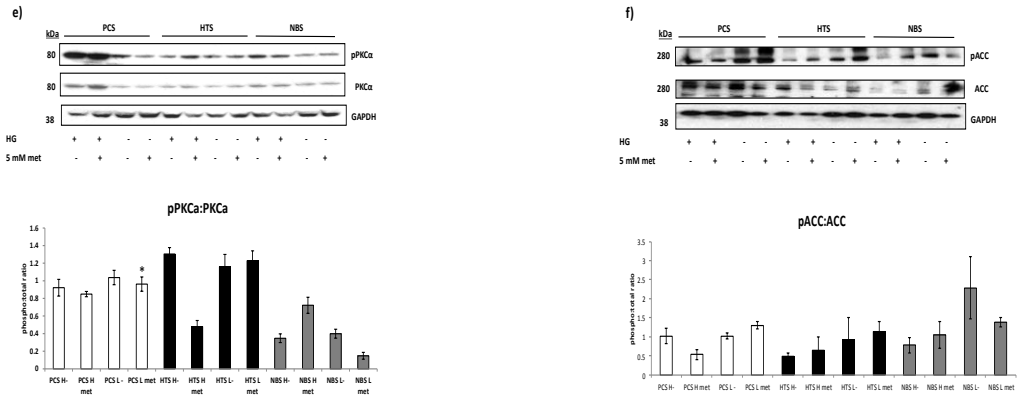


Figure 29: Effects of metformin on AMPK associated signaling in HG and LG conditions. a).Metformin upregulates AMPK activity in fibroblasts in a dose-dependent manner. Graph compares ratio of phosphorylated to total AMPK; results are from 3 independent experiments ± SEM. b-e). Effects of 5 mM metformin on phosphorylation of b)AMPK, c) Akt, d) mTor and e) PKCα. Fibroblasts were grown in media containing high glucose (25 mM) or low glucose (5 mM) for. Results are

with metformin (**Figure 29C)**. Metformin downregulated mTOR activation in HTS and NBS fibroblasts in high glucose conditions (**Figure 29D**). Metformin also downregulated PKCα in HTS fibroblasts in high glucose conditions (**Figure 29E**). No significant changes in ACC phosphorylation were observed (**Figure 29F**). These results indicate that metformin increases AMPK activity in fibroblasts, and might inhibit cell proliferation through mTOR inhibition.

Figure 30

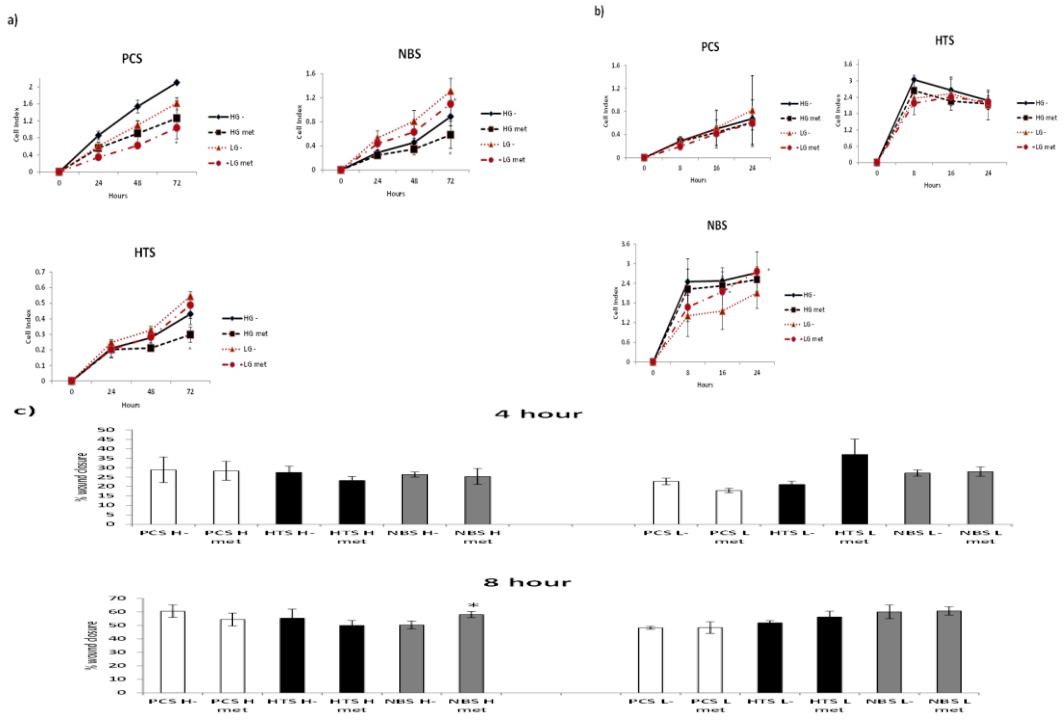


Figure 30: Metformin alters real time fibroblast proliferation and wound healing in HG and LG conditions. a). Effects of metformin on fibroblast proliferation determined by xCELLigence . *: p<0.05, **: p<0.001 compared to untreated. N=3 SEM. b). Fibroblast migration measured using xCELLigence. *: p<0.05, **: p<0.001 compared to untreated. N=3 SEM. c). Effects of metformin on wound healing. Wound healing was observed over 8 hours and percent closure determined by comparing to the initial time point. Results are from 4 independent experiments ± SEM. *: p<0.05 compared to untreated controls analyzed by Student's t-test.

Metformin impedes cell proliferation: Our previous data showed inhibition of mTOR with metformin treatment under high glucose conditions (**Figure 29C**). To observe the effects of metformin on cell proliferation we treated cells with or without 5 mM metformin and monitored proliferation using the xCELLigence system. In all cell lines we observed significant inhibition of proliferation with metformin treatment over 72 hours in all cell lines (for PCS p= 0.01 LG, for HTS p =0.01, for NBS p = 0.02 HG, 0.004 LG). HTS fibroblasts also displayed lower proliferation index values than non-burn and control lines (**Figure 30A**). Metformin did not significantly alter fibroblast migration

over 24 hours, but migration was significantly increased in NBS over 24 hours in LG conditions (0.02) (**Figure 20B**). This suggests that while metformin inhibits fibroblast proliferation, it might promote cell migration and wound healing.

Scratch assays were used to measure cell migration and wound closure over 8 hours (**Figure 30C**). At 8 hours metformin slightly increased wound healing in NBS cells in high glucose (p =0.022). This suggests that metformin does not greatly augment wound healing in high or low glucose conditions.

Regulation of mitochondrial oxidative phosphorylation by metformin is dependent on cell type: Fibroblasts were treated with or without 5 mM metformin, and mitochondrial oxygen consumption was measured using Oxygraph 2k (Oroboros Instruments, Innsbruck, Austria). Under low glucose conditions metformin upregulated oxygen consumption in HTS and NBS dermal fibroblasts (p=0.01), and PCS was upregulated under high glucose conditions (p=0.04), (**Figure 31A and B**). Inhibition of mitochondrial complexes II-V demonstrated upregulation of oxygen flux in PCS, HTS and NBS dermal fibroblasts under both high and low glucose conditions (p = 0.02, 0.03, and 0.009 in HG, p = 0.03, 0.01, and 0.03 in LG) following metformin treatment (**Figure 31C**). HepG2 hepatocytes treated with or without 5 mM metformin were used as a positive control. A significant decrease in complex I activity was observed in HepG2 cells (p =0.007).

Figure 31

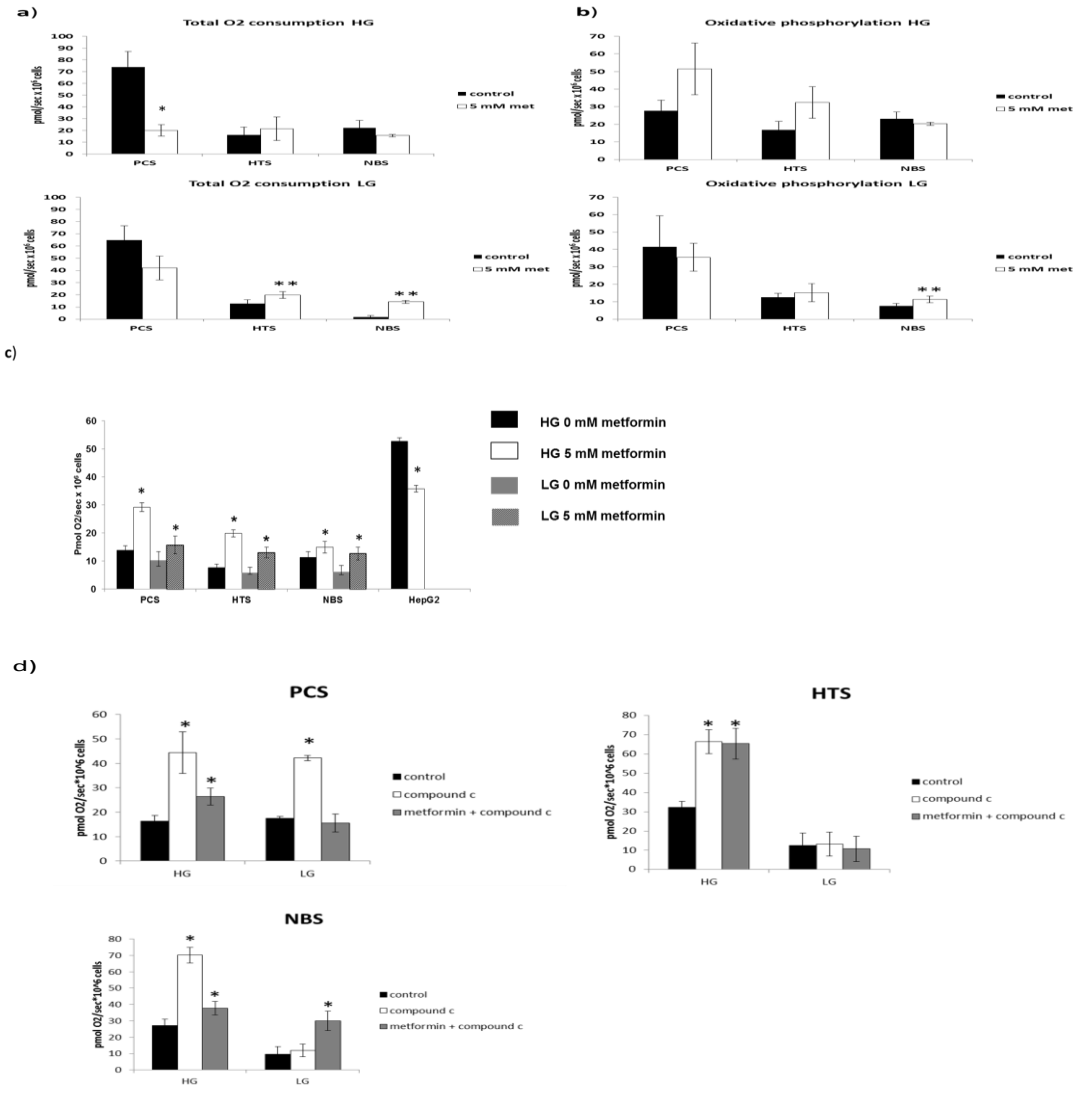


Figure 31: Metformin alters fibroblast mitochondrial activity in HG and LG conditions. Total oxygen consumption was measured in a), oxidative phosphorylation in b). and oxygen flux in c) using Oxygraph 2k (Oroboros Instruments). Results are from 3 independent experiments ± SEM. *: $p<0.05$, **: $p<0.01$ compared to untreated controls analyzed by Student's t-test. d). Effects AMPK inhibition on complex I activity. Fibroblasts were treated ± 5 μM of AMPK inhibitor Compound C for 24 hours, then ± 5 mM metformin for 24 hours before analysis using Oxygraph 2k. Results are from 4 independent experiments, * :$p < 0.05$ using one way ANOVA.

To determine if the effects of metformin on fibroblast oxygen utilization are dependent on AMPK activity we treated fibroblasts with 5 μM of AMPK inhibitor Compound C 24 hours prior to metformin treatment (**Figure 31D**). We observed that Compound C alone and combined with metformin increased oxygen flux activity in PCS fibroblasts under

high glucose ($p = 0.007$ and 0.014), while Compound C increased oxygen flux in low glucose ($p=0.04$). Under high glucose conditions Compound C alone and with metformin increased oxygen flux in burn patient derived fibroblasts ($p = 0.037$ and 0.025 in HTS, $p= 0.01$ in NBS). Under low glucose conditions Compound C and metformin increased oxygen flux in NBS ($p = 0.03$). These results indicate that both metformin and AMPK activity regulate oxygen flux, and the effects of metformin may be independent of AMPK activation.

Effects of metformin on mitochondrial biogenesis: To determine whether the effects observed in mitochondrial oxidative phosphorylation were due to increases in mitochondrial biogenesis total cellular mitochondria was assessed by treating fibroblasts with 75 nM MitoTracker Green™ following 5 mM metformin treatment. A slightly significant effect ($p = 0.043$) in reduction of total mitochondria in NBS cells grown under low glucose conditions (**Figure 32A**), suggesting that metformin does not alter cellular mitochondrial production in fibroblasts.

Since burn injury has been linked with mitochondrial dysfunction (2), we measured several genes related to mitochondrial function and insulin utilization (78, 138-140) to determine whether metformin impacted fibroblast mitochondria. Metformin decreased PGC-1α gene expression under high glucose conditions ($p = 0.02$), but increased PGC-1α gene expression in low glucose conditions for PCS and HTS fibroblasts ($p = 0.01$). Porin was upregulated by metformin in NBS in low glucose, and Mfn2 upregulated in PCS and NBS in high glucose conditions (**Figure 32B**). PGC1-α and Mfn2 levels were also affected, indicating that burn injury affects mitochondrial function in fibroblasts in and outside of the initial injury site (**Figure 32B**). These results suggest that while metformin

does not alter total mitochondrial production in fibroblasts, burn injury affects the transcription of mitochondrial biogenesis genes, which is further attenuated by metformin and glucose levels.

Figure 32

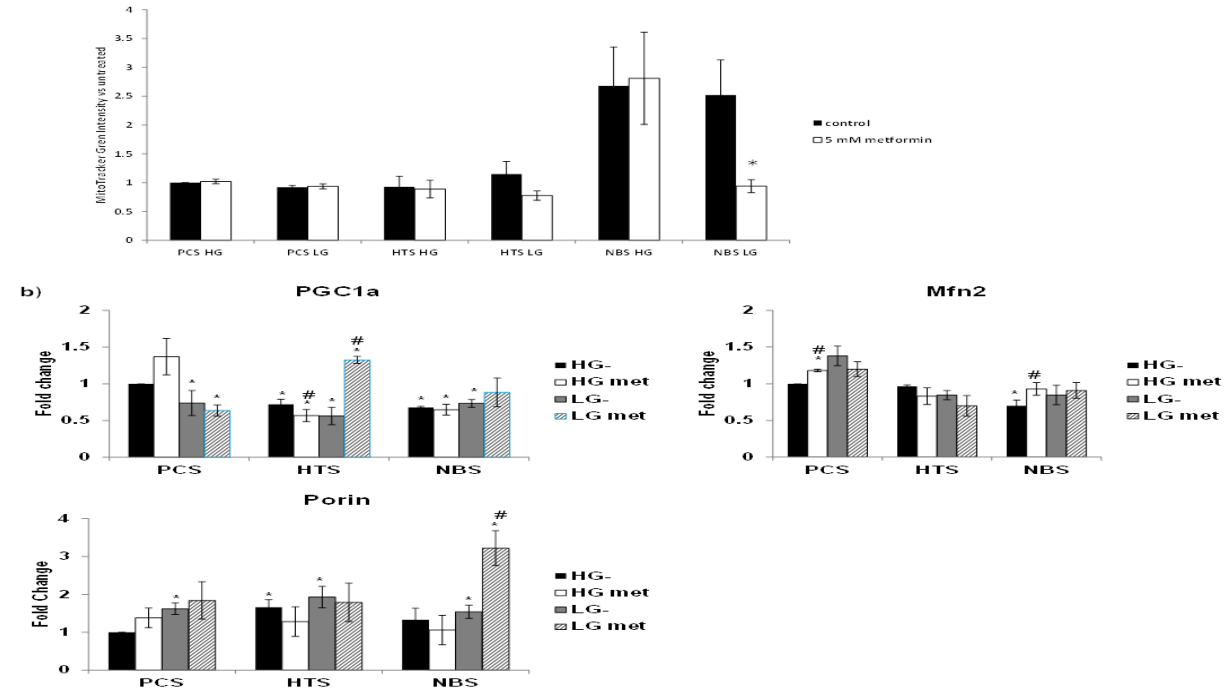


Figure 32: Effects of metformin on mitochondrial proliferation in HG and LG conditions. a). Total mitochondrial proliferation measured by MitoTracker Green™. Results are from 3 independent experiments ± SEM. b). Expression of PGC-1α, porin, and Mfn2 mitochondrial biogenesis genes measured by quantitative real time PCR. *: $p<0.05$ compared to untreated controls analyzed by Student's t-test, #: $p<0.05$ compared to PCS high glucose. N=3 ± SEM.

Metformin lowers fibroblast ATP production: Existing data suggests that metformin lowers cellular energy status by reducing ATP production through complex I inhibition in hepatocytes (27), but might raise ATP production in muscle (75). To determine the effects of metformin on fibroblast ATP production we obtained lysates from cells treated with or without 5 mM metformin and analyzed them for ATP content. Metformin decreased ATP production in PCS and HTS fibroblasts only under low glucose conditions ($p = 0.009$ for PCS, $p = 0.026$ for HTS) (**Figure 33**).

Figure 33

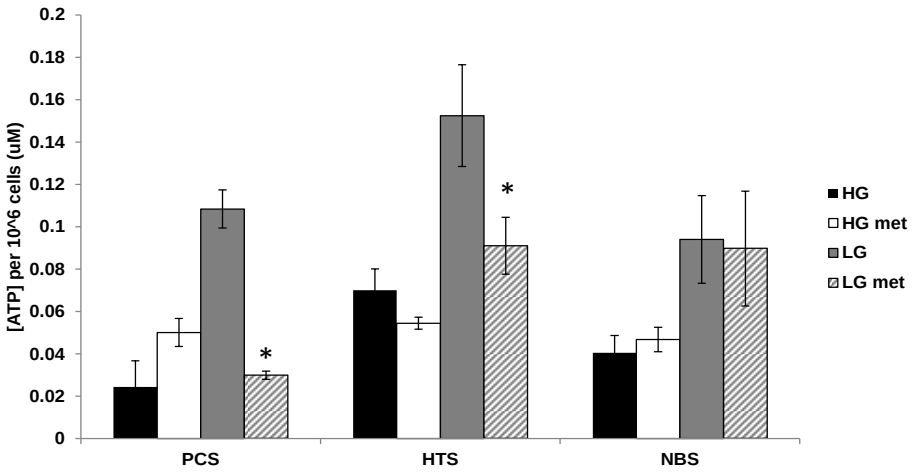


Figure 33: Effects of metformin on ATP production in HG and LG conditions. Intracellular ATP content was measured using ATP Assay kit (Abcam) and readings measured at 572 nm. Results are from 3 independent experiments ± SEM. *: $p<0.05$ compared to untreated controls analyzed by Student's t-test.

AMPK inhibition does not alter collagen secretion: We previously found that metformin reduced total collagen secretion in low glucose conditions (**Figure 26**). To determine if the effects of metformin on collagen secretion were dependent on AMPK we treated fibroblasts ± 5 μM Compound C for 24 hours, followed by 5 mM metformin for an additional 24 hours in low glucose (5 mM) conditions. No significant effects on total collagen secretion were observed **(Figure 34)**, indicating that collagen secretion was regulated solely by SMAD3 activity independent of AMPK.

Figure 34

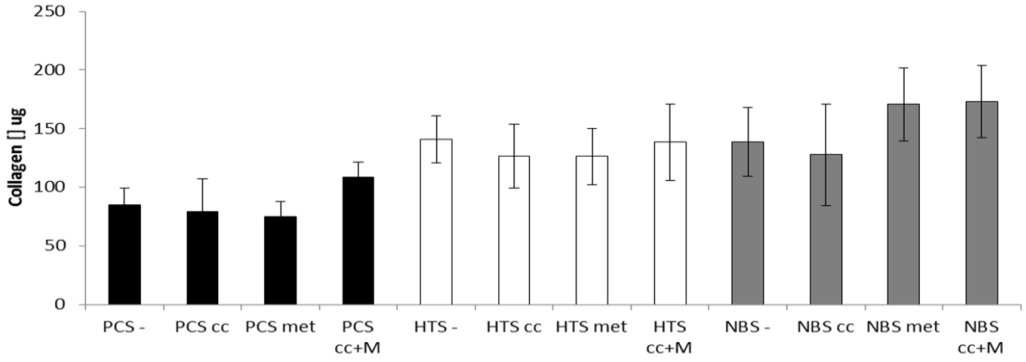


Figure 34: Effects of AMPK inhibition on collagen secretion. Fibroblasts were grown in low glucose conditions, treated ± 5 µM Compound C for 24 hours, then ± 5 mM metformin and subjected to collagen isolation, concentration, and analysis. N= 3 ±SEM

Metformin does not affect glucose transporter expression: We next determined if metformin treatment affected glucose transporter (GLUT1) gene expression (**Figure 35**). No significant changes were observed in GLUT1 gene expression with or without metformin, suggesting that metformin does not increase glucose transporter expression in fibroblasts.

Figure 35

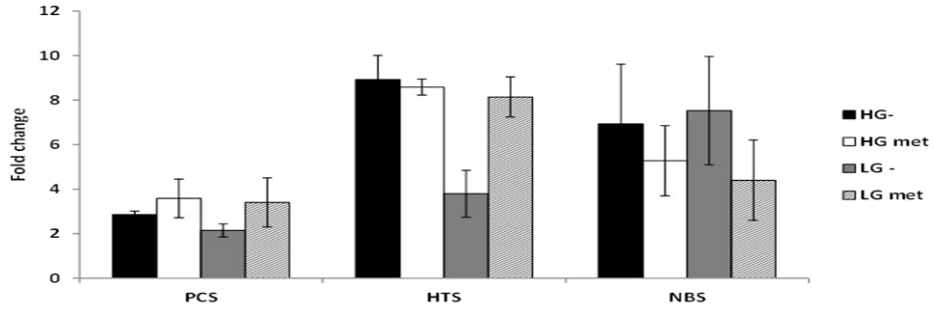


Figure 35: Effects of metformin on GLUT1expression. Fibroblast GLUT1 mRNA levels measured by quantitative real time PCR. N=3 ± SEM.

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169. van der Bliek AM, Shen Q, Kawajiri S. Mechanisms of mitochondrial fission and fusion. *Cold Spring Harb Perspect Biol* 2013;5(6).

Vita

Michael David Wetzel was born on August 11, 1979, to David and Patricia Wetzel in Grand Rapids, MI. He spent most of his life in San Antonio, TX. The following is a listing of his accomplishments:

Education

2011-present: Ph.D. candidate: University of Texas Medical Branch at Galveston. Cell Biology graduate program.

2006: M.S.: Cellular and Structural Biology. University of Texas Health Science Center at San Antonio. May 2006.

2002: B.S.: Biomedical Science. Texas A&M University, College Station, TX. December 2002. GPR: 3.45

Research Experience

- 2012-present: Graduate Assistant in Department of Cell Biology and Shriners Hospital for Children, UTMB. Mentor: Celeste Finnerty, PhD.
- 2013-present: Associate Faculty member for Critical Care and Emergency Medicine F1000
- 2009-2011: Research Assistant at Baylor College of Medicine, Houston, TX. Supervisor: Dario Marchetti, Ph.D.
- 2009-2011: Head technician of circulating tumor cell core facility at Baylor College of Medicine
- 2006-2008 : Senior Research Assistant in UTHSCSA Department of Vascular Surgery. Supervisor: Paula K. Shireman, M.D.
- 2003-2006: M.S student at University of Texas Health Science Center at San Antonio. Mentor : Lily Q. Dong, Ph.D.
- 2005: Twice served as *ad hoc* reviewer for *American Journal of Physiology.*

- 2002: Texas A&M University: Department of Veterinary Anatomy and Public Health. I worked on a project which involved determining immune responses to different antibiotics in bovine milk samples.

Awards and Honors.

- Cell Biology Graduate Program Outstanding Service Award Third Place, 2016
- Sponsored membership in AAAS/Science Program for Excellence in Science, 2016
- GSBS Associates Travel Award Scholarship, 2015
- Cell Biology Graduate Program Outstanding Service Award First Place, 2015
- Janet and David Niesel Scholarship Endowment, 2014
- Wound Healing Society Foundation Travel Scholarship, 2014.
- Armand J. Guarino Award for Excellence in M.S. Studies, 2006.
- Excellence in M.S. Studies Award in Cellular and Structural Biology, 2006.
- Poster presentation in UTHSCSA Annual Graduate Student Symposium, 2006.
- Best M.S. Student Poster Presentation: UTHSCSA Cellular and Structural Biology Departmental Retreat, 2005.
- Best M.S. Student Poster Presentation: UTHSCSA Cellular and Structural Biology Departmental Retreat, 2004.
- Named to Educational Communications Inc. *The Chancellor's List* for outstanding graduate students, 2004.
- Inducted into Beta Beta Beta National Biological Sciences Honor Society,2002
- Inducted into Texas A&M University Chapter of Phi Eta Sigma Undergraduate Honor Society, 1999.

Publications

- Prasai A, El Ayadi A, Mifflin RC, **Wetzel MD**, Andersen C, Redl H, Herndon DN, Finnerty CC. Characterization of Adipose Derived Stem Cells Following Burn Injury. Accepted by *Stem Cell Reviews and Reports*, 2017.
- Mascarenhas D, El Ayadi A, **Wetzel M.D.**, Prasai A, Mifflin R, Jay J.W., Herndon D.N, Finnerty C. C. Effects of the Nephrilin Peptide on Post-Burn Glycemic Control, Renal Function, Fat and Lean Body Mass, and Wound Healing. Accepted by *International Journal of Burns and Trauma*, 2016.
- Melton D.W., Roberts A.C., Wang H, Sarwar Z., **Wetzel M.D.**, Wells J.T.,Porter L., Berton M.T., McManus L.M., Shireman P.K.. Absence of CCR2 results in an inflammaging environment in young mice with age-independent impairments in muscle regeneration. Accepted by *Journal of Leukocyte Biology*, 2016.
- Wang, CZ, El Ayadi, A, Prasai, A, **Wetzel, MD**, Finnerty, CC, Enkhbaatar, P, Herndon, DN, Ansari, NH. Amelioration of Burn Induced Inflammation and Epithelial Cell Death in Rats by Topically Applied Metal Chelator. Submitted 2016.
- Zhang L, Ngo JA, **Wetzel MD**, Marchetti D. Heparanase mediates a novel mechanism in lapatinib-resistant brain metastatic breast cancer. *Neoplasia* 2015 17(1): 101-113.
- Zhang L, Ridgway LD, **Wetzel MD**, Ngo J, Yin W, Kumar D, Goodman JC, Groves MD, Marchetti D. The identification and characterization of breast cancer CTCs competent for brain metastasis. *Sci Trans Med* 2013 15(180):180ra48.
- Ridgway, LD, **Wetzel, MD**, Ngo, JA, Epstein, AE, Marchetti, D. Heparanase - induced GEF-H1 signaling regulates the cytoskeletal dynamics of brain metastatic breast cancer cells. *Mol Can Res* 2012; (6):689-702.
- Ridgway LD, **Wetzel MD**, Marchetti D. Heparanase Modulates Shh and Wnt3a Signaling in Human Medulloblastoma Cells. *Exp Ther Med*. 2011 2(2):229-238.
- Ridgway LD*, **Wetzel MD***, Marchetti D. Modulation of GEF-H1 induced signaling by heparanase in brain metastatic melanoma cells. *J Cell Biochem*. 2010 111(5):1299-309. *Contributed equally to this work
- Liu M, Zhou L, Xu A, Lam KS, **Wetzel MD**, Xiang R, Zhang J, Xin X, Dong LQ, Liu F. A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. *Proc Natl Acad Sci* U S A. 2008 Nov 25;105(47):18302-7.

- Wang, C., Mao, X., Wang, L., Liu, M., **Wetzel, MD**., Guan, K., Dong LQ., and Liu, F. Adiponectin Sensitizes Insulin Signaling by Reducing p70 S6 Kinase-mediated Serine Phosphorylation of IRS-1. *J Biol Chem* 2007 282, 7991-7996.

Pending publications

- **Wetzel M.D.**, Porter C., El Ayadi A., Bergmann J.S., Prasai A, Herndon D.N., Finnerty C.C.. Metformin alters fibroblast metabolic activity and mitochondrial oxidative phosphorylation. Manuscript to be submitted to *Wound Healing and Regeneration*, 2016.
- **Wetzel M.D.,** Jay J.W., El Ayadi A., Prasai A., Herndon D.N., Finnerty C.C. Metformin induced alteration of fibroblast epithelial-mesenchymal transition (EMT) in burn patient derived fibroblasts. Manuscript in preparation.
- **Wetzel M.D**., Herndon D.N, Finnerty C.C .Metformin and Burn Injury: A Review. .Review Article

Abstracts and Invited Presentations

- **M.D. Wetzel,** D.N. Herndon, C.C. Finnerty. Metformin and AMPK inhibition modulate mitochondrial activity in burn patient derived fibroblasts. Poster presentation at American Burn Association Symposium, May 2016. Las Vegas, NV
- **M.D. Wetzel,** A. El Ayadi, D.N. Herndon, C.C. Finnerty. Role of oxandrolone in androgen and glucocorticoid receptor signaling in fibroblast and myoblast cell lines. Oral presentation at Wound Healing Society Symposium, April 2016. Atlanta, GA
- **M.D. Wetzel,** D.N. Herndon, C. C. Finnerty. Effects of metformin and AMPK inhibition on burn patient derived fibroblast mitochondrial activity and collagen secretion. Poster presentation at Poster Presentation at Annual Forum for Translational Medicine, March 2015. Galveston, TX
- **M.D. Wetzel,** D. Scruggs, D.N. Herndon, C.C. Finnerty. Fenofibrate alters AMPK signaling pathways and cell proliferation in dermal fibroblasts. Poster presentation at Wound Healing Society Symposium, April 2015. San Antonio, TX
- **M.D. Wetzel,** C. Porter, D.N. Herndon, C.C. Finnerty. Biguanide Drugs Reduce Hyperglycemia in Fibroblast Cells through alteration of Mitochondrial Oxidative Respiration. Oral presentation at Cell Biology Symposium, March 2015. Galveston, TX
- **M.D. Wetzel,** D.N. Herndon, C.C. Finnerty. Metformin Alters Expression of EMT Genes in Burn Patient Derived Fibroblasts. Poster Presentation at Annual Forum for Translational Medicine, Feb 2015. Galveston, TX

- A. Ali, C.C. Finnerty, O.E. Suman, D.N. Herndon, **M.D. Wetzel**, L.S. Sidossis, C. Porter. Mitochondrial Complex I Activity in Children with Burn Injury Treated with and without Metformin. Poster Presentation at Shock Society Meeting, July 2014. Charlotte, NC
- **M.D. Wetzel,** C. Porter, D.N. Herndon, C.C. Finnerty. Effects of Metformin on Improving AMPK Signaling and Mitochondrial Function in Burn Patient Derived Fibroblasts. Singleton Surgery Society presentation, June 2014. Galveston, TX
- **M.D. Wetzel**, J.S. Bergmann, A. Ali, D.N. Herndon, C.C. Finnerty. Metformin Down-Regulates Epithelial-Mesenchymal Transition (EMT) in Fibroblasts from Burned Patients. Poster presentation at Metabolism, Diet, and Disease meeting, May 2014. Washington, DC
- **M.D. Wetzel,** C. Porter, D.N. Herndon, C.C. Finnerty. Novel Effects of Biguanide Drugs on Reducing Hyperglycemia in Burn Patients through Alteration of Oxidative Respiratory Pathway. Oral presentation at Wound Healing Society Symposium, April 2014. Orlando, FL
- **M.D. Wetzel,** C. Porter, D.N. Herndon, C.C. Finnerty. Biguanide Drugs Reduce Hyperglycemia in Fibroblast Cells through alteration of Mitochondrial Oxidative Respiration. Oral presentation at Cell Biology Symposium, March 2014. Galveston, TX
- **M.D. Wetzel,** C. Porter, D.N. Herndon, C.C. Finnerty. Novel Effects of Biguanide Drugs on Reducing Hyperglycemia Through Alteration of Oxidative Respiratory Pathway. Poster presentation at Annual Forum for Translational Medicine, Feb 2014. Galveston, TX
- J.S. Bergmann, **M.D. Wetzel**, P.G. Nainar, D.N. Herndon, C.C. Finnerty. A New Device and Method for Performing the Scratch-Wound Assay on Cell Monolayers. Poster at American Society of Cell Biology, Dec. 2013. Washngton, DC
- D. Sun, **M. D. Wetzel**, L. L. Waite, J. E. Michalek, L. M. McManus, P. K. Shireman. Enviornmental Factors Determine In Vivo Myogenic Progenitor Cell Function. Abstract at 3rd Annual Academic Surgical Congress, Feb 2008. Huntington Beach, CA

<u>Volunteer and Mentoring Work</u>

- 2015-2016: Mentor for Research and Design program, UTMB and Ball High school, Galveston, TX.

- 2014-2015: Secretary of Society for Cell Biology, Department of Cell Biology, UTMB, Galveston, TX.

- 2013-2014: Mentored Acara Turner and Darah Scruggs, high school students in the UTMB summer research program projects at Shriner's Hospital, Galveston, TX.

- 2012-present: Associate faculty member for F1000 Prime, F1000.com/prime.

- 2012-2013: Cell Biology Departmental Representative in UTMB Student Government Association, UTMB, Galveston, TX.

- 2005: Mentor for UTHSCSA Department of Pharmacology Summer Undergraduate Research Fund Program. San Antonio, TX

- 2002: Volunteer at Boys and Girls Club of Bryan, TX.

- 2001-2002: Officer in Texas A&M Biomedical Science Association, College Station, TX.

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