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AMPK, TUBERIN, AND mTOR: NOVEL BIOLOGICAL PATHWAYS LINKING DIABETES TO COLORECTAL CANCER

by
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AN ABSTRACT OF THE THESIS OF

Sarah Toufic Abu Merhi for Master of Science
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Title: AMPK, tuberin, and mTOR: Novel biological pathways linking diabetes to colorectal cancer

Background: Diabetes and cancer are prevalent diseases whose incidence is increasing worldwide. Recent studies found that people with diabetes were more likely to be diagnosed with colorectal cancer. While diabetes and cancer share many risk factors, the biological links between the two diseases are poorly characterized.

Aim: In this study, we aimed to determine the role of AMPK, tuberin, mTOR and their crosstalk with the NADPH oxidase subunits Nox1 and Nox4 in normal versus cancer colon epithelial cells in their response to high glucose (HG), high insulin (HI) or their combination. We also wanted to determine if treatment of colon cancer cells with the AMPK activator AICAR, the mTOR inhibitor rapamycin, or their combination reverses HG-induced cell proliferation.

Methods: Western blot analysis was performed to study the protein expression of the NADPH oxidases Nox1 and Nox4, fibronectin, AMPK, tuberin, and mTOR. The peroxide-sensitive fluorescent probe 2′, 7′-dichlorodihydrofluorescin (DCF) diacetate and dihydroethidium (DHE) were used to measure intracellular and extracellular ROS production respectively. Wound healing, MTT, and invasion assays were performed to assess cancer migration, proliferation, and invasion respectively.

Results: We have evidence that HG or HI increases reactive oxygen species (ROS) production in both normal human epithelial colon cells (NCM356) and human epithelial colon adenocarcinoma cells (CaCo2). Of a greater interest, the basal level of ROS production is enhanced in colon cancer cells. Treating CaCo2 cells with either HG or HI inactivates adenine monophosphate kinase (AMPK) and up-regulates NADPH oxidases Nox1 and Nox4-induced ROS production. In addition, HG or HI increases fibronectin expression, an indicator of epithelial to mesenchymal transmission (EMT), induces the loss of function of the tumor suppressor gene, tuberous sclerosis complex 2, encoding tuberin, and activates the mTOR pathway. HG or HI also enhances cancer cell migration, proliferation, and invasion. Pharmacologic activation of AMPK by 5-aminoimidazole-4-carboxamide-1-riboside AICAR or inhibition of mTOR by rapamycin restores AMPK and tuberin phosphorylation/activity, down-regulates Nox1, Nox4, and fibronectin expression, decreases mTOR phosphorylation/activity, and down-regulates cell migration, proliferation, and invasion

Conclusion: Our results uncover a novel and critical role for AMPK, tuberin, and mTOR in cancer cell proliferation and extracellular matrix accumulation in response to high glucose or insulin; this pathway is through an oxidative stress-dependant mechanism. Our work also sets the stage to additional studies for identifying new therapeutic approaches to treat cancer development/progression in diabetes.

CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

LIST OF ABBREVIATIONS

DM: Diabetes Meallitus

CRC: Colorectal Cancer

IGF-1: Insulin-like Growth Factor

HbA1c: Hemoglobin A1C

AP: Adenomatous Polyps

ROS: Reactive Oxygen Species

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NOX: NADPH oxidase

VEGF: Vascular Endothelial Growth Factor

FBS: Fetal Bovine Serum

ECM: Extracellular Matrix

PBS: Phosphate Buffered Saline

P/S: Penecillin/Streptomycin

AMPK: Adenosine-monophosphate protein kinase

AICAR: AminoImidazole-4-CarboxAmide Riboside

mTOR: mammalian-target-of-rapamycin

TSC: Tuberous sclerosis complex

GTP: Guanosine triphosphate

Rheb: Ras homology enriched in brain

GAP: GTPase-activating protein

INTRODUCTION

A. Diabetes Mellitus (DM)

1. Definition and Types

Diabetes mellitus (DM) is considered as the most common metabolic abnormality worldwide. It occurs through two different primary disease processes, type 1 (insulin-dependent) and type 2 (non-insulin dependent), with different patient characteristics (Alberti *et al*., 1998). These two subtypes of DM have fundamentally different metabolic and hormonal characteristics. Type 2 DM is characterized by impaired insulin release and/or decreased hepatic and extrahepatic insulin sensitivities where as type 1 DM is characterized by the cessation of insulin biosynthesis due to the autoimmune destruction of insulin-producing pancreatic beta cells (Efendic and Ostenson, 1993;Ostenson, 2001),

2. Complications of Diabetes Mellitus

Diabetes Mellitus, particularly the more common type 2 DM, results in many serious long term micro and macro vascular complications including cardiovascular, retinal, and nerve disease, along with chronic renal failure and high tendency for infections. Cardiovascular complications are considered the most prominent in DM where approximately two-thirds of affected people die from heart disease or stroke (Centers for Disease Control and Prevention, 1999).

B. Diabetes and Cancer

While it is widely recognized that DM is associated with a high risk of cardiovascular and micro vascular complications, the association of DM with increased risk of cancer, independent of its association with obesity is still not well established (Chowdhury, 2010). Many epidemiological studies have conveyed frequent co-occurrence of diabetes and cancer suggesting an association of DM with specific types of solid tumors including ovarian (Swerdlow *et.al*, 2005), pancreatic (Wideroff *et al.*, 1997), liver (Ogunleye *et al*., 2009), renal (Smith *et al*., 2008), breast (Wideroff *et al.*, 1997), melanomas (Smith *et al*., 2008), and cancers of the urinary tract, stomach, cervix, endometrium, and colorectum (Zendehdel *et al*., 2003). These cancers are more commonly linked to type 2 DM associated with obesity, insulin resistance, and hyperglycemia. Therefore, patients with type 2 DM are more likely to develop cancer - and to die from it - than members of the general population making cancer now a newly identified serious complication of DM (Smith and Gale, 2009).

C. Diabetes and Colorectal Cancer (CRC)

Colorectal cancer (CRC) is the third leading cause of cancer-related death in the Western world with 655,000 deaths per year (Center *et al.,* 2009). And , in 2002, at least 171 million patients were diagnosed with DM worldwide, and it is expected that by the year 2030, the number will almost double; DM is projected to affect 366 million people worldwide (Wild *et al*., 2004). A recent meta-analysis showed that patients with type 2 DM have a 30% increased risk for CRC versus the general population (Giouleme, 2011). Moreover, a large retrospective study conducted in 2006 reported

that there is a significantly elevated risk for proximal CRC in diabetic men (Limburg *et al*., 2006; Ren *et al*., 2009), diabetic women (Elwing *et al.,* 2006), or both sexes (Yang et al., 2005). Interestingly, type 2 DM predisposes patients to an increased risk for proximal and distal CRC (Limburg *et al*., 2006), or proximal and distal colonic and rectal cancers (Yang *et al.,* 2005).

1. Pathophysiological Mechanisms Linking Diabetes to CRC

DM and CRC share common etiologic factors including obesity, sedentary lifestyle, and diet .This led to the hypothesis that there might be a correlation between DM and CRC.

a. Insulin-like Growth Factor (IGF-1)- Hyperinsulinemia Theory

Several pathophysiological mechanisms have been postulated to clarify the possible relation between DM and CRC including the insulin-like growth factor (IGF1)-hyperinsulinemia theory. Berster and Goke (2008) hypothesized that elevated insulin- in an effort to overcome peripheral insulin resistance- and free IGF-1 levels support the proliferation of colon cells, thereby leading to a survival benefit, resulting in CRC. This was demonstrated by the increased insulin receptor density in the cancer cell lines in comparison to normal colon epithelium. Furthermore, insulin leads to increased bioavailability of IGF-1 (Berster and Göke, 2008). Both Insulin and IGF-1 enhance proliferation, or inhibit apoptosis of colon epithelial cells, leading to carcinogenesis. Thus, it seems possible that diabetic patients might be at increased risk of CRC, and

normal colon cells acquire neoplastic characteristics, displaying cancerous transformation (Giouleme, 2011). Moreover, hyperinsulinemia is further augmented by exogenous application of insulin. Chronic insulin therapy has been associated with an increased CRC risk among type 2 diabetic patients (Chung *et al.*, 2008 & Yang *et al.*, 2004). Distinctively, a three-fold risk increase for patients with insulin-dependent type 2 DM in comparison to the general population has been observed (Nagel and Göke, 2006).

b. Hemoglobin A1C (HbA1c) and CRC

In addition, glucose level has been allied with CRC. Elevated hemoglobin A1C (HbA1c) has been proven as an independent predictor of aggressive clinical behavior in patients with CRC (Giouleme, 2011). In a retrospective review of 625 patients with type 2 DM diagnosed with colonic adenomatous polyps (APs), HbA1c levels were evaluated as an index of glycemic control over the year that preceded the diagnosis of APs. Among patients with poorly controlled diabetes (HbA1c >7.5%), a drastically increased occurrence of right –sided polyps, a greater number of polyps, more advanced polyps, and a younger age of presentation was noted (Siddiqui *et al.*, 2008). However, based on a Norwegian study, diabetes does not seem to affect the short-term survival or the overall cancer-specific survival in patients with CRC. Cardiac diseases and higher age contributed to the shorter general survival in diabetic patients suffering from CRC in comparison to non-diabetics (Jullumstrø *et al.*, 2009).

2. Reactive Oxygen Species (ROS) in Diabetes and CRC

a. Roles and Sources of ROS

Reactive Oxygen species (ROS) have evolved to play an important role in housekeeping physiologic and cellular processes. ROS and free radical formation affect pathways involved in innate immunity, cell and tissue growth, angiogenesis, salt and fluid homeostasis, biochemical reactions, apoptosis, etc. NADPH oxidase (NOX) enzymes are a family of heme-containing transmembrane proteins whose central function is ROS production (Bedard *et al.,* 2007). They intervene in many significant physiological and pathological processes including cell signaling, inflammation and mitogenesis (Brar *et al.,* 2002; Geiszt, 2006). Diabetes and hyperglycemia enhance the generation of ROS (Eid *et al.,* 2010). Along with ROS generated from mitochondrial respiratory chain, NADPH oxidase-derived ROS have been shown to greatly contribute to the injury of various organs including colon (Bedard & Krause, 2007). A number of homologs of the NADPH oxidase catalytic subunit (Nox2) have been known. These enzymes are involved in various biological processes including proliferation, migration, contraction, fibrosis and apoptosis. Along with Nox2, Nox1 and Nox4 have been shown to be also expressed in colonic tissues (Bedard & Krause, 2007).

b. Role of NADPH Oxidase (NOX) enzymes in Cancer

In addition to the non-malignant cells, cancer cells also produce ROS; in tumors, reactive oxygen metabolites can act as signaling molecules to enhance cell survival over apoptosis (Szatrowski and Nathan, 1991; Storz, 2005). Gene expression of the NOX family is organ-specific for both malignant and non-malignant tissue. NADPH

oxidase Nox1-generated hydrogen peroxide can trigger an ‘angiogenic switch’ including the induction of angiogenic factors, such as the vascular endothelial growth factor (VEGF), that promote tumor cell vascularization and proliferation (Arbiser *et al.*, 2002). Also in the gastrointestinal tract, expression of Nox1 is significantly increased in colon cancers compared with adjacent normal bowel mucosa (Juhasz *et al*., 2009). However, the mechanisms underlying the enhanced expression of Nox1 in human tumors remain to be fully elucidated. Nox4-mediated ROS have been shown to prevent apoptosis and promote tumor cell growth in pancreatic cancer cells (Mochizuki *et al.,* 2006; Vaquero *et al.,* 2004). However, and despite these studies, our understanding of the role(s) of the NOX family of genes and their mechanism of action in the development and growth of human cancer and especially in colon cancer is limited.

c. ROS-dependant enhancement of cell proliferation

Many studies have documented a role for ROS in cell proliferation (Laurent *et al.,* 2005; Milovanova *et al*., 2005; Zhu *et al.,* 2006). In most of these studies, there was evidence that the source of mitogenic ROS was an NADPH oxidase. Many in vitro studies based on either antisense or siRNA suppression suggest a role for Nox1 and Nox4 in smooth muscle cell proliferation (Menshikov *et al.,* 2006; Sturrock *et al.,* 2005). Nox1 was also described to play a main role in cell proliferation, and the enzyme was even referred to as "mitogenic oxidase 1" (Lambeth, 2004). Lambeth also mentioned that NOXs expression in fibroblasts failed to produce transformation indicating that NOXs role in cell proliferation is organ and tissue specific.

3. Adenosine-monophosphate protein kinase (AMPK) in Diabetes and Cancer

Serine/threonine protein kinase 11 (STK11) or LKB1 is one among those tumor suppressors which is also a regulator of metabolic signaling. It responds to changes in cellular energy balance [adenosine triphosphate (ATP) levels](Carling , 2004; Hardie, 2003) and governs whole body insulin sensitivity (Carling, 2004; Viollet *et al.*, 2003). In cells with excess adenosine monophosphate due to altered energy homeostasis, serine/ threonine protein kinase (STK11) phosphorylates the adenosine monophosphate-dependent kinase AMPK (Shaw *et al.,* 2004). This latter plays a central role in regulating cellular energy status (Hardie, 2004). Moreover, several genes involved in metabolic and insulin signaling were associated with both colon and rectal cancer (Slattery *et al.,* 2010). STK11 inactivation is the genetic basis of Peutz-Jeghers syndrome, a familial colorectal polyp disorder in which patients are predisposed to early-onset cancers in other tissues (Boudeau *et al.,* 2003). In addition, PRKA is the gene coding for AMPK. AMPK is a heterotrimeric complex comprising a catalytic α-subunit (gene subunits α 1 and α 2) and regulatory β (β1 and β2) and γ (γ1, γ2, and γ3) subunits (Hardie *et al.,* 2003). α1 and γ2 have been associated with colon cancer. γ2 subunit has been also correlated with rectal cancer (Slaterry *et al.,* 2010). More interestingly, the α1 and γ2 subunits have been associated with both glucose and lipids metabolism (Xu et *al.,* 2005; Eid *et al.,* 2010).

a. Activation of AMPK

The AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that is switched on by a rise in the AMP: ATP ratio; a high

cellular ratio of AMP: ATP is a signal reflecting that the energy status of the cell is compromised. AMPK is switched on either by cellular stresses that interfere with ATP production or by stresses that increase ATP consumption. The kinase is also regulated by more physiological stimuli. In pancreatic β-cells, low glucose activates AMPK in the same range of concentrations over which it inhibits insulin release (Salt et al., 1998). Another physiological metabolic stress that activates AMPK by increasing ATP consumption is exercise in skeletal muscle (Winder and Hardie, 1996). AMPK is also activated by receptors coupled to phospholipase C via the G protein Gq (Kishi et al., 2000; Kim *et al*., 2007) and by the adipocytokines leptin (Riboulet-Chavey *et al.,* 2008) and adiponectin (Kim *et al.,* 2007)

Compound 5-aminoimidazole-4-carboxamide riboside (AICAR) also tends to activate AMPK. This adenosine analog is taken up into cells and converted by adenosine kinase to the monophosphorylated nucleotide 5-aminoimidazole-4-carboxamide-1-d-ribofuranosyl-5′-monophosphate (ZMP). ZMP mimics all of the activating effects of AMP on the AMPK system, although it is much less potent than AMP itself. Nevertheless, in most cells, it accumulates to sufficiently high concentrations that it activates AMPK without disturbing cellular levels of AMP, ADP, or ATP (Corton *et al.,* 1995; Aschenbach *et al.,* 2002).

In addition, the AMPK system is a probable target of the major anti-diabetic drug metformin; it activates AMPK by a mechanism that involves phosphorylation by the upstream kinase but with no alteration in the cellular AMP: ATP ratio (Fryer *et al.,* 2002). Retrospective epidemiological evidence (Pollak, 2010; Decensi *et al.,* 2010) suggest that type II diabetics receiving metformin have lower cancer incidence and mortality than those not receiving this agent, with some reports showing approximately

50% reduction. AMP stimulation is only observed if the intact aßγ complex, and not the isolated kinase domain (which lacks the AMP-binding sites), is used as the substrate (Hawley *et al.*, 2003). This shows that the effect is due to binding of AMP to the substrate, AMPK, and not to the upstream kinase.

b. Role of AMPK in Diabetes and Cancer

Activation of AMPK down regulates anabolic pathways such as fatty acid and cholesterol biosynthesis, yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation, glucose uptake and glycolysis; these metabolic changes would be beneficial in subjects with type II diabetes (Hardie, 2003). However, AMPK signaling modulates multiple biological pathways, such as protein synthesis (Lee *et al.,* 2007), autophagy (Matsui *et al.,* 2007), and apoptosis (Russell *et al.,* 2004). Although activation of AMPK is likely, in the short term, to reduce ATP consumption and thus to protect cells from transient metabolic stresses, sustained activation of the enzyme entrains a sequence of events ultimately leading to programmed cell death (Hardie *et al.,* 1998). Work on many cell lines, including HT-29 colon cancer (Su *et al.,* 2007), has implicated AMPK activation in cell death and proapoptotic activity (Kefas *et al.,* 2003). However, the downstream mechanism of action needs more investigation. A particularly interesting downstream target of LKB1/AMPK recently identified is tuberin. The LKB1/AMPK/tuberin pathway negatively regulates the target of rapamycin (TOR) that is implicated in protein synthesis, cell survival and tumorigenesis by the fact that rapamycin inhibits tumor growth (Menon *et al.*, 2008).

4. The tuberin/mTOR pathway in diabetes and cancer

a. Structure and Properties of Hamartin-Tuberin complex

Tuberous sclerosis is a genetic multisystem disorder characterized by widespread hamartomas in several organs, including the brain, heart, skin, eyes, kidney, lung, and liver. The affected genes are TSC1 and TSC2, encoding hamartin and tuberin respectively. The hamartin—tuberin complex inhibits the mammalian-target-of-rapamycin (mTOR) pathway, which controls cell growth and proliferation (Curatolo *et al.*, 2008). The gene products of hamartin and tuberin form a physical and functional complex in which hamartin functions as a membrane-tethering anchor protein; it interacts with tuberin via its coiled–coiled domain and stabilizes tuberin (Inoki & Guan, 2009). This latter functions as the catalytic subunit to promote GTP hydrolysis of the small GTPase Rheb, a small G-protein activator of mTOR (Van Slegtenhorst *et al.*, 1998; Aspuria & Tamanoi, 2004). Loss of function mutations in either the TSC1 or TSC2 gene lead to abnormal up-regulation of mTOR signaling and are associated with the pathogenesis of many diseases especially diabetic complications and cancer (Fujishita *et al.*, 2009).

b. Tuberin in Diabetes and Cancer

Activation of the nutrient-sensing kinase, AMPK, under energy starvation condition leads to decreased activity of hamartin and tuberin complex as a GTPase-activating protein (GAP) toward the small GTPase Rheb (Inoki *et al.*, 2003). GTP-bound Rheb then activates mTORC1 by antagonizing FKBP38, an endogenous mTOR inhibitor (Bai *al* et., 2007). However, the mechanism of action of TSC2 and its crosstalk

with other proteins in the signaling pathway leading to colon cancer in diabetes still needs to be elucidated.

c. Structure and Downstream Effectors of mTOR complex

The mammalian target of Rapamycin (mTOR) is a serine/threonine kinase conserved from yeast to mammals existing in two distinct multi-protein complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Dann *et al.*, 2007). mTORC1 is a rapamycin-sensitive complex and consists of Raptor, mLST8, PRAS40 and mTOR (Loewith *et al.*, 2002; Sancak *et al*., 2007; VanderHaar *et al*., 2007). Rapamycin suppresses phosphorylation of the mTORC1 substrates such as p70S6 kinase/S6 kinase 1 (S6K1) and mRNA translational initiation repressor 4E-binding protein 1 (4E-BP1) (Um *et al.*, 2006). On the other hand, mTORC2 contains Rictor, mSin1, mLST8, PRR5 and mTOR (Loewith *et al.*, 2002; Frias *et al.*, 2006; Pearce *et al.*, 2007), and directly phosphorylates S^{473} of the protein kinase B/Akt and S^{422} of the serum- and glucocorticoid-induced protein kinase1 (SGK1) (Sarbassov *et al*., 2005; Garcia-Martinez & Alessi et al., 2008). mTORC2 is largely rapamycin-resistant (Sarbassov *et al.*, 2005).

d. Role of mTORC1 Pathway in Diabetes and Cancer

In response to growth factors and nutrient availability such as amino acids and glucose, mTORC1 is activated and regulates a wide array of cellular processes, including protein translation, transcription, mRNA splicing, cell cycle and autophagy (Wullschleger *et al.*, 2006). The best-characterized mTORC1 function is its role in the protein synthesis, especially translation that associates with cell growth.

Hyperactivation of mTORC1 pathway has also been postulated as an important contributing factor for tumorigenesis (Inoki & Guan, 2009). It was reported that the mTORC1 pathway is activated in about 40% of colorectal cancer patients (Nozawa *et al*., 2007). A study conducted on Apc mutant mice having polyps conveys that the mTORC1 pathway is activated, accompanied by an elevated level of mTOR protein, and the treatment with RAD001, an mTORC1 inhibitor, suppresses the growth of these polyps. This study suggests an important role of mTORC1 pathway in colorectal cancer, as well as a therapeutic possibility for mTORC1 inhibitors in its treatment (Fujishita *et al*., 2009). mTOR activity is negatively regulated by the heterodimeric complex consisting of tuberin and hamartin. Phosphorylation of TS serves as an integration point for a wide variety of environmental signals that regulate mTORC1 (Sarbassov *et al.,* 2005). However, the role of tuberin/mTORC1 pathway in colon tumorigenesis in DM remains largely unknown.

D. Hypothesis

We hypothesize that diabetes-like conditions (HG and/or HI) inactivate(s) AMPK. AMPK inactivation up regulates Nox1 and Nox4 expression, which in turn inactivate tuberin. Tuberin inactivation leads to mTORC1 pathway activation. mTOR plays important roles in increased tumor development and tumor burden in diabetic patients (Figure 1). To explore our hypothesis, we aim in this study to determine the role of AMPK and tuberin/mTOR and their crosstalk with the NADPH oxidase subunits Nox1 and Nox4 in normal versus cancerous colon epithelial cells in their response to the diabetogenic high glucose (HG), diabetogenic high insulin (HI) or their combination. We also aim to determine if treatment of colon cancerous cells with the AMPK activator AICAR, the mTORC1 inhibitor rapamycin, or their combination reverses the effect of HG, HI, or their combination.

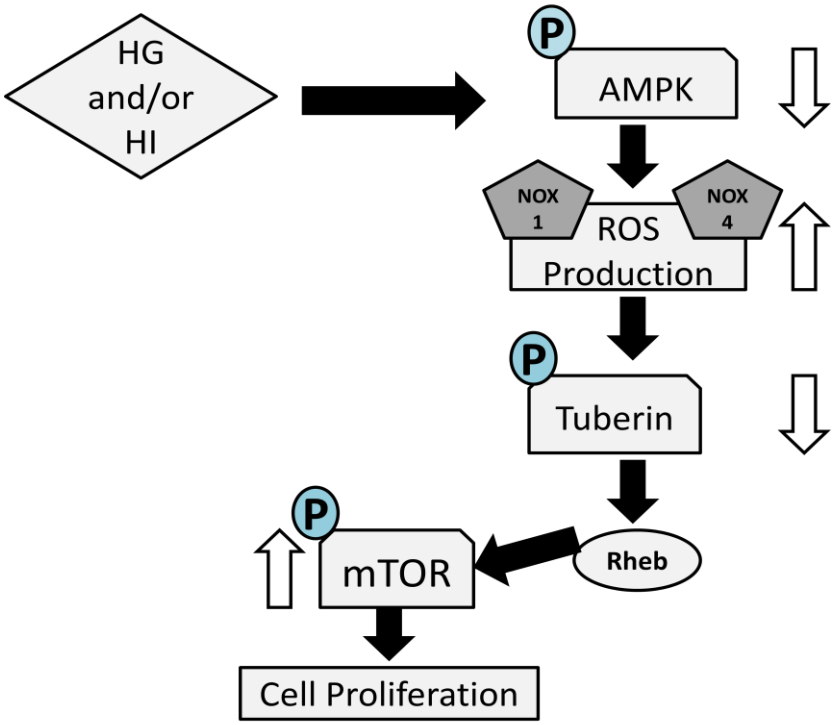


Figure 1: Proposed hypothesis of diabetes /cancer link

MATERIALS AND METHODS

A. Cell lines

The *in vitro* studies were performed using established normal human epithelial colon cell line NCM 356 (INCELL Corporation, San Antonio, TX) and established human epithelial colon adenocarcinoma cell line (CaCo2). Both cell lines were cultured in Dulbecco's modified Eagles medium (1000mg/l glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), and 2% N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES). The cell lines were maintained in a humidified incubator at 37ºC and 5% CO_2. Cell treatment was performed with 25mM glucose (HG), 500µM insulin (HI) or their combination in the absence of serum. All the treatment duration was for 72 hours. CaCo2 cells were also pretreated for an hour with the AMPK activator AICAR (1.5mM), mTORC1 inhibitor Rapamycin (25nM) or their combination.

B. Western blotting analysis

NCM 356 and CaCo2 cells were grown to near confluency and serum deprived for 24 hours. All incubations were carried out in serum-free Dulbecco's modified Eagles medium (DMEM) at 37°C according to the experimental design. The cells were lysed using RIPA buffer consisting of 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 150mM NaCl, 50mM Tris HCL, 100mM EDTA, 1% Tergitol (NP40), and 1% of protease and phosphatase inhibitors. The cell lysates were centrifuged at 13,600 g for 30 minutes at 4°C. Protein concentration in the supernatants was assessed by Lowry's method for protein measurement. For immunoblotting, 50µg of proteins

were separated on 8% or 10% Polyacrylamide Gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk in Tris-buffered saline and then incubated with rabbit polyclonal anti-Nox1 and anti-Nox 4 (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-fibronectin (1:1000, sigma-aldrich), rabbit polyclonal anti-phospho-Thr ^{172} AMPKα (1:500,Cell Signalling) , rabbit polyclonal anti-phospho-Ser ^{1387} tuberin (1:500, Cell Signalling), and rabbit polyclonal anti-phospho-Ser ^{2448} mTOR (1:500, cell signalling) antibodies. The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). Protein bands were visualized by enhanced chemiluminescence reagent (Roche Applied Science). Densitometric analysis was performed using Image J software.

C. Detection of intracellular ROS

The peroxide-sensitive fluorescent probe 2′, 7′-dichlorodihydrofluorescin (DCF) diacetate (Molecular Probes) was used to measure intracellular ROS. Normal and cancerous cells were grown in 12-well plates and serum-deprived for 24 h. Immediately after 72 hours of treatment, cells were washed with PBS containing Ca^{2+} and Mg^{2+} and then loaded with 50 μmol/l DCF diacetate dissolved in PBS for 30 min at 37°C. DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured in a multiwell fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific**).**

D. Cell proliferation

Cell proliferation of human colon adenocarcinoma cells was assessed by MTT Cell Proliferation Kit I (Roche Applied Science), a colorimetric assay for the non-radioactive quantification of cell proliferation and viability. CaCo2 cells were seeded and grown in 96 well tissue culture plate with a final volume of 100µl culture medium per well (5×10^4 cells/ml), in a humidified atmosphere (37ºC, 5% CO2). Serum deprived for 24 hours, cancerous cells were treated according to the experimental design for 72 hours.10µl of the MTT labeling reagent (final concentration of 0.5mg/ml) was then added to each well, and the microplate was incubated for four hours. After this incubation period, purple formazan salt crystals were formed. These salt crystals became soluble by adding 100µl of the solubilization solution and incubating the plate overnight. The solubilized formazan product was spectro-photometrically quantified using an ELISA reader (Multiskan Ex) at a wavelength of 590nm. Each condition was counted in duplicate. Proliferation graphs were plotted as percentage of the control, i.e. CaCo2 cultured alone without treatment (normal glucose), adjusted to 100%.

E. Wound healing assay

Wound healing assay is designated to assess cell migration and proliferation. CaCo2 cells were seeded in 60 mm dishes (Corning, USA) and allowed to attach, spread, and form a confluent monolayer. Serum deprived for 48 hours, a sterile p200 pipet tip was used to scratch and remove cells from a discrete area of the confluent monolayer to form a cell-free zone into which cells at the edges of the wound can migrate. CaCo2 cells were then treated according to the experimental design. Images of cell movement at different magnifications were captured using a digital camera (Mpeg

movie EX) before and after treatment of 72 hours. The area of closure was analyzed using the Image J software.

F. Cell invasion

To study cell migration and invasion, the Millipore QCM ™ 8µm 24-well Chemotaxis Assay kit was used. Micro porous membrane inserts (8µm pore size) were fitted in sterile 24-well tissue culture plates, which contain the treatment in its wells. A volume of 250 µL of a cell suspension containing 0.5×10^6 cells/ml was added into each insert. The plate was then incubated for 24 hours (37℃ and 5% CO2) allowing cells to migrate through the pores, to the other side of the membrane. Cells that migrated toward the treatment were stained with a cell stain solution, and extracted to the lower chamber through the extraction buffer. 100µL of the solution was added to a 96 well plate and quantified by spectrophotometer (Multiskan Ex**)** at a wavelength of 560 nm. Cell invasion graphs were plotted as percentage of the control, i.e. CaCo2 cultured alone without chemoattractants, adjusted to 100%.

G. Cellular DNA fragmentation ELISA

Programmed cell death (apoptosis) of human colon adenocarcinoma cells was assessed by the Cellular DNA Fragmentation ELISA kit (Roche Applied Science). CaCo2 cells were seeded in 12-well tissue culture plates with a final volume of 500µl culture medium per well (1×10^5cells/ml). Reaching 70-80% confluency, CaCo2 cells were serum deprived for 24 hours and then treated according to the experimental design for 72 hours. The assay is based on the quantitative "sandwich enzyme immunoassay" (ELISA) principle, using two mouse monoclonal antibodies directed against DNA and

BrdU. In the first step of this procedure, 100µl of anti-DNA coating solution containing anti-DNA antibody was adsorptively fixed in the wells of a microplate, which was incubated overnight at 4℃. In the second step, and before stopping the treatment by 12hours, CaCo2 cells were treated with BrdU-labeled DNA fragments (15µM) to allow its binding to the immobilized anti-DNA antibody. In the third step, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed on the surface of the microplate by microwave irradiation for 5 minutes. This procedure improves the accessibility of the antigen BrdU for detection by the antibody. In the final step, 100µl/well of anti-BrdU-peroxidase conjugate (anti-BrdU- POD) was added to react with the BrdU incorporated into the DNA for 90 minutes at RT. After removal of unbound peroxidase conjugates three times with a washing buffer, the amount of peroxidase bound in the immune complex was photometrically determined when 100µl of substrate solution was added into each well. Absorbance was measured at a wavelength of 450nm against a reference wavelength of 650 nm using an ELISA reader (Multiskan Ex). Apoptosis graphs were plotted as percentage of the control, i.e. CaCo2 cultured alone without treatment adjusted to 100%.

H. Live imaging

Extracellular ROS production was assessed by dihydroethidium (DHE) staining (Molecular Probes) with a working concentration of 2 µmol/l. For live cell imaging, CaCo2 cells were seeded on Mat Tek confocal dishes, and when reaching 60-70% confluency, they were serum starved for 24 hours, and then treated according to the experimental design for 72 hours. After the incubation period, the cells were labeled with DHE staining for 30 minutes at 37 ºC. Fluorescence was measured at a wavelength

of 560 nm using a Zeiss LSM710 laser confocal microscope (Carl Zeiss) equipped for live cell imaging. Images were taken from different fields and at different magnifications. Quantification was done using the Zen Light Software

I. Statistical analysis

Results are expressed as mean ± S.E from three to four independent experiments. Statistical significance is asessed by student's unpaired ttest. Siginifance is determined as probability (p) <0.05.

RESULTS

A. ROS production

1. High glucose, high insulin, or their combination induce ROS production

Hyperglycemia and/or hyperinsulimia in diabetes elicit cellular responses that contribute to diabetic complications. Since diabetes induces oxidative stress including NADPH-dependent superoxide generation in many organs, we studied the effect of high glucose (HG, 25 mM), high insulin (HI, 500 μM insulin) or their combination (as would be encountered in type II DM) on intracellular ROS generation using the peroxide-sensitive fluorophore DCF in NCM 356 and CaCo2 cells. Our results show that HG, HI, or their combination increase superoxide production in both NCM 356 and CaCo2 (Figure 2). It is also important to highlight that the basal level of ROS production is significantly increased in CaCo2 when compared to NCM 356 cells (Figure 3).

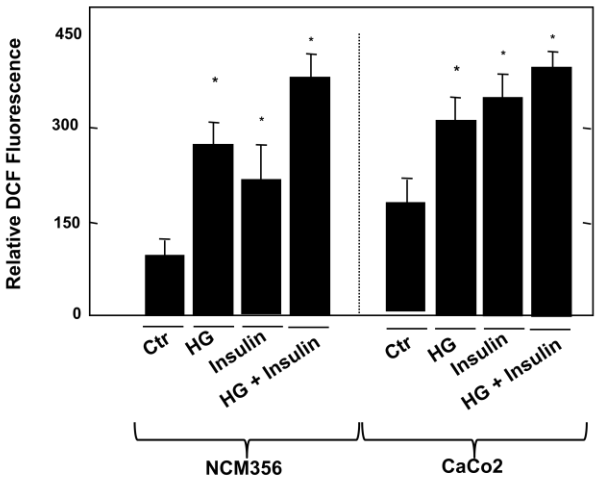


Figure 2. High glucose, high insulin, or their combination induces ROS production. Intracellular ROS measured using the peroxide-sensitive fluorophore DCF in human cultured colon epithelial cells (NCM 356) and human epithelial colon adenocarcinoma cells (CaCo2) treated with HG (25mM), HI (500µM), or their combination for 72h. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control.

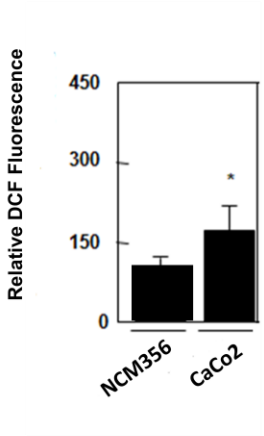


Figure 3. ROS basal level in CaCo2 is higher than in NCM356. Intracellular ROS assessed using the peroxide-sensitive fluorophore DCF in human cultured colon epithelial cells (NCM 356) and human epithelial colon adenocarcinoma cells (CaCo2) without treatment. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control.

2. Basal Nox1 and Nox4 protein expression is upregulated, AMPK/tuberin phosphorylation/activity is decreased and fibronectin expression is increased in CaCo2 cells.

Western blotting was done on both normal and cancerous colon cells. Our data show an increase in Nox1 and Nox4 protein expression in cultured epithelial adenocarcinoma cells (CaCo2) compared to the normal non-cancerous colon cells (NCM 356)(Figure 4A). AMPK phosphorylates tuberin on its activating site Ser^{1387}

In CaCo2 cells, we observe a decrease in AMPK phosphorylation on its activating site, Thr^{172}, which can explain the decrease of tuberin phosphorylation on its activating site Ser^{1387} and tuberin expression (Figure 4B). More importantly, our data was paralleled by an increase in fibronectin as compared to normal colon cells (Figure 4C). These results suggest Nox1 and/or Nox4-dependent ROS production is enhanced in cancer leading to increased fibronectin expression and this increase can be due to inactivation of the AMPK/tuberin pathway.

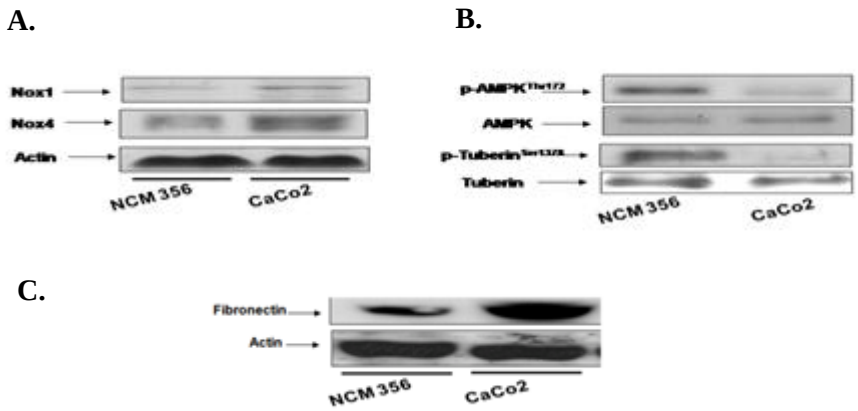


Figure 4. Up-regulation of NADPH oxidases Nox1 and Nox4, decreased phosphorylation/activity of AMPK and tuberin, and increased fibronectin expression in cancerous cells. Immunoblot of protein extracts from human cultured epithelial colon cells (NCM 356) and human epithelial colon adenocarcinoma cells (CaCo2) incubated until confluency with NG. *A*, representative Western blot of Nox1, Nox4, and β-actin levels. *B*, representative Western blot of phospho-Thr^{172} AMPK, AMPK, phospho-Ser^{1387} tuberin and tuberin. *C*, representative Western blot of fibronectin and β-actin levels. A single experiment was performed.

3. High glucose, high insulin or their combination increases fibronectin expression, up-regulates Nox1 and Nox4 protein expression, decreases AMPK and tuberin phosphorylation/activity and increases mTOR phosphorylation/activity in (CaCo2)

Since we are interested in diabetes-induced cancer progression and to correlate our findings with the diabetic state, we studied in the first set of experiments the effect of HG (25mM), HI (500µM), or their combination on ROS production in CaCo2 cells. Our findings convey, after densitometry analysis, that HG, HI or their combination up-regulates NADPH oxidases Nox 1 and Nox 4 protein expression (Figure 5A, *A-C*) and this is associated with significant increase in fibronectin expression (Figure 5A, *D and E*). Also, treatment with HG, HI or their combination noticeably inactivates AMPK/tuberin assessed by the decrease of AMPK/tuberin phosphorylation on its activating site Thr^{172} and Ser^{1387} respectively, and activates mTOR by increasing mTOR phosphorylation on its activating site Ser^{2448} (Figure 5B, *F-J*). These results suggest that HG, HI, or their combination increases the susceptibility of colorectal cancer to extracellular matrix accumulation (increased fibronectin expression) and these effects are mediated through an AMPK/NADPH/tuberin/mTOR pathway.

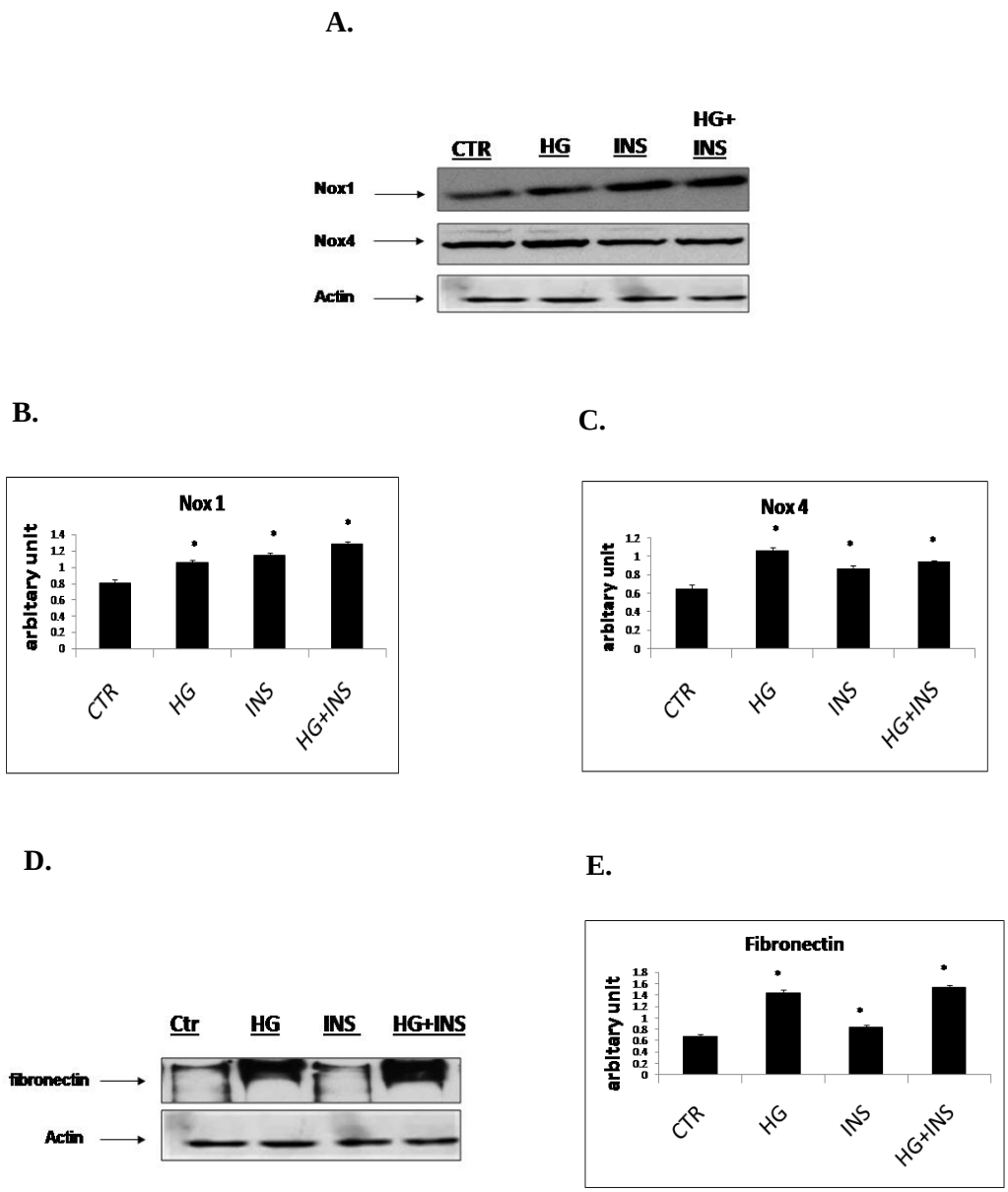


Figure 5A. High glucose, high insulin, or their combination up-regulates Nox1 and Nox4, and induces fibronectin expression. Epithelial colon adenocarcinoma cells were serum-deprived for 24 h and treated with high glucose (25mM), high insulin (500µM), or their combination for 72 h. *A,* representative Western blot of Nox1, Nox4, and β-actin levels. *B and C,* histograms of Nox1 and Nox4 respectively after densitometry analysis using Image J software. *D,* representative Western blot of fibronectin and β-actin levels. *E,* histogram of fibronectin after densitometry analysis. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control.

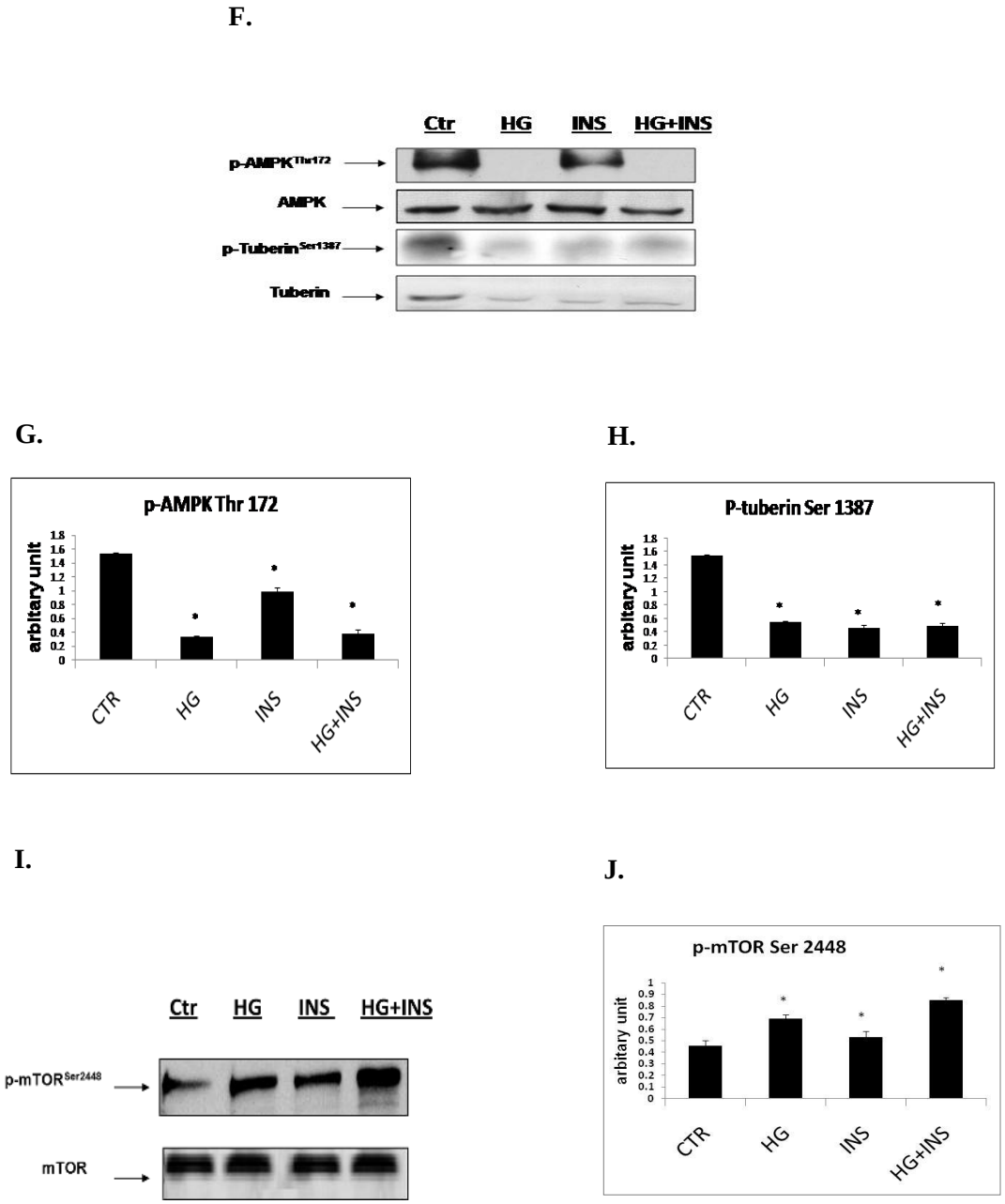


Figure 5B. High glucose, high insulin, or their combination decreases AMPK and tuberin phosphorylation/activation, and increases mTOR phosphorylation/activity. Epithelial colon adenocarcinoma cells were serum-deprived for 24 h and treated with high glucose (25mM), high insulin (500µM), or their combination for 72 h. *F*, representative Western blot of phospho-Thr^{172} AMPK, AMPK, phospho-Ser^{1387} tuberin, and tuberin. *G and H*, histograms of phospho-Thr^{172} AMPK and phospho-Ser^{1387} tuberin respectively after densitometry analysis. *I*, representative Western blot of phospho-Ser^{2448} mTOR and mTOR. *J*, histogram of phospho-Ser^{2448} mTOR after densitometry analysis. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control.

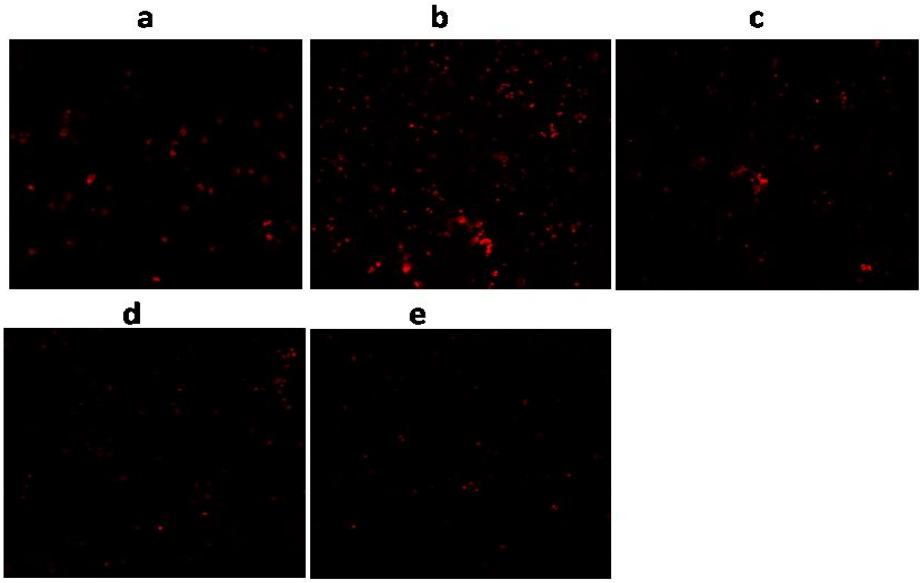
4. Activation of AMPK and inhibition of mTOR decrease ROS production, fibronectin expression, and NADPH oxidases protein expression in CaCo2.

In order to further dissect the mechanism of how HG enhances the deleterious effect of cancer and to study the role of AMPK/tuberin and mTOR in the progression of cancer, CaCo2 cancerous cells were pretreated for an hour with the pharmacologic AMPK activator AICAR (1.5mM), mTOR inhibitor rapamycin (25nM), or their combination in the presence of HG for 72h.

a. Activation of AMPK and inhibition of mTOR decrease ROS production

Extra cellular ROS production was assessed through dihydroethidium (DHE) staining (Molecular Probes) using confocal microscopy technique. Our results show a significant increase in ROS production in CaCo2 treated with HG when compared with CaCo2 cells exposed to NG (Figure 6, *A, compare panel b with panel a, and B*). More importantly, treatment of CaCo2 with the AMPK activator AICAR, the mTOR inhibitor rapamycin, or their combination results in a significant decrease of ROS production induced by HG (Figure 6, *A, compare panel c, d, e, respectively with panel b, and B*).

A.



B.

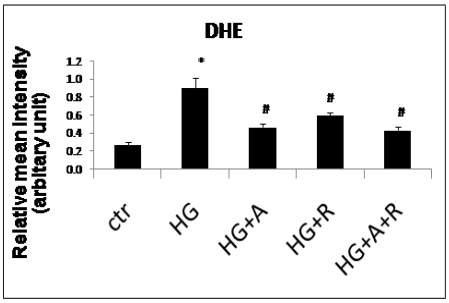


Figure 6. Activation of AMPK/tuberin and inhibition of mTOR decrease ROS generation. Extra cellular ROS assessed using dihydroethidium (DHE) staining. *A*, representative fluorescence images of CaCo2 incubated with NG (a), HG (b), HG pretreated for 1h with AMPK activator AICAR (c), mTOR inhibitor rapamycin (d), or their combination (e) . Incubation period for 72h. Difference is appreciated in the fluorescent density of CaCo2 with NG (b) and CaCo2 treated with HG (b). This difference was not seen in CaCo2 treated with AICAR (c), rapamycin (d), or their combination. *B, histogram* analysis representing fluorescence intensity of CaCo2. All values are the mean ± S.E. from two independent experiments.*, $p < 0.05$ *versus* the control; #, $p < 0.05$ *versus* HG treatment.

b. Activation of AMPK/tuberin and inhibition of mTOR down-regulate fibronectin and NADPH oxidases protein expression

To examine the role of AMPK/tuberin and mTOR on fibronectin and NADPH oxidases Nox1 and Nox4 protein expression, western blot analysis was performed on lysate from CaCo2 cells pretreated for 1h with the pharmacologic AMPK activator AICAR, mTOR inhibitor rapamycin, or their combination in the presence of HG for 72h. It has been shown that AMPK phosphorylates tuberin on its activating site Ser^{1387}. In CaCo2 cells, treatment with the AMPK activator AICAR restores tuberin phosphorylation on Ser^{1387} compared to CaCo2 incubated with HG only (Figure 7, *A and B*); this phosphorylation/activition is also showed when CaCo2 cells were pretreated with rapamycin or the combination of AICAR and rapamycin (Figure 7, *A and B*). More importantly, our findings convey, after densitometry analysis, that AICAR, rapamycin, or their combination significantly down-regulate NADPH oxidases Nox 1 and Nox 4 protein expression compared to CaCo2 cells exposed to HG only (Figure 7, *C-E*). Our results also show that treatment of CaCo2 cells with AICAR or combination of AICAR and rapamycin significantly decreases fibronectin expression; whereas as, rapamycin alone shows a mild decrease in fibronectin expression; however this decrease is statistically significant (Figure 7, *F and G*). This suggests that AMPK/tuberin activation and mTOR inhibition may attenuate the effect of high glucose in increasing the susceptibility of colorectal cancer progression by reducing extra cellular matrix accumulation (fibronectin expression) and down-regulating Nox1/Nox4 derived ROS production.

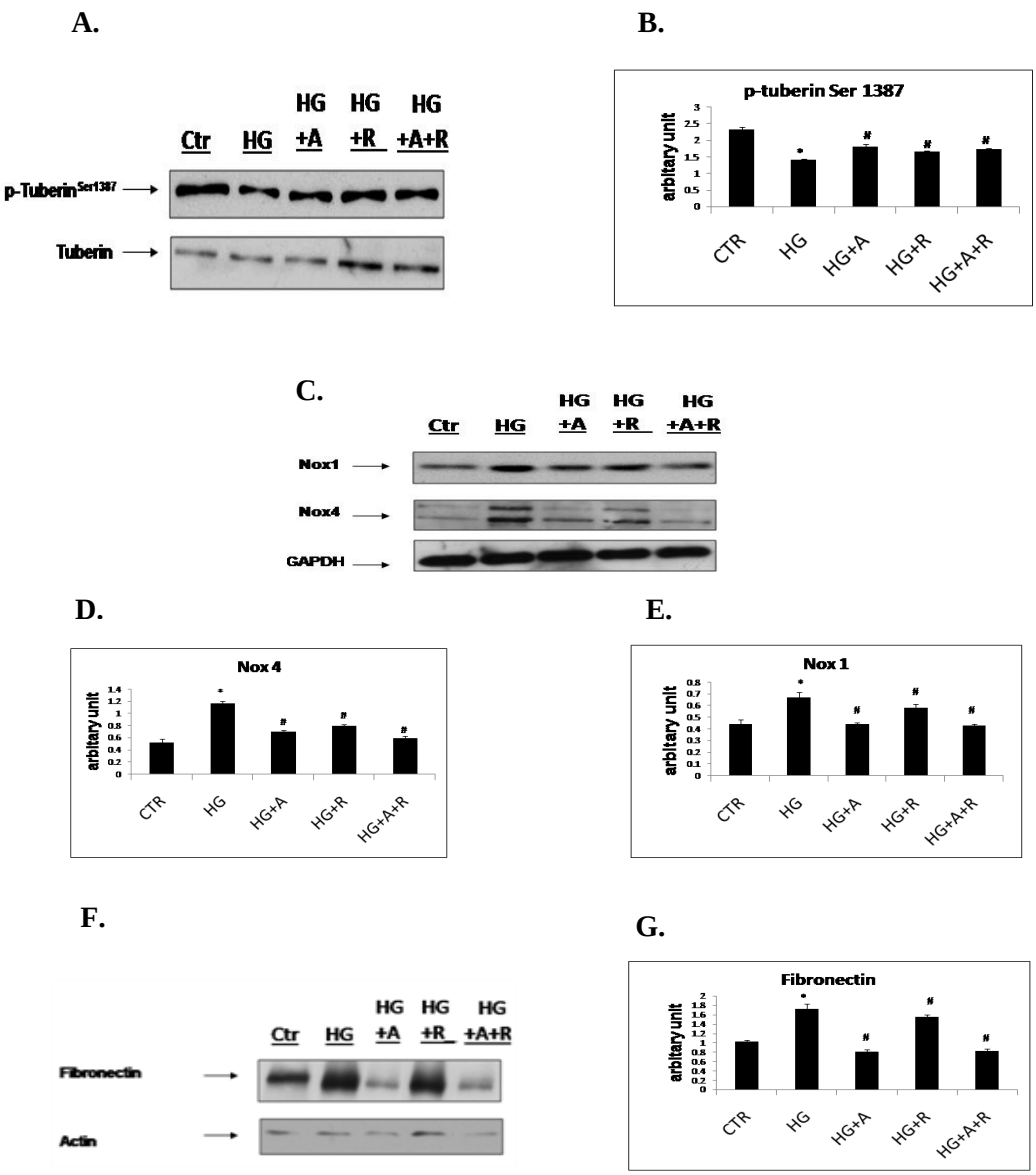


Figure 7. AMPK/tuberin activation and mTOR inhibition down-regulate Nox1, Nox4, and fibronectin protein expression. Human cultured epithelial colon adenocarcinoma cells were serum-deprived for 24h and pretreated with the AMPK activator AICAR (1.5mM), mTOR inhibitor rapamycin (25nM), or their combination for 1 h before incubation with HG. *A*, representative Western blot of phospho-Ser^{1387} tuberin and tuberin. *B*, histogram of phospho-Ser^{1387} tuberin after densitometry analysis using Image J software. *C*, representative Western blot of Nox1, Nox4, and GAPDH levels. *D and E*, histograms of Nox1 and Nox4 respectively after densitometry analysis. *F*, representative Western blot of fibronectin and β-actin levels. *G*, histogram analysis of fibronectin after densitometry analysis. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control; #, p <0.05 *versus* HG.

B. Cell proliferation, migration, and invasion

In the second set of experiments, and since we are interested in how diabetes may enhance the progression of cancer, we wanted to assess the role of the previously defined signaling pathway AMPK/tuberin/mTOR on cell proliferation, migration, and invasion on cultured epithelial colorectal adenocarcinoma cells (CaCo2).

1. Effect of high glucose, high insulin, or their combination on cancer cell proliferation

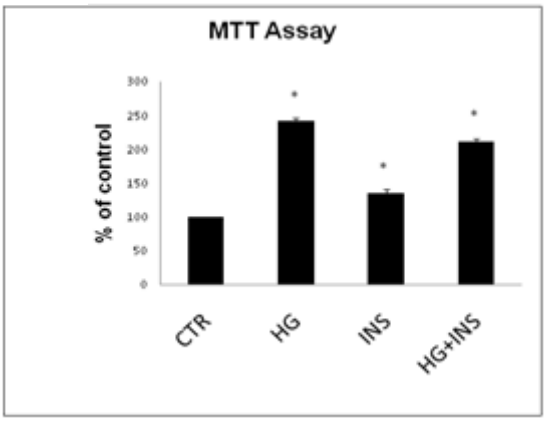
a. High glucose, high insulin, or their combination induce cancer cell proliferation

To investigate the effect of high glucose, high insulin, or their combination on the proliferation of cultured epithelial colorectal adenocarcinoma cells, MTT Cell Proliferation Kit I (Roche Applied Science) was used. Results show that HG, HI, or their combination induces CaCo2 proliferation and growth compared to CaCo2 incubated with NG (Figure 8A).

b. AMPK activation and mTOR inhibition reduce cancer cell proliferation

In parallel experiments, we wanted to see the effect of AMPK and mTOR on cancerous cell proliferation in the presence of HG. For that, CaCo2 cells were pretreated for an hour with the pharmacologic AMPK activator AICAR (1.5mM), the mTOR inhibitor rapamycin (25nM), or their combination and then exposed to 25mM of glucose (HG) for 72h. As shown in Figure 8B, treatment with AICAR, rapamycin, or their combination significantly reduces HG- induced CaCo2 cell proliferation, suggesting that AMPK activation or/and mTOR inhibiton may attenuate cancer progression in diabetic state.

A.



B.

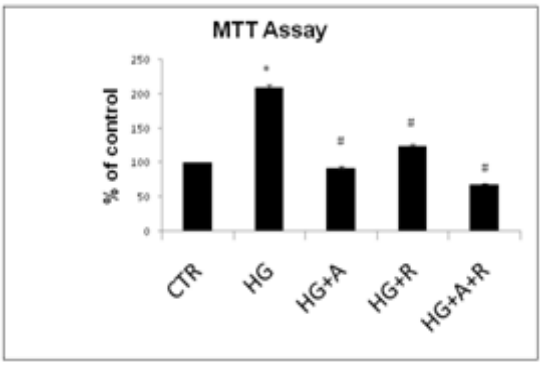
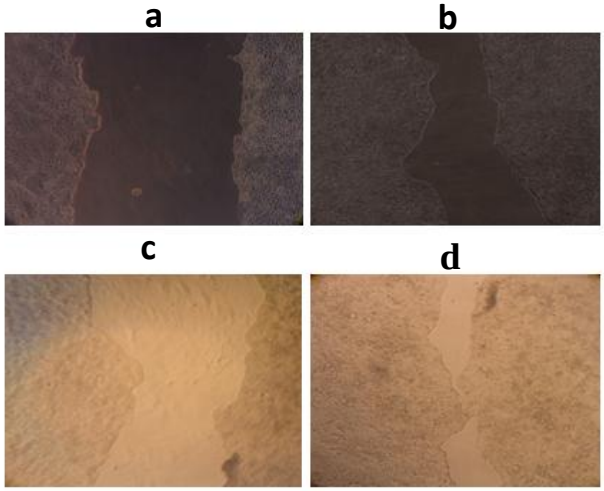


Figure 8. AMPK activation and mTOR inhibition regulate cell proliferation induced by HG, HI, or their combination. Cultured human colon adenocarcinoma cells (CaCo2) were serum-starved for 24 h and treated with high glucose (25mM), high insulin (500µM), or their combination for 72 h. In parallel experiments, CaCo2 cells were pretreated with the AMPK activator AICAR (1.5mM), mTOR inhibitor rapamycin (25nM), or their combination for 1 h before incubation with HG for 72 h. HG, HI, or their combination induced cell proliferation *(A)*, whereas AICAR, rapamycin, or their combination regulated HG-induced cell proliferation to control level and even lesser *(B).* All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control; #, $p < 0.05$ *versus* HG treatment.

1. High glucose, high insulin, or their combination induce cell migration

Cell migration was assessed by the still commonly used wound healing assay (Sudarshan *et al.,* 2011). Our results convey that when cancerous cells were treated with high glucose (25mM), high insulin (500µM), or their combination, the area separating the two edges of the wound decreases after 72 hours compared to cancerous cells exposed to normal glucose (5mM); CaCo2 treated with HG, HI, or their combination migrated more toward the wound to even closure of the wound compared to the control (Figure 9, *A, compare panel b, c, d respectively with panel a, and B*). These results show that HG, HI, or their combination enhances cancerous cells migration, which might possibly exacerbate cancer progression.

A.



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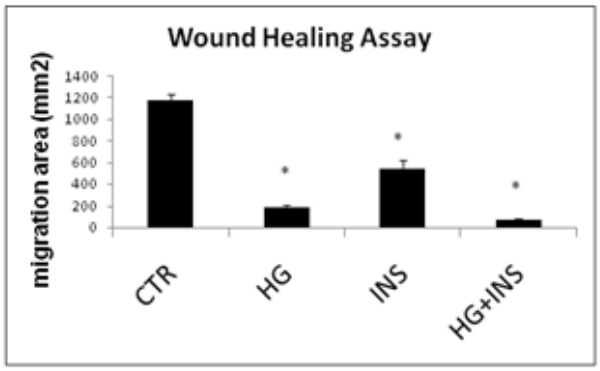

Figure 9. High glucose, high insulin, or their combination enhances the migration of cancerous cells. Wound healing assay used to assess cell migration. *A*, images of human cultured epithelial colon adenocarcinoma cells treated with high glucose (b), high insulin (c), or their combination (d) and compared to control with normal glucose (a). *B*, histogram representing the migration area measured in (mm^2). Images taken at 5x magnification and migration areas measured using Image J software. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control.

3. AMPK activation and mTOR inhibition reduce cancer cell invasion induced by HG.

To further investigate if the diabetogenic level of glucose increases cancer cell invasion and to elucidate its mechanism of action, cellular invasion was examined through the Millipore QCM ™ 8µm 24-well Chemotaxis Assay kit according to the manufacturer protocol. Our results show that HG significantly induces cancer invasion; incubation of CaCo2 with the AMPK activator AICAR (1.5mM), the mTOR inhibitor rapamycin (25nM), or their combination significantly reversed the effect of HG-induced invasion (Figure 10). These data may indicate that HG aggravates colon cancer by enhancing invasion. This latter mechanism might be regulated by activating AMPK and inhibiting mTOR in cancerous cells.

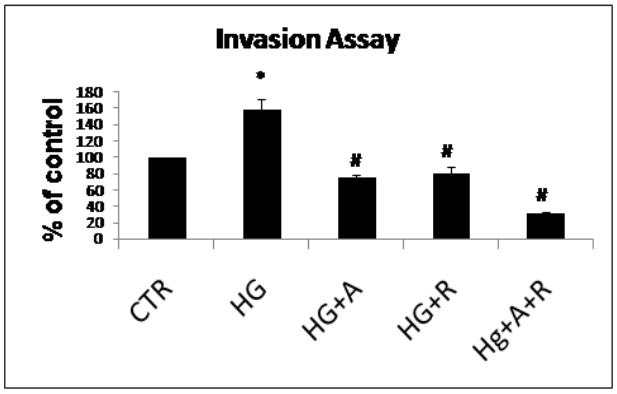

Figure 10. AMPK activation and mTOR inhibition regulate HG-induced cancer cell invasion. Histogram representing the percentage of CaCo2 cells that have invaded the ECVs monolayer after treatment and as compared to control. CaCo2 cells were pretreated with the AMPK activator AICAR (1.5mM), mTOR inhibitor rapamycin (25nM), or their combination for 1 h before incubation with HG for 72 h. AICAR, rapamycin, or their combination regulated HG-induced cell invasion. All values are the mean ± S.E. from three independent experiments. *, $p < 0.05$ *versus* the control; #,$p < 0.05$ *versus* HG treatment.

C. AMPK activation induces apoptosis in cancer

We next examined the role of AMPK and mTOR in apoptosis of CaCo2 cancerous cells induced by HG. To study apoptosis, cellular DNA Fragmentation ELISA kit (Roche Applied Science) was used. As expected, exposure of CaCo2 like other cell lines with HG augments DNA fragmentation (Figure 11). Incubation of CaCo2 with the AMPK activator AICAR (1.5mM) mimics more significantly the effect of HG and induces apoptosis in cancerous cells, whereas pharmacologic inhibition of mTOR by rapamycin (25nM) shows to have minimal effect in inducing apoptosis and even lessens the effect of AICAR (Figure 11). This implicates that AMPK in cancer induces apoptosis for regulating and reducing cancer cell proliferation. On the other hand, rapamycin with a working concentration of 25nM shows to have minimal effect in inducing apoptosis of CaCo2.

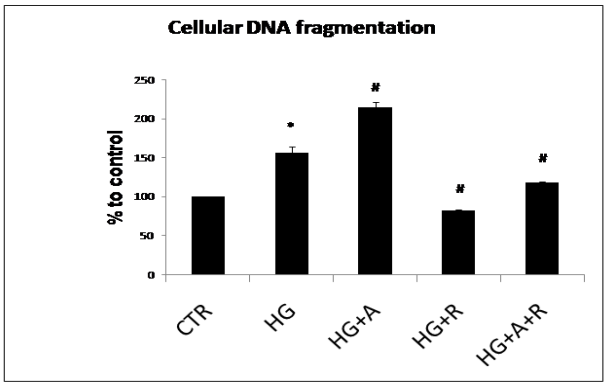

Figure 11. AMPK enhances apoptosis in cancer. Histogram representing percentage of CaCo2 cells that underwent apoptosis and treated with AICAR (1.5mM), rapamycin (25nM), or their combination for 1 h before incubation with HG for 72 h as compared to control with NG. AICAR treatment enhanced apoptosis of cancerous cells. Rapamycin showed minimal effect in inducing CaCo2 apoptosis. All values are the mean ± S.E. from three independent experiments.*, $p < 0.05$ *versus* the control; #, $p < 0.05$ *versus* HG treatment.

DISCUSSION

Both diabetes mellitus and cancer are prevalent diseases whose incidence is increasing worldwide. Many epidemiological studies have conveyed frequent co-occurrence of diabetes and cancer suggesting an association of DM with specific types of solid tumors including colorectum (Zendehdel et al., 2003). Patients with type 2 DM have a 30% increased risk for colorectal cancer versus the general population (Giouleme, 2011). While diabetes and cancer share many risk factors, the biological links between the two diseases are poorly characterized. In this study, we uncovered novel biological pathway that suggests an explanation for diabetes-induced cancer development and progression; mainly, mediated through an AMPK/NADPH/TSC2/mTOR pathway.

Cancer cells, like other non-malignant cells, have enhanced ROS production. In tumors, reactive oxygen metabolites can act as signaling molecules to promote cell survival over apoptosis (Storz, 2005; Szatrowski & Nathan, 1991). We have conveyed that cancer itself is a major source of ROS production since it is significantly increased in CaCo2 cancerous cells compared to normal NCM 356 non-cancerous cells (Figure 3). ROS-generating NADPH oxidases are highly organ-specific for both malignant and non-malignant tissue. It was shown that in the gastrointestinal tract, expression of Nox1 is significantly increased in colon cancer compared to adjacent normal bowel mucosa (Juhasz et al., 2009). In other studies, in pancreatic cancer cells, Nox4-mediated ROS have been shown to prevent apoptosis and promote tumor cell growth (Mochizuki et al., 2006; Vaquero et al., 2004). Despite these studies, the mechanisms underlying the effect of Noxs in cancer and more importantly the role of these enzymes in diabetes-induced cancer development and progression is poorly studied and understood.

On the other hand, AMPK phosphorylation on its activating site Thr^{172} maintains its activity (Hawley et al., 1996). AMPK is a key nutrient and energy sensor in cells and it is activated under compromised energy status (Fitzgerald et al., 2012). We find that AMPK activity is lower in CaCo2 compared to NCM 356 and this is assessed by decreased phoshorylation of AMPK on its activating site Thr^{172} (Figure 4B). These results are consistent with recently published work, done in our collaborators lab, showing that AMPK activity is lesser in cultured renal cell carcinoma (RCC) cells compared to normal renal epithelial cells (Fitzgerald et al., 2012). The interaction between ROS and AMPK is stimulus and tissue specific. During hypoxia, mitochondria-generated ROS activate AMPK (Emerling et al., 2009). Similarly, during exercise, NADPH oxidase-derived ROS induce AMPK activation (Moir et al., 2010). Concerning tissue specificity, in pancreatic β-cells, HG activates AMPK and enhances the production of ROS, resulting in loss of mitochondrial membrane potential (Kim et al., 2007). On the other hand, in umbilical vein endothelial cells, activation of AMPK increases the expression of the antioxidant manganese superoxide dismutase and inhibits HG-induced intracellular and mitochondrial ROS production (Kukidome et al., 2006), suggesting that activated AMPK may suppress oxidative stress. Similarly, in renal podocytes, it has been recently demonstrated that AMPK activation down-regulates Nox4 expression (Eid et al., 2010). These observations highlighting that activated AMPK down-regulates Noxs expression are consistent with our results that show up-regulation of Nox1 and Nox4 in CaCo2 compared to NCM 356 (Figure 4A); these observations are paralleled by decreased phosphorylation/activation of AMPK in CaCo2 cells (Figure 4B).

In our study, we also show that high glucose, high insulin, or their combination increases the susceptibility of the epithelial colon adenocarcinoma cell line (CaCo2) to produce ROS more than the normal colon cells (Figure 2). This is paralleled by the inactivation of AMPK/tuberin (Figure 5B, *F-H*), up-regulation of Nox1 and Nox4 (Figure 5A, *A-C*) associated with increased fibronectin expression (Figure 5A, *D and E*), and activation of mTOR (Figure 5B, *I and J*). Our data suggest that high glucose or high insulin induced Nox1 and/or Nox4-dependent ROS production can lead to increased fibronectin expression and this induction can be due to inactivation of the AMPK/tuberin and activation of mTOR pathway as we may see in our result section. To further support our data, activation of AMPK by AICAR and the inhibition of mTOR by rapamycin decrease ROS production (Figure 6, *A and B*), restore tuberin phosphorylation/activity (Figure 7, *A and B*), attenuate Nox1/Nox4-dependant ROS production (Figure 7, *C-E*), and decrease fibronectin expression (Figure 7, *F and G*) induced by high glucose or high insulin. However, the mechanism by which AMPK or mTOR regulates Nox1/Nox4 is still poorly characterized and needs further investigation.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase and is a key regulator of protein translation/synthesis. This signaling molecule occupies a significant role in cell growth and proliferation (Schmelzle & Hall, 2000; Fingar *et al.* 2002). The mTOR pathway is activated by amino acids and by growth factors such as PDGF, epidermal growth factor (EGF) and insulin (Motoshima et al., 2006). Several groups have reported that activation of AMPK suppresses mTOR signalling. AMPK directly phosphorylates mTOR at Thr-2446 to reduce S6K1 phosphorylation by insulin, suggesting the inhibition of mTOR action (Cheng *et al.* 2004). In this study, our data

show that HG or/and HI induce(s) cancer cell proliferation (Figure 8A), migration (Figure 9), and invasion (Figure 10). Results also demonstrate that activation of AMPK by AICAR and inhibition of mTOR by rapamycin regulate cancer cell proliferation (Figure 8B) and invasion (Figure 10). Furthermore, in response to HG, HI, or their combination, and as mentioned before, immunoblot analysis reveals noticeable inactivation of AMPK/tuberin by decreasing AMPK/tuberin phosphorylation on its activating site Thr^{172} and Ser^{1387} respectively and activation of mTOR by increasing mTOR phosphorylation on its activating site Ser^{2448}. This suggest that HG or/and HI induced- cancer proliferation is possibly mediated through AMPK/TSC2/mTOR pathway. Our data also indicates that AMPK activation and mTOR inhibition regulate cell proliferation and invasion highlighting further the mechanism that may lie behind diabetes induced cancer progression.

In this study, we also established that AMPK activation in cancer exerts a pro-apoptotic effect resulting in decreased cancer cell proliferation. Our group has previously demonstrated that inactivation of AMPK in podocyte mediates the pro-apoptotic effect of HG and is necessary for podocyte apoptosis (Eid et al., 2010). In fact, in cancer, our results using the pharmacologic AMPK activator AICAR indicate that AMPK enhances apoptosis and even more significantly than HG (Figure 11). Although HG induces apoptosis, the MTT cell proliferation assay reveals that HG or/and HI significantly enhance(s) cell proliferation (percentage of increase in HG-induced proliferation is greater than the percentage of increase in HG-induced apoptosis, compare Figure 11 to Figure 8A). This may indicate that both mechanisms HG-induced proliferation and apoptosis occur in cancer, however, proliferation may be dominating over apoptosis. Our data also suggest that AMPK activation in cancer enhances

apoptosis to regulate HG or HI induced-cancer cell proliferation. These results are consistent with the already published data showing that although activation of AMPK is likely, in the short term, to reduce ATP consumption and thus to protect cells from transient metabolic stresses (Hardie et al.,1998), sustained activation of this signaling molecule entrains a sequence of events eventually leading to programmed cell death (Chavey et al., 2008). It is also noteworthy to mention that rapamycin suppresses phosphorylation of S6 kinase at nano-molar concentrations; however, at higher micro-molar doses it induces apoptosis in several human cancer cell lines (Yellen et al., 2011). This explains why the 25nM of rapamycin in our study does not induce apoptosis of CaCo2.

The limitation that was identified during the course of this study is the high concentration of glucose (25mM) used to mimic the diabetogenic state. Other studies have considered 11mM as the diabetogenic glucose concentration and have shown that this concentration promotes cancer proliferation and migration (Masur et al., 2011). So, in the future work, it would be interesting to compare these two concentrations of glucose and to examine their impact on cancer proliferation and migration

Our data in this study identify a novel promising pathway linking diabetes to colorectal cancer. Our *in vitro* studies support a good part of our hypothesis that HG or/and HI (mimicking Type I and Type II diabetes meallitus) modify cancer cell proliferation through AMPK/Noxs/tuberin/mTOR pathway. Our observations suggest that AMPK activation or mTOR inhibition may characterize an adjunct therapy to control cell growth in diabetes-induced cancer. However, in the next step, more work is required to assess how diabetes may induce and also aggravate colorectal cancer. This aim will be definetely approached and more elaborated through *in vivo* model.

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