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Diabetes and colorectal cancer : epidemiological and physiopathological studies

Fatima Mohsen Mroueh

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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
ED 414-Ecole Doctoral des Sciences de la Vie et de la Santé

THÈSE présentée par :
Fatima MOHSEN MROUEH

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Discipline/ Spécialité : Doctorat Sciences Médical

**Diabète et Cancer Colorectal : Épidémiologie et
Physiopathologie**

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For faith gives constant peace to our souls and holds us up everytime we fall, for we know that faith in GOD will always give us strength to fulfill our goals, for that and so much more, I am eternally grateful.

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To you and to my parents, I dedicate this thesis.

Fatima Mohsen Mroueh

University of Strasbourg

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ABSTRACT

FATIMA MOHSEN MROUEH FOR DOCTORATE OF PHILOSOPHY

MAJOR: BIOMEDICAL SCIENCES

DIABETES AND COLORECTAL CANCER: EPIDEMIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES

Diabetes is a chronic systemic malfunction characterized by persistent metabolic disturbances that culminate in a high rate of micro- and macrovascular events to which cancer was recently annexed. In fact, diabetes inflates colorectal cancer (CRC) risk by 1.2-1.5 folds leaving the patients with increased aggressiveness and poorer 5-year survival. The mechanisms that contribute to the onset and development of these complications are still poorly defined even though they are known to orbit reactive oxygen species (ROS) as a common origin. CRC progress has been linked to hyperglycemia and hyperinsulinemia. However, the cellular and molecular pathways involved in diabetes-induced CRC progression are still underdeveloped. In this study, we show that AMPK/mTORC1 pathway is deregulated in both diabetes and CRC. This was paralleled by an elevation in the expression of the NADPH oxidase Nox4 leading to an increase in ROS production. Additionally, our group and others have also described the role of mTORC1 pathway in the progression of diabetic complications. Yet, the exact mechanism of mTORC1 activation is not yet fully described. We hypothesize that oxidative stress generating ROS, secondary to alteration in the level and activity of the NADPH oxidase Nox4 is augmented in diabetes and contributes to the progression of CRC. The resulting oxidative stress leads to the alteration in the signaling of the AMPK/mTORC1 pathways culminating in an exacerbated aggressiveness in cancer cell behavior and colon polyp formation. From a fundamental perspective, this project will allow the identification of novel molecular mechanisms involved in diabetes-induced CRC progression and from the clinical angle it will allow the development of effective therapeutic strategies to reverse or slow the progression of CRC in diabetic patients.

DIABÈTE ET CANCER COLORECTAL :

ÉPIDÉMIOLOGIE ET PHYSIOPATHOLOGIE

Le diabète et le cancer colorectal sont des maladies fréquentes dont les taux d'incidence augmentent dans le monde, à cause des facteurs de risques du style de vie, notamment le régime alimentaire, l'inactivité physique et l'obésité, qui jouent tous un rôle pivot dans l'étiologie de ces deux maladies.

Des études épidémiologiques donnent de fortes preuves que les sujets porteurs d'un diabète ont un fort risque de développer un cancer, notamment une tumeur solide. Une grande étude rétrospective menée en 2006 a rapporté un sur-risque significatif de cancer colorectal proximal chez l'homme diabétique (Limburg et al., 2006, Oh et al., 2008, Ren et al., 2009), la femme diabète (Elwing et al., 2006) ou tout sexe confondu (Yang et al., 2005). De même, le traitement insulinique régulier a été trouvé associé avec un risque accru de tumeur colorectale parmi des patients diabétiques de type 2 (Chung et al., 2008, Yang et al., 2006), surtout chez diabétique insulino-requérant (Nagel et al., 2006). Les dérivés réactifs de l'oxygène (Reactive Oxygen species, ROS ou radicaux libres) sont reconnus pour jouer un rôle important dans l'entretien physiologique cellulaire. Les cellules cancéreuses, comme les cellules non-cancéreuses, produisent des ROS. Dans les tumeurs, les métabolites actifs de l'oxygène peuvent agir comme des molécules de signalisation pour favoriser la survie cellulaire sur l'apoptose (Rayjean et al., 2005, carling et al., 2004). Il a été démontré que les ROS médiés par le Nox4 préviennent l'apoptose et favorise la croissance des cellules tumorales dans les cancers du pancréas (Viollet et al., 2003, Shaw et al., 2004). De plus, plusieurs rapports décrivent une augmentation de la quantité de 8-oxodG dans les cellules mononuclées and dans l'urine des patients diabétiques de type 1 et 2 (Slattery et al., 2010).

L'Adenosine monophosphate-activated protein kinase (AMPK) joue un rôle central dans la régulation du statut énergétique de la cellule. Plusieurs études ont associé ses sous-unités $\alpha 1$ et $\gamma 2$ tant au cancer colorectal qu'au métabolisme lipidique et glucide, c'est-à-dire au diabète (Mochizuki et al., 2006). L'AMPK peut être phosphorylée et activée dans différents tissus par les

agonistes des β -adrenorecepteurs, tels que les agents pharmacologiques comme la metformine ou AICAR (Mochizuki et al., 2006).

Une cible d'aval de la LKB1/AMPK particulièrement intéressante a été récemment identifiée, la TSC2 (tubérine). La voie LKB1/AMPK/TSC2 régule négativement la cible de la rapamycine (TOR), impliquée dans la synthèse des protéines, la survie cellulaire et la tumorigénèse par le fait que la rapamycine inhibe la croissance cellulaire.

En réponse aux facteurs de croissance et à la présence des nutriments comme les acides aminés et le glucose, mTORC1 est activé et régule un grand nombre de processus cellulaires, incluant la traduction des protéines, la transcription, l'épissage de mRNA, le cycle cellulaire et l'autophagie (Wullschleger et al., 2006). L'hyperactivation de la voie mTORC1 a été supposée être un facteur important de la tumorigénèse (Inoki et al., 2009). Il a été rapporté que la voie TORC1 était activée chez environ 40% des patients porteurs de cancer colorectal (Nozawa et al., 2007). La phosphorylation de TSC2 sert de point d'intégration à une grande variété de signaux environnementaux qui régulent mTORC1 (Sarbasov et al., 2005). Néanmoins, le rôle de la voie TSC2/mTORC1 dans la tumorigénèse d'un cancer du côlon dans le diabète reste encore largement inconnu.

Dans cette étude, nous espérons révéler le lien biologique entre diabète et cancer du côlon. Le diabète peut influencer le processus néoplasique au travers de multiples mécanismes, incluant l'hyperglycémie, l'insulinorésistance et l'hyperinsulinémie. Nous espérons donc déterminer le rôle de l'AMPK, du mTOR et de leur action croisée, sur les sous-unités Nox1 et Nox4 des oxydases NADPH dans les cellules normales et dans les cellules épithéliales de cancer du côlon, et ce dans leur réponse à l'hyperglycémie, l'hyperinsulinémie et la combinaison des deux.

Nous avons la preuve que dans la cellule cancéreuse épithéliale du cancer colorectal, le traitement hyperglycémique inactive l'adénine monophosphate kinase (AMPK), active la voie mTOR/S6Kinase et favorise la production de DNA muté, 8-oxodG (**Fig. a**).

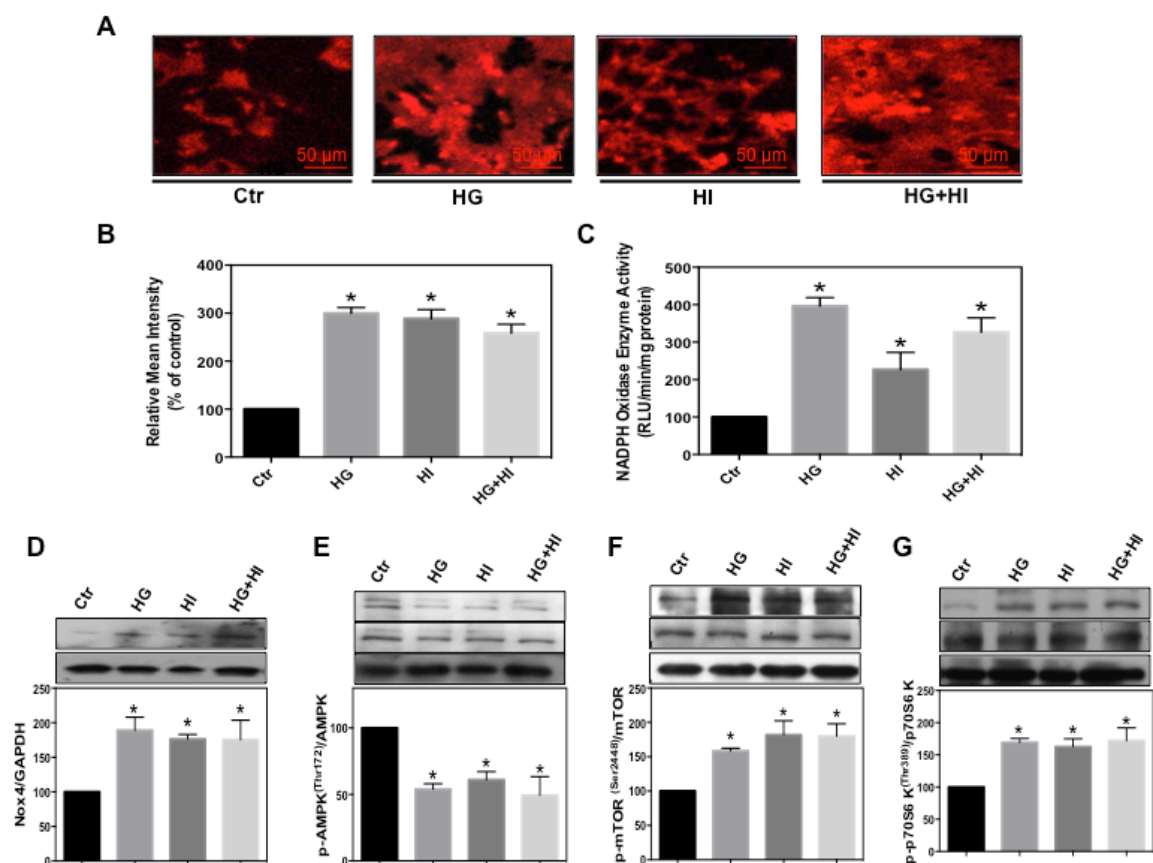


Figure a. L'effet du taux de glucose élevé sur les cellules cancéreuses est dû à la production de ROS dépendante du NADPH et la voie AMPK/mTOR est impliquée. Les cellules HT-29 ont été traitées avec HG (25 mM), HI (500 nM) ou une combinaison des deux pendant 72 heures. (A) Les figures représentatives de la coloration DHE montrent que HG, HI ou leur combinaison induisent la production de ROS. (B) Histogramme montrant la coloration DHE des cellules cancéreuses, ROS extracellulaire a été mesurée après une incubation avec DHE (5 μ M) pendant 30 minutes à 37°C. (C) Le test d'activité NADPH oxydase des cellules HT-29 a été mesurée en utilisant le test Lucigénine. (D), (E), (F) et (G) immuno-empreintes représentatives et histogrammes de Nox4, phospho-Th172 AMPK, AMPK, phospho-Ser2448 mTOR, mTOR, phospho-Thr389 p70S6 kinase, et p70S6 kinase résultant du lysats des cellules cancéreuses. Toutes les valeurs sont la moyenne \pm S.E. d'au moins trois expériences indépendantes. *, $p < 0,05$ par rapport au témoin.

Nous montrons aussi que l'inactivation de l'AMPK régule positivement Nox4, qui ensuite vont activer mTOR. Ces observations étaient associées avec une augmentation de la prolifération cellulaire, de la migration et de l'envahissement des cellules cancéreuses (**Fig. b**).

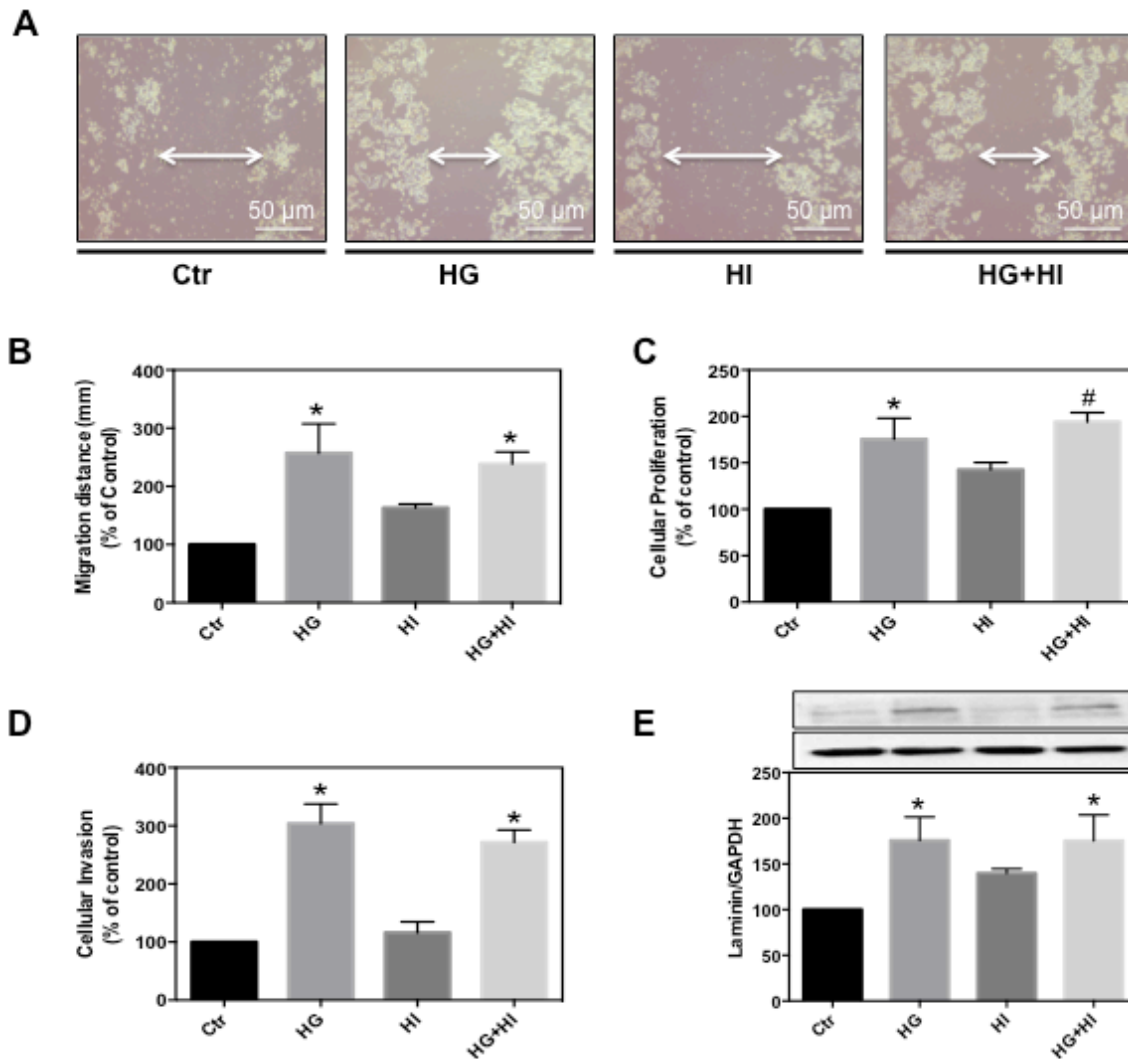


Figure b. Effet de HG, HI ou leurs combinaisons sur les cellules cancéreuses. Les cellules HT-29 ont été traitées avec HG (25 mM), HI (500 nM) ou une combinaison des deux pendant 72 heures. (A) Les figures représentatives du test de migration effectué en permettant aux cellules de croître jusqu'à la confluence et induction d'une égratignure dont la distance est mesurée avant et après le traitement. (B) Histogramme du test de migration. (C) essai de prolifération MTT. (D) Un test d'invasion cellulaire effectué en utilisant des inserts de culture cellulaire de 8 µm. Les cellules envahissant la membrane poreuse ont été colorés par la coloration H&E et comptés sous un microscope optique. (E) immuno-empreinte représentative de la laminine et GAPDH résultant de lysats des cellules cancéreuses. Toutes les valeurs sont la moyenne \pm S.E. d'au moins trois expériences indépendantes. *, $p < 0,05$ par rapport au témoin.

Le traitement par metformine, un puissant activateur de l'AMPK ou par rapamycine, un inhibiteur de mTORC1, renverse les changements biochimiques et comportementaux observés dans les cellules cancéreuses traitées par hyperglycémie, insuline ou par combinaison des deux (Fig c).

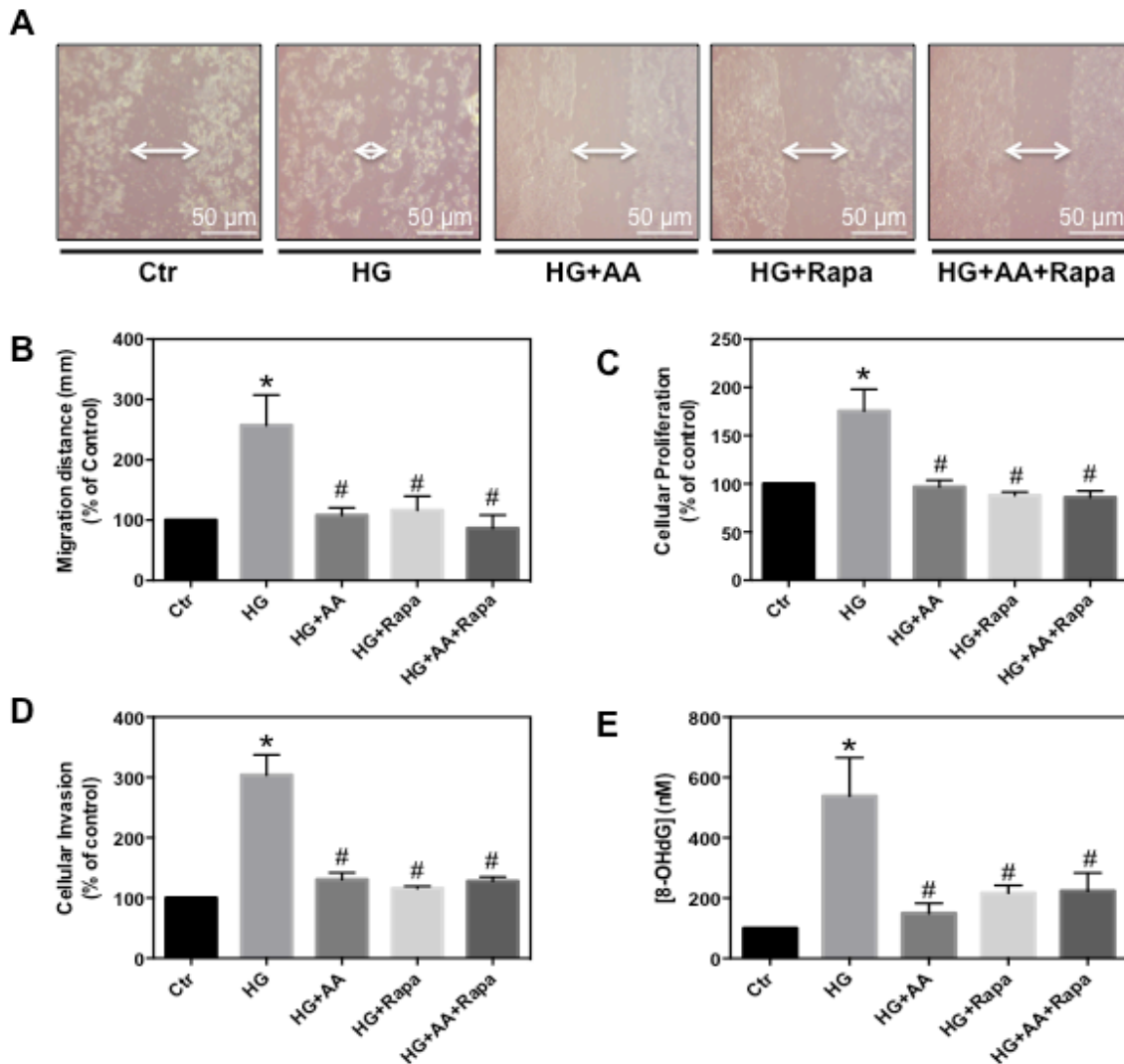


Figure c. L'activation de l'AMPK et/ou l'inhibition de mTOR réduit la lésion des cellules cancéreuses et la production de 8-oxodG. Les cellules HT-29 ont été traitées avec du HG (25 mM) seul ou avec de la metformine (5 mM) de l'AICAR (1.5 mM), de la rapamycine (25 nM), ou une combinaison des deux pendant 72 heures. (A) Figures représentatives du test de migration dans les cellules cancéreuses avec l'histogramme (B) montrant la distance de migration des cellules cancéreuses en pourcentage du contrôle. (C) Test MTT évaluant la prolifération des cellules cancéreuses. (D) Essai d'invasion cellulaire montrant une diminution de l'invasion des cellules cancéreuses par traitement à la metformine et / ou à la rapamycine. (E) Histogramme montrant la concentration d'adduits 8-oxodG dans les cellules cancéreuses; L'ADN a été extrait des cellules traitées en utilisant la méthode high salt et le calcul de la concentration en 8-oxodG a été fait en référence à une courbe standard de 8-OHdG. Toutes les valeurs sont la moyenne \pm S.E. d'au moins trois expériences indépendantes. *, $p < 0,05$ par rapport au témoin. #, $p < 0,05$ versus HG.

Ces effets étaient aussi observés avec l'utilisation de Nox4 CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), qui agit en éteignant l'expression du gène Nox4 (**Fig. d**).

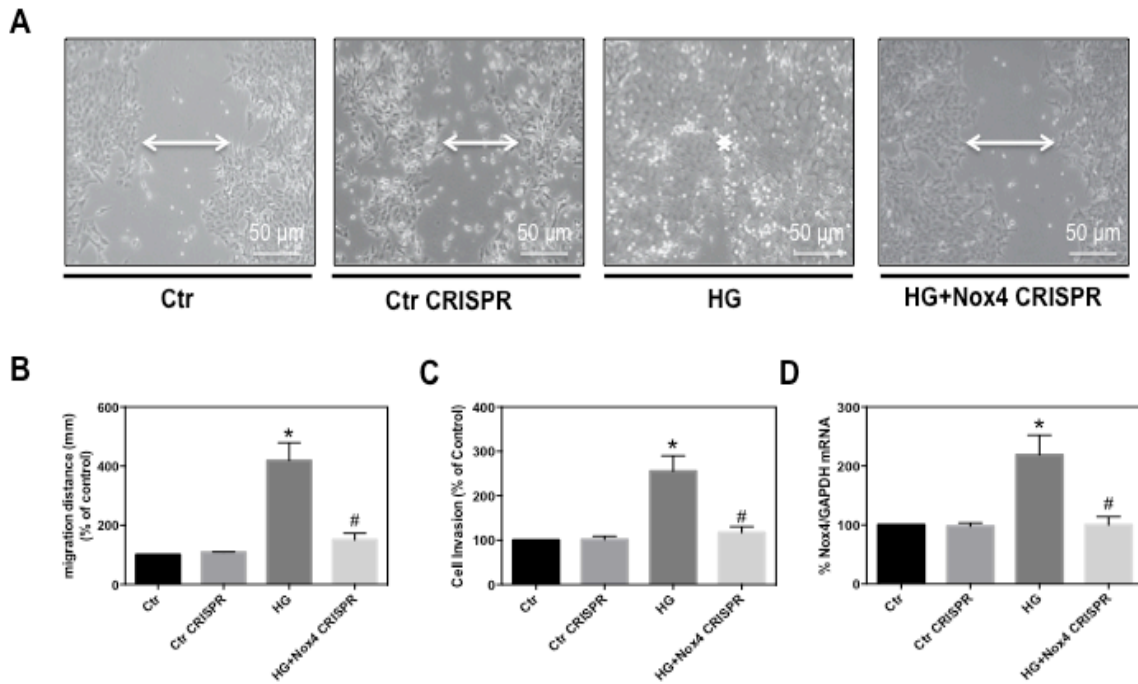


Figure d. Nox4 médie la production du ROS induit par le taux élevé du glucose ainsi que la lésion des cellules cancéreuses. Les cellules CaCO2 ont étéensemencées à faible confluence et ensuite transfectées avec soit le témoin CRISPR, soit le Nox4 CRISPR traité avec ou sans HG (25 mM). Aucune différence significative n'a été observée entre les cellules non traitées (témoin) et les cellules transfectées avec le témoin CRISPR. (A) Figures représentatives de l'essai de migration montrant une diminution de la distance de migration des cellules traitées par HG lors du knockdown de l'expression du gène Nox4. (B) Histogramme du test de migration. (C) Essai d'invasion montrant une diminution de l'invasion du cancer lors de knockdown Nox4. (D) histogramme montrant les niveaux d'ARNm relatifs de Nox4; Les cellules traitées avec HG + Nox4 CRISPR ont montré une diminution de l'expression du gène Nox4 par rapport aux cellules traitées par HG. Toutes les valeurs sont la moyenne \pm S.E. d'au moins trois expériences indépendantes. *, $p < 0,05$ par rapport au témoin. #, $p < 0,05$ versus HG.

Dans le modèle murin de cancer colorectal (souris APC), nous avons trouvé une activation de la voie mTOR/S6Kinase chez la souris diabétique, en parallèle d'une augmentation des niveaux de production de ROS, au travers d'un mécanisme dépendant de NADPH (**Fig. e**).

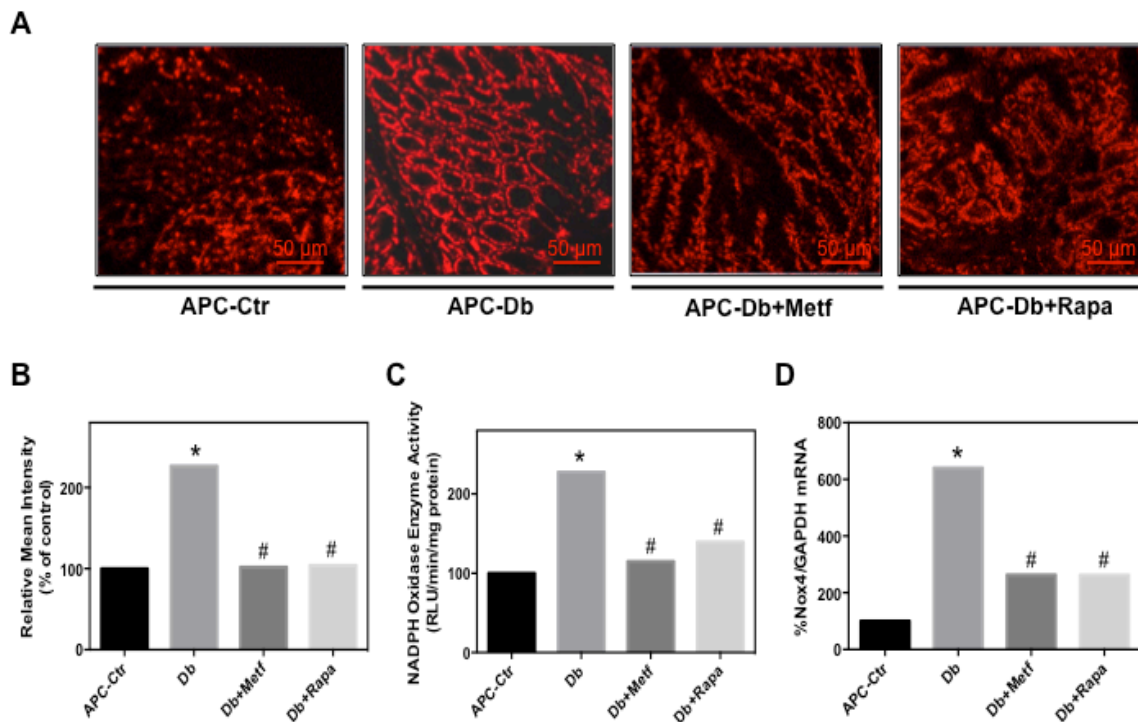


Figure e. Les traitements à la metformine et à la rapamycine réduisent la production de ROS et les adduits de 8-oxodG chez les souris APC. Des souris APC diabétiques de type 1 induites par STZ ont été traitées avec soit du véhicule, soit de la metformine (150 mg / kg / jour), soit de la rapamycine (0,5 mg / kg / jour) administrée par injection intrapéritonéale pendant 6 semaines alors que les souris control ont reçu seulement le véhicule. Des sections du tissu frais du côlon (4-5 µm d'épaisseur) des souris APC ont été incubées pendant 1 heure à 37 °C avec une coloration DHE 15 µM. Les images ont été prises directement à l'aide d'un microscope confocal à balayage laser. (A) Images représentatives de la coloration à la DHE des tissus du côlon. (B) Histogramme montrant le résultat de la coloration DHE de 4 souris par groupe. (C) Essai d'activité de la NADPH oxydase. (D) Niveaux d'ARNm relatifs de Nox4. Toutes les valeurs sont la moyenne ± S.E. de 4 souris par groupe. *, $p < 0,05$ contre les souris témoins C57 traitées avec le véhicule. #, $p < 0,05$ contre les souris APC diabétiques de type 1 induites par la STZ et traitées avec le véhicule.

Ces observations indiquent un rôle important de AMPK et mTOR dans la progression du diabète. De plus, l'activation de AMPK ou le blocage de la voie mTORC1 chez la souris APC diminue la production de ROS, inverse la mutation OGG1 et l'accumulation dans le côlon de 8-oxo-dG et diminue le développement tumoral (**Fig. f**).

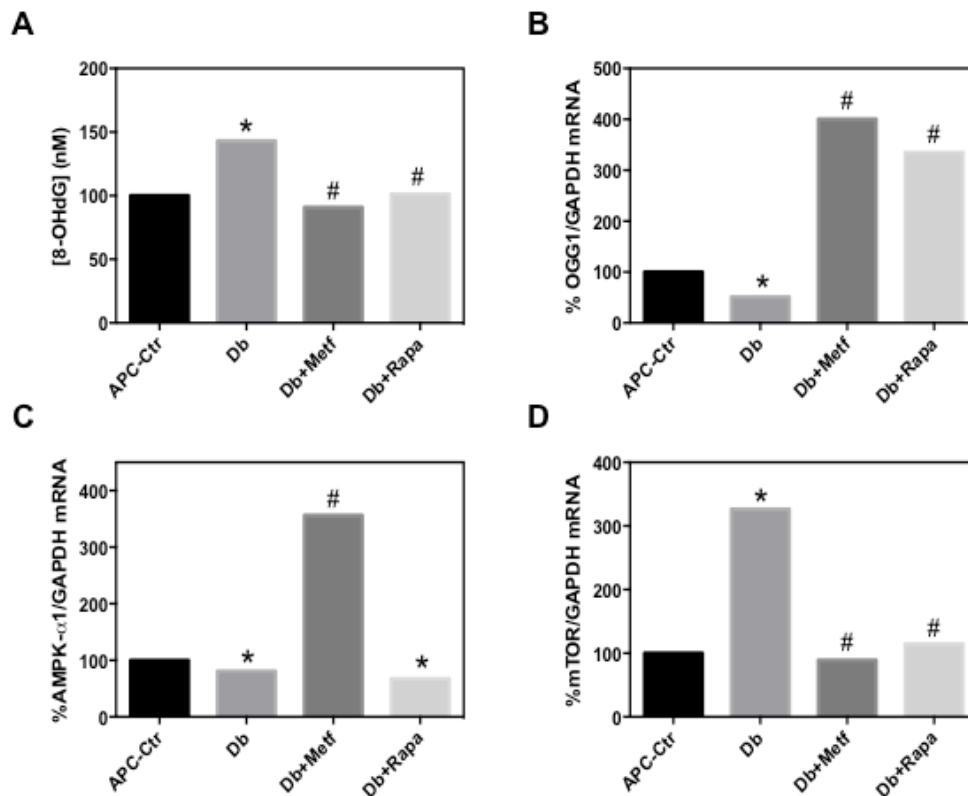


Figure f. Les traitements à la metformine et à la rapamycine réduisent la production de ROS et les adduits de 8-oxodG chez les souris APC. Des souris APC diabétiques de type 1 induites par STZ ont été traitées avec soit du véhicule, soit de la metformine (150 mg / kg / jour), soit de la rapamycine (0,5 mg / kg / jour) administrée par injection intrapéritonéale pendant 6 semaines alors que les souris control ont reçu seulement le véhicule. (A) La concentration de 8-oxodG diminue de manière significative dans les tissus du côlon des souris traitées avec la metformine ou la rapamycine par rapport aux souris diabétiques. Niveaux d'ARNm relatifs d'OGG1 (B); l'enzyme qui reconnaît et excise les adduits de 8-oxodG, AMPK- α 1 (C) et mTOR (D). Toutes les valeurs sont la moyenne \pm S.E. de 4 souris par groupe. *, $p < 0,05$ par rapport aux souris témoins APC traitées avec le véhicule. #, $p < 0,05$ contre les souris APC diabétiques de type 1 induites par la STZ et traitées avec le véhicule.

Nos résultats identifient une nouvelle voie prometteuse reliant diabète et cancer colorectal. Nos études *in vitro* et *in vivo* sont en faveur de notre hypothèse que l'hyperglycémie et/ou l'hyperinsulinémie (mimant un diabète type 1 ou 2) modifient le comportement de la cellule cancéreuse au travers de la voie AMPK/Nox4/mTOR (**Fig. g**). Nos observations suggèrent que l'activation de l'AMPK ou l'inhibition de mTOR pourrait servir de base à un traitement adjuvant visant à contrôler le comportement des cellules cancéreuses dans les cancers induits par le diabète.

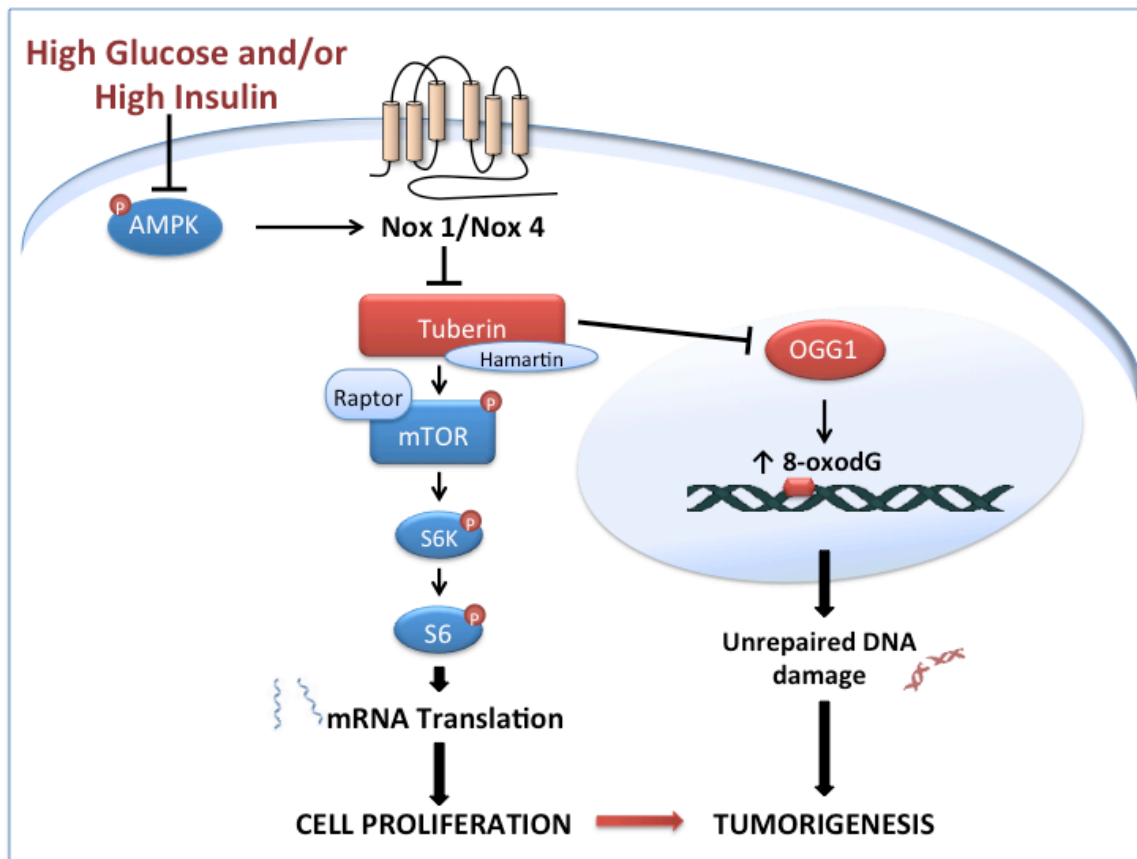


Figure g. Mécanisme proposé de la progression du cancer du côlon induite par le diabète. Le diabète conduit à l'activation de Nox4 à travers l'inactivation du AMPK. Cela conduit à une inhibition de la tuberculine et donc à une activation de la voie mTORC1 / p70S6K, ce qui entraîne une augmentation de la prolifération cellulaire et de l'agressivité. L'inactivation de la tuberculine inhibe également l'activité de l'OGG1, ce qui entraîne une accumulation de lésions de l'ADN qui contribuent à la tumorigenèse.

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ABBREVIATIONS

AA:	AMPK activator
AICAR:	5-amino-4-imidazole carboxamide ribonucleoside
AMPK:	5'-Adenosine monophosphate-activated Protein Kinase
APC:	Adenomatous Polyposis Coli
CRC:	Colorectal Cancer
DCF:	2',7'-dichlorofluorescein diacetate
DHE:	Dihydroethidium
GI tract:	Gastrointestinal tract
GLUT:	Glucose transporter
HG:	High Glucose
HI:	High Insulin
IGF:	Insulin-like growth factor
LKB1:	Liver Kinase B1
Metf:	Metformin
mTOR:	Mammalian Target of Rapamycin
MTT:	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
Nox:	NADPH oxidase
OGG1:	8-oxoG-DNA glycosylase
8-oxodG:	8-Oxo-2'-deoxyguanosine
PARP:	poly-ADP ribose polymerase

ROS: Reactive Oxygen Species
Rapa: Rapamycin
STK11: Serine Threonine Protein Kinase
STZ: Streptozotocin
TSC: Tuberin-encoding gene

PREAMBLE

Diabetes is a major public health problem that affects about 9% of the world population. The incidence of diabetes has increased immensely in the past 10 years. This alone makes it an epidemic disease. The world atlas of diabetes states France as one of the leading European countries with an increasing incidence of diabetes, with a prevalence of 7.4% in adults between 20 and 70 years. Furthermore, diabetes incidence has increased tremendously during the last years in the Lebanese population with an increased prevalence of diabetes of 12.2 % in the adult population.

Diabetes is associated with a number of micro and macro-vascular complications considered to be the major cause of morbidity and mortality in diabetic patients. The mechanisms that contribute to the onset and development of these complications are poorly defined.

The main part of my PhD work is concentrated towards diabetes-induced CRC progression keeping in mind that 75% of diabetic experience problems in their gastrointestinal (GI) tract. Colorectum cancers are actually among the frequently reported in diabetic patients with a risk ranging from 1.2 to 1.5 where patients with poorly controlled glucose levels have been reported to have more aggressive CRCs, an earlier onset, greater use of exogenous insulin, and a poorer 5-year survival. Consequently, both hyperglycemia and hyperinsulinemia have been linked to colorectal tumorigenesis. Nevertheless, the metabolic and mitogenic mechanisms by which glucose or insulin signaling induce neoplastic growth and malignancies have not been clearly understood.

Taking into account the lack of the scientific knowledge about the mechanisms leading to CRC progression in diabetes, **we tailored my thesis work toward dissecting the cellular and**

molecular mechanisms leading to CRC cancer progression and cancer cell aggressiveness in diabetes.

The metabolic abnormalities of diabetes especially hyperglycemia have been described to play a pivotal role in the pathogenesis of diabetic complications including colorectal cancer. Several studies, including those from our group, showed that glucose induces oxidative stress by increasing ROS production. The increased superoxide production prompted by the rise in glucose levels is now considered to be the unifying key mediator of colorectal cancer progression in diabetes. Nevertheless, antioxidant approaches alone do not prevent or reverse cancer, and the use of a general ROS inhibitor or scavenger was described to be detrimental for organ function, that's why in this thesis project we decided to identify the major source of ROS implicated in the thrive of colorectal cancer.

The NADPH oxidases of the Nox family are a group of enzymes whose only function is the production of ROS. The Nox family includes five members, Nox1, Nox2, Nox3, Nox4 and Nox5 in addition to the dual oxidases Duox1 and Duox2, each with tissue and disease- specific regulation. Others and we have previously highlighted the clear coalition between NADPH oxidase-induced oxidative stress and diabetic complications, but the implication of NADPH oxidase, Nox4 as a source of oxidative stress in colon cancer is unknown. Several studies have described that NADPH oxidases can also regulate a series of downstream effectors like the mechanistic/ mammalian target of Rapamycin (mTOR) signaling pathways.

The aims of my thesis are:

Aim 1.

We look forward to determine the role of AMPK and tuberlin/mTOR and their crosstalk with the NADPH oxidases subunit Nox4 in normal versus cancerous colon epithelial cells in their response to high glucose (HG), high insulin (HI) or their combination. Our hypothesis is that the selective inhibition of Nox4 or the mTORC1 pathway *in vitro*, or the activation of the AMPK pathway may attenuate CRC aggressiveness at the molecular and cellular levels.

Aim 2.

We intend to unveil the mechanism by which diabetes accelerates tumor development and tumor burden *in vivo*. Thus, inhibition of mTORC1 or activation of AMPK may reverse damage at the molecular and phenotypic levels.

INTRODUCTION

A. Diabetes Mellitus: General Overview

Diabetes Mellitus (DM) is a chronic, systemic malfunction that is characterized by elevated blood glucose levels (Neville, R.F. & Sidawy, A.N. 2012). Metabolic disturbances as a result of DM may be classified as either Type 1 DM or Type 2 DM. Type I DM is marked by an autoimmune destruction of pancreatic β -cells resulting in a deficiency in insulin production. Type II DM is the more prominent form of DM constituting up to 75% of all cases and is characterized by a deficient cellular response to insulin production (International Diabetes Federation, 2015). The commonly experienced and clinically relevant symptoms of both types include frequent thirst, polyuria, tiredness, lack of interest and concentration blurred vision, tingling sensation or numbness in the hands or feet, weight loss/gain, which is sometimes accompanied with polyphagia, and poor wound healing (American Diabetes Association, 2010).

Persistent hyperglycemia is associated with complications affecting the diverse organs and organ systems (American Diabetes Association, 2015). These effects may be classified as damage to the macrovasculature such as cardiovascular disorders, dyslipidemia, and hypertension in addition to skin infections. Whereas microvasculature injury manifests as nephropathies that may lead to renal failure, retinopathy that may lead to blindness and neuropathy with peripheral and autonomic nervous system malfunction (DCCT, 1993; Ali et al., 2013). By cause of its complications, diabetes has been attributed as an incurable disorder that requires a lifetime of treatment and thus, DM presents individuals with a socio-economical burden that exceeds other diseases, with a sufficient proportion of care needed in developed and developing countries (Kaul, et al. 2013; The Economist Intelligence Unit 2007 and National

Diabetes Clearinghouse 2012). Additionally, the poor management of diabetes may result in life-threatening consequences such as ketoacidosis or a nonketotic hyperosmolar syndrome which may lead to stupor, coma and, in the absence of effective treatment, death (Assal, J.P., & Groop, L. 1999). Most importantly, the symptoms associated with DM are often undetected, and consequently pathological and functional alterations induced by hyperglycemia may develop before a diagnosis is issued (Assal, J.P., & Groop, L. 1999).

1. Diabetes in Numbers

According to the International Diabetes Federation, it is estimated that DM affects 415 million of the world adult population (International Diabetes Federation, 2015). The incidence of the diabetes epidemic is on the rise and it is expected to rise beyond 592 million in 2035 (Guariguata et al., 2014). According to the World Atlas of Diabetes, France is one of the leading European countries with increased incidence of diabetes. In 2015, 7.4% of the adult population was diagnosed with diabetes which is equivalent to 3.3 million cases (International Diabetes Federation, 2015). Furthermore, in the Middle East and North Africa region (MENA), the prevalence of diabetes has increased due to many factors mainly urbanization and lifestyle changes. In 2013, countries in the MENA region were among the areas with the highest rates of diabetes worldwide, whereby 9.2% of the adult population was diagnosed which is equivalent to 34.6 million people. This number is expected to increase to almost 67.9 million in 2035. In fact, Lebanon ranks the 7th in the top 10 countries for prevalence in the MENA region bearing 14.99% of diabetic cases (Majeed, et al. 2014). In a population-based study, predominantly type II DM was found to be a leading cause of mortality among the Lebanese population, with 8.5% issued a diagnosis (Costanian, et al. 2014).

2. Classification of Diabetes

Diabetes can be classified into 4 categories: Type I DM, Type II DM, gestational diabetes and other specific types however, the most prevalent forms of diabetes are Type I and Type II DM (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). In the following section, a brief review of each type will be provided.

a. Type I Diabetes Mellitus

Insulin Dependent Diabetes Mellitus (IDDM) or juvenile-onset diabetes is commonly referred to as Type I DM (American Diabetes Association, 2004). This form develops as a result of the autoimmune destruction of the insulin-producing β -cells of the pancreas, thus leading to insulin deficiency (Atkinson, M.A., & Maclaren, N.K. 1994; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). It is reported that Type I DM is associated with a genetic predisposition; however, there have been speculations that environmental factors may play a role in inducing autoimmune destruction of β -cells, but these are still poorly understood. Type I DM accounts for about 10% of patients with diabetes and is most commonly reported to occur in children and teenagers (Harcourt et al., 2013) although it may appear during adulthood as well. This disease is affiliated with a sudden onset whereby the absence of insulin production leaves patients dependent on exogenous insulin administration for their survival (Richesson et al., 2013; Alberti, K.G., & Zimmet, P.F. 1998). Despite active research, Type I diabetes has no cure, and carries the constant threat of devastating complications.

b. Type 2 Diabetes Mellitus

Previously referred to as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or adult-onset diabetes mellitus (American Diabetes Association, 2004), this form of diabetes is

characterized by insulin resistance in peripheral tissues and a relative insulin deficiency (Harcourt et al., 2013). It is the more prevalent form of diabetes mellitus and accounts for about 85% of the cases worldwide (Harcourt et al., 2013; Mayfield, J. 1998). Type II diabetes is often associated with a strong genetic component, yet the risk of incidence increases with age, obesity, and lack of physical activity, which suggests that environmental factors are important contributors to the disease (Alberti, K.G., & Zimmet, P.F. 1998; Yamamoto-Honda et al., 2016). In fact, studies have shown that obesity itself or increased percentage of body fat in the abdominal region is tightly correlated with insulin resistance (Kahn, B.B., & Flier, J.S. 2000; Kissebah et al., 1982).

Although insulin levels may seem normal or high in type II diabetic patients, the defective insulin secretion is insufficient to compensate for insulin resistance and hyperglycemia (Polonsky et al., 1996; Harcourt et al., 2013). In contrast to type I diabetic patients, individuals with type II diabetes rarely depend on insulin treatments to survive. While there is currently no cure for type II diabetes, the condition can be managed through lifestyle modifications and pharmacological therapy (Wing et al., 1994; Richesson et al., 2013). However, strict management is difficult to maintain and despite the advances made, the risk of complications remains associated with a ten-year-shorter life expectancy (Melmed et al., 2011).

c. Gestational Diabetes

This type of diabetes is a major medical complication implicated during pregnancy that is by cause of glucose dysfunction in women with onset or first recognition during pregnancy (Panel, I.C. 2010). In some women, hyperglycemia usually appears in most women after delivery which places them at increased risk of developing diabetes mellitus later in life (Coustan et al.,

1993; Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). The prevalence of gestational diabetes ranges from 1% to 14% and this is population-dependent with certain ethnicities being at a higher risk (American Diabetes Association, 2004; Engelgau et al., 1995; Alberti, K.G., & Zimmet, P.F. 1998).

d. Other Specific Types

Diabetes may further manifest as other types which may be genetically defined or associated with other diseases or drugs such as: exocrine pancreas disease (ex. pancreatitis), viral infections, endocrinopathies (ex. Cushing's syndrome), and insulin receptor antibodies (Assal, J.P., & Groop, L. 1999).

3. Diabetic Complications and the Role of Hyperglycemia

Diabetic complications largely contribute to the morbidity and mortality of the disease and affect a wide range of organs (DCCT, 1993; UKPDS, 1998). The major macrovascular complications comprise cardiovascular diseases resulting in myocardial infarction and cerebrovascular disorder manifesting as strokes. Whereas the major microvascular complications manifest as nerve dysfunction, predominantly the triopathies: nephropathy that may lead to renal failure, retinopathy with potential blindness, and neuropathy with increased risk of foot ulcers, amputation, Charcot joints, and features of autonomic dysfunction (DCCT, 1993; Ali et al., 2013). However, one of the complications that has been increasingly shown to be associated with diabetes is cancer (De Bruijn et al., 2013; Shikata et al., 2013; Shi & Hu., 2014). Numerous epidemiological, observational and cohort studies have established the risk of cancer development in diabetic individuals with either Type I or Type II DM (Huxley et al., 2005; Shu et al., 2010; Yang et al., 2010; Harding et al., 2015) . Furthermore, diabetes being a current

epidemic predicted to rise in incidence in the future poses as a burden on diabetic individuals with the risk of cancer development.

Diabetes/Hyperglycemia has been reported in the literature to be associated with the pathophysiology of the disease and the development of complications. The International Diabetes Federation recognizes three targets for diabetic patients to pay particular attention to for proper management of diabetes. These are the glycated hemoglobin, fasting plasma glucose, and postmeal glucose levels (Ceriello & Colagiuri., 2008). Numerous clinical studies have been conducted examining the implications of glycemic control on the prognosis of diabetic patients. The studies have shown glycemic management to play a role in attenuating the progression of complications in type I diabetic patients (DCCT, 1993; EDIC, 1999; Nathan et al., 2014). As for type II diabetes, numerous studies have shown the implications of a wider array of metabolic disorders that are further involved in the etiology of the disease such as obesity, hypertension, dyslipidemia, inflammation, and insulin resistance (Callaghan et al., 2012). Yet, hyperglycemia remains one of the factors that trigger the onset of complications that upon onset may be irreversible or incurable. Consequently, intensive efforts currently work on investigating the pathways through which hyperglycemia inflicts tissue injury in respective organs.

One of these initiatives involved intervention trials that were performed to investigate the effects of intensive glycemic control on complications in recruited type II diabetic patients (UKPDS, 1998; ADVANCE, 2008; ACCORD, 2008). The UKPDS trial reported that the risk of microvascular complications and myocardial infarction is reduced upon glucose lowering (Holman et al., 2008; UKPDS, 1998). However, others such as the ACCORD and the ADVANCE trials were halted due to increased risk of hypoglycemia as well as no amelioration that is of significance in the strictly controlled group (Duckworth et al., 2009; ADVANCE, 2008;

ACCORD, 2008; Callaghan et al., 2012; Ismail-Beigi et al., 2010). These trials indicate that rigid metabolic control is challenging and often fraught with other complications (Nathan et al., 2014). Thus, the comprehension of hyperglycemia and the cellular mechanisms inflicted is essential to target the onset and progression of diabetic complications as well as apprehend any interplay with other key factors to develop novel therapeutic and preventative approaches to diabetes.

a. Macrovascular Complications

Extensive clinical evidence has established diabetes as a major risk factor for the onset and progression of all manifestations of cardiovascular disease (CVD), including myocardial infarction, stroke, peripheral arterial disease, cardiomyopathy and heart failure (Buse et al., 2007; Fox et al., 2007; Kannel, W.B., & McGee, D.L. 1979; Emerging Risk Factors Collaboration, 2010). Actually, it is estimated that CVD accounts for 65% of the mortality seen in subjects with diabetes (Grundy et al., 1999; Laing et al., 2003). However, the pathophysiology of diabetes-induced cardiovascular diseases is poorly understood, but research suggests potential mechanisms that include hyperglycemia, oxidative stress, impaired endothelial function and inflammation (Hayat et al., 2004; DCCT, 1993, EDIC, 1999; UKPDS, 1998; ACCORD, 2008; ADVANCE, 2008; Palmieri et al., 2003; Tan et al., 2014; Lowe et al., 2014; Schkwijk et al., 1999). Consequently, strategic glycemic and blood pressure control, lifestyle interventions as well as anti platelet therapy are necessary to lower the risk of cardiovascular disorders in diabetes, in addition to novel adjunct therapies, based on the latest research discoveries (Buse et al., 2007).

b. Microvascular Complications

Diabetic retinopathy is the leading cause of blindness among adults aged 20–74 years (Fong et al., 2004). After 20 years of the onset of diabetes, it is estimated that almost all patients

with type I diabetes and more than 60% of patients with type 2 diabetes will develop retinopathy (Klein et al., 1984; Hirai et al., 2011; Kempen et al., 2004). Briefly, diabetic retinopathy is characterized by hyperglycemia-induced intramural pericyte death and thickening of the basement membrane which contributes to blood-retinal barrier alterations and increased vascular permeability in the early stages (Frank, R.N. 2004). However, with disease progression, vascular closure and degeneration of retinal capillaries contribute to ischemia (Bresnick et al., 1976). This in turn progresses the disease to the proliferative phase where neovascularization and accumulation of fluid within the retina, referred to as macula edema, result in visual impairment (Frank, R.N. 2004).

Another microvascular complication that contributes to premature morbidity and mortality in diabetic patients is diabetic nephropathy. Nephropathy is often characterized by renal hypertrophy, matrix protein accumulation tubulointerstitial fibrosis, and glomerular injury especially podocyte injury which contribute to the eventual decline in glomerular filtration rate in humans and experimental models of diabetes (Wolf, G., & Ziyadeh, F.N. 2007). Affecting kidney function, nephropathy leads to the accumulation of extracellular matrix proteins, including fibronectin, collagen or laminin in the tubular compartment and finally, tubulointerstitial fibrosis. Tubular cells are a primary target of hyperglycemia and chronic exposure to elevated blood glucose levels contributes to the tubulointerstitial changes seen in overt DN (Jones et al., 1999; Gilbert and Cooper, 1999). Numerous studies showed the implications of hyperglycemia in vitro on podocytes, proximal tubular cells and extracellular matrix protein accumulation (Eid et al., 2013b,c; Liu et al., 2008; Morcos et al., 2002; Ford et al., 2013; Mariappan et al., 2008). Morphology in addition to cellular physiology is affected by hyperglycemia (Pagtalunan et al., 1997; Meyer et al., 1999; Wolf et al., 2005; Huber et al., 2003;

Schiffer et al., 2004). As a result of these injuries, cellular functions including adhesion, migration, survival, differentiation, and growth are affected (Pankov et al., 2002). This further exacerbates renal function, inflicting injury on the glomerular filtration barrier leading to the urinary excretion of albumin that advances to proteinuria, which is a clinically relevant and significant prognostic risk factor for kidney disease (De Zeeuw et al., 2004; Ruotsalainen et al., 1999; Shono et al., 2007; Rincon-Choles et al., 2006) ,

c. Cancer in Diabetes

Among the leading causes of mortality worldwide, cancer prevalence is predicted to increase by 70% globally (World Health Organization, 2017). In Europe and the Middle East, cancer presents as the second significant and among the top four causes of morbidity and mortality respectively, with the most commonly occurring types being cancers of the lung, breast, prostate, and colorectum (World Health Organization, 2014; Ferlay et al., 2015). Several risk factors integrate with the risk of cancer incidence in patients, namely physical and chemical carcinogens such as UV, radiation, tobacco and alcohol, and biological carcinogens such as infections. Other life-style based risks exist such as age, body-mass index, obesity, weight, poor diet, smoking and sedentary to low physical activity (World Health Organization, 2017; American Cancer Society, 2017). Some of these risk factors are shared with the 12th leading cause of death worldwide, diabetes. Thus, speculations to the extent to which diabetes may contribute to cancer development among patients were raised.

In a meta-analysis study, cancer incidence among individuals with preexisting diabetes was reported to be associated with a higher all-cause mortality risk relative to non-diabetics (Lopez et al., 2006). This was further observed in a Swedish cohort study where fasting glucose and post-load glucose levels were used to assess the diabetic state of individuals. The data

showed a significant increase in risk of site-specific cancers such as pancreatic, melanoma and urinary tract cancers for both genders with abnormal fasting glucose levels even after adjustment of bodyweight-mass indexes (Stattin et al., 2007). Similarly, diabetes was shown to also be associated with kidney and gastric cancer risk with a stronger association in women, although men may be equally affected since some studies have not controlled for obesity, which is a major risk factor especially for kidney cancer (Larsson & Wolk., 2011; Ge et al., 2011). Cancers of the liver and pancreas were also shown to be strongly associated with diabetes, which poses as a threat that further exacerbates the etiology and pathological progression of the disease (Vigneri et al., 2009; Suh & Kim, 2011; Johnson et al., 2012).

As for gender-specific cancers, meta-analysis studies also showed an elevated risk in breast and endometrial cancers which were frequently reported in diabetic women (Friberg et al., 2007; Larsson et al., 2007). However, hyperglycemia was reported to be associated with a reduced risk of prostate cancer in cohort and case-controlled studies of diabetic male subjects (Harding et al., 2015; Kasper & Giovannucci, 2006; Onitilo et al., 2014; Roujun et al., 2016). In another study, glycated hemoglobin levels and glucose levels were accounted for as an estimation of the glycemic control of recruited individuals. The study examined the implications of glucose-reducing treatments within the scope of hyperglycemia and cancer risk. The data identified hyperinsulinemia (also known as secondary diabetes) as a diabetic factor rather than hyperglycemia as a contributor to an elevated risk of colon cancer among type II diabetic subjects in response to insulin treatment (Onitilo et al., 2014). Finally, although most of the epidemiological evidence links the prevalence of cancer to the more prevalent form of diabetes, type II, a growing body of data, however limited, indicates an elevated risk among type I patients as well (Zendehdel et al., 2003; Gordon Dseagu et al., 2013; Carstensen et al., 2016).

Nevertheless, the mechanisms by which cancer progression occurs in diabetes are still under investigation.

Diabetogenic cancer onset has been shown to develop by cause of numerous pathways. Metabolic disorders associated with diabetes such as hyperglycemia, hyperinsulinemia and inflammation have been described to be key modulators of neoplastic growth (Asterholm et al., 2016; Giovannucci et al., 2010; Noto et al., 2013; Ryu et al., 2014; Wojciechowska et al., 2016). This multifactorial pathogenesis is dependent on mediators such as cytokines, hormones and growth factors that play a prominent role in metabolic signaling and function. The coupled diabetic duo, glucose and insulin, influence mitogenic and oncogenic pathways that are normally tightly regulated.

Insulin and the Insulin-like growth factor (IGF) receptors have been shown to be expressed on the surface of most cancerous cells. In a breast cancer cell line, it was shown that the downregulation of IGF receptor expression in cancerous cells elevated their sensitivity to insulin, and reduced the malignancy, proliferation and metastatic potential (Zhang et al., 2007). IGF receptors have also been shown to play a role in hyperinsulinemia-dependent carcinogenesis. Through its indirect effects, exogenous insulin attenuates the levels of IGF binding protein production by the liver, which in turn leads to the increased availability of biologically active IGF levels in the blood, further enhancing the proliferative and mitogenic effect on neoplastic growth (Giovannucci, E. 2001; Pollak, M., 2008; Weinstein et al., 2009).

Additionally, diabetes and cancer reciprocate influence onto pathways that aggravate the complexity of the disease. With glucose being the central biological linkage that integrates with both etiologies, the Warburg hypothesis actually explains the energetics of tumorigenesis and suggests the significance of insulin-independent glucose uptake by cancerous cells and their

reliance on glycolysis as a powerhouse for cellular survival (Warburg, O., 1956; Vander Heiden et al., 2009; Yun et al., 2009; Hanahan & Weinberg, 2011). Furthermore, hyperglycemia has been reported to influence protein expression profiles and growth factors that are associated with proliferation such as increased glucose transporters, GLUT1 and GLUT 3, expression, epidermal growth factor, protein kinase C, peroxisome proliferator-activated receptor gamma and cyclin-dependent kinases in a variety of site-specific cancers (Okumura et al., 2002; Hahn et al., 1998; Han et al., 2011; Masur et al., 2011). Besides hyperglycemia, hyperinsulinemia too influences the hypoxic response of tumors through the hypoxia-inducible transcriptional factor 1- α which activates certain anti-apoptotic pathways and triggers angiogenesis (Semenza, G., 1999; Catrina et al., 2004; Lee et al., 2004). As for the metastatic and aggressive potential of tumors, hyperglycemia is described to inflict phenotypical and epigenetic changes, such as epithelial-mesenchymal transitions, while altering oxidative and oncogenic pathways that are dependent on reactive oxygen species (ROS) (Cascio et al., 2008; Rojas et al., 2009; Iwatsuki et al., 2010; Li et al., 2011; Masur et al., 2011; Dong et al., 2013).

Diabetes is a metabolic disorder that is associated with complex metabolic ‘organs’ such as adipose tissue and lipids. This imparts a risk in inflammatory cytokine signaling changes that manifest as inflammation. Such factors include adipokines which have been reported to be associated with insulin resistance and diabetes, that influence proinflammatory cytokine (interleukins and tumor necrosis factor) release (Van Kruijsdijk et al., 2010; Park et al., 2010; López-Jaramillo et al., 2014). In a recent study, the roles of the adipokines were examined for their insulin-modulating effect in diabetes. The results were indicative of fluctuating levels of certain adipokines in the serum of diabetic patients, and were reported to play a role in mesotheliomas, gastric cancer, as well as colon cancer (Szydło et al., 2016). These complex

pathways have been linked to tumorigenesis and tumor invasive potential as well as an overall weakened immunity in patients (Yu et al., 2009). Together, this suggests that diabetes-associated manifestations such as hyperglycemia and hyperinsulinemia may contribute to and facilitate carcinogenesis especially with chronic, poor glucose management in diabetic patients (Krone & Ely., 2005; Stattin et al., 2007; Zhou et al., 2010; Masur et al., 2011). In the same spirit, such pathways reiterate and emphasize the impact of diet on disease prognosis and more importantly, show how cancer may indeed be a lifestyle-associated disease (Algire et al., 2008; Pollak, M., 2009; Park et al., 2010).

For the scope of this thesis, the focus will be on the diabetic colorectum, its physiology and significant contribution to the diabetic etiology as well as colorectal cancer (CRC) as one of the major complications concomitant with diabetes onset. The discussion will also explore the effect of specific treatments for diabetes that are associated with an increased risk of carcinogenesis.

B. The Gastrointestinal Tract: Physiology and Pathophysiology in Diabetes

1. Overview of Metabolic subdivisions in Glucose Metabolism

The digestive system and its supplementary organs synergistically suffice the metabolic requirements of the body while maintaining a homeostatic and tightly regulated physiology. The gastrointestinal (GI) tract is specialized to mediate this process through its anatomically and physiologically distinct subdivisions. The GI tract opens at the mouth and esophagus, pouches into the stomach and then constricts to form the muscular intestinal tube. The intestinal tube is further subdivided into duodenum, jejunum and ileum which comprise the small intestine, and the colon which is also known as the large intestine. The colon is spatially segmented and

divided into cecum, ascending, transverse, descending and sigmoid colon, which is finally followed by the rectum that terminates at the anus.

Segments of the GI tract are embedded within a meshwork of endocrine and exocrine, digestive and accessory organs with complex circulatory, ductal and neural networks that integrate to mediate different stages of the digestive process. Upon meal intake, anticipatory events excite neural networks that perfuse the GI system leading to gastric activity and secretions for digestion. Gastric emptying into the intestines is a process that is regulated and works collaboratively with the incretin response (Holst et al., 2016; Kim & Egan, 2008; Sanyal, D., 2013). Thus, gastric emptying ensures a constant and stabilized entry of nutrients into the intestine. This entry is regulated by and dependent on nervous motor stimulation of the gastric lining as well as hormonal secretions by the stomach and small intestine such as ghrelin, somatostatin, cholecystokinin, secretin, glucagon-like peptide 1, gastric inhibitory peptide and peptide YY (Holst et al., 2016; Levin et al., 2006; Sanyal, D., 2013; Wettergren et al., 1993). More specific digestive processes occur in the small intestine after which, absorption and nutrient deposition takes place. From this stage and onwards, the fate of glucose released from diet components during digestion is determined by the liver, enteric nervous system, pancreatic secretions, enteroendocrine cells, colon and gut microbiota (Holst et al., 2016) .

Glucose may be absorbed and transported through glucose transporters expressed on enterocytes lining the intestinal epithelium or by sodium-glucose cotransporter 1 (Kellert et al., 2001). Together with an increased blood flow post-digestion, this allows for nutrient allocation mainly to the liver, the primary site of action for insulin whereby glucose uptake takes place (Ramnanan et al. 2012). The rise in glucose concentrations during transport in plasma leads to an increased recruitment of glucose transporters in the periphery with the simultaneous release of

insulin (Schwartz et al., 2013). More importantly, glucose balance and control is intimately integrated with neuroendocrine signals and controlled through various neural networks such as vagal nervous and hypothalamic cross-talks between the gut and the central nervous system (Grill & Hayes., 2012). The duodenal and jejunal K-cells in addition to L-cells of the jejunum, ileum and colon are chemosensors along the gut epithelium and respond to nutrient availability (Gribble & Reinmann., 2016). K and L cells secrete gastric inhibitory peptide as well as glucagon-like peptide 1 respectively. Together, these incretin gut hormones may further regulate pancreatic insulin and glucagon responses to post-prandial glucose in what is actually reported to contribute to half the insulin secretory response throughout the body (Kim & Egan., 2008). Gastric inhibitory peptide for instance triggers a glucose-dependent secretion of insulin, whereas glucagon-like peptide 1 halts gastric emptying and glucose production by inhibiting glucagon release, thus playing a role in insulin phasic secretion. This is achieved by increasing the transcription of PDX-1, a homeobox involved in the transcription of the insulin gene, as well as glucokinase 2 and GLUT2 expression (Sanyal, D., 2013).

More importantly, in addition to regulating blood glucose levels, the incretins contribute to pancreatic β cell function and survival by preventing apoptosis and triggering proliferation (Sanyal, D., 2013). Glucose absorption may also occur in the colon however, the bulk of glucose absorbed is fermented by bacterial symbionts. The fermentation products include fatty acids which may either be absorbed, combusted or used for gluconeogenesis (Puertollano et al., 2014). The gut microbiota therefore play a homeostatic role such that any alteration in flora leads to lipid and glucose dysmetabolism , in addition to the disruption of the intestinal barrier as well as gut endocrine dysregulation that may contribute to diabetes, obesity, and inflammatory

complications (Karlsson et al., 2013; Musso et al., 2010; Wei et al., 2012; Vermorken et al., 2016).

2. The Diabetic Colorectum

The GI is a common target in diabetes. It is estimated that 75% of diabetic subjects experience GI dysfunction which is attributable to numerous etiologies. The clinically relevant manifestations of a diabetogenic GI system include nausea, pain, greedy colon (constipation), dysphagia, diarrhea and reflux (Bytzer et al, 2001; Samsom et al, 2003). Additionally, extensive research has shown the implications of myoenteric loss and degeneration, biomechanical, morphometric remodeling or alterations, gastroparesy and motor deficits throughout the GI tract (Chandrasekharan & Srinivasan, 2007; Guo et al, 2004; Liao et al, 2006; Zhao et al, 2003; Zhao et al, 2013) besides morphological changes such as the cellular count, integrity, and activity of cryptic goblet cells and neurons (Mantle et al., 1989; Ettarh & Carr, 1997; Buttow et al., 1997; Romano et al., 1996; Zaroni et al., 1997; Furlan et al., 2002).

Briefly, the colon and rectum form the final components of the digestive tract. They are suspended within a layer of connective tissue with circulatory, nervous and lymphatic innervations. The innermost layer of the large intestine is made of epithelial cells, connective tissue and a muscular layer, collectively known as the mucosa. The submucosa is known to be affiliated with nerve endings and mucus glands whereas the muscularis propria to be a thick muscular layer surrounded by the serosa, a layer exclusive to the colon. The colon serves the digestion process by absorbing nutrients and water, while products are carried via peristaltic muscular contractions towards the rectum, where undigested particles are stored as waste.

Colorectal physiology has been increasingly shown to be vulnerable to inflammation, infection, colitis, inflammatory bowel disease, constipation, diverticulitis and diverticulosis,

polyp formation and cancer (Asterholm et al., 2016; Tanaka et al., 2009; Wang et al., 2016; Zeng et al., 2016; Han et al., 2016). In fact, all of these are bridged to a number of risk factors such as sedentary life styles, poor habits such as alcohol consumption and tobacco use, diet, obesity, genetic mutations, and most importantly diabetes (Asterholm et al., 2016; Cespedes et al., 2016; Dąbrowski et al., 2016; Sugimachi et al., 2016; Takami et al., 1994; Zeng et al., 2016). With special emphasis on colonic complications and the overlapping risks with diabetes, colon pathophysiology has been shown to be especially influenced by diabetes and its complex pathologies (Giouleme et al., 2011; Giovannucci, E., 2007; Jin, T., 2008; Siegman et al., 2016; Sugimachi et al., 2016; Tan et al., 2016; Yu et al., 2016).

Numerous studies in experimental models of diabetes have reported the implications of diabetes in colon remodeling and malfunction. The data revealed dimensional increases in area and dilation concurrent with significant reductions in muscular tunic, colonic wall thickness, muscular fiber tone and neuronal myenteric innervation and population (Zhao et al., 2009; Furlan et al., 2002; Roldi et al., 2009; Chandrasekharan et al., 2011; Siegman et al., 2016). In a recent study, Siegman et al., (2016) described the effects of diabetes in Streptozotocin-treated Sprague-Dawley rats. Muscles cells from the colonic mucosa were reported to undergo hypertrophy characterized by elevated DNA levels and extracellular matrix protein (such as collagen) expression under hyperglycemia. These structural modifications were paralleled with muscular stiffness, which contributes to poor dilatory capacity and physiological dysfunction (Siegman et al., 2016).

In another study, hyperglycemia was reported to influence colonic enteric neurons through the induction of cellular apoptosis and altered neurochemical coding which were correlated with colonic motor malfunction (Chandrasekharan et al., 2011). These pathologies

were further linked to the generation of colonic oxidative stress observed as increased superoxide dismutase activity and reduced glutathione levels. The impact of oxidative stress was shown to be ameliorated in response to treatment with an antioxidant, α -lipoic acid (Chandrasekharan et al., 2011). Impaired glucose metabolism presents the intestinal tract with a vicious cycle of self-inflicting injury. For instance, Chen et al., (2012) reported an increased production of advanced glycated end products alongside their respective receptor expression in the diabetic colon. Such alterations may modulate the intestinal digestive activity and epithelial cell function, exacerbating the implications of hyperglycemia on the GI tract (Chen et al., 2012; Zhao et al., 2013). These end products have also been linked to oxidative stress in diabetes (Singh et al., 2014). Additionally, diabetes being associated with a malfunctioned bowel transit activity may induce colonic damage possibly through excessive exposure to carcinogenic agents (Wong et al., 2012). Taken together, diabetes-induced pathologies of the colon raise the risk of injury to the colonic lining, glands, and cellular composition, and eventually may lead to the development of CRC, a significant pathology of the GI tract. Thus, we next expand upon the correlation between diabetes and CRC.

3. Colorectal Cancer Staging and Oncogenesis

As mentioned earlier, epidemiological studies provide strong evidence that diabetic subjects are at a significantly higher risk of developing numerous forms of cancer and solid tumors, at a higher rate of occurrence and progression relative to the general population (Sciacca et al., 2013; Swerdlow et al., 2005; Wideroff et al., 1997; Ogunleye et al., 2009; Smith & McKay, 2008; Zendehdel et al., 2003). Colorectum cancers are actually among the frequently reported in diabetic patients with a risk ranging from 1.2 to 1.5 (Vigneri et al., 2009). Indeed, the

impact of diabetes on CRC patients' disease-free and overall survival and recurrence of the malignancy was reported to be worse in comparison to non-diabetic patients (Jeon et al., 2013).

There are three phenotypic forms of CRC that are currently described: non-cancerous, pre-cancerous and cancerous. CR non-cancerous growths are non-metastatic and often asymptomatic. They are benign tumors that arise from the colonic lining and pouch into the lumen, and are often referred to as polyps. These polyps may be hyperplastic (as a result of an inflammatory condition), hamartoma (as a result of a hereditary condition) and lipoma (originating from adipocytes) (Tanaka, T., 2009; American Cancer Society, 2013). Polyps may further develop a pre-malignant phenotype known as adenomas which eventually turn into a cancerous tumor that can grow, infiltrate the various colonic linings and metastasize. The most commonly occurring cancerous tumor of the colon is known as adenocarcinoma representing almost 95% of the clinically observed cases and these tend to develop from gland cells of the colonic mucosa (American Cancer Society, 2013). Adenomas mainly arise in the form of pre-existing polyps that are epithelial in nature with abnormal cryptic cell proliferation (Tanaka, T., 2009). Moreover, there are additional types of cancerous growths in the colon, however, these are rare and are typical of the soft tissue, lymph nodes, and nearby tissues surrounding the colorectum. Some of these tumors may be neuroendocrine growths as well. Consequently, there are clinically defined stages of CRC progression and these stages depend on the degree of colonic lining and vascular invasion as well as metastasis into other organ systems (American Cancer Society, 2013).

Briefly, according to the American Society of Clinical Oncology, stage 0 CRC involves cancerous growths that occur from the inner mucosa to the muscularis propria, in the epithelium or connective tissue. Stage I is characterized by malignant growth spanning the submucosa to the

thick muscularis propria. As for stage II and III CRC, it involves growths spanning the outer layers, namely the subserosa to the pericolorectal tissues including muscular layers and lymph node invasion with slight metastatic potential. Whereas in the final stages of CRC, stage IV is relatively advanced and defined by the complete infiltration through the colonic lining, to the lymph nodes, unrestricted to any layer, and characterized by maximal metastatic potential to distant organs (**Fig. 1**) (American Society of Clinical Oncology, 2013).

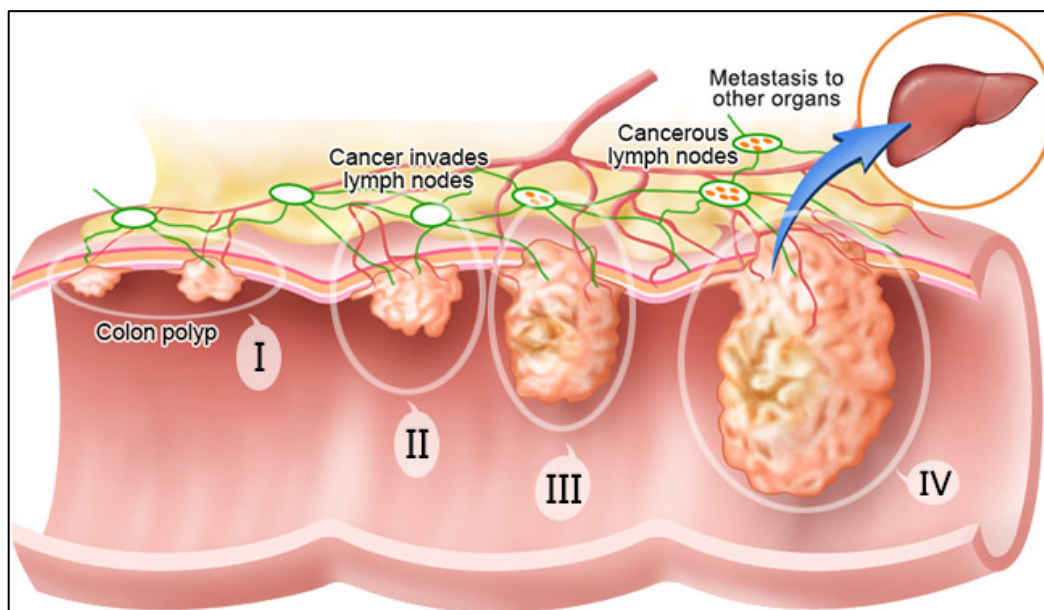


Figure 1. Colorectal cancer transformation happens at various stages 1 to 4. Stage 1 is the least advanced, and involves the innermost layers of the colorectum. Stage 2 is defined as the growth extends beyond the wall of the colorectum into pericolonic layers. Stage 3 is more advanced and the cancer spreads to local lymph nodes. Stage 4 is the most lethal and the cells metastatize to distant organs of the body. Adapted from: Modern Cancer Hospital Guangzhou, 2012 (<http://www.asiancancer.com/cancer-topics/new-intestinal/>).

The oncologic pathogenesis of CRC is a multi-factorial etiology and much research goes to unravel the mechanisms through which benign growths turn into malignancies. Numerous studies show that CRC tumor development and malignant transformations possess genetic and epigenetic components that govern cellular pathways. One of the most important genes

contributing to adenoma formation and responsible for the autosomal-dominant disease known as familial adenomatous polyposis is the adenoma polyposis coli (APC) gene (Powell et al., 1993). The APC gene triggers colonic adenoma development throughout the colon of these patients at a young age (**Fig. 2**) (Powell et al., 1993).

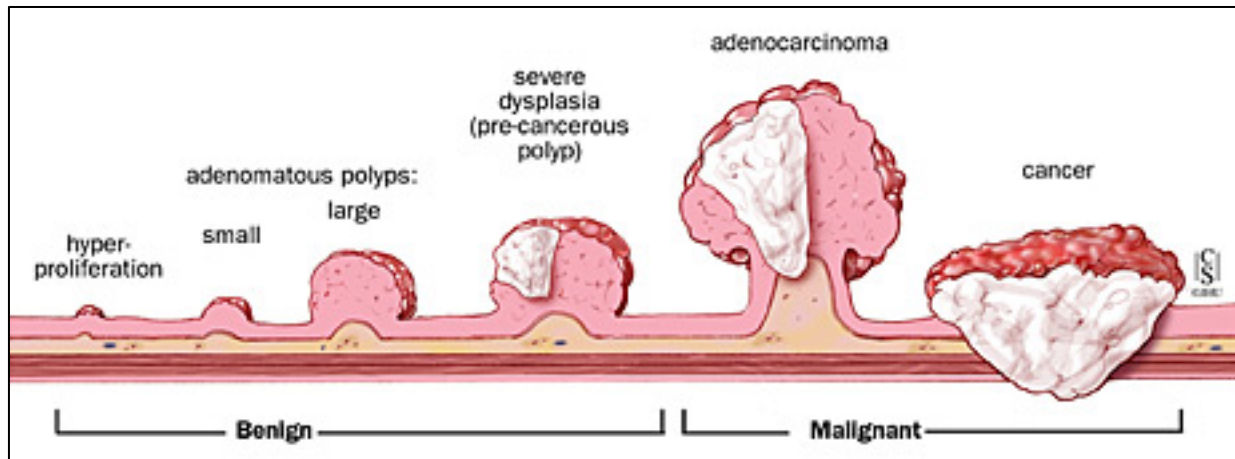


Figure 2. Neoplastic transformation from primary to metastatic tumors. CRC progression starts when cells lining the colon undergo uncontrolled proliferation and escape cellular death. These growths within the colon, known as polyps, are initially precancerous. Polyps with severe dysplasia look like cancer but are not yet cancerous. These tumors then undergo genetic mutations that render them malignant (adenocarcinoma) and in advanced stages an invasive cancer.

Adapted from: Bowel Cancer Australia, 2017 (<https://www.bowelcanceraustralia.org/community-fundraising>).

The APC gene codes for a tumor-suppressor that plays a prominent role in WNT/ β -catenin/TCF signaling pathways (Morin et al., 1998), Notch signaling (van Es et al., 2005), and interacts with cyclins and cyclin-dependent kinases (Arber et al., 1996; Alao, J. P., 2007; Firestein et al., 2008), and the p53 cell guardian in the late stages (Baker et al., 1990; Conlin et al., 2005). Mutations in the APC gene have been linked to CRC progression in the early stages (Shih et al., 2001) and have been described to prevent the apoptotic death of colonic neoplastic growth in the early phases by conferring cyclin D1 over expression (Arber et al., 1996). This

mutation is one of numerous others among chromosomal instability abnormalities that involve the RAS pathway which mediates transition to malignant carcinomas of CRC (Malumbres, M., & Barbacid, M. (2003; Guerrero et al., 2000; Imamura et al., 2012; Loupakis et al., 2009), AMPK pathway which is the energy sensor of cells that is indirectly influenced and interacts with the K-ras, WNT, mitogenic MAPK, and PI3K/mTOR/AKT pathways involved in CRC initiation (Horst et al., 2012; Baba et al. 2010^a; Deming et al., 2013; Samuels, Y., & Velculescu, V. E., 2004) and HIF-1 α in collaboration with cyclooxygenases in the pathology of CRC (Baba et al., 2010; Kaidi et al., 2006).

A second distinctive mechanism of CRC tumorigenesis involves defects in the DNA Mismatch Repair system which is pivotal for proofreading and nucleotide repair during replication (Thomas et al., 1996). These defects are further associated with microsatellite instability (Ricciardiello et al., 2005). These represent molecular pathways whereby the aforementioned repair system is disabled exacerbating the rate of mutations typically described in another form of CRC, hereditary nonpolyposis CRC (Thomas et al., 1996). Two genes of significance in the mismatch repair system include the human homologues MSH and MLH which are prone to germline mutations that elevate the risk of carcinogenesis and epigenetic silencing facilitating CRC tumorigenesis (Parsons et al., 2012; Kane et al., 1977; Veigl et al., 1998). Likewise, loss of function mutations in Sma genes isoforms and the subsequent mutations in TGF- β receptor II have been shown to promote tumor development as well as enhance metastatic phenotypes of CRC (Takayama et al., 2006; Zhang et al., 2010; Eppert et al., 1996). Several other genes involved in DNA mismatch repair system have been reported in CRC pathogenicity such as the BAX tumor suppressor, cyclin D1, PIK3CA (reviewed by Colussi et al., 2013) and interestingly, the insulin growth factor type 2 receptor (Calin et al., 2000).

Multiple pathways seem to be mediators of the oncogenic transformations of adenomas to carcinomas in CRC, and ongoing genetic-based studies are being conducted to investigate the implications of specific genetic mutations. Inflammatory pathways, micro RNA based pathologies, and hypermethylation of CpG dinucleotide sequences of genes that are involved in cell cycle regulation and cellular proliferation and apoptosis as well as aggressiveness and metastatic capabilities of tumors that have been linked to CRC onset and progression (Reviewed by Colussi et al., 2013).

4. The Role of Hyperglycemia/Hyperinsulinemia in Colorectal Cancer Progression

Several of the pathways by which CRC progresses are intimately affected by hyperglycemia/diabetes. Within this context, extensive research indicates that an association exists between elevated glucose levels or glycated hemoglobin levels and the predisposition to CRC malignancies (Yu et al., 2016; Suchanek et al., 2016; Asterholm et al., 2016; Lee et al., 2017; Vasconcelos-dos-Santos et al., 2017). Indeed, clinical studies reported that patients with poorly controlled Type II DM have more right sided and advanced CRCs, a younger age of presentation, greater use of exogenous insulin, and a poorer 5-year survival (Siddiqui et al., 2008). In another study, epidemiological data revealed the relationship between fasting serum glucose and DM and the risk of all cancers as well as specific cancers in men and women. The study found that elevated fasting serum glucose concentrations and DM are risk factors for the development of cancer in several tissues including colon cancer (Jee et al., 2005). The age-adjusted incidence and mortality rate for colon cancer in this study were increased in both genders too (Jee et al., 2005). Whereas treatments with glucose-lowering agents in diabetic patients with developed CRC have showed an improved survival outcome (Park et al., 2016; Paulus et al., 2016). Together these data imply that hyperglycemia plays a significant role in

diabetes-induced cancer onset. However, further diabetes-associated abnormalities, such as hyperinsulinemia, are shown to also be involved in colorectal tumorigenesis as well (Currie et al., 2013; Li et al., 2013; Rosato et al., 2016; Lu et al., 2017). This was observed in a retrospective cohort study whereby Type II DM patients going through insulin therapy were followed to investigate CRC occurrence. The data from this cohort indicated a positive correlation between duration of exogenous insulin administration and CRC risk (Yang et al., 2004). Emphasis on insulin signaling is placed alongside hyperglycemia due to the complications that arise as a result of insulin treatments. In type 2 diabetes, insulin levels become exaggerated mainly due to peripheral resistance, which leads to the overproduction of insulin in a deleterious and cyclic manner. Insulin administered exogenously further exacerbates these outcomes, leading to CRC as epithelial cells of the colon gradually acquire characteristics typical of neoplasia (Giouleme et al., 2011). Indeed, a significant and threefold increase in risk of CRC development has been reported among type 2 diabetic patients that are dependent on insulin (Nagel & Göke, 2006). Nevertheless, the metabolic and mitogenic mechanisms by which glucose or insulin signaling induce neoplastic growth and malignancies have not been clearly understood.

Extensive research has identified the role of the IGF in hyperinsulinemic CRC pathophysiology. Increased levels of IGF have been described to be concomitant with elevated insulin levels and consequently, studies have shown that CRC cells possess a high expression of IGF receptors compared to normal colonic epithelial cells (Berster & Göke, 2008; Bach, & Rechler, 1992). These findings were suggestive of an insulin-mediated effect in CRC tumorigenesis. The mechanisms of this effect were poorly elucidated, however, early studies in colon cancer cell line have indicated the stimulatory effect on insulin on HT-29 cell growth (Björk et al., 1993). Studies in endocrine cells lining the intestines have shown that insulin-

mediated proliferation via an mTOR pathway in collaboration with two major pathways influenced by the APC gene as well, namely the WNT and β -catenin pathways (Sun & Jin, (2008; Yi et al, 2008). Current *in vitro* studies in a human colon cancer cell line showed that insulin also triggered proliferation and metastasis through activating matrix metalloproteinase expression through the insulin receptor-dependent induction of the MAPK and PI3K/AKT pathways (Lu et al., 2017). The contribution of insulin to the pathogenesis of CRC cells was further validated in another study that examined the implications of insulin signaling on myotubularins (Weidner et al., 2016). Myotubularins are phosphoinositide phosphatases that have been shown to possess tumor-suppressor-like activities by downregulating growth-factor stimulated activation of AKT and ERK pathways that are central to cellular proliferation (Weidner et al., 2016). Further studies in human CRC cell lines showed that insulin reduced the expression of myotubularins and thus enhanced tumorigenicity and proliferative potential in CRC (Weidner et al., 2016).

Other recent studies point to the involvement of the hexosamine biosynthetic pathway in CRC; a central pathway that is an intracellular energy and nutrient sensor which is involved in glucose metabolism (Lucena et al., 2016; Vasconcelos-dos-Santos et al., 2017). This pathway was also reported to be altered in other cancerous malignancies (Munkley et al., 2016) via facilitating epithelial-mesenchymal transitions (Alisson-Silva et al., 2013; Lucena et al., 2016). The alteration of this pathway leads to the enhanced modification and glycation of cellular proteins which also correlated with tumorigenicity and migratory potential of malignant cells (Munkley et al., 2016). Indeed, growing evidence lends support to increased glycosylation of lipids and proteins in response to high glucose activity, and this contributes to the malignant transformation of neoplastic growth in CRC by modulating oncologic phenotypes such as

cellular signaling, proliferation and migration (Holst et al., 2015). Moreover, in a study that examined the role of post-translational alteration of glycoproteins, the data suggested that the knockdown of the MGAT5 gene (responsible for the synthesis of branched glycans) reduced adenoma size in CRC mouse models and increased the likelihood of survival. This study further revealed that MGAT5 knockdown decreased the ability of CRC stem cell renewal capabilities, thus contributing to the attenuation of CRC progression (Guo et al., 2014), adding to the significance of maintaining protein and lipid integrity for normal cellular function and proliferation. Despite these findings, the pathogenicity of hyperglycemia/hyperinsulinemia in tumorigenesis of colorectal cancer remains poorly investigated and studies conducted are limited. The mechanistic approach is still unknown and has yet to be revealed. In the next section, oxidative stress will be highlighted within the context of diabetes as the final mediator common to the pathways involved in the pathogenesis of diabetic complications.

C. Reactive Oxygen Species and the Oxidative Status in Diabetes and its Complications

1. Cellular Sources of Reactive Oxygen Species and their Role in Homeostatic Maintenance

Reactive Oxygen species (ROS) are bioactive molecules that contain oxygen, and are produced as byproducts of ongoing biochemical cellular reactions. Several forms have been identified, and they include nitrogen based free radical species such as nitric oxide and peroxynitrite as well as superoxide free radicals (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen. ROS have evolved to play an important role in house-keeping physiologic and cellular processes and therefore, are crucial entities for cellular physiology that are involved in gene expression, cell signal transduction, cell and tissue growth and proliferation, host defense and innate immunity and homeostatic maintenance (Turpaev, K. T. 2002). ROS and free radical

formations also influence angiogenesis, salt and fluid homeostasis, biochemical reactions, apoptosis, etc. (Bedard et al., 2007).

Intracellular ROS signaling is tightly regulated at a specific homeostatic set point via antioxidant defense mechanisms that neutralize the bioactive radicals. The antioxidant system includes enzymes like Superoxide Dismutase (SOD), glutathione peroxidase (GPx), and catalase, as well as non enzymatic compounds such as α -tocopherol (vitamin E), β -carotene, ascorbate (vitamin C), and glutathione (Bursell et al., 1999; Haak et al., 2000; Packer et al., 2001; Xu et al., 2014). An imbalance between ROS generation and the ability of the antioxidant defense mechanism to neutralize the excess ROS and their intermediates, or repair the resulting damage, is considered deleterious and may lead to oxidative stress (Dröge, W. 2002). This is defined as the state whereby ROS overwhelms cellular defenses and leads to injury through lipid and protein oxidation, dysmetabolism, activation of intracellular signaling and transport pathways, and ultimately programmed cell death (Vincent et al., 2004).

Hyperglycemia has been shown to disrupt the oxidant-antioxidant balance by triggering persistent ROS production (Cameron, N. E., & Cotter, M. A., 1999; King, G. L., & Loeken, M. R. 2004; Singh et al., 2014), and lowering the antioxidant defenses (Laight et al., 2000). As a result, antioxidant therapy approaches have been studied and shown to act at different levels, inhibiting the formation of ROS, scavenging free radicals or increasing the antioxidants defense enzyme capabilities. While some animal studies showed that antioxidant treatment prevents or slows the development of diabetic complications in animal models of diabetes, human studies failed to correlate with the data seen in diabetic animals (Vincent, A.M. 2011; Pop-Busui et al., 2013). Furthermore, recent studies show that total blockade of ROS can be in some cases deleterious (De Zeeuw et al., 2013). Subsequently, a new strategy based on identifying the

cellular sources of ROS in a disease specific manner was advanced in order to promote the use of source specific antioxidants in the pathobiology of diabetes and its complications. In fact, a number of enzymes have been reported to be implicated in ROS production (**Fig. 3**). These include nicotinamide adenine dinucleotide phosphate oxidase (NADPH Noxes), cytochrome P450 monooxygenase, nitric oxide synthase, lipoxygenase, cyclooxygenase, and xanthine oxidase (Niedowicz, D. M., & Daleke, D. L. 2005). Additional ROS production has been described to be generated by intracellular glucose metabolism via glucose autoxidation, mitochondrial oxidative phosphorylation, and the production of advanced glycation end products (Niedowicz, D. M., & Daleke, D. L. 2005). Studies have since aimed to examine the specialized contribution of individual sources of ROS to the pathogenesis of various diseases, including diabetes and its complications.

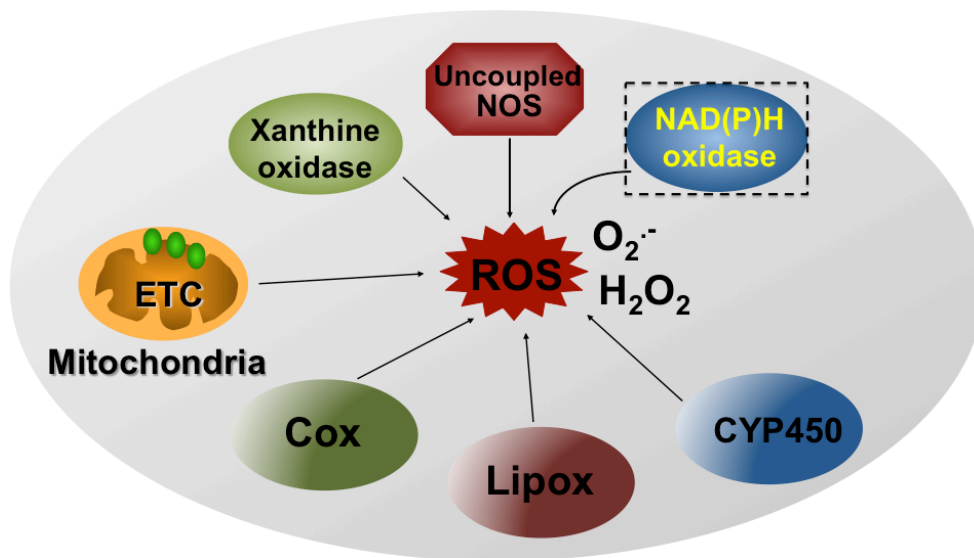


Figure 3. Potential sources of Reactive Oxygen Species (ROS) in cells. The mitochondrial electron transport chain, xanthine oxidase, uncoupled NOS, NAD(P)H oxidases, lipoxygenases, cyclooxygenases and cytochrome P450 monooxygenase, produce ROS in different organs of the body including the GI tract.

2. Oxidative Stress in Diabetic Complications

Diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species (ROS) (Eid et al., 2010). Diabetes in all its forms is characterized by impaired insulin-stimulated glucose uptake in adipose and muscle tissues leading to the consequential elevation in circulating blood glucose levels (Brownlee, M. 2001), and the subsequent uptake by insulin-independent tissues. As a result, the unregulated intracellular flux of glucose leads to ROS overproduction. In fact, long-standing hyperglycemia is reported to be associated with increased systemic and cellular oxidative stress, which is currently accepted to be the common pathway for cellular injury, leading to the onset and progression of diabetic complications (Baynes, J.W., & Thorpe, S.R. 1999; Feldman et al., 1997; Giugliano, D., & Ceriello, A. 1996; Kowluru, R.A., & Kennedy, A. 2001).

With regards to diabetes onset, there are different mechanisms that have been described to contribute to the formation of ROS. For instance, two pathways of importance that were reported to mediate the pathogenesis of diabetic retinopathy, diabetic atherosclerosis, diabetic nephropathy, diabetic neuropathy and cardiomyopathy, are the polyol pathway and glycation reactions that produce advanced glycation end products (AGEs) (Yadav et al., 2012; Lee, A.Y., & Chung, S.S. 1999; Vikramadithyan et al., 2005; Liu et al., 2011; Song et al., 2003; Hao et al., 2015; Berner et al., 2012; Oldfield et al., 2001; Wada et al., 2001; Ma et al., 2009) especially in the diabetogenic intestinal tract (Chen et al., 2012; Zhao et al., 2013). Similarly, a number of enzymes have been shown to be associated specifically in diabetic complications such as the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidases or NOXs) (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013; Eid et al., 2016; Gray et al., 2013; Ago et al., 2004), cytochrome P450 monooxygenase (Eid et al., 2009; Eid et al., 2014; Wang et al., 2011),

lipoxygenase (Suzuki et al., 2015 ; Obrosova et al., 2010), cyclooxygenase (Retailleau et al., 2010 ; Kellogg et al., 2008), and xanthine oxidase (Miric et al., 2013; Romagnoli et al., 2010). However, ongoing studies aim to delineate the pathophysiological interaction between oxidative stress and diabetes to better understand the mechanisms by which increased oxidative stress accelerates the onset and development of diabetes-induced complications.

Extensive research has shown that ROS overproduction poses a pathogenic state in multiple organs and is the common feature to the pathogenesis of diabetic complications (Lambeth Krause, K. H., & Clark, R. A., 2008; Naziroglu et al., 2012; Eid et al., 2009; Eid et al., 2010; Eid et al., 2013; Nayernia et al., 2014; Kowluru, A. R., & Mishra, M. 2015; Filla, A. L., & Edwards, L. J. 2016; Eid et al., 2016). Oxidative stress was shown to inflict injury in diabetic retinopathy through the overproduction of ROS via inflammation, the polyol pathway, accumulation of AGEs, the hexosamine pathway flux, NADPH oxidases and protein kinase C (PKC) activation (Brownlee, M. 2005). Studies in our laboratory and others have shown the role of oxidative stress in mediating podocyte injury, apoptosis, and depletion in early stage diabetic nephropathy as well as albuminuria (Hardie, D.G., & Carling, D. 1997; Ha, H., & Lee, H.B. 2000; Brownlee, M. 2001; Kuroki et al., 2003; Ha, H., & Lee, H.B. 2000; Brownlee, M. 2001; Kuroki et al., 2003). Likewise, ROS production has been shown to be associated with diabetic neuropathy leading to nerve dysfunction (Obrosova et al., 2005; Hamilton et al., 2013; Oh et al., 2012; Song et al., 2003). However, numerous studies have shown that inhibition of some of the ROS sources independently ameliorated oxidative stress in diabetes-induced renal, cardiovascular and other system injuries (Lambeth, J. D., Krause, K. H., & Clark, R. A. 2008; Eid et al., 2009; Eid et al., 2010; Wu et al., 2012). Despite the fact that these studies are promising, further investigations are essential to better understand the sources of ROS altered in

a disease-specific manner and the mechanisms involved in the etiology so as to develop a therapeutic approach.

3. Oxidative Injury and Carcinogenesis

ROS play a central role in intracellular signaling that may be a double-edged sword. ROS may play a role in cellular senescence or cellular survival depending on a variety of factors and signaling pathways affected, which are actually susceptible to endogenous or exogenous sources of ROS, conferring support to the environmental contribution to carcinogenesis (Valko et al., 2006; Benz & Yau, 2008; Sosa et al., 2013). Consequently, cells may either maintain molecular interactions and remain anti-tumorigenic entities, or transform into oncogenic cancer cells (Valko et al., 2006). Such alterations exacerbated by ROS production by cancerous cells alter several pathways that mediate oncogenesis and malignant transformations (Ishikawa et al., 2015; Chio & Tuveson, 2017). In this regard, macromolecular modifications are one of the entities affected by ROS that lead to cellular structural and functional damage (Acharya et al., 2010). Lipids may be affected via peroxidation, or may be modified to form oxysterols that alter cellular membrane dynamics. The oxidation of proteins similarly affects cellular activity, and this tends to be an irreversible injury (Thanan et al., 2014). However, oxidative stress further induces injury through nuclear and mitochondrial genetic material mutations (Sabharwal & Schumacker, 2014; Ishikawa et al., 2015). Indeed, oxidative damage has been reported to be underlying the mechanism of carcinogenesis in several cancers (Prasad et al., 2017; Li et al., 2011; Li et al., 2013; Deep et al., 2016; Auyeung & Ko, 2017), via facilitating genomic instability (Dizdaroglu & Jaruga, 2012; Cadet & Wagner, 2014) and by being a DNA mutagen (Shibutani et al., 1991; Ogrunc et al., 2014; Ishikawa et al., 2015). Such mutations are mediated by certain oxidative species with precise and specific interactions. For instance, superoxides tend

to affect iron-sulfur clusters whereas hydrogen peroxides may target cysteine residues on proteins (Winterbourn & Metodiewa, 1999; Poole, L. B., 2015). These alterations may influence protein-DNA interactions (Velu et al., 2007). As for hydroxyl moieties, this form of ROS is capable of interactions with sugar entities that are part of DNA bases adding to the significance of oxidative stress and its role in triggering DNA damage (Dizdaroglu & Jaruga, 2012). Thus, ROS may induce DNA strand nicks, lesions, base modifications, and impaired DNA repair mechanisms. In this manner, ROS elevate the risk of inducing genetic mutations in genes central for cell cycle, cell death and metabolic regulation (Bhatt et al., 2010; Reuter et al., 2010; Chew, S. H., & Toyokuni, S. 2015; Zhong et al., 2017).

Promutagenic ROS-dependent DNA damage and base modifications are thought to be introduced via misincorporation of DNA bases, for example, due to the presence of unrepaired DNA adducts, or by slippage of DNA polymerase during replicative by-pass (Rodriguez et al., 2013).

3.a. 8-oxodG and Oxidative Stress

One of the most frequently generated DNA modifications, and observed hallmarks of oxidative DNA damage, is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) (Swenberg et al., 2010) although other forms have been reported such as 8-hydroxydeoxyguanosine (8-OHdG) (Marnett, J., 2000; Kryston et al., 2011). 8-OHdG is known to significantly contribute to oncogenesis, and has been reported to be dramatically increased in breast cancer tissues obtained from human patients (Matsui et al., 2000). Similar observations were reported by other groups that linked 8-OHdG elevated levels to renal cell carcinoma (Okamoto et al., 1994) as well as colon cancer tumors (Olivia et al., 1997). However, 8-oxodG tends to be the major form encountered that marks oxidative injury of DNA; this is by virtue of the relatively steady state in

the cell at an approximate frequency of 2,400 (Swenberg et al., 2010). Any alteration may thus be directly linked to pathology. For instance, increased 8-oxodG content was reported in mononuclear cells and in urine samples from Type 1 and Type 2 diabetic patients (Hinokio et al., 2002). More importantly, 8-oxodG adducts formed by ROS and their accumulation are primarily deleterious because they result in GC to TA transversions and lead to cellular damage (Cheng et al., 1992; Bruner et al., 2000; Tsuruya et al., 2003). Thus, 8-oxodG is a quantitatively major form of oxidative DNA damage and its ability to induce mutations by DNA adducts such as 8-oxodG is well documented (Efrati et al., 1999). Consequently, the role of DNA repair genes associated with cancer is of major significance since the efficacy of DNA repair may determine the susceptibility to carcinogens.

Extensive literature describes a number of major DNA repair pathways, including homologous recombination, non-homologous end joining, base excision repair, mismatch repair, single-strand annealing and nucleotide excision repair. With regards to 8-oxodG in DNA, these adducts are repaired primarily via the DNA base excision repair pathway (Smart et al., 2006). The enzyme that recognizes and excises 8-oxodG from cellular genetic material is 8-oxoG-DNA glycosylase (OGG1); however there is little information on the cellular and molecular mechanisms of OGG1 regulation (Mitra et al., 2001). Research conducted on the OGG1 gene cloned approximately 2.0 Kb of OGG1 promoter and a nuclear factor-YA (NF-YA) has been identified as a transcription factor that binds to a consensus sequence in the OGG1 promoter. More importantly, it was reported that loss of heterozygosity at the OGG1 allele leads to loss of OGG1 function which in turn contributes to tumorigenesis (Hung et al., 2005). Actually, the OGG1 gene is found somatically mutated in certain cancers (Hung et al., 2005; Srivastava et al., 2009; Zhang et al., 2013; Duan et al., 2012; Das et al., 2016) and is highly polymorphic among

humans. Actually, one of the most commonly occurring polymorphisms of OGG1 is the Ser326Cys substitution of a serine with a cysteine (Bravard et al., 2009), and these polymorphisms in the OGG1 gene have been frequently described in CRC (Pardini et al., 2008; Przybylowska et al., 2013; Canbay et al., 2011; Su et al., 2014; Lai et al., 2016). However, the impact of OGG1 polymorphisms and their mechanisms affected remain poorly investigated.

3.b. OGG1 and Diabetes

In diabetes, oxidative stress is one of the major mediators of tissue and cellular injury. At the molecular level, there have been studies that suggested the association between hyperglycemia and impairment in the DNA repair system. For instance, in one study, strong evidence showed the role of insulin in regulating the synthesis of the xeroderma pigmentosum-D (XP-D), a DNA repair enzyme which plays a pivotal role in nucleotide excision repair. Evidently, long-term exposure to glucose at a high concentration may result in downregulation of the insulin-dependent increase in XP-D mRNA levels, increasing the extent of DNA damage (Merkel et al., 2003). In the same manner, hyperglycemia has also been implicated in the dysregulation of OGG1.

With emphasis on OGG1, numerous studies have examined the significance of OGG1 and DNA repair in diabetes. Diabetes-induced elevation of 8-oxodG levels in the renal mitochondria from diabetic animals was reported to be normalized in response to insulin treatment, suggesting an insulin-dependent mechanism of modulation (Kakimoto et al., 2002). In a recent study, hearts of type-1 diabetic mice were shown to exhibit elevated OGG1 expression however with a relatively poor activity accompanied by increased adduct levels in mitochondrial DNA. The investigators also showed the role of O-GlcNAcylation in the blockade

of OGG1 activity, indicative of a hyperglycemia-mediated role in diabetic cardiomyopathy pathogenesis (Cividini et al., 2016). As for the diabetic kidney, *in vitro* and *in vivo* studies conducted in the renal epithelial cells as well as the renal cortex reported a reduction in OGG1 activity and an increase in oxidized DNA which may contribute to diabetic nephropathy through an AKT-induced upregulation and activation of Tuberin (Simone et al., 2008). These data were further verified by a recent study that showed the impact of the protective effect of AMPK pathway activation on OGG1 expression and activity restoration (Habib et al., 2016). On a final note, the role of OGG1 in maintaining cellular energy and metabolism was described in a study that utilized OGG1 double-knockout animals. High-fat diet fed animals were shown to exhibit a deficiency in OGG1 activity in addition to hyperinsulinemia and resistance to glucose that elevated the risk for metabolic dysfunction (Sampath et al., 2012). However, the influence of chronic and long-term hyperglycemia on OGG1 expression or activity remains unknown. Nevertheless, results from these studies indicate that high glucose and insulin deficiency may contribute to DNA damage and repair in diabetes (Dandona et al., 1996; Simone et al., 2008).

Besides the functional role of OGG1, there have been findings that correlated the specific Ser326Cys polymorphism in the OGG1 gene with diabetes pathobiology (Daimon et al., 2009; Thameem et al., 2010). Such polymorphisms may increase the susceptibility to mutagens and impaired DNA repair which may contribute to genomic instability in diabetic patients and, as a consequence, to cancer. While extensive literature supports the evidence that in DM there is an accumulation of products of damage to DNA and RNA, the mechanism(s) by which the oxidized DNA accumulates or is efficiently removed by cellular DNA repair pathways has not been elucidated. DNA repair genes, of which there are over 125, have many functions, serving to recognize and repair DNA damage and protect cells from errors of incorporation during DNA

replication. Loss of function of DNA repair genes predisposes cells to further genetic alterations as a result of diminished repair capacity, a form of genomic instability that can lead to cancer. These types of DNA repair genes are considered tumor suppressor and/or “caretaker genes” (Levitt & Hickson, 2002). Together, these observations provide strong rationale to explore the role of oxidative stress as a pathogenic mechanism predisposing to mutagenesis and colorectal cancer in diabetes.

D. NADPH oxidases in Diabetes

1. The Family

NADPH oxidases (NOX) are a family of heme-containing proteins whose sole purpose is the generation of reactive oxygen species (ROS) across biological membranes. Members of the human NOXs include: Nox1, Nox2, Nox3, Nox4, Nox5, DUOX1 and DUOX2, each with distinct activation mechanisms and a differential expression that is tissue-dependent (**Fig. 4**) (Bedard & Krause, 2007).

NOXs are known to be transmembrane proteins with common and conserved structural properties. These features include: 1) a NADPH-binding site at the COOH terminus, 2) a flavin adenine dinucleotide (FAD)-binding region in proximity of the COOH-terminal transmembrane domain, 3) six conserved transmembrane domains, and 4) four highly conserved heme-binding histidines (Bedard & Krause, 2007). It is generally accepted that under physiological conditions, NADPH oxidases are expressed at relatively low level of constitutive activity and critical physiological and pathological processes including cell signaling, inflammation and mitogenesis (Brar et al., 2002; Geiszt, M., 2006). However, stimuli such as cytokines (Rustenhoven et al., 2016), growth factors (Brandes et al., 2001), hyperlipidemia (Miller et al., 2010), and

hyperglycemia (Eid et al., 2010) can induce NADPH oxidase enzymatic activity both acutely and chronically which in turn disrupts homeostatic set points and results in a wide range of disorders.

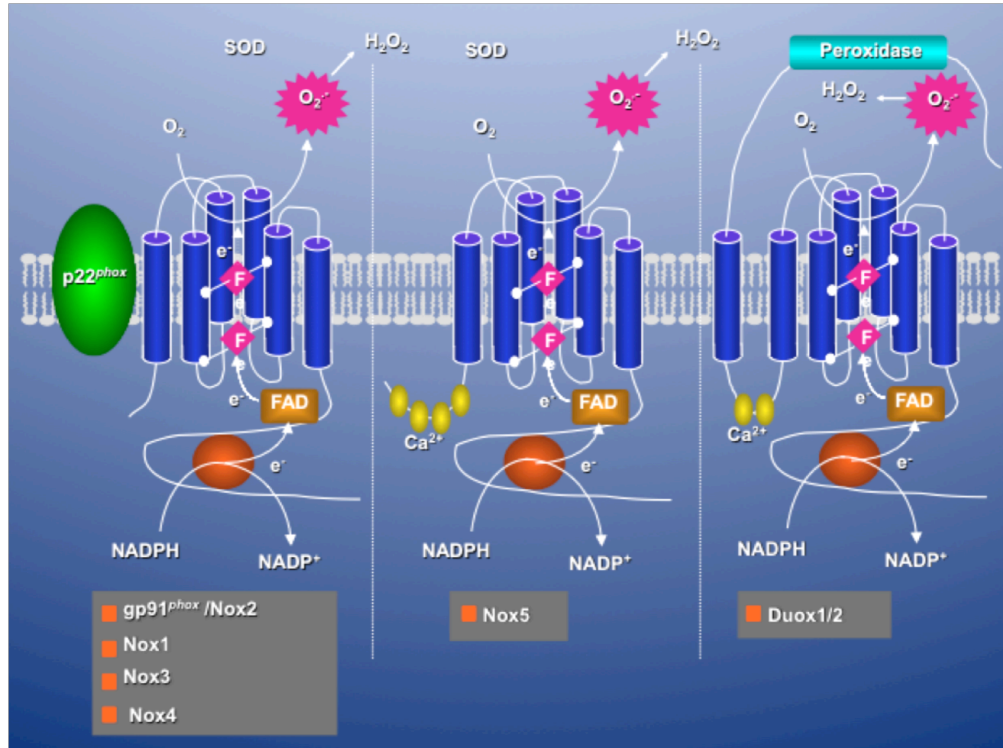


Figure 4. Structure of the NADPH oxidases family of enzymes. NADPH oxidase or Noxes have been described to be transmembrane proteins and share common structural properties that are conserved for all family members. These features include: 1) a NADPH-binding site at the COOH terminus, 2) a flavin adenine dinucleotide (FAD)-binding region in proximity of the COOH-terminal transmembrane domain, 3) six conserved transmembrane domains, and 4) four highly conserved heme-binding histidines. NADPH oxidases transfer electrons sequentially from NADPH to FAD, to the heme groups and finally to oxygen.

Of concern to this dissertation, NADPH oxidase-derived ROS have been shown to play a significant role in injury to various organs including colon, in addition to ROS generated from mitochondrial respiratory chain (Bedard & Krause, 2007). Moreover, a number of homologs of the phagocyte NADPH oxidase catalytic subunit (Nox2) have been identified, and these enzymes are known to participate in a number of biological processes including proliferation, migration, contraction, fibrosis and apoptosis (Bedard & Krause, 2007). In colonic tissues, Nox2, Nox1 and

Nox4 are reported to be expressed collectively (Bedard & Krause, 2007), and these isoforms will be the major focus throughout this work. Consequently, we next highlight the role of Nox1 and Nox4 in the pathophysiology of diabetes and colorectum.

2. NADPH Oxidases Nox 1 and Nox 4 in Diabetic Complications

One of the most extensively studied and largely expressed isoforms of NADPH oxidases within colon epithelium is Nox 1 (Rokutan et al., 2008). Nox 1 which has also been detected in the brain (Sorce, S., & Krause, K.H. 2009), vascular smooth muscle, uterus, prostate, bone and retina (Brandes et al., 2014) is described to be a 55–60 kDa protein. It requires p22phox, NoxO1 (Nox Organizer 1), NoxA1 (Nox Activator 1), and the small GTPase Rac for its activation. Like all other Nox members, Nox1 contains six transmembrane domains and conserved motifs corresponding to binding sites of heme, flavin, and NADPH. Moreover, Nox1 has been described to be located bound to plasma membranes or localized to the nucleus, cytosol, endoplasmic reticulum (ER) and mitochondria (Bedard, K., & Krause, K.H. 2007; Brandes et al., 2014).

Although Nox1-derived ROS production has been reported to have various functions in cell signaling, cell growth (Ibi et al., 2006), differentiation and apoptosis, angiogenesis (Arbiser et al., 2002), in addition to blood pressure regulation (Gavazzi et al., 2006), extensive studies have attributed a role of Nox1- dependent ROS generation in organ damage with implications in diabetic complications. Studies have shown that increased expression of Nox1 induces vascular dysfunction in the aorta of diabetic animals (Wendt et al., 2005), and plays a key role in diabetes–accelerated atherosclerosis (Gray et al., 2013). The silencing or pharmacological inhibition of Nox1 has been shown to provide renoprotection in diabetic nephropathy (Gorin et

al., 2015; Zhu et al., 2015). Recent evidence also indicates that Nox1 selective inhibition preserves human and mice beta cell function in diabetes (Weaver et al., 2015). These data suggest a pathogenic feature of Nox1 in diabetes and diabetic complications.

As for Nox4, findings have reported that it is expressed ubiquitously in many cell types including kidney mesangial (Gorin et al., 2003) and epithelial cells (Eid et al., 2009, 2013a), smooth muscle cells (Hilenski et al., 2004), endothelial cells (Ago et al., 2004), fibroblasts (Cucoranu et al., 2005), keratinocytes (Chamulitrat et al., 2004), osteoclasts (Yan et al., 2001), neurons (Vallet et al., 2005), and hepatocytes (Carmona-Cuenca et al., 2006) as well as the colon (Bauer et al., 2013). Nox4 is unique among all other catalytic Nox subunits in that it is thought to be constitutively active and that it only requires the membrane subunit p22phox for its ROS producing activity (Bedard, K., & Krause, K.H. 2007; Brandes et al., 2014). Thus, Nox4 mRNA formation alone determines ROS production, a process now considered to be tissue and disease specific. Besides being predominantly localized in the plasma membrane, Nox4 has been also described to be expressed in the ER, in the nucleus and in the mitochondria (Nauseef, W.M., 2008; Desouki et al., 2005; Block et al., 2009).

Physiologically, Nox4 is reported to be involved in a number of homeostatic processes. In fact, Nox4-derived ROS have been implicated in cellular senescence (Schilder et al., 2009), apoptosis (Pedruzzi et al., 2004), survival (Vaquero et al., 2004), insulin signaling (Mahadev et al., 2004) and differentiation (Yang et al., 2001). Nevertheless, the constitutively active Nox4 isoform, which produces low levels of ROS under basal conditions, can be upregulated in many chronic diseases, such as cardiovascular diseases (Pedruzzi et al., 2004), cancer (Vaquero et al., 2004), and kidney disease (Gorin et al., 2003) in addition to diabetes (Eid et al., 2009; Eid et al.,

2010; Eid et al., 2013a; Eid et al., 2016). Indeed, Nox4 has been increasingly shown to play a critical role in micro- and macro- vascular complications of diabetes.

Previous work by our group has shown the role of Nox4 in diabetic nephropathy and podocyte depletion in diabetic experimental animal models (Eid et al., 2010, Eid et al., 2013a, Eid et al., 2016). The downregulation of Nox4 expression *in vitro* and *in vivo* was found to significantly ameliorate podocyte apoptosis and albuminuria indicating a pathogenic role for Nox4. Additional studies reported that the pharmacological inhibition of Nox4 using a dual Nox1/ Nox4 inhibitor reduces renal pathology in type 1 and type 2 diabetes, as reflected by normalized albuminuria, mesangial expansion, tubular dystrophy and glomerulosclerosis (Gorin et al., 2015; Sedeek et al., 2013). Likewise, a pathological role of Nox4- derived ROS has been suggested in diabetic retinopathy as well. Studies on cultured retinal capillary endothelial cells and in db/db mice showed a rise in Nox4 expression *in vitro* and in the retinas of db/db mice, covertly indicating a promising therapeutic potential in diabetic retinopathy (Li et al., 2010; He et al., 2013). As for diabetic cardiomyopathy, controversial results have been reported regarding the exact role of Nox4. The importance of Nox4 in the atheroprotective response of vascular smooth muscle was investigated in diabetic Apoe (-/-) mice deficient for Nox4. The data showed that a genetic deficiency in Nox4 expression was deleterious and may further exacerbate diabetes-induced cardiac dysfunction (Di Marco et al., 2016). In contrast, other studies investigated the detrimental role of Nox4 in cardiac injury. *In vivo* studies on diabetic Apoe (-/-) mice deficient for Nox4 have shown that Nox4 may be involved in diabetes-induced endothelial dysfunction as well as vascular stiffness (Paradis et al., 2016). Similarly, it was shown that the upregulation of Nox4 in the myocardium causes cardiac remodeling (Zhao et al., 2015). The debate as to whether Nox4 insults or compliments the cardiovascular system is ongoing and

extensive *in vivo* studies are pivotal to progress further. On a final note, the involvement on Nox1 and Nox4 in diabetic neuropathy are ongoing and currently being investigated in our laboratory. However, previous studies have linked the involvement of NADPH oxidases in diabetic neuropathic progression via the nonspecific inhibition of NADPH oxidases. The findings showed an amelioration of neurophysiological deficits and vascular conductance in an STZ-induced type 1 diabetic rat model, suggesting a damaging role of the Nox family in neurovascular abnormalities brought about by diabetes (Cotter, M. A. & Cameron, N. E. 2003). However, these compounds inhibit other enzymes beside NADPH oxidases, such as nitric oxide synthase, xanthine oxidase and cytochromes P450 family of enzymes (Vincent et al., 2011). The work in our laboratory has accordingly used available small-molecule Nox1/Nox4 inhibitors from the pyrazolopyridine chemical series. The findings are indicative of a Nox1 and Nox4 in peripheral nerve pathophysiology in diabetes (Eid, S et al., 2017; unpublished data).

3. NADPH Oxidases in the Colon and Carcinogenesis

Cancer cells, like other non-malignant cells, produce ROS. In tumors, these reactive oxygen metabolites can act as signaling molecules to promote cell survival over apoptosis (Storz, P., 2005; Szatrowski & Nathan, 1991). Moreover, in malignant and non-malignant tissue, the expression of the NOX family of genes is described to be highly organ-specific (Kamiguti et al., 2005; Roy et al., 2015). The Nox1 and Nox4 isoforms are majorly expressed in colon cells. In fact, colonic epithelial as well as gastric cells Nox1 expression induces ROS production that is essential to maintain gut motility and mucosa secretion (Kojima et al., 2002), and to contribute to host defenses (Krause, K. H., 2004). However, in colon cancer, and although findings have been inconclusive and controversial, a rising body of data reports that Nox1 is overexpressed (Rokutan et al., 2006) and is reported to contribute to malignancy by mediating extracellular

matrix-degrading invadopodia of tumor cells and thus, invasion of CRC cells (Gianni et al., 2010). Additionally, Nox1 overexpression is thought to confer resistance to the neoplastic growth despite radiative stimuli (Rokutan et al., 2006). Further studies showed that Nox1-generated hydrogen peroxide can trigger an “angiogenic switch” that includes the induction of angiogenic factors, such as the vascular endothelial growth factor (VEGF), and this promotes tumor cell vascularization and proliferation (Arbiser et al., 2002). Also in the gastrointestinal tract, expression of Nox1, as well as NoxA1 and NoxO1, is significantly increased in colon cancers compared with adjacent normal bowel mucosa (Juhász et al., 2009).

Although Nox1 is the predominant isoform in the gastrointestinal tract, Nox4 has in the same manner been shown to be prominent in tumorigenicity. Indeed, Nox4 has been shown to play a role in carcinogenesis in several cancers. For instance, Nox4-mediated ROS is reported to prevent apoptosis and promote tumor cell growth in pancreatic cancer cells (Mochizuki et al., 2006; Vaquero et al., 2004), melanoma cancer cell proliferation and growth (Yamaura et al., 2009), breast cancer cells (Tobar et al., 2010), glioblastoma cancer cells (Mondol et al., 2014) and colon cancer cells (Bauer et al., 2013). Nox4 in these studies is shown to contribute, like Nox1, to tumor cell resistance, metastasis and oncotic transformation. Indeed, in CRC patients overexpressing Nox4, findings have correlated Nox4 overexpression with a poorer prognosis in CRC patients (Lin et al., 2017) in concordance with a higher likelihood of metastatic growth (Zhang et al., 2014), angiogenicity (Helfinger et al., 2016) and apoptotic death (Zhu et al., 2013). Interestingly, Nox4 overexpression has been also dubbed as a prognostic factor in metastatic CRC predictive of relapse in Stage II and III CRC and highly associated with metastatic profiles of tumors relative to primary stages (Bauer et al., 2013; Lin et al., 2017). In a study conducted by Bauer et al, results were indicative of the implication of Nox4-dependent cellular motility and

thus, a Nox4 mediated contribution to aggressiveness of tumors in CRC by modulating cytoskeletal regulating proteins (Bauer et al., 2014). The mechanism through which Nox4 regulates cellular motility in CRC was further investigated and described to progress through a TGF- β -activated protein tyrosine phosphatases axis (Zhang et al., 2015). In the same study, Zhang et al provided evidence for Nox1 and Nox4 interplay through their differential expression throughout CRC progression, with Nox1 playing a role in early stage adenoma, while Nox4 expression peaking upon transition to a malignant form (Zhang et al., 2015).

However, despite these studies, our understanding of the role(s) of the NOX family of genes in the development and growth of human cancer and especially in colon cancer is limited, nevertheless the role of hyperglycemia in CRC tumorigenesis. Recent studies have shown the influence of Nox1 and Nox4 isoforms on the NF κ B pathway prior to tumor appearance (Wang et al., 2011). The inhibition of Nox1 *in vitro* was shown to correlate with an attenuated growth of colon cancer cells and animal models of CRC, in addition to the elevation of phosphatase protein activity associated with the blockade of the cellular cycle and the dephosphorylation of MAPK (Doroshov et al., 2013; Juhasz et al., 2017). Furthermore, in a recent study Joo et al showed the role of growth receptor bound protein 2 or Grb2, and the Cbl proteolytic pathway in regulating the Nox Organizer 1 (NoxO1) in CRC progression. The findings of the study show an elevated stability of NoxO1 that is associated with profound ROS production through Nox1 that is associated with tumorigenesis, concomitant with stimulation via the epidermal growth factor (EGF) (Joo et al., 2016). This study highlighted the mechanism of activation of Nox1 that lead to CRC. However, in another investigation, EGF-stimulated CRC progression was further dissected in human colon cancer cells lines *in vitro*. The findings were indicative of the pathologic role of EGF in inducing the expression of heme oxygenase which is through a NADPH oxidase induced

oxidative stress and ROS production through the activation of c-Src, AKT and NF- κ B pathways that terminates in colon cancer cell proliferation (Lien et al., 2014).

For the scope of this work, we aim to examine the mechanisms involved in oncogenesis and the role of NADPH oxidases in the development of colon cancer in diabetes through at least two mechanisms: ROS-dependent DNA damage and ROS-dependent enhancement of cell proliferation. On a final note, oxidative stress has been shown to aid in angiogenesis in metastatic colon cancers through factors that are regulated by the mammalian target of rapamycin (mTOR) pathway (Auyeung & Ko, 2017). Consequently, the following section will be directed onto the mTOR pathway, as well as the AMPK pathway which is known to be influenced by oxidative injury.

E. AMPK: Physiology and Pathophysiology in Diabetes

1. The Physiological Roles of AMPK

The PRKA gene, more commonly referred to as Adenosine Monophosphate-activated Protein Kinase (AMPK), is a heterotrimeric serine/threonine kinase complex (Emerling et al., 2009) that consists of a catalytic α -subunit and regulatory β - and γ -subunits. These units are encoded by seven genes: two for α (α 1 and α 2), two for β (β 1 and β 2), and three for γ (γ 1, γ 2, and γ 3), and their respective expression is described to occur in a tissue-specific manner. At the activation site, AMPK phosphorylation on its threonine residue Thr¹⁷² localized to the activation loop of the α -subunit, leads to the activation of AMPK which allows it to act as a fuel detector and as a master switch for the regulation of glucose and lipid metabolism (**Fig. 5**) (Hardie & Carling, 1997; Hardie et al., 1994; Kemp et al., 1999; Mitchelhill et al., 1994; Tsuboi et al., 2003; Carling, 2004; Hardie, 2004; Eid et al., 2010; Ronnett et al., 2009).

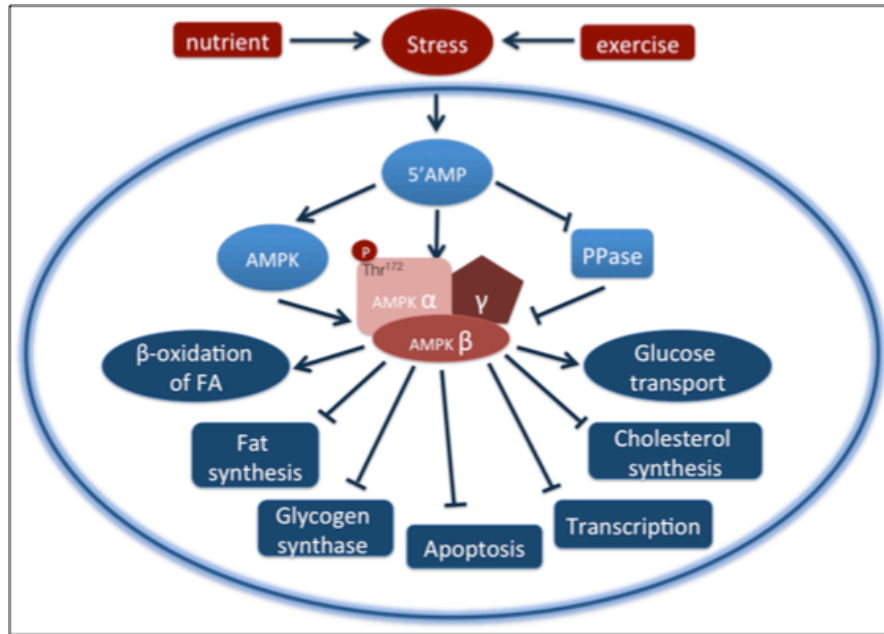


Figure 5. AMP-activated protein kinase: a cellular energy sensor. In response to cellular stress leading to depletion in ATP levels, AMPK is activated managing several functions in the cell such as apoptosis, transcription, glucose transport...

Additionally, the activation of AMPK inhibits lipid and glycogen synthesis, and subsequently triggers fatty acid oxidation and glycolysis. Thus, AMPK switches the cell from energy-storing to energy-releasing under conditions where ATP is limited and restores the energy balance (Emerling et al., 2009). This metabolic modulator is therefore known to be a key player in lipid and glucose metabolism, as well as protein synthesis, autophagy defined as the central degradative process in cells in which autophagosomes break down macromolecules and organelles during nutrient and energy starvation (Jensen et al., 1979), cell survival (Faulkner et al., 2005), and redox reaction homeostasis (Jeon, S., 2016) through the interaction with various and multiple pathways. In this context, the AMPK pathway is also well regulated through mechanisms that target the catalytic α -subunit and its phosphorylation at the Thr¹⁷². This activation is dependent on ADP/ATP and AMP/ATP ratios indicative of the cellular energy status (Hardie, D. G., 2004, Hardie et al., 2012), as well as phosphorylating kinases and

dephosphorylating phosphatases (Jeon, S., 2016). For instance, upon binding to AMP, AMPK undergoes a conformational change that facilitates the phosphorylation of the α -subunit (Polekhina et al., 2005). Moreover, AMPK can be phosphorylated and activated in various tissues by hormones that act through Gq receptors (Russell et al., 2004, Kim et al., 2007) and by adiponectin (Kim et al., 2007), leptin (Riboulet-Chavey et al., 2008), and pharmacological agents such as β -adrenoreceptor agonists (Gorin et al., 2004), metformin (Mu et al., 2001), AICAR (Eid et al., 2010, Aschenbach et al., 2002) and thiazolidinediones (TZDs) (Abboud et al., 1982).

One of the significant kinases associated with AMPK regulation is Liver Kinase B1 (LKB1), which is also known as Serine Threonine Protein Kinase (STK11). LKB1 is a tumor suppressor gene that responds to changes in cellular energy balance or ATP levels (^aCarling, D., 2004, Hardie, D. G., 2003). More importantly, STK11 or LKB1 is known to govern whole body insulin sensitivity (^bCarling, D., 2004, Violet et al., 2003) such that in cells with elevated levels of AMP due to altered energy homeostasis, STK11 phosphorylates AMPK (^aCarling, D., 2004, Shaw et al., 2004). Insulin is currently emerging through extensive research to be involved in the AMPK pathway indirectly through a mediating pathway, the AKT pathway (Hawley et al., 2014). This mechanism remains poorly understood, however, it is indicative of an underlying role especially in the context of diabetes. In fact, several studies revealed that under high glucose concentrations or nutrient excess, AMPK may 'switch off' in several cell types (Eid et al., 2010; Chowdhury et al., 2011; Tsuboi et al., 2003; Mountjoy & Rutter, 2007), and contribute to pathological states, two of which are diabetes and cancer (Jeon, S., 2016). Despite that, the specific role of AMPK and its modulators in diabetes is not fully understood.

2. AMPK in Diabetic Complications

In diabetes, the $\alpha 1$ and $\gamma 2$ subunits of AMPK have been associated with glucose and lipid metabolism (Eid et al., 2010; Xu et al., 2005) as well as glucose transport (Wright et al., 2004) and uptake in a manner that does not depend on insulin (Winder & Hardie, 1999). However, the relationship between AMPK and diabetes is complex and remains under investigation. Studies have insofar shown that AMPK largely influences autophagy as part of sensitizing skeletal muscles to insulin uptake (Yao et al., 2016) as well as at times of starvation in pancreatic beta cells and hepatocytes at the metabolic level (Yao et al., 2016). Defected AMPK activation modulates cellular autophagic responses to energy and nutrient availability through mechanisms that remain poorly understood. However, this pathway is a significant contributor to cellular homeostasis, and research reinforces the impact of AMPK dysfunction at the molecular level especially under chronic and prolonged hyperglycemic phases. In fact, several studies linked AMPK signaling with the forms of diabetes and various complications.

For instance, in diabetic cardiomyopathy, a study in experimental STZ-induced diabetic rats showed cardiac hypertrophy through the reduction in cardioprotective aldehyde dehydrogenase. The results of this study further showed the reduction in autophagy-associated proteins *in vitro* in cardiomyoblasts and their restoration upon treatment with an aldehyde dehydrogenase activator. However, a potential role of AMPK in autophagosome function was depicted when this effect was not possible in the presence of compound C, an AMPK inhibitor. These findings are indicative of the central role of AMPK in restoring autophagy in diabetic cardiomyopathy (Guo et al., 2015). Similar findings were reported in another study that showed how AMPK restored autophagy via dissociating BCL2 and beclin-1 protein complexes in cardiomyocytes (He et al., 2013). Additionally, in another experimental study, *in vitro* in

cardiomyocytes and *in vivo* testing of diabetes-induced ischemia, showed how AMPK modulated autophagosome formation and function through adiponectin, a protective molecule that seems to be underexpressed in diabetes. The results of this study indicate that AMPK-dependent association with Beclin-2 and autophagosome formation occurred upon the pharmacological stimulation of the adiponectin receptor, which in turn reduced infarct size and injury (Wang et al., 2017).

More importantly, AMPK plays a role in modulating oxidative stress. Several studies support the interaction between ROS and AMPK and show it to be stimulus and tissue specific. During hypoxia, mitochondria-generated ROS activate AMPK (Juhász et al., 2009). Similarly, during exercise, NADPH oxidase-derived ROS induce AMPK activation (Mochizuki et al., 2006). Concerning tissue specificity, in pancreatic β -cells, HG activates AMPK and enhances the production of ROS, resulting in loss of mitochondrial membrane potential (Vaquero et al., 2004). 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 (Kamiguti et al., 2005) suggesting that activated AMPK may suppress oxidative stress. As for insulin signaling, studies have highlighted the role of AMPK in enhancing insulin uptake by muscles and endothelial cells in response to AMPK activation as well as reversing oxidative injury (Ruderman et al., 2003).

Our group has previously investigated AMPK in renal podocytes and the findings provide evidence that AMPK largely influences podocyte renal function upon activation with AMPK agonists. The findings described a reversal of hyperglycemia-induced elevations in ROS production concomitant with apoptotic cell death since the expression of the cell guardian, p53, is compromised (Eid et al., 2010). However, in another study, AMPK activation and the

consequent reduction in diabetic renal injury hallmarks (such as albuminuria, matrix accumulation and inflammation) were reported to undertake an elevation in mitochondrial-dependent biogenesis and ROS production (Dugan et al., 2013).

In the same spirit, the role of AMPK in diabetic neuropathic complications was evident in experimental models of diabetes. Hyperglycemia has been known to trigger the switching off of AMPK and of silent information regulator T1 (SIRT1) which is an NAD^+ sensor that functions as a deacetylase of histones and proteins including PGC-1 α . Its inactivation results in impaired peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) activity and diminished mitochondrial activity. In type 1 diabetic mice, the expression of phosphorylated AMPK (p-AMPK), and PGC-1 α was decreased significantly (Chowdhury et al., 2011; Bao & Sack, 2010). Thus, hyperglycemia-induced loss of the AMPK/SIRT/PGC-1 α cascades lead to a deficiency in functional mitochondria that ultimately triggered neuronal death, a hallmark of severe diabetic peripheral neuropathy (Emerling et al., 2009).

3. AMPK in Cancer Biology

Like diabetes and its associated metabolic states, cancer cells, and other non-malignant cells, enhance ROS production in tumors such that, reactive oxygen metabolites can act as signaling molecules to promote cell survival over apoptosis (Bedard & Krause, 2007, Storz, P., 2005). Such compromised energy status may be indicative of a malfunctioning energy sensing, and thus, the AMPK pathway may be directly implicated in the pathology. There are studies that report a peculiar effect of AMPK in cancer, which is the induction of autophagy via supraphysiological activation of AMPK by drugs reported in lung adenocarcinoma, hepatocellular carcinomas and colorectal cancer (^bWang et al., 2011; Xiao et al., 2013; Imamura

et al., 2001; Zheng et al., 2013; Vara et al., 2011; Sato et al., 2007). However, there are countless studies that provide evidence of the AMPK pathway's association in tumorigenesis such as in melanomas and lymphomas, in addition to gastric, pancreatic, thyroid and colorectal cancers (Park et al., 2006; Kim et al., 2012; Hardie, D. G., 2013; Yue et al., 2014; Sui et al., 2014; Faubert et al., 2015). Our collaborators specifically showed that AMPK activity is reduced in cultured renal cell carcinoma cells compared to normal renal epithelial cells (Arbiser et al., 2002). However, AMPK seems to impose contradictory effects during carcinogenesis, depending on the context, energy status, and type of cell.

AMPK has been dubbed a 'conditional suppressor or a contextual oncogene' that interacts with insurmountable tumor specificity (Zadra et al., 2015). Actually, by cause of its complex interaction with numerous pathways, AMPK loss or gain in function may directly integrate with oncogenic and transforming factors, as has been described in *in vivo* studies. For example, in lymphoma pathologies, the knockout of AMPK's α subunit induced metabolic alterations that lead to the development of neoplastic growth (Faubert et al., 2013). By contrast, in head and neck carcinomas, AMPK knockdown promoted the induction of apoptosis of malignant growths in response to cetuximab, a potential treatment. These results are indicative of sustained AMPK expression in promoting survival of cancer cells (Li et al., 2015). Several other studies examined AMPK *in vitro* in experimental cell lines. Studies conducted on hepatoma, prostate, and breast cancer cells showed that AMPK activation via AICAR, a pharmacological agonist, elevated the expression of cyclin-dependent kinase inhibitors that are tumor suppressors (Imamura et al., 2001; Rattan et al., 2005; Xiang et al., 2004). Particularly in gender specific cancers, such as breast and prostatic cancers, AMPK activity was associated with preventing oncogenic transformation of cancer stem cells that aid in metastatic migrations (Chou et al.,

2014). In another study, the attenuation of AMPK cascades via cytochrome-dependent activity in breast cancer was associated with tumor proliferation (Rodriguez & Potter, 2013). Indeed, depending on the context, AMPK loss or gain in function effects are dramatically different suggesting that there are complex pathways that cross paths with AMPK (Luo et al., 2010). For instance, studies have shown that the loss of function of targets upstream of AMPK such as LKB1, also influence carcinogenesis together with AMPK inducing an oncogenic metabolic profile (Hardie & Alessi, 2013; Faubert et al., 2014). Studies that investigate these interactions are of major importance since they aid in comprehending the many faces of AMPK in pathogenic states.

As far as this work is concerned, AMPK in colorectal cancer is still poorly understood. There have been studies that showed the associations of colon cancer with the $\alpha 1$, $\gamma 2$ and $\beta 1$ subunits of AMPK (Slattery et al., 2010; Vetvik et al., 2014). In fact, in a study conducted by Baba and colleagues, the authors detected a predictable positive prognostic outcome in colorectal cancer patients with an elevated expression of AMPK $\alpha 1$ (Baba et al., 2010^b). By contrast, colorectal tumors were shown to possess an elevated expression of the $\beta 1$ subunit in comparison to adjacent mucosal tissue (Vetvik et al., 2014) indicative of the multi-faceted implication of AMPK in colorectal cancer. Overall, activation of AMPK at its Thr-172 phosphorylation site was assessed both *in vitro* in CRC cell lines (Tripodi et al., 2012) and *in vivo* in xenografted animals models of CRC (Valtorta et al., 2014). The data were suggestive of a positive effect of AMPK activation in adenocarcinoma whereby apoptotic death of CRC cells was induced upon treatment with an AMPK activator (Kim et al., 2007), in addition to retarding tumorigenic growth via the blockade of tubulin polymer formation (Tripodi et al., 2012). This anti-tumorigenic potential was reflected by an attenuated glucose uptake and metabolism in CRC

animals (Valtorta et al., 2014). Additional studies have also provided evidence of the beneficial capacity of AMPK activation especially at an advanced and metastatic stage in patients with CRC (Zulato et al., 2014).

Interestingly, in a study conducted by Kim et al, AMPK activity was assessed within the context of adipocytokines and lipid biogenesis, *in vitro* in colon cancer cell lines, as well as tissue biopsies from colon cancer patients (Kim et al., 2010). The findings of this study were indicative of the anti-tumorigenic role of AMPK in colon cancer. It was reported that adiponectin suppressed colon cancer cell proliferation via cell cycle arrest induction (Kim et al., 2010). This was demonstrated to be dependent on AMPK activation as well as cyclin-dependent kinase inhibitor expression. These cellular events were concurrent with reduced lipogenic gene expression indicative of a restored lipid homeostasis which is otherwise deficient in proliferating cancer cells, and finally, apoptotic death (Kim et al., 2010). In the same spirit, another recent study showed the facilitative effect of AICAR-induced AMPK activation in colorectal cancer cell response to 5-fluorouracil, a commonly used chemotherapeutic in the treatment of CRC patients both *in vitro* and *in vivo* in a xenograft model, as well as apoptosis induction (Sui et al., 2014). In another study, it has been suggested that AMPK activation occurs in colon cancer cells that are nutrient-deprived, which leads to autophagy as a bypass to countereffect the depletion of cellular reserves typical of constantly proliferating cells, and thus, in this way, AMPK activation contributes to colorectal tumor survival, however in at least the early phases of tumorigenesis (Sato et al., 2007).

The current data available regarding AMPK and its mechanisms of action in CRC carcinogenesis remains controversial and inconclusive. Moreover, the specific role of AMPK within a hyperglycemic environment has yet to be elucidated. There have been some

investigations that studied the effects of glucose lowering drugs on CRC survival or risk among diabetic patients. Sui and colleagues further examined the role of metformin, a glucose lowering pharmacological agent used in the treatment of diabetes, on CRC cells. The findings report a nullified effect in HT-29 cells whereby changes in cell proliferation and apoptotic death were nonexistent (Sui et al., 2014). These results seem to contradict with others that are suggestive of a beneficial and somewhat protective effect of metformin against CRC in diabetic patients (DeCensi et al., 2010; Lee et al., 2012; Higurashi et al., 2016; Pernicova & Korbonits, 2014; Sui et al., 2014; Zadra et al., 2015).

Regardless, additional studies are crucial to unveil the mechanistic pathways through which AMPK interacts in diabetes, especially within the context of CRC. Although the downstream effectors and mechanisms of action require further investigation, a particularly interesting downstream target of LKB1 and AMPK recently identified is Tuberin (TSC2). As mentioned earlier, LKB1 and its affiliation with CRC has previously been examined. However, taken together, the LKB1/AMPK/TSC2 pathway axis has been reported to interact with and negatively regulate the target of rapamycin (TOR) that is implicated in protein synthesis, cell survival and tumorigenesis by the fact that rapamycin inhibits tumor growth (Menon & Manning, 2008). In cancer, AMPK and the mammalian target of rapamycin (mTOR) interaction has been extensively described in several studies. The following section will consequently be devoted to the mTOR pathway within the context of diabetes and carcinogenesis.

F. The Role of mTOR Signaling in Diabetes

1. The Physiology of the mTOR Pathway

The mammalian target of rapamycin (mTOR) is a well-conserved pathway that evolved in cells for the purpose of efficiently coordinating nutritional and energetic supply and demand,

allowing them to survive and grow even when environmental resources become limited. mTOR specializes in integrating diverse environmental signals and translating these cues into cellular processes that govern cellular growth, survival and metabolism (Laplante & Sabatini, 2012).

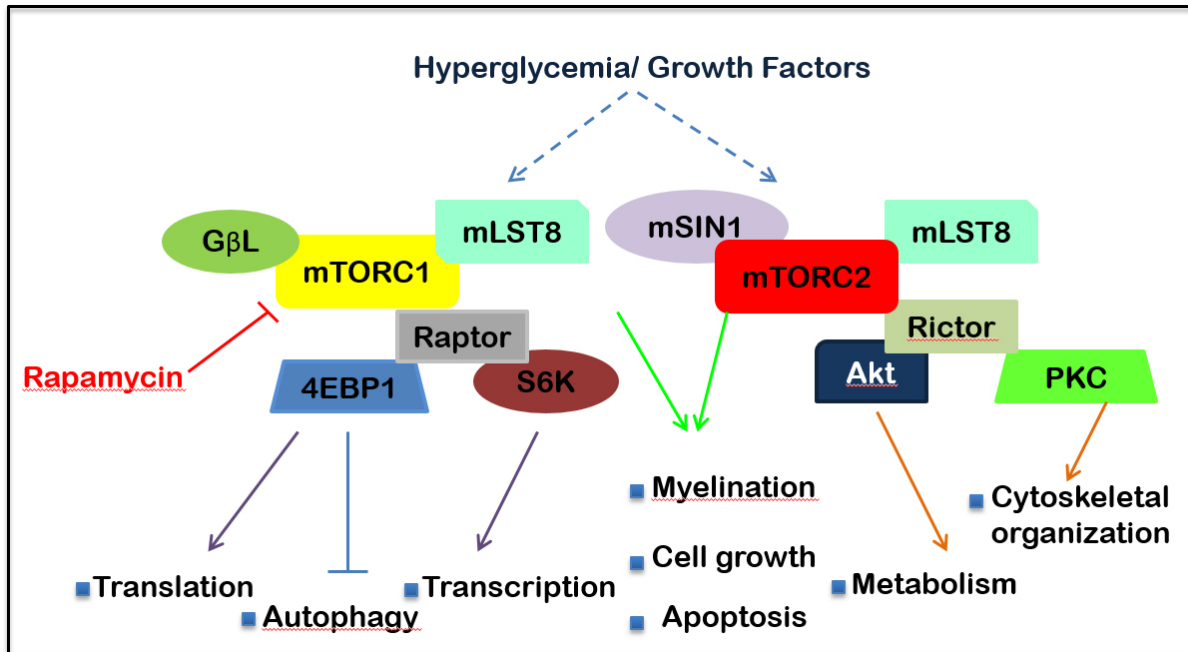


Figure 6. mTOR signaling pathway. mTOR pathway, a master regulator of cellular growth, survival and metabolism is altered in diabetes and diabetic complications.

Basically, mTOR is a serine/threonine protein kinase that interacts with several protein associations to form two complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) that differ based on their sensitivities to rapamycin, as well as their distinct upstream inputs and downstream targets (**Fig. 6**). Studies have shown that the acute treatment with rapamycin directly interacts and inhibits cellular mTORC1 with a null influence on mTORC2 signaling. However, emerging data reported that long-term rapamycin treatment is capable of mTORC2 function reduction by compromising complex assembly to variable degrees depending on cell type (Sarbasov et al., 2006). For the purpose of this thesis, we will cover mTORC1 downstream

effectors, major mTORC1 upstream signals, and cellular functions regulated by the mTORC1 signaling pathway.

mTORC1 with its core components: the catalytic mTOR subunit, mammalian lethal with sec-13 (mLST8) and the regulatory-associated protein of mammalian target of rapamycin (raptor) mediates protein synthesis and cell size through its downstream effectors. The best characterized downstream effectors of mTORC1 are p70S6 Kinase (P70S6K) and 4E-binding protein 1 (4E-BP1). Upon phosphorylation and activation by mTORC1, P70S6K and 4E-BP1 promote mRNA biosynthesis as well as translational initiation and elongation (Ma, X.M., Blenis, J. 2009). In addition to regulating protein synthesis, mTORC1 controls other anabolic processes including synthesis of lipids required for cell membrane formation and energy storage (Li et al., 2010; Laplante, M., & Sabatini, D.M. 2012). It also mediates nucleotide synthesis and suppresses autophagy. Thus, by integrating diverse upstream inputs, mTORC1 drives anabolic and inhibits catabolic cellular processes. Indeed, mTORC1 is able to sense and respond to a broad array of upstream signals including growth factors, stress, energy status, oxygen, and amino acids (Cornu et al., 2013). In particular, mTOR activity is negatively regulated by the heterodimeric complex consisting of tuberin (TSC2) and hamartin (TSC1). Phosphorylation of tuberin serves as an integration point for a wide variety of environmental signals that regulate mTORC1 (Sabatini, D.M. 2006). Importantly, phosphorylation of tuberin by AMP-activated protein kinase (AMPK) maintains its tumor-suppressor activity and prevents the activation of mTORC1 (Eid et al., 2013a).

As for mTORC2, little is known about the mTORC2 physiology and pathophysiology. mTORC2 comprises mTOR, mammalian stress-activated map kinase-interacting protein 1 (mSIN1), mammalian lethal with sec-13 (mLST8), and the rapamycin-insensitive companion of

mTOR (Rictor). The downstream effector of mTORC2 is Akt which is phosphorylated at its Serine473 when fully active (Sarbasov et al., 2005). Akt regulates cellular processes such as metabolism, cytoskeletal organization, survival, apoptosis, growth, and proliferation by further phosphorylating other downstream effectors (Wullschleger et al., 2006). Unlike mTORC1, mTORC2 signaling is insensitive to nutrients but is thought to respond to growth factors such as insulin (Zinzalla et al., 2011). In fact, the involvement of mTOR in diabetic complications has demonstrated in work previously done in our laboratory as well as others, which will be elaborated on in the next section.

2. mTOR and Pathologies of Diabetes

A short-term activation of mTORC1 signaling upon availability of environmental resources and within a physiological range is essential for anabolic processes, energy storage, and consumption, as well as normal cell/tissue growth. However, a persistent activation of mTORC1 signaling plays a role in panoply of injurious pathways and has been shown to be involved in diabetes onset and progression (Zoncu, R., Efeyan, A., & Sabatini, D. M. 2011). Mounting evidence has described an increased basal mTORC1 activity in both genetic and diet-induced animal models of obesity and pre-diabetic disorders (Khamzina et al., 2005; Turdi et al., 2011). Importantly, it is thought that hyperglycemia rather than growth factors may be the major player in activating mTORC1 signaling in diabetes and diabetic complications. Numerous studies have suggested that inhibition of the mTORC1 pathway with Rapamycin may have protective effects on the diabetic kidney in both type 1 and type 2 diabetic animal models (Eid et al., 2013a; Lloberas et al., 2006; Yang et al., 2007). In fact, Eid et al showed that Rapamycin administration significantly reduces NADPH-oxidase dependent oxidative stress, inhibits renal

or glomerular hypertrophy, decreases podocyte apoptosis, and attenuates mesangial expansion and albuminuria.

In diabetes-related cardiovascular diseases, ongoing efforts are attempting to pharmacologically target mTOR signaling in via rapamycin. Their efforts were shown to be effective in reducing the risk for major cardiovascular events in diabetic patients with coronary artery disease (Baumgart et al., 2007; de Waha et al., 2011). In fact, in more recent *in vivo* studies, rapamycin treatment prevented cardiac dysfunction, attenuated oxidative stress and altered the expression of antioxidant and contractile proteins in type 2 diabetic mice (Das et al., 2014).

As for mTORC2 involvement in diabetic complications, it has been recently reported that mTORC2 mediates mesangial cell hypertrophy in diabetic nephropathy, thus suggesting a therapeutic potential of mTORC2 inhibition (Das et al., 2016). In line with these observations, we have previously demonstrated the implications of mTORC2 through an Akt dependent pathway to play a key role in diabetic kidney injury. The rictor/mTORC2 pathway was shown to induce podocyte apoptosis *in vitro* and *in vivo* as well as enhance NADPH- dependent oxidative stress in the diabetic kidney (Eid et al., 2016).

However, the role of mTORC2 signaling in the pathology of diabetes-associated cardiovascular injury remains inconclusive. Recent data show that a relatively low dose of chronic rapamycin treatment prevented cardiac dysfunction in type 2 diabetic mice by mTORC1 inhibition, while preserving mTORC2 signaling, thus suggesting a cardioprotective role of mTORC2 in diabetes (Das et al., 2014; Das et al; 2015). Further studies showed that these cardioprotective effects may be mediated by Akt activation (Lin et al., 2014), although other studies report that the

constitutive activation of Akt in cardiomyocytes impairs GLUT4-mediated glucose uptake (Zhu et al., 2013).

In diabetic retinopathy, mTORC2 has been involved and studied extensively. Akt is thought to be a key survival factor that protects retinal cells at the initial stages of diabetic retinopathy (Reiter et al., 2006). Akt downregulation has been particularly associated with retinal, endothelial and neuronal cell death induction in the diabetic milieu (Huang et al., 2015; el-Remessy et al., 2005; Park et al., 2014). Yet, parallel studies reported that an increase in Akt signaling pathway in retinal endothelial cells may contribute to the pathogenesis of diabetic retinopathy (Huang, Q., & Sheibani, N. 2008; Yadav et al., 2012).

3. Carcinogenesis and mTOR

mTOR being the cellular commander, either driving cellular processes towards anabolism or catabolism, is also an oncogenic transformer that contributes to carcinogenesis. In fact, mTOR has been described to be a modulator of cell survival, growth, proliferation, differentiation and migration, processes that are dysregulated in cancer. Studies have extensively shown that mTOR regulators such as RHEB, PTEN and TSC are also altered in cancers (Huang et al., 2008; Zoncu et al., 2011; Campa et al., 2011; Naguib et al., 2011; Demark-Wahnefried et al., 2012). For instance, the phosphorylation of TSC2 serves as an integration point for a wide variety of environmental signals that regulate mTORC1 (Sarbasov et al., 2005). These pro-carcinogenic effects are facilitated by S6Kinase and 4EBP-1 which facilitate protein translation, as well as other pathways that feed into the cross-talk such as AKT, AMPK, and HIF-1 α .

In lung cancer cells, mTORC1 was shown to regulate input from HIF-1 α and LKB1 such that the downregulation of mTORC1 permitted control of tumor growth (Faubert et al.,

2014). Similarly, in head and neck carcinomas, hyperactivation of mTOR has been linked to hypoxic states that are characterized by elevated levels of a REDD1, a negative regulator of mTOR (Schneider et al., 2008). In CRC, mTOR inhibition via a combination of phytochemicals was shown to be pro-apoptotic in colon cancer cell lines via the reduction of cyclin D1 and c-Myc expression (D'Angelo et al., 2014). As for hepatocellular cancers, it was reported that anticancerous anthraquinone administration *in vitro* was associated with hyper-activating TSC complexes inhibiting mTOR at its activating sites and triggering entry into cell cycle arrest (Chiang et al., 2010). Despite these studies, the role of TSC2/mTORC1 pathway in colon tumorigenesis in DM remains largely unknown. In a recent study, Din and colleagues reported that AMPK activation occurred in response to aspirin treatment in CRC cells that subsequently inhibited mTOR which promoted autophagic cellular response activation. This effect was additive with metformin administration (Din et al., 2012).

G. Hypotheses and Aims

Diabetes targets numerous organ systems and leads to deleterious consequences among which are the micro- and macrovascular injuries. In recent years, the onset of solid tumors including CRC was classified as one of the new diabetic complications. Despite the ability of glycemic control to partially turn down diabetes-induced injury, such control is hard to achieve and the onset of complications is inevitable. Thus, the comprehension of the mechanistic progression of diabetes-induced CRC progression is central to identify novel therapeutic targets. Our group has previously shown that, in diabetic nephropathy and neuropathy, there is a clear coalition between NADPH oxidase-induced oxidative stress and kidney cellular injury as well as Schwann cell injury. The major aim in my thesis work was to understand the mechanisms leading to diabetes-induced CRC progression.

Rational: To our knowledge, this is the first study that sheds the light on the role of NADPH oxidases, specifically Nox4, in diabetes-induced CRC aggressiveness. More importantly in this thesis project we identified a potential crosstalk between NADPH oxidases and other signaling pathways, AMPK and mTOR, known to play a role in the physiology and pathophysiology of several diseases (**Figure 7**). Nonetheless, their role in diabetes-induced CRC has not been described.

Aim 1.

We look forward to determine the role of AMPK and tuberin/mTOR and their crosstalk with the NADPH oxidases subunit Nox4 in normal versus cancerous colon epithelial cells in their response to high glucose (HG), high insulin (HI) or their combination.

Aim 2.

We aim to explore the mechanism by which diabetes accelerates tumor development and tumor burden and whether treatment with the AMPK activator, metformin, the mTORC1 inhibitor rapamycin, or the blockade of the NADPH oxidase Nox4, retards the development of colorectal cancer in diabetes.

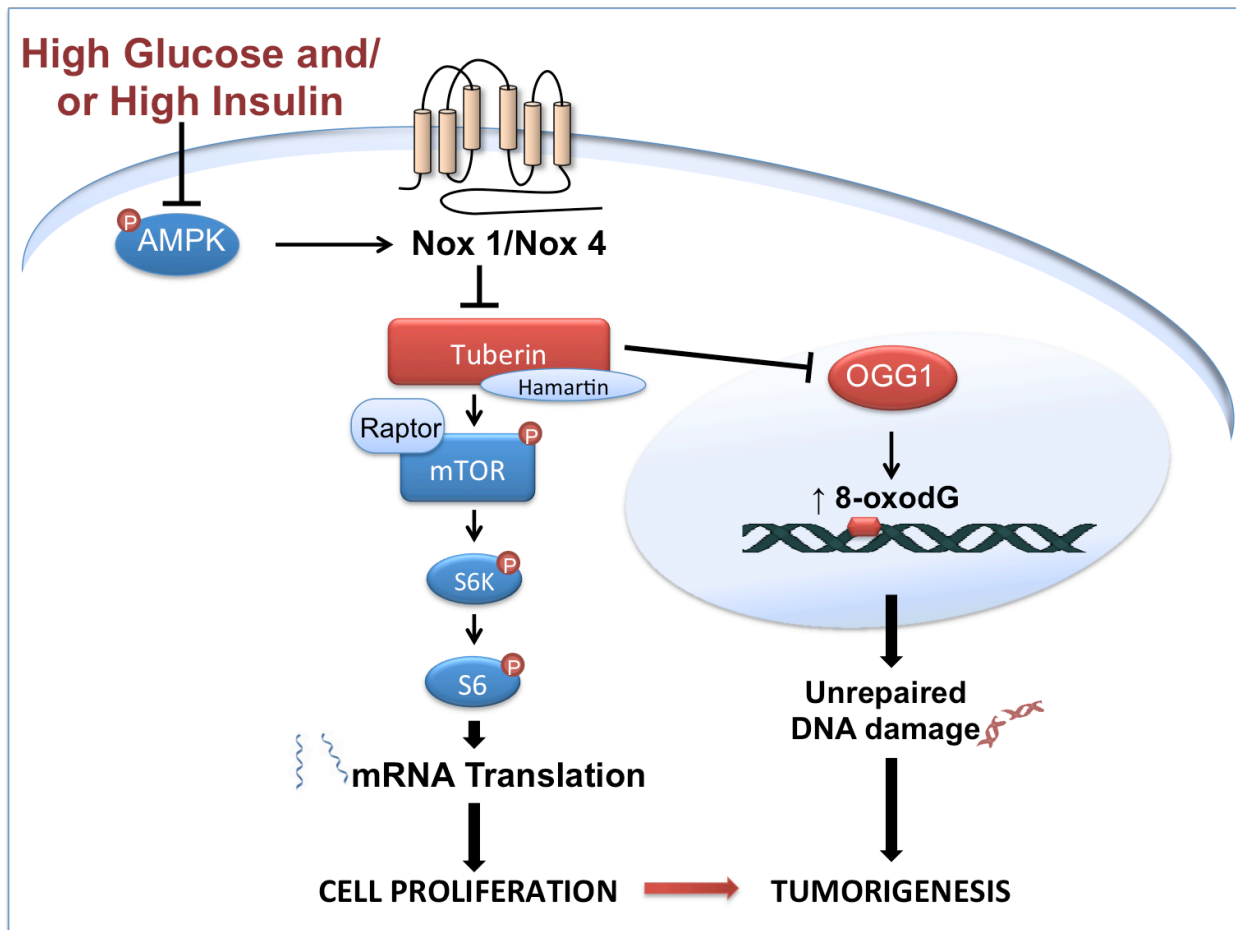


Figure 7. Hypothesis of the study. Hyperglycemia/hyperinsulinemia leads to an upregulation of Nox4 through downregulation of AMPK activity. This leads to an inhibition of Tuberin and thus an activation of the mTORC1/p70S6K pathway resulting in increased cellular proliferation and aggressiveness. Tuberin inactivation also inhibits OGG1 activity leading to accumulation of DNA damage further contributing to tumorigenesis.

MATERIALS AND METHODS

Animal Models

All animal procedures were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and were approved by the institutional animal care and use committee at the American University of Beirut. An animal model of colorectal cancer was used in this study, the C57BL/6-*Apc*^{tm1Tyj}/J (The Jackson Laboratory, Bar Harbor, ME). The APC (adenomatosis polyposis coli) mice possess a germline heterozygous deletion of the *Apc* gene (15 coding exons located on 5q22.2) that is flanked by loxP sites. This mutation renders them prone to tumor formation similar to APC^{Min} animals and thus, APC mice serve as a great model of colon cancer (The Jackson Laboratory, 2017). In addition, the C57BL/6J strain was used as a background for the APC mice. The C57 mice are the most widely used inbred strain that possess a permissive background for maximal expression of most mutations. These mice are also prone to diet-induced obesity, and type-2 diabetes.

In our study the C57 mice were used as a control and Streptozotocin (STZ)- induced APC and C57 mice were used as type 1 diabetic models.

APC and C57 male mice, six-eight- week old and weighing around 23-25 g, received 3 consecutive 100 mg/kg body weight intra-peritoneal injections of STZ (Sigma-Aldrich, Steinheim, Germany) dissolved in sodium citrate buffer (0.01 M, pH 4.5). Controls received similar injections of citrate buffer. Glucose measurement was performed 10 days after the STZ injection and blood was obtained via tail vein punctures and a glucometer (Accucheck, Roche). Mice with a fasting blood glucose ≥ 250 mg/dl were considered diabetic. Blood glucose levels were monitored weekly and were significantly different in diabetic animals relative to control

littermates. STZ, that has a preferential toxicity towards the pancreatic β cells, is widely used to induce both type 1 and type 2 diabetes in rodents (Jeffy et al., 2016).

Six-week old APC mice weighing 23-25g, were purchased from Jackson Laboratories, JAX stock #009045 (The Jackson Laboratory, Bar Harbor, ME, Carraro et al., 2014, Cheung et al., 2010, Jacks et al., 2010, Schneidar et al., 2014, Nieuwenhuis & Vasen et al., 2007, Soravia et al., 1998). C57BL/6J mice weighing 23-25 g were purchased from the animal care facility at the American University of Beirut (Beirut, Lebanon). APC and C57 mice were deemed diabetic when they exhibited blood glucose levels ≥ 250 mg/dl (**Table 2**).

Diabetic animals were given one of the following treatments:

- 150 mg/kg body weight of metformin, an activator of the AMPK signaling pathway, administered daily by intraperitoneal injection.
- 0.5 mg/kg body weight of rapamycin (Rapa), an mTORC1 inhibitor, administered three times a week by intraperitoneal injection.

For both strains of mice (APC and C57), the animals were grouped into three subsets: I, control mice; II, diabetic mice; III, diabetic mice treated with metformin; IV, diabetic mice treated with rapamycin. Treatment doses were based on previous studies performed by our group (Eid et al., 2013).

All animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and had standard chow and water access. Treatments were administered for 6 weeks and after euthanizing the colons were extracted, cleaned with saline solution, and the number as well as the volume of the polyps were assessed under a dissecting microscope.

B. Cell Culture and Transfection

HT-29 cells and Caco2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) containing 5 mM glucose normal glucose (NG), or treated with 500 nM Insulin (HI) or 25 mM glucose (HG) for 72 hours in the presence or absence of AMPK activators (1.5 mM AICAR or 5 mM metformin), and/or 25 nM Rapamycin (mTORC1 inhibitor). Both AICAR and Metformin act similarly as AMPK upregulators. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. For the RNA interference experiments, a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas9) system for Nox4 was purchased from Santa Cruz Biotechnology, Inc. CRISPR (0.1 ug/uL) was introduced into the cells by a single transfection using Oligofectamine or Lipofectamine 2000, as previously described by (Eid et al., 2013). Control CRISPR (nontargeting CRISPR, 0.1 ug/uL) was used as a negative control.

C. mRNA analysis

mRNA was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method (Eid et al., 2009). Total RNA was isolated from colon tissues using TRIzol reagent (QIAzol Lysis Reagent, QIAGEN Sciences, Maryland, USA). mRNA expression was quantified using a Realplex mastercycler (Eppendorf, Westbury, NY) with SYBR green dye and mouse RT² qPCR Primers in the table below:

Primers	Sequences
Nox1	F: 5'-AAATGAGGATGCCTGCAACT- 3' R: 5'-GGGTCAAACAGAGGAGAGCTT- 3'
Nox4	F: 5'-ACCCTCCTGGCTGCATTAG- 3' R: 5'- AAAACCCTCGAGGCAAAGAT-3'

mTOR	F: 5'-AAGCCCGTGATGAGAAGAAG-3' R: 5'-GGGCTGTTCTCATTGCTCTC-3'
AMPK- α 1	F: 5'-GTGGAACCCTCCCTTTTGAT-3' R: 5'-ATCTTTTATTGCGGCCCTCT-3'
Tuberin	F: 5'-GACTGTTGAAGTGGTGGGTGT-3' R: 5'-TGTGTTCGATGCAGGAGGA-3'
OGG1	F: 5'-GTGCCCGCTATGTACGTG-3' R: 5'-TCTGGACCCTCACCTTGG-3'
GAPDH	F: 5'-GTGGACCTCATGGCCTACAT-3' R: 5'-TGTGAGGGAGATGCTCAGTG-3'

Table 1. List of Primers

D. Western Blot Analysis

Homogenates from colon tissues were prepared in 200 μ l of radioimmune precipitation assay buffer containing 20 mmol/l Tris·HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l Na₃VO₄, 1 mmol/l PMSF, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin, and 1% NP-40. Homogenates were incubated for two hours at 4°C and centrifuged at 13,000 rpm for 30 min at 4°C.

Cultured human HT-29 and Caco2 cells were grown to near confluence in 100 mm dishes and serum-deprived for 12 h. All incubations were carried out in serum-free DMEM containing 1% FBS at 37°C for 72h. The cells were then lysed in radioimmune precipitation buffer at 4°C for two hours. The cell lysates were then centrifuged at 13 000 rpm for 30 min at 4°C.

Proteins in the supernatants were measured using the Lowry Protein Assay. For immunoblotting, proteins (40-60 μ g) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with rabbit polyclonal anti-Nox4 (1:500, Santacruz), rabbit polyclonal anti-Nox1 (1:500, Santacruz), rabbit polyclonal anti-p-mTOR^{Ser2448}, anti-mTOR (1:1000, cell signaling), rabbit polyclonal anti p-p70S6K^{Thr389}, rabbit

polyclonal anti p70S6K (1:500, Cell Signaling), rabbit polyclonal anti-p-Tuberin^{Ser1387}, anti-Tuberin (1:500, Cell signaling), rabbit polyclonal anti-p-AMPK^{Thr172}, anti-AMPK (1:500, Abcam), rabbit polyclonal anti-OGG1 (1:500, R&D), and rabbit polyclonal anti-Laminin (1:1000, Abcam). The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:20000). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using National Institutes of Health Image software.

E. NADPH Oxidase Activity

NADPH oxidase activity was measured in HT-29 and Caco2 cells grown in serum-free medium or in colon tissue homogenates. Cultured cancer cells were washed once with cold PBS and scraped from the plate in the same solution followed by centrifugation at 800 rpm, 4°C, for 10 min. The cell pellets were resuspended in lysis buffer containing 20 mmol/l KH₂PO₄, pH 7.0, 1 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, and 0.5 µg/ml leupeptin. Proteins were extracted from colon tissues using cooled mortar and pestle by smashing the frozen tissue parts and suspending the remnants in the lysis buffer. To start the assay, 20 µg of homogenates were added to 50 mmol/l phosphate buffer, pH 7.0, containing 1 mmol/l EGTA, 150 mmol/l sucrose, 5 µmol/l lucigenin, and 100 µmol/l NADPH. Photon emission expressed as relative light units was measured every 30 s for 10 min in a luminometer. A buffer blank (<5% of the cell signal) was subtracted from each reading. Superoxide production was expressed as relative light units per milligrams of protein. Protein content was measured using a Bio-Rad protein assay reagent.

F. Detection of Intracellular ROS by DHE Staining

Dihydroethidium (DHE) or hydroethidine is a cell-permeable compound that, upon entering the cells, interacts with superoxide anion to form oxyethidium (Zhao et al., 2003), which in turn interacts with nucleic acids to emit a bright red color detectable by fluorescent microscopy. Thus, DHE stain allows the detection of intracellular reactive oxygen species (ROS).

HT-29 or Caco2 cells were seeded in 6-well plates (Corning, USA), each containing a coverslip, and serum starved for 12 hours. The cells were then treated according to the experimental design for 72 hours and a 5 μM DHE stain (Life Technologies, USA) was added and the cells were incubated in the dark at 37°C for 30 minutes.

Colon tissue sections (4-5 μm thick) were incubated with a 10 μM DHE stain for 1 hour at 37°C. After the incubation period, images were taken using the Laser Scanning Confocal Microscope (Zeiss, Germany) at a 20X magnification. The results were quantified using Zen software and graphs representing the intensity of DHE stains were drawn as a percentage of the control. At least 3 different images were taken for each condition.

G. *In vitro* Detection of Intracellular ROS by DCF

The peroxide-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein (DCF) diacetate (Molecular Probes) was used to measure intracellular ROS as previously described (Eid et al., 2009). Cells were grown in 12- well plates and serum-deprived for 12 h. Cells were then treated according to the experimental design for 72 hours. Immediately before the experiments, cells were washed with Hank's buffered salt solution containing Ca^{2+} and Mg^{2+} and then loaded with 20 $\mu\text{mol/L}$ DCF diacetate dissolved in Hank's buffered salt solution 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 fl).

H. Cellular Proliferation by MTT Assay

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 cellular proliferation, viability and metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. HT-29 and Caco2 cells were seeded in 96 well plates until 50-60% confluence. Serum deprived for 12 hours, cancerous cells were then treated according to the experimental design for 72 hours. After treatment, the MTT labeling reagent (0.5mg/ml) was added to each well, and the microplate was incubated for 3-4 hours at 37°C. During this incubation period, the NAD(P)H-dependent cellular oxidoreductase enzymes reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to the insoluble formazan crystals, which have a blue-purplish color. These salt crystals become soluble by adding the solubilizing solution and incubating the plate overnight. The solubilized formazan product was finally spectrophotometrically quantified using an ELISA reader (Multiskan Ex) at a wavelength of 590 nm. Proliferation graphs were plotted as a percentage of the control, i.e HT-29 or CaCo-2 cells cultured alone without treatment (normal glucose), adjusted to 100%. Each condition was counted in triplicate, and three independent experiments were performed but only one representative graph is shown.

I. Wound Healing Assay

The Wound Healing assay is designed to assess cellular migration. HT-29 or Caco2 cells were seeded in 60 mm dishes (Corning, USA). The cells were allowed to attach, spread, and

form a confluent monolayer after which they were serum deprived for 12 hours. Using a sterile tip, a scar was induced in the form of a straight line in the middle of the dish from one side to another allowing the removal of 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. HT-29 or 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) or a light microscope (Zeiss, Germany) before and after the 72-hour treatment. Results were analyzed using the Image J software by measuring the migration distance/wound area. Three independent experiments were performed but only one representative graph is shown.

J. Cell Invasion Assay

Microporous membrane inserts are widely used for cell migration and invasion assays. The most widely accepted of which is the Boyden Chamber assay. The Boyden Chamber system uses a hollow plastic chamber, sealed at one end with a porous membrane. This chamber is suspended over a larger well that may contain culture medium and/or chemo-attractants. We used the cell culture inserts (Corning, USA) suitable for a 24- well plate with a transparent PET membrane and 8.0 μm pore size appropriate for cancer cells as it is the case of HT-29 and Caco2 cells.

For assessment of cancer cell invasion, the inserts were placed in a 24-well plate (Corning, USA) and the inner chamber was coated with Matrigel (BD Biosciences, USA). The assay was then initiated by adding the cells, suspended in serum-free medium, to the upper chamber. To the lower chamber, 500 μL of complete media containing 10% FBS, acting as a chemo-attractant, were added. Treatments were added according to the experimental design and the cells were incubated at 37°C for 72 hours after making sure that no air bubbles were trapped

at the interphase. The cancer cells will invade the matrigel layer and migrate to the opposite side of the membrane. The cells were finally fixed using 4% Formaldehyde and stained with Hematoxylin and Eosin. The final step involved counting the stained cells under a light microscope. Graphs representing the number of invading cells were plotted as a percentage of the control, i.e. HT-29 or Caco2 cells cultured alone without treatment (normal glucose), adjusted to 100%. Three independent experiments were performed but only one representative graph is shown.

K. 8-Oxo-dG Concentration Assay

8-OH-dG is a well-known biomarker for the detection of oxidative DNA damage and oxidative stress. In our experiments we used the HT-8-oxo-dG ELISA Kit II (Gaithersburg, Maryland) that allows the quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA.

HT-29 and Caco2 cells were grown in 6-well tissue culture plates (Corning, USA) until 50-60% confluence followed by serum starvation overnight and then treatment for 72 hours according to the experimental conditions. The next step involved extracting DNA from the cells using the high salt DNA extraction method. After treatment, the cell pellet was collected and 600 μ L of TNES buffer (5% of 1 M Tris-HCl pH=8, 20% of 0.5 M EDTA pH=8, 8% of 5 M NaCl, 5% of 10% SDS, 62% double distilled water) were added in addition to 350 μ g of Proteinase K and then left to digest overnight at 55°C with gentle shaking. Then 5 M NaCl was added to each sample and the supernatant was collected after a 15-minute centrifuge at 13000 rpm. Thread like DNA was finally visible after the addition of 100% EtOH and a final centrifuge allowed the collection of the DNA pellet which was re-suspended in nuclease-free water with gentle shaking overnight at 37°C. Subsequently, we initiated the assay procedure that involved preparing an 8-OHdG standard curve for calculation of the 8-oxodG concentration in the samples used. An 8-OHdG monoclonal antibody was added to bind competitively to 8-oxo-dG immobilized on pre-

coated wells and in a sample solution. Detection of the retained antibody was performed using an HRP conjugate and TACS-Sapphire™ colorimetric substrate. Finally, adding a 0.2 M HCl solution stopped the reaction and the absorbance was read directly at 450nm.

L. Statistical analysis

For the *in vitro* work, results are expressed as percentages of the control \pm standard errors (SE). Statistical significance was assessed by one-way ANOVA (ANOVA), followed by Tukey's post test when more than two variables were analyzed. Two group comparisons were performed by Student's t-test. Statistical significance was determined as a probability (P value) of less than 0.05. All statistical analysis was performed with Prism 6 software (GraphPad Software).

For the *in vivo* work, statistical analyses included an inferential step using Bayesian techniques. For the estimates, we used Markov chains and Monte Carlo (McMC) integrations, and chose prior distributions to be nearly conjugated situations. Unless the diagnoses for convergence gave clues to the contrary, we used 3 Markov chains with separated starting points, a burn-in of 10,000 for each chain and 50,000 more iterations with a thinning of 12 for building a total sample of 10,000 iterations on which the Monte Carlo integrations are used to retrieve characteristics of posterior distributions (mean, 95% posterior credible interval). The analyses were carried on using the R software (with *ad hoc* packages, e.g. R2jags).

For modelling continuous variables (for example measurements of [8-oxodG]), we considered that the values of observation i , Y_i , are normally distributed with a mean μ_i and a precision τ (inverse of variance) common to all observations. Then μ_i (linear predictor) was additively structured according to the following equation:

$$a_1 + a_2I(diabetic_i = 1) + a_3I(metformin_i = 1) + a_4I(rapamycin_i = 1),$$

where:

- $I(X=1)$ stands for a dummy variable of which the value is 1 if $X=1$ and 0 otherwise
- $diabetic_i$, $metformin_i$, and $rapamycin_i$ are covariates for the group belonging to observation i (resp. non treated diabetic, metformin treated and rapamycin treated). Thus, a_2 , a_3 , and a_4 are the parameters indicating the difference in Y means between groups;
- a_1 is a parameter standing for the grand mean of the model (mean value of Y for controls)
- we assume a vague normal prior with a 0 mean and a variance of 1,000 on all the 4 parameters to be estimated.

For dealing with inflated variances and important differences in Y measurements (ratio of 1/10) we centred and reduced all the values of Y , by subtracting its mean and dividing by its standard deviation. In the previous model, several indexes can be monitored:

1. Differences between groups of Y mean: by monitoring a_2 (differences between diabetic and control groups), a_3 (differences between metformin treated and control groups) and a_4 (differences between rapamycin treated and control groups)
2. Differences between treated groups and non treated diabetic groups: for example $a_3 - a_2$ (differences between metformin and non treated diabetic groups)
3. Ratios: for example: a_1 is the Y mean for control and $a_1 + a_2$ the Y mean for the diabetic group so the monitoring of $\frac{a_1+a_2}{a_1}$ is the ratio between the Y mean for the diabetic group and the Y mean for the control group
4. Probabilities: the number of times across iterations that for example a_4 is positive gives a probability; the probability that the mean of Y in the rapamycin group is higher than the mean of Y in the control group.

When the Y to be modelled cannot be assumed normally distributed (for example PCR reported as percentage with respect to control or Western Blot as ratio), we had to change the likelihood of the data from normal distribution to Gamma distribution. This change had two main consequences:

1. Gamma distribution is parameterized with two quantities α and β of which the direct interpretation is meaningless. Thus we re-parameterized this distribution using its mean μ and its precision φ using $\begin{cases} \alpha = \mu^2 \varphi \\ \beta = \mu \varphi \end{cases}$. In this case the linear predictor is on a log-scale (link for Gamma distribution in generalized linear models) and hence, comparing with the previous normal situation, this is $\log(\mu)$ which was additively structured with the same parameters as before.
2. since the linear predictor is on the log-scale, for retrieving posterior distribution of effects, we had to back-transform the parameters and monitor for example $\exp(a_1)$, Y mean for controls.

RESULTS

A. Effect of HG and/or HI treatment on normal and adenocarcinoma colon cell lines

Hyperglycemia and/or hyperinsulinemia in diabetes elicit cellular responses that contribute to diabetic complications. Previous findings from our group have shown that diabetes induces oxidative stress through a NADPH-dependent superoxide generation mechanism (Eid et al., 2010, Eid et al., 2013, Eid et al., 2016). For that reason, we were interested to study the effect of high glucose (HG, 25 mM), high insulin (HI, 200 μ M insulin) or their combination (as would be encountered in type 2 DM) on intracellular ROS generation using the peroxide-sensitive fluorophore DCF.

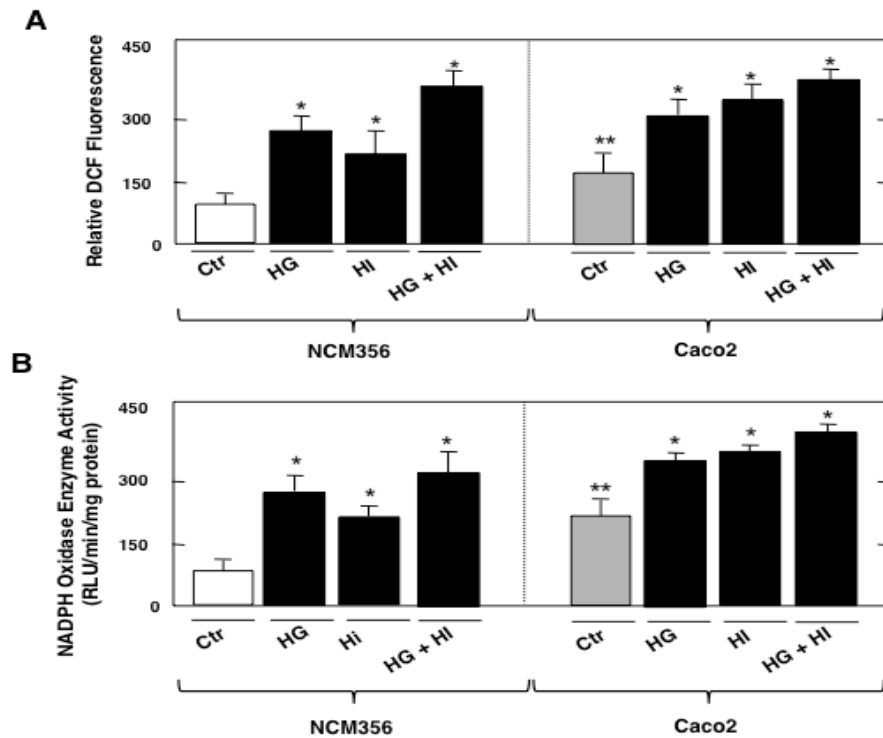


Figure 8. HG, HI, or their combination induces ROS production and NADPH oxidase activity in both normal and adenocarcinoma colon cells. (A) Intracellular ROS measured using the peroxide-sensitive fluorophore DCF in human cultured colon epithelial cells (NCM 356) and human epithelial colon adenocarcinoma cells (Caco2) treated with HG (25mM), HI (500 μ M), or their combination for 72 hours. (B) NADPH oxidase activity assay in NCM356

and Caco2 cells measured using Lucigenin assay. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the normal or cancer control cells. **, $p < 0.05$ versus the normal control cells.

Furthermore, we evaluated the involvement of the NADPH oxidases family of enzymes in this process. For that purpose, a human cultured colon epithelial cell line, NCM 356, and human epithelial colon adenocarcinoma cells (Caco2) were used and treated as described above.

Our results show that HG, HI or their combination increase superoxide production (**Fig. 8A**) and this increase is accompanied by an increase in NADPH oxidase activity (**Fig. 8B**). These results were observed in both normal and carcinogenic colon cell lines. Interestingly, these findings indicate that ROS production, through activation of the NADPH oxidases, may play an important role in colon cancer and that HG, HI or their combination increase ROS production with a higher incremental susceptibility in cancerous cells.

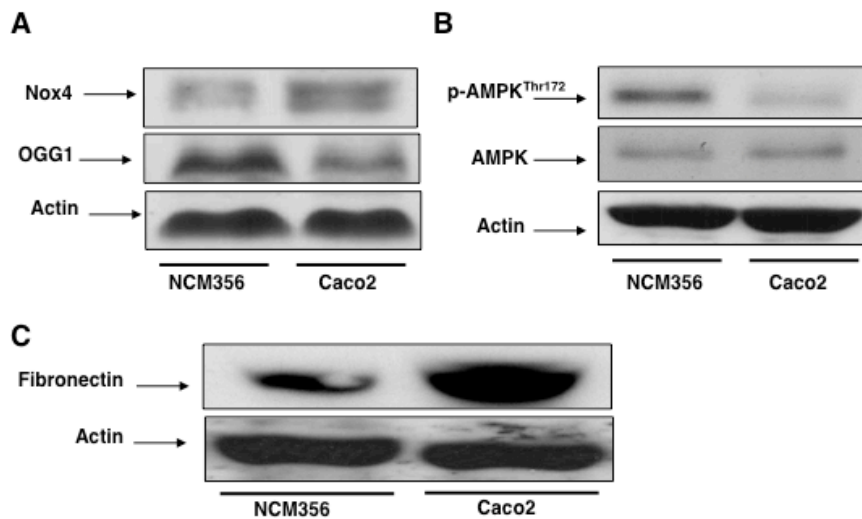


Figure 9. Colon Cancer induces NADPH oxidase Nox4 and fibronectin expressions and downregulates AMPK and OGG1 protein levels. Immunoblots of protein extracts from human cultured epithelial colon cells (NCM 356) and human epithelial colon adenocarcinoma cells (Caco2) incubated until confluence with low glucose (5mM). (A) Representative Western blots of Nox4, OGG1, and β -actin levels. (B) Representative Western blots of phospho-Thr172 AMPK, AMPK, and β -actin levels. (C) Representative Western blots of fibronectin and β -actin levels.

Additionally, in cultured epithelial colon adenocarcinoma cells (Caco2), our data show that NADPH-dependent ROS production is associated with an increase in Nox4 protein expression and a decrease in OGG1 protein levels (Fig. 9A). We also observe a decrease in AMPK phosphorylation on its activating site, Thr-172, and an increase in fibronectin expression (Fig. 9C). These results suggest that cancer induced Nox4-dependent ROS production can lead to an accumulation of extra cellular matrix proteins as well as an inactivation of the AMPK pathway that, by our speculation, could be altering directly or indirectly the mTOR signaling pathway in carcinogenic colon cells.

B. Effect of diabetes-induced Nox-dependent ROS production in colon cancer cells

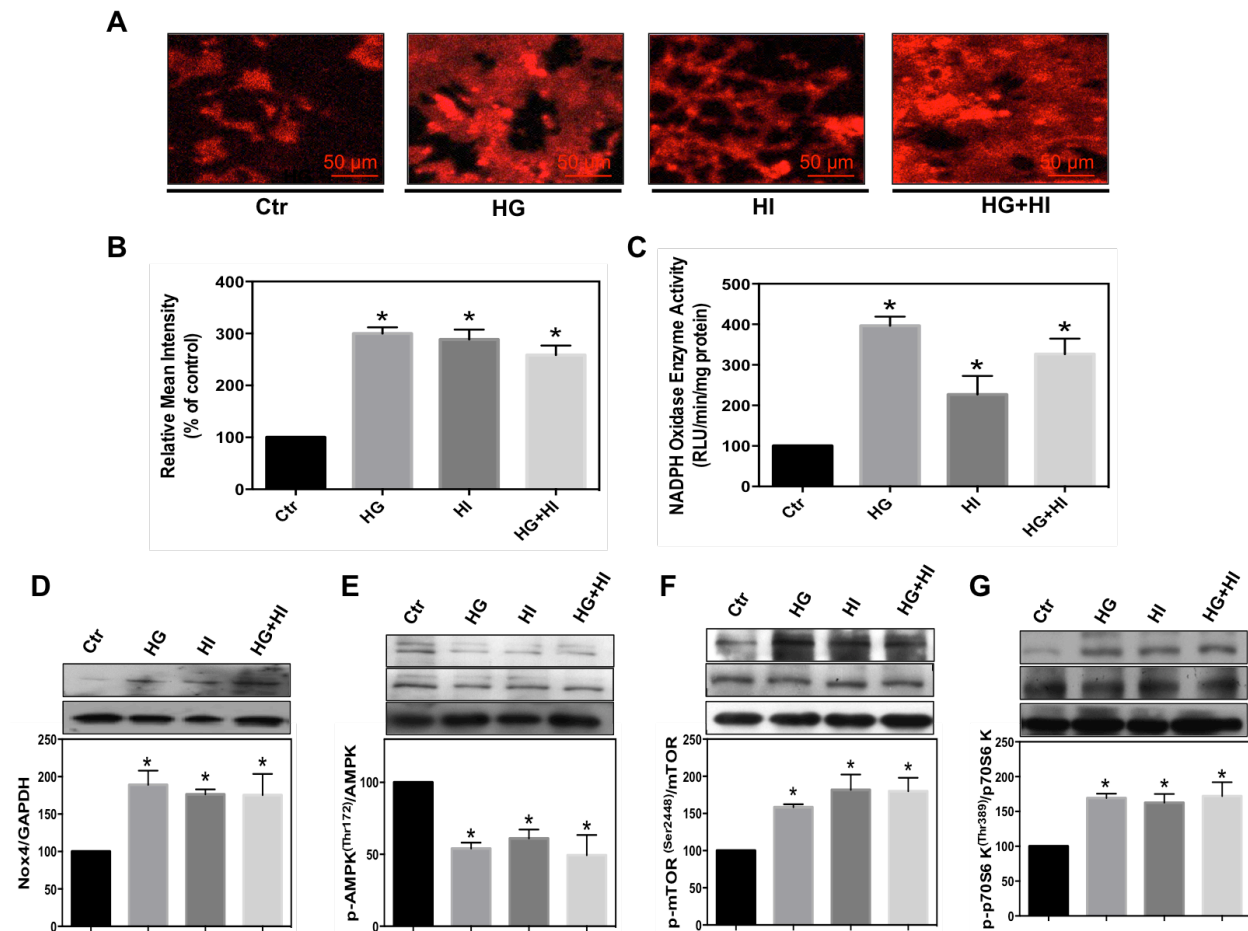


Figure 10a. Effect of HG on cancer cells is through NADPH-dependent ROS production and the AMPK/mTOR pathway is involved. HT-29 cells were treated with HG (25 mM), HI (500 nM), or a combination

of both for 72 hours. (A) Representative figures of the DHE staining, HG, HI or their combination induces ROS production. (B) Histogram showing the DHE staining of cancer cells, extracellular ROS was measured after an incubation with DHE (5 μ M) for 30 minutes at 37°C. (C) NADPH oxidase activity assay in HT-29 cells was measured using the Lucigenin assay. (D), (E), (F), and (G) Representative immuno blots and histograms of Nox4, phospho-Th172 AMPK, AMPK, phospho-Ser2448 mTOR, mTOR, phospho-Thr389 p70S6 Kinase, p70S6 Kinase and GAPDH resulting from cancer cell lysates. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control.

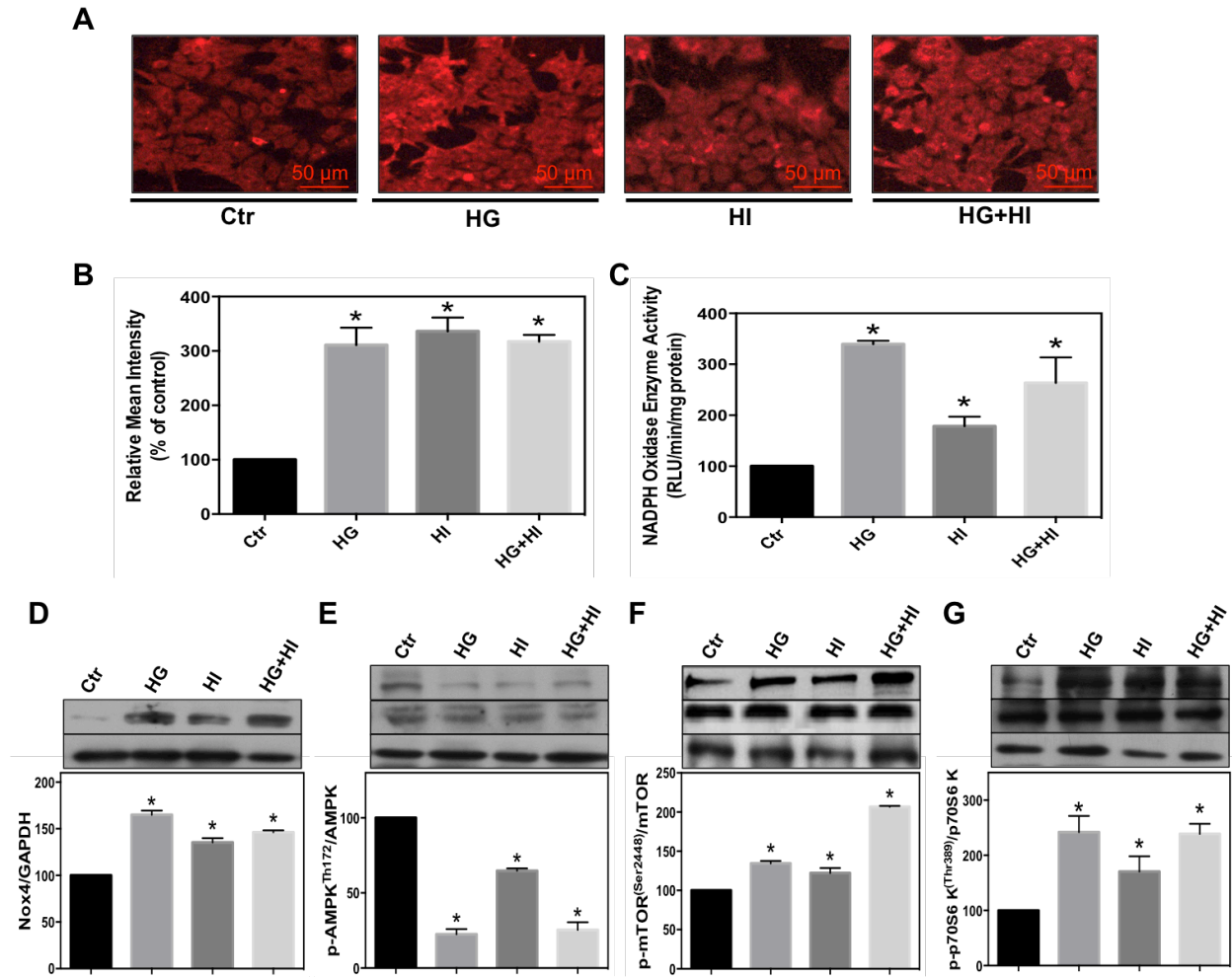


Figure 10b. Effect of HG on cancer cells is through NADPH-dependent ROS production and the AMPK/mTOR pathway is involved. Caco2 cells were treated with HG (25 mM), HI (500 nM), or a combination of both for 72 hours. (A) Representative figures of the DHE staining, HG, HI or their combination induces ROS production. (B) Histogram showing the DHE staining of cancer cells, extracellular ROS was measured after an incubation with DHE (5 μ M) for 30 minutes at 37°C. (C) NADPH oxidase activity assay in Caco2 cells was measured using the Lucigenin assay. (D), (E), (F), and (G) Representative immuno blots and histograms of Nox4, phospho-Th172 AMPK, AMPK, phospho-Ser2448 mTOR, mTOR, phospho-Thr389 p70S6 Kinase, p70S6 Kinase and GAPDH resulting from cancer cell lysates. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control.

Hyperglycemia and/or hyperinsulinemia in diabetes elicit cellular responses that contribute to diabetic complications through the induction of NADPH-induced-oxidative stress in many organs. For that reason we studied the effect of high glucose (HG, 25 mM), high insulin (HI, 200 μ M insulin) or their combination on extracellular ROS generation using Dihydroethidium staining in two different types of human epithelial colon adenocarcinoma cells; the HT-29 and Caco2 cell lines. Our results show that HG, HI, or their combination increase superoxide production in both HT-29 (**Fig. 10a-A&B**) and Caco2 cell lines (**Fig. 10b-A&B**). This was paralleled by an increase in NADPH-oxidase activity (**Fig. 10C**) and an upregulation of Nox4 protein expression (**Fig. 10D**).

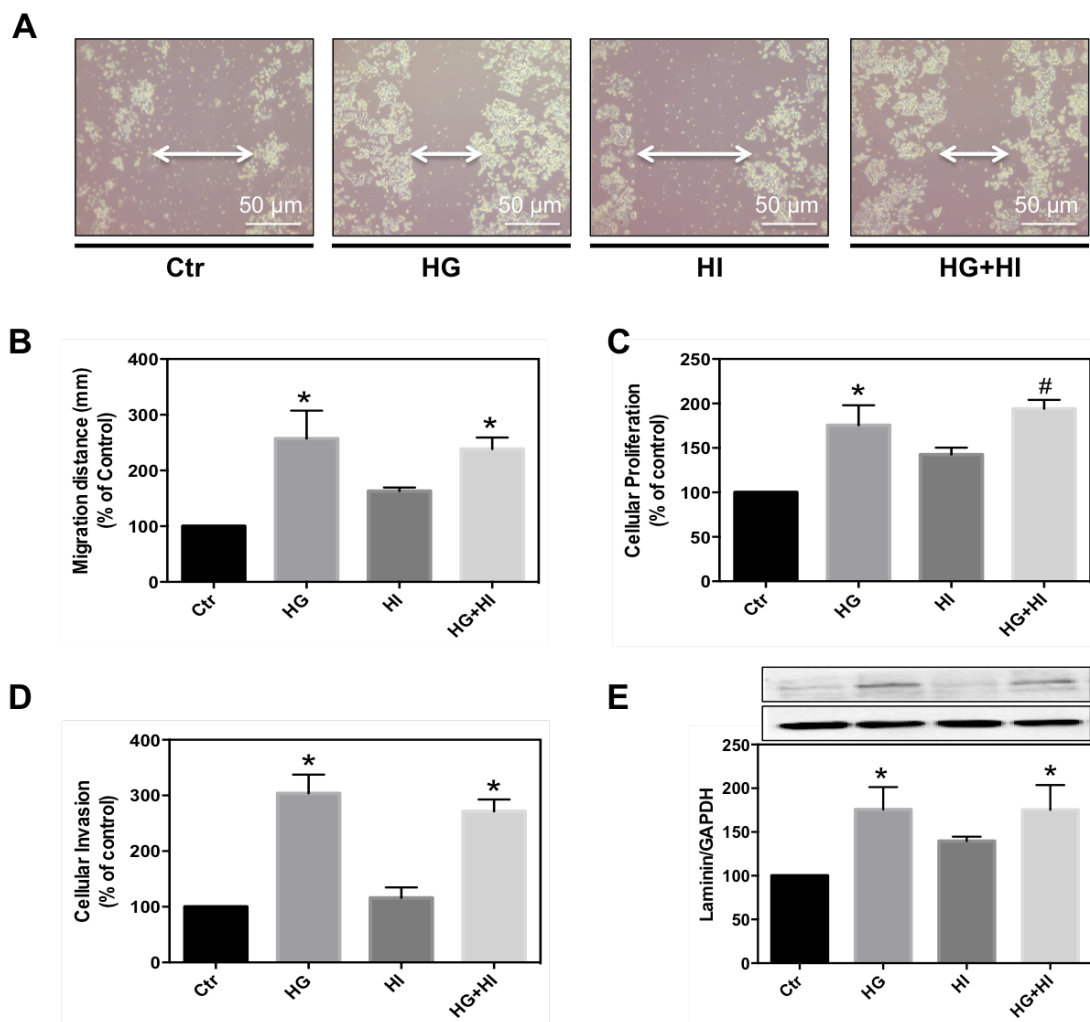


Figure 11a. Effect of HG, HI or their combination on cancer cells. HT-29 cells were treated with HG (25 mM), HI (500 nM), or a combination of both for 72 hours. (A) Representative figures of the migration assay performed by allowing the cells to grow till confluence and inducing a scratch of which the distance is measured before and after treatment. (B) Histogram of the migration assay. (C) MTT proliferation assay. (D) Cellular invasion assay performed using 8 μ m cell culture inserts and the cells invading the porous membrane were stained by H&E staining and counted under a light microscope. (E) Representative immuno Blot of Laminin and GAPDH resulting from cancer cell lysates. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control.

More importantly, our findings revealed a major role for AMPK inactivation in HG and/or HI-induced cellular injury (**Fig. 10E**). In fact, upon HG and/or HI treatment, mTOR along with its downstream effector p70S6 Kinase were activated as observed by the increase in the protein expressions of their activating phosphorylation sites (**Fig. 10F&G**). Our results were confirmed in both HT-29 (**Fig. 10a**) and Caco2 (**Fig. 10b**) cell lines. Insulin, however, had a less pronounced effect in the Caco2 cells than the HT-29 cells. These findings suggest a pivotal role for the NADPH oxidase enzyme, Nox4, in the HG and/or HI induced ROS production in colon cancer cells and highlight the important involvement of the AMPK/mTOR pathway in our system.

In order to correlate our findings with the diabetic state and assess cancer cell behavior in such environment, we investigated the influence of a diabetic milieu on cancer cell migration, proliferation, invasion, as well as extra cellular matrix accumulation. Our data show that in the presence of HG for 72 hours, both HT-29 and Caco2 cells migrated faster to fill up the gap created by the induced scratch (**Fig. 11A&B**). Additionally, under the same treatment, colon cancer cells exhibited an increased proliferation rate (**Fig. 11C**) and potential to invade the reconstituted basement membrane known as Matrigel Matrix (**Fig. 11D**).

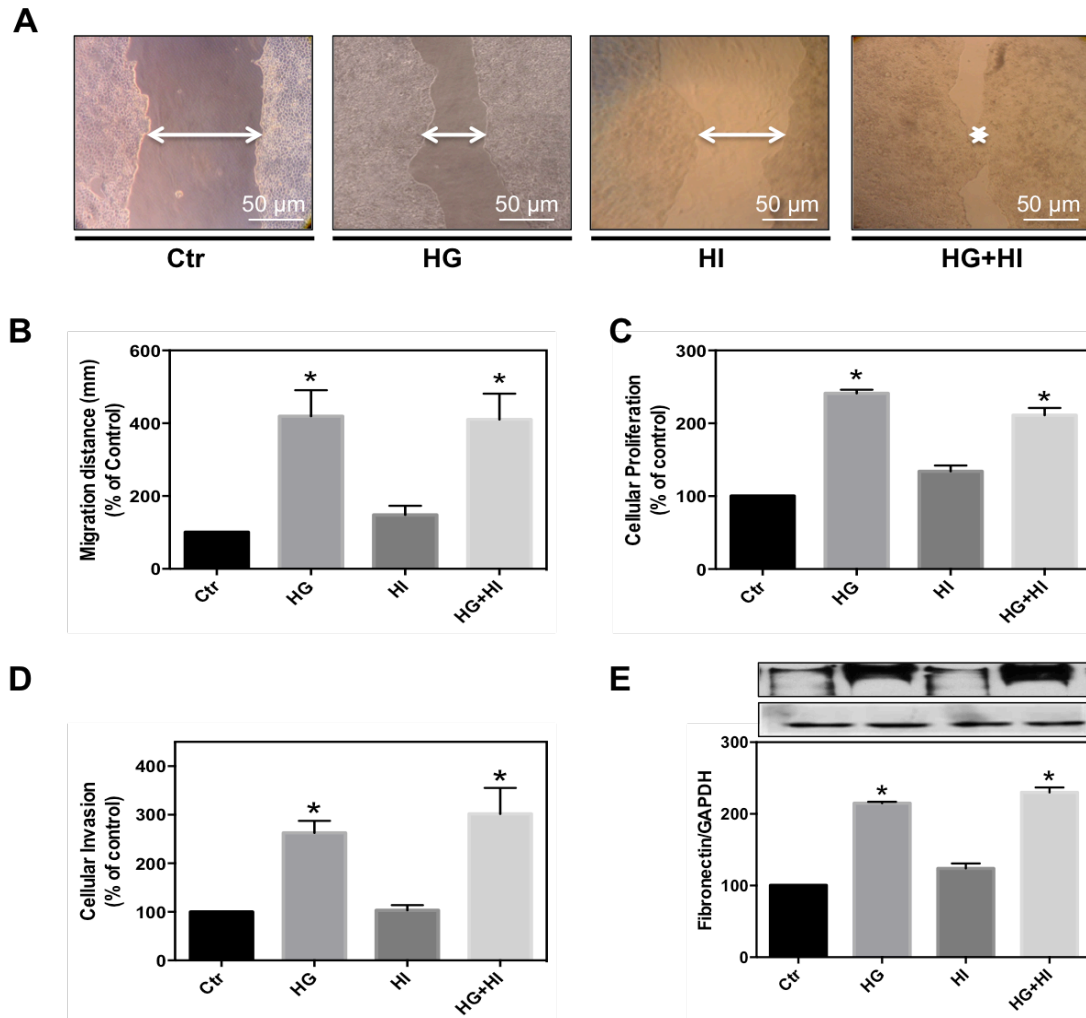


Figure 11b. Effect of HG, HI or their combination on cancer cells. Caco2 cells were treated with HG (25 mM), HI (500 nM), or a combination of both for 72 hours. (A) Representative figures of the migration assay performed by allowing the cells to grow till confluence and inducing a scratch of which the distance is measured before and after treatment. (B) Histogram of the migration assay. (C) MTT proliferation assay. (D) Cellular invasion assay performed using 8 μ m cell culture inserts and the cells invading the porous membrane were stained by H&E staining and counted under a light microscope. (E) Representative immuno Blot of fibronectin and GAPDH resulting from cancer cell lysates. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control.

Laminin and fibronectin, two main constituents of the extra cellular matrix of cells, displayed a rise in their protein expressions upon high glucose treatment (**Fig. 11E**). However, HI treatment did not generate the same effect as HG. These results show that HG can alter the behavior of cancer cells rendering them more aggressive and can cause the accumulation of

extracellular matrix proteins such as Laminin (**Fig. 11a-E**) and Fibronectin (**Fig. 11b-E**). Taken together, our results highlight a more pronounced effect of HG on colon cells' phenotypic alteration when compared to HI treatment. Therefore, in the rest of the experiments, our work focused on the signaling mechanism altered by HG and its effect on CRC cell injury.

C. AMPK activation and/or mTOR inhibition reverse HG effect in colon cancer cells.

To further confirm the role of the AMPK/mTOR pathway in the HG-induced ROS production, HT-29 and Caco2 cells were treated with a pharmacological AMPK activator, or an mTOR inhibitor, or their combination for 72 hours. In both HT-29 and Caco2 cells, the activation of AMPK reduces HG-induced ROS production (**Fig. 12A&B**) as well as NADPH oxidase enzyme activity (**Fig. 12C**).

Our results also show that the inhibition of mTOR had no effect on AMPK phosphorylation/activity (**Fig. 12D**) confirming the fact that mTOR acts downstream of AMPK. As expected, AMPK upregulation reduced HG-induced mTOR/p70S6K phosphorylation/activity also ensuring the upstream signaling role of AMPK over the mTOR/p70S6K pathway (**Fig. 12E&F**).

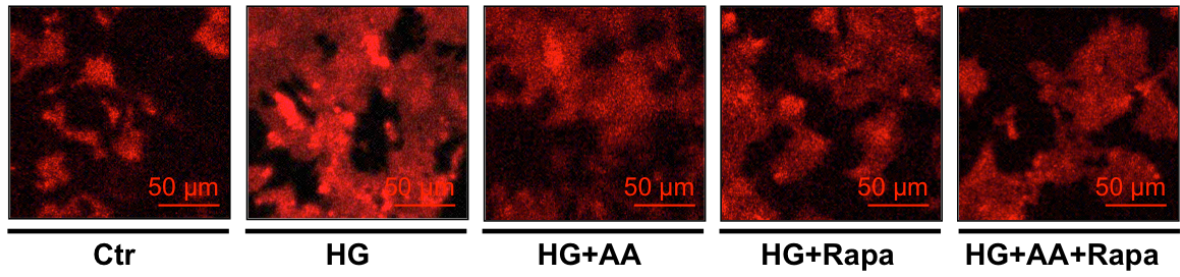
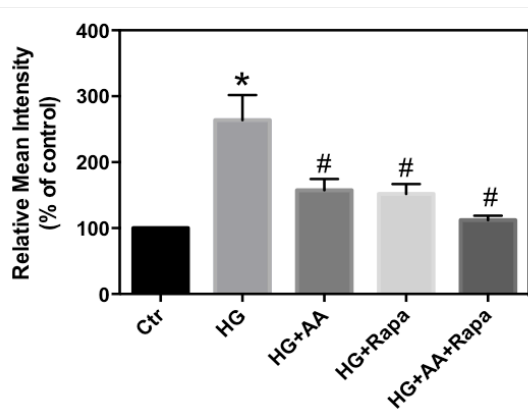
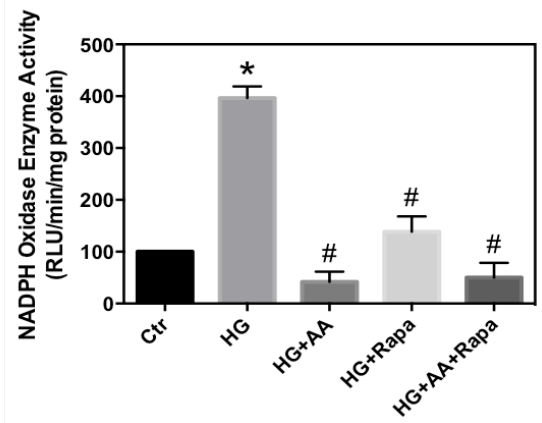
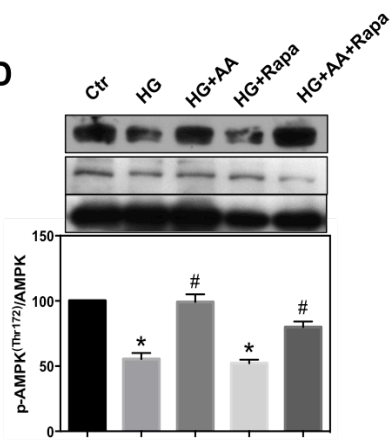
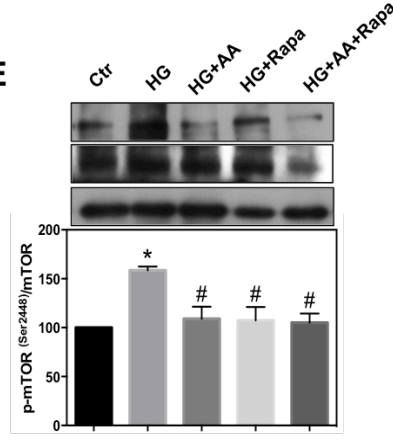
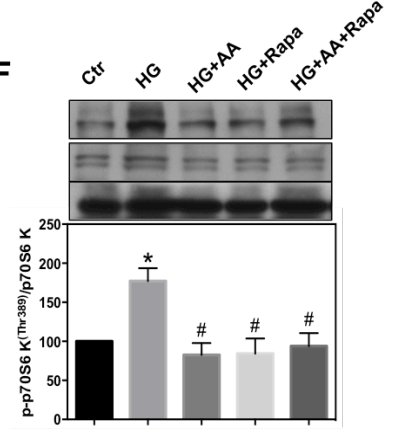
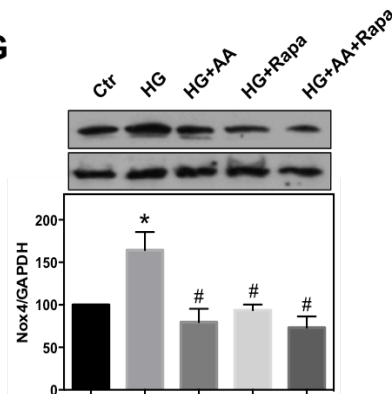
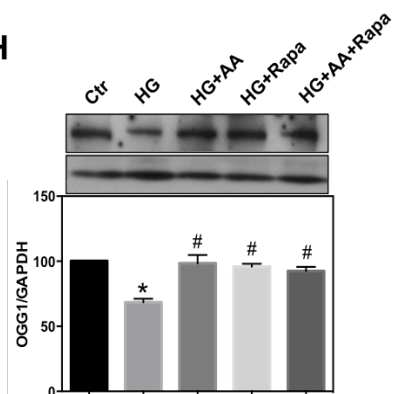
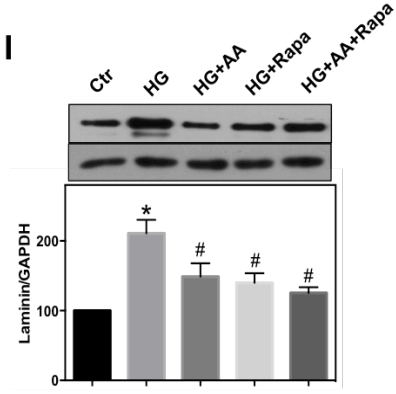
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Figure 12a. Activation of AMPK and/or inhibition of mTOR reverse HG effect in cancer cells. HT-29 cells were treated with HG (25 mM) alone or with AMPK activators (AA)-Metformin (5 mM) or AICAR (1.5 mM), rapamycin (25nM), or a combination of both for 72 hours. Both AICAR and Metformin act similarly as AMPK upregulators. (A) Representative figures of DHE staining of cancer cells along with the histogram (B) showing a decrease in ROS production after treatment. (C) Histogram of NADPH oxidase activity of cancer cells. (D)-(I) Histograms and representative immuno blots resulting from cancer cell lysates of phospho-Th172 AMPK, AMPK, phospho-Ser2448 mTOR, mTOR, phosphoThr389-p70S6 Kinase, p70S6 Kinase, Nox4, OGG1, Laminin, as well as GAPDH protein expression. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

In parallel, our results demonstrate that AMPK activation and/or mTORC1 inhibition attenuate Nox4 protein expression in cancer cells (**Fig. 12G**), thus suggesting a cross-talk between the AMPK/mTORC1 pathway and the NADPH oxidase (Nox4) responsible for the ROS over-production. We also show that HG treatment led to a decrease in OGG1 levels (**Fig. 12H**), keeping in mind that OGG1 is the enzyme responsible for recognizing and excising 8-oxodG.

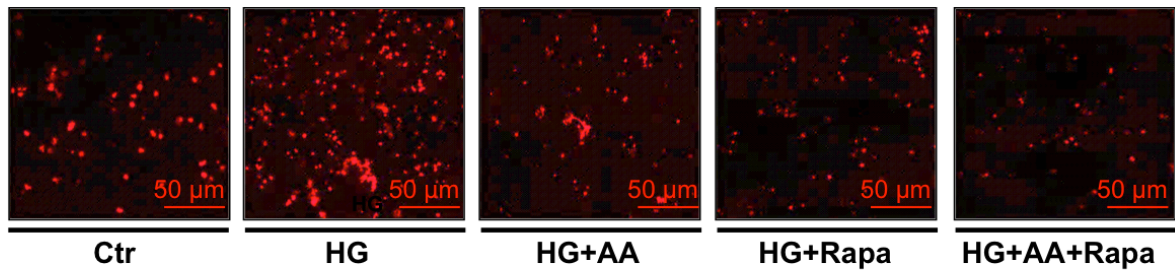
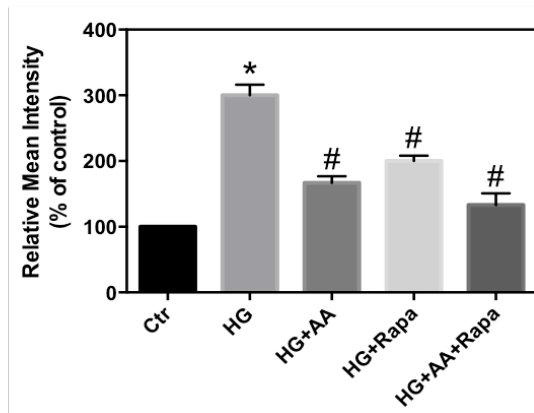
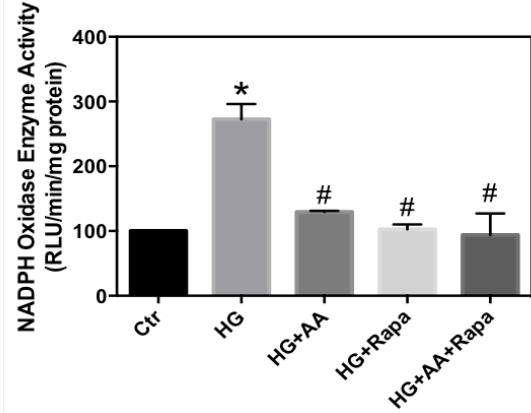
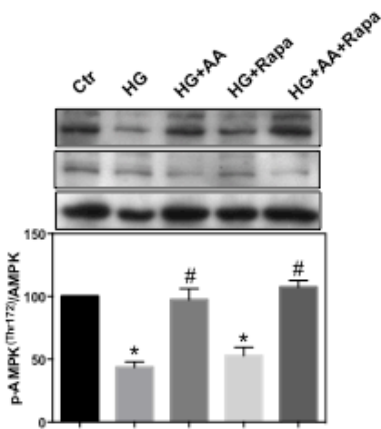
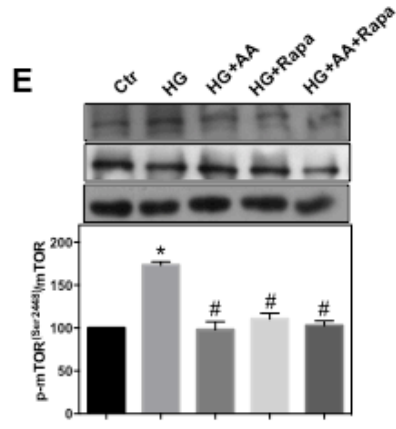
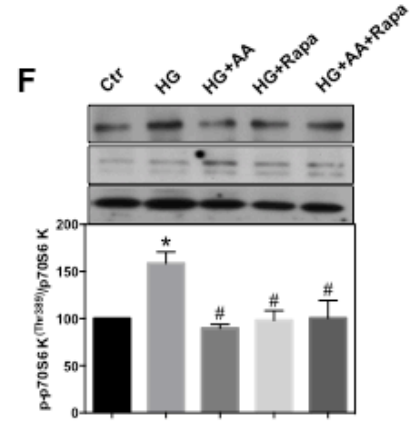
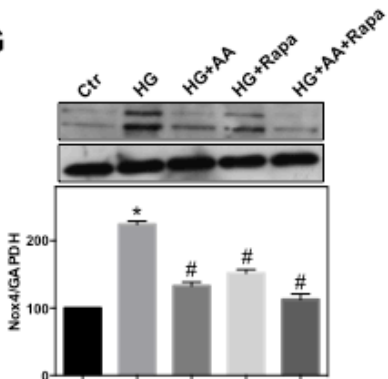
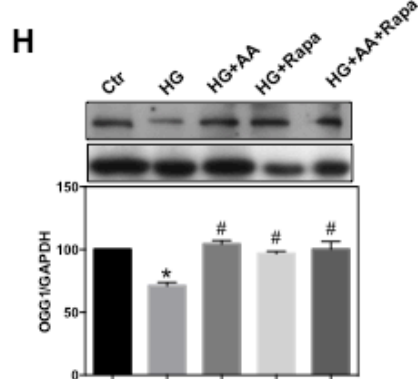
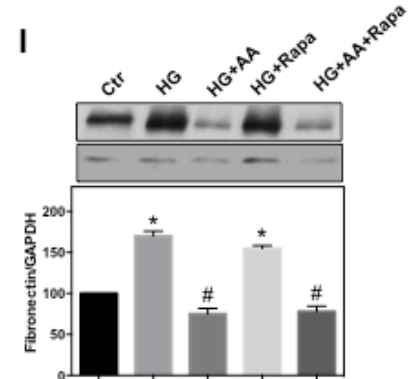
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Figure 12b. Activation of AMPK and/or inhibition of mTOR reverse HG effect in cancer cells. Caco2 cells were treated with HG (25 mM) alone or with AMPK activators (AA)-Metformin (5 mM) or AICAR (1.5 mM), rapamycin (25nM), or a combination of both for 72 hours. Both AICAR and Metformin act similarly as AMPK upregulators. (A) Representative figures of DHE staining of cancer cells along with the histogram (B) showing a decrease in ROS production after treatment. (C) Histogram of NADPH oxidase activity of cancer cells. (D)-(I) Histograms and representative immuno blots resulting from cancer cell lysates of phospho-Th172 AMPK, AMPK, phospho-Ser2448 mTOR, mTOR, phosphoThr389-p70S6 Kinase, p70S6 Kinase, Nox4, OGG1, Fibronectin, as well as GAPDH protein expression. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

In the same spirit, our *in vitro* pharmacological treatments attenuated Laminin (**Fig. 12a-I**) as well as Fibronectin (**Fig. 12b-I**) protein expressions reversing the effect of HG. These findings suggest that AMPK activation and mTOR inhibition may attenuate the effect of high glucose in increasing the susceptibility for colorectal cancer progression by reducing extra cellular matrix accumulation (Laminin and fibronectin expression), down-regulating Nox4-derived ROS production and restoring OGG1 expression *in vitro*.

D. Activation of AMPK and/or inhibition of mTOR reduce cancer cell injury and 8-oxodG productions.

To further dissect the mechanism by which HG/diabetes induces cancer development and progression, we assessed the role of the AMPK/mTOR signaling pathway on cellular proliferation, migration, and invasion in cultured epithelial colon adenocarcinoma cells (HT-29 and Caco2).

Our results show that treatment with AMPK activators, rapamycin, or their combination significantly reduced HG- induced cancer cell migration (**Fig. 13A&B**), proliferation (**Fig. 13C**), and invasion (**Fig. 13D**). Our findings also show that these treatments were able to attenuate 8-oxodG concentrations in the DNA of colon adenocarcinoma cells (**Fig. 13E**). Taken together, these data suggest that AMPK activation and/or mTOR inhibition may attenuate colon cancer

progression in colon adenocarcinoma cells. In fact, a suspected cross-talk between Nox4 and AMPK/mTORC1 pathways could be alleviating the oxidative damage inflicting colon cancer cells when bathed in a diabetic milieu. This pathway must also embrace OGG1 whose efforts are indispensable for alleviating ROS-induced DNA mutagenesis.

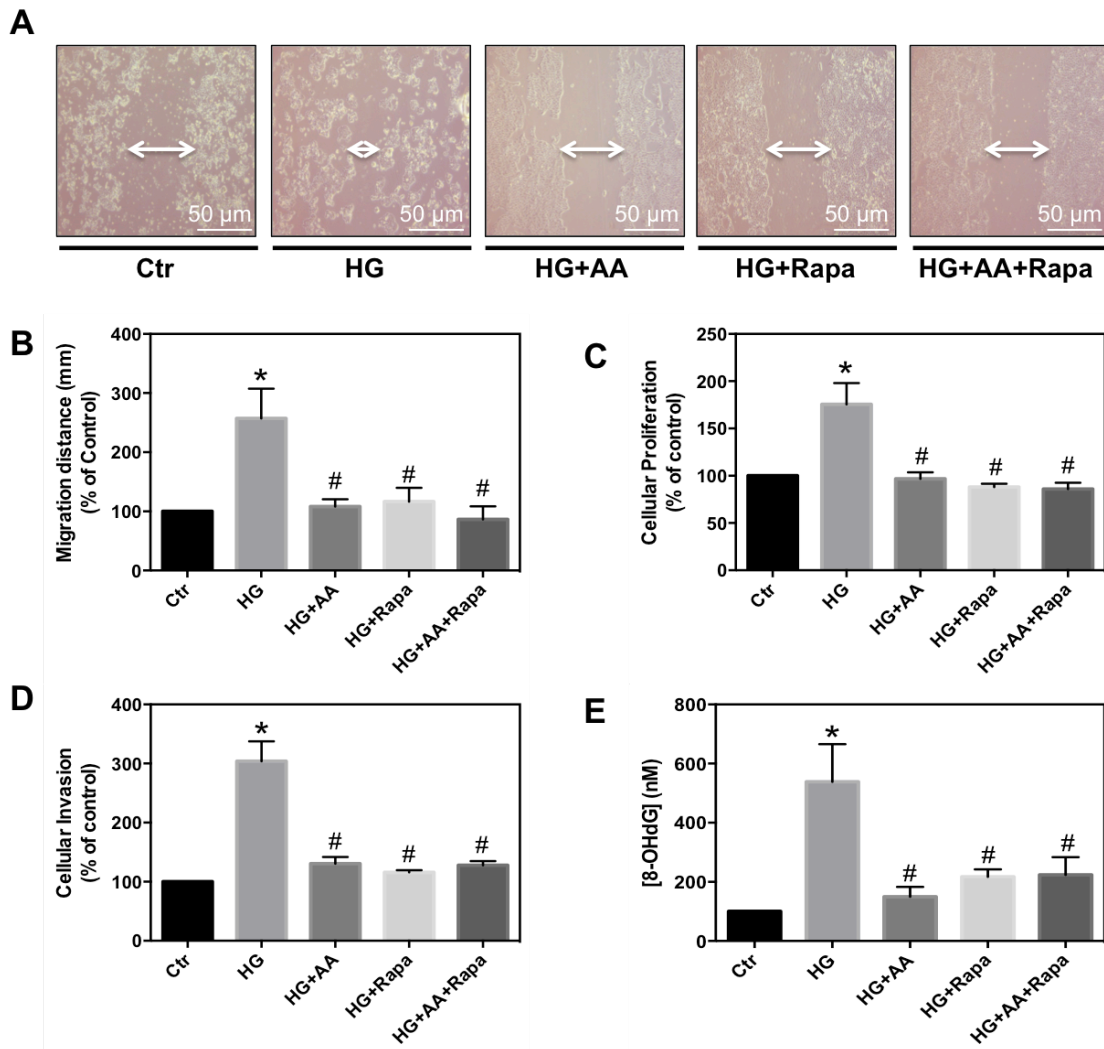


Figure 13a. Activation of AMPK and/or inhibition of mTOR reduce cancer cell injury and 8-oxodG production. HT-29 cells were treated with HG (25 mM) alone or with AMPK activators (AA)-Metformin (5 mM) or AICAR (1.5 mM), rapamycin (25nM), or a combination of both for 72 hours. Both AICAR and Metformin act similarly as AMPK upregulators. (A) Representative figures of the migration assay (scratch assay) in cancer cells along with the histogram (B) showing migration distance of cancer cells as percent of the control. (C) MTT assay assessing cancer cell proliferation. (D) Cellular invasion assay showing a decrease in cancer cell invasion upon metformin and/or rapamycin treatment. (E) Histogram showing the concentration of 8-oxodG adducts in cancer cells; DNA was extracted from the treated cells using the high salt method and calculation of the 8-oxodG

concentrations was done in reference to an 8-OHdG standard curve. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

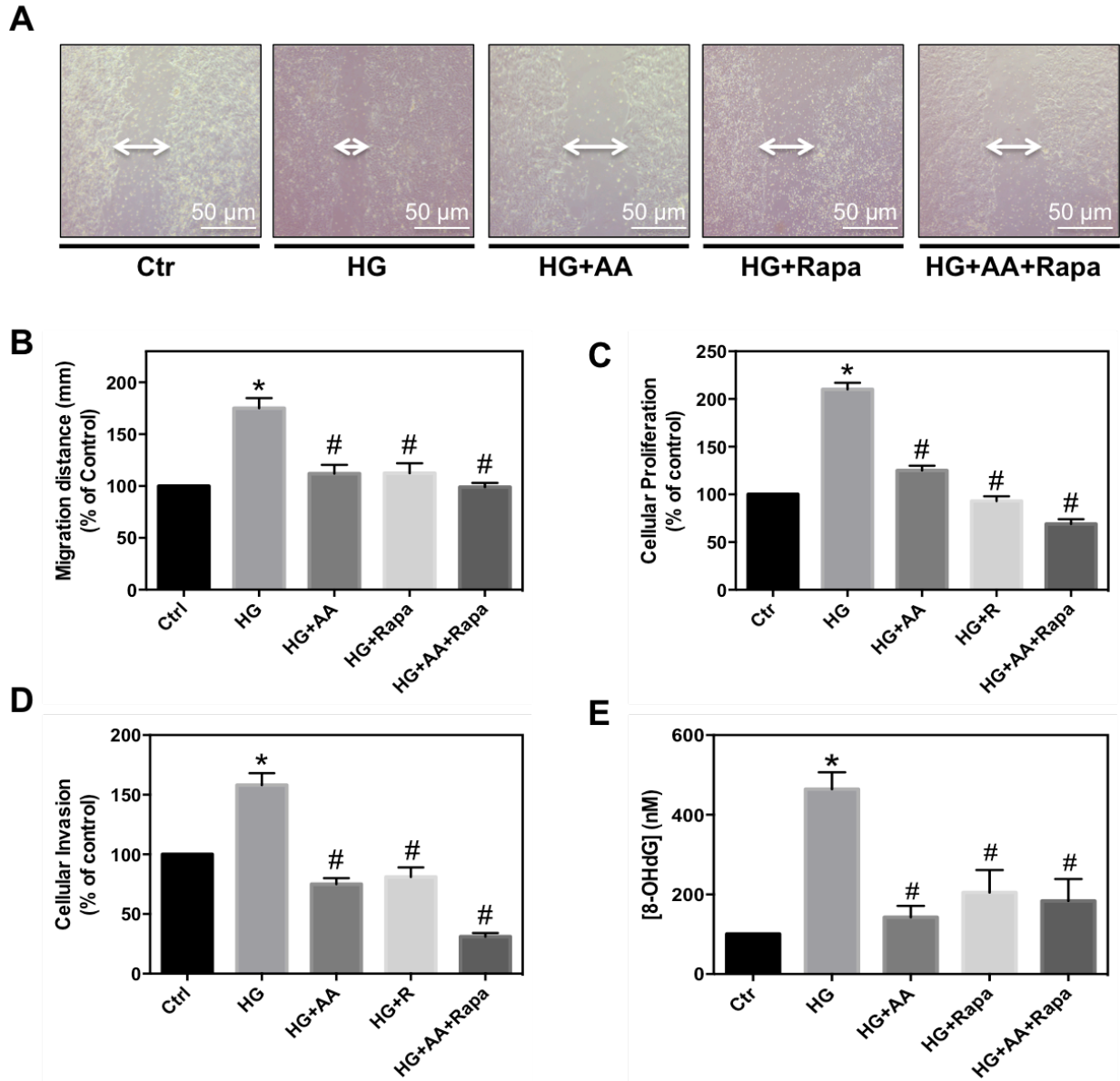


Figure 13b. Activation of AMPK and/or inhibition of mTOR reduce cancer cell injury and 8-oxodG production. Caco2 cells were treated with HG (25 mM) alone or with AMPK activators (AA)-Metformin (5 mM) or AICAR (1.5 mM), rapamycin (25nM), or a combination of both for 72 hours. Both AICAR and Metformin act similarly as AMPK upregulators. (A) Representative figures of the migration assay (scratch assay) in cancer cells along with the histogram (B) showing migration distance of cancer cells as percent of the control. (C) MTT assay assessing cancer cell proliferation. (D) Cellular invasion assay showing a decrease in cancer cell invasion upon metformin and/or rapamycin treatment. (E) Histogram showing the concentration of 8-oxodG adducts in cancer cells; DNA was extracted from the treated cells using the high salt method and calculation of the 8-oxodG concentrations was done in reference to an 8-OHdG standard curve. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

E. Nox4 mediates HG-induced ROS production and cancer cell injury.

To further investigate the role of the NADPH oxidase (Nox4) in promoting cancer cell injury, we transfected the HT-29 and Caco2 cells with Nox4 CRISPR/Cas9 KO Plasmid to knockdown Nox4 gene expression. Control CRISPR/Cas9 Plasmid was also used as a negative control to evaluate the specificity of CRISPR/Cas9 KO Plasmid system.

Our results show no significant change in Nox4 expression when the cancer cells were transfected with the control CRISPR. However, transfection with Nox4 CRISPR attenuated the relative mRNA levels of Nox4 induced by HG treatment (**Fig. 14G**) validating the usage of our genetic inhibitors. Cancer cells transfected with the Nox4 CRISPR exhibited a reduction in HG-induced extracellular ROS production (**Fig. 14A&B**) in parallel with a decrease in NADPH oxidase activity (**Fig. 14C**). Knockdown of Nox4 was also successful in attenuating the super migratory (**Fig. 14D&E**) and invasion (**Fig. 14F**) capabilities of the colon adenocarcinoma cells upon HG treatment. These findings further stress the role of Nox4 in mediating HG-induced ROS production and cancer cell injury.

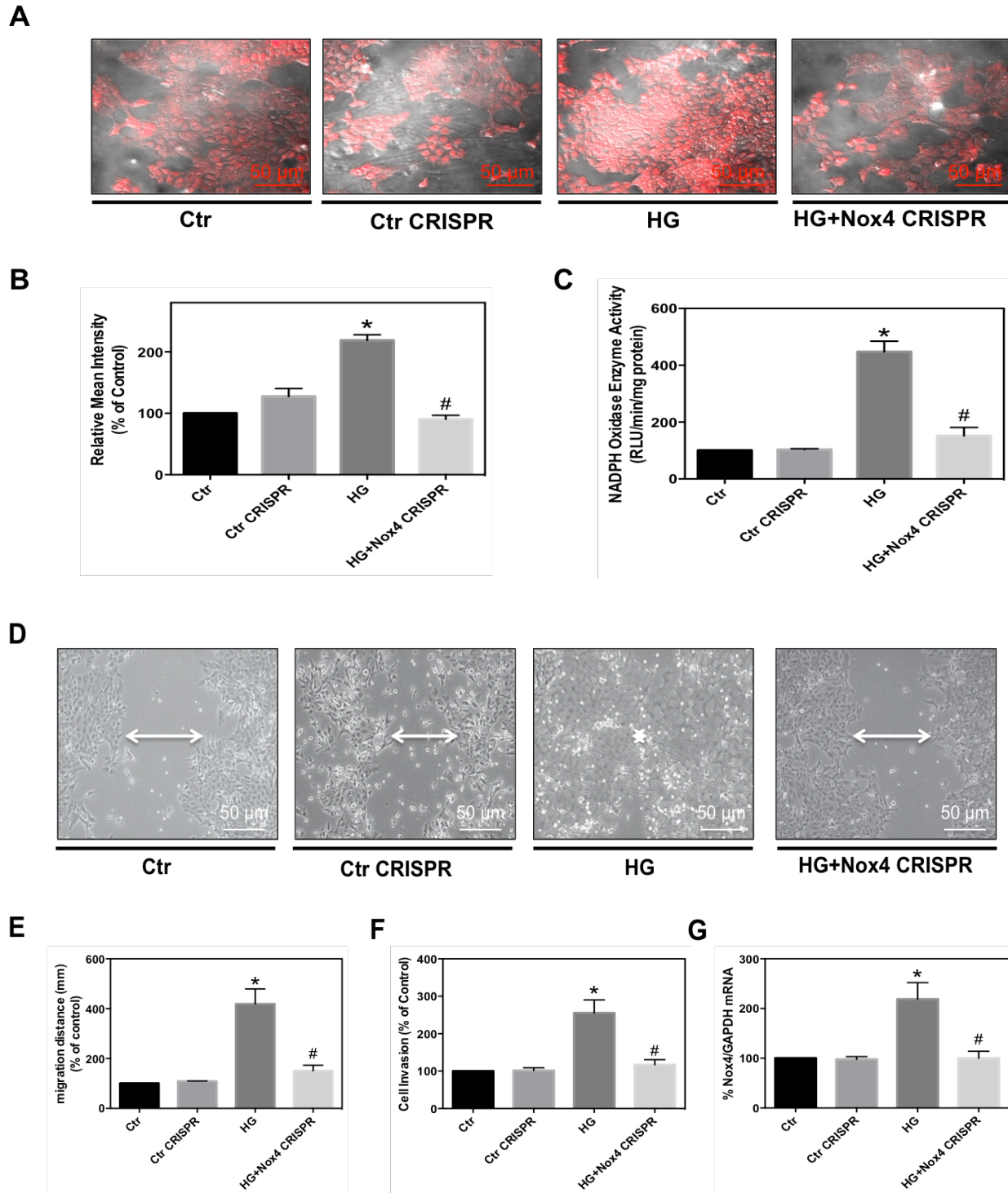
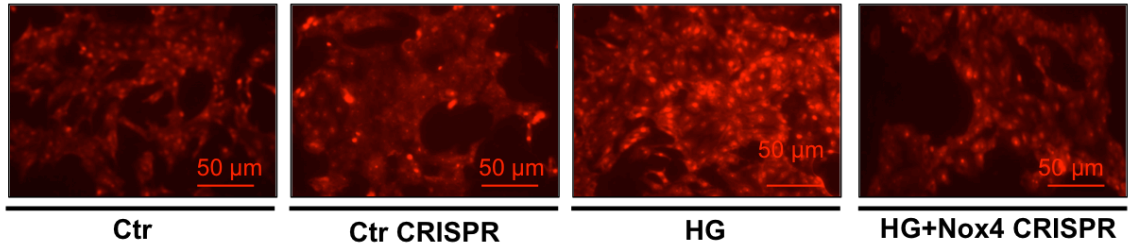


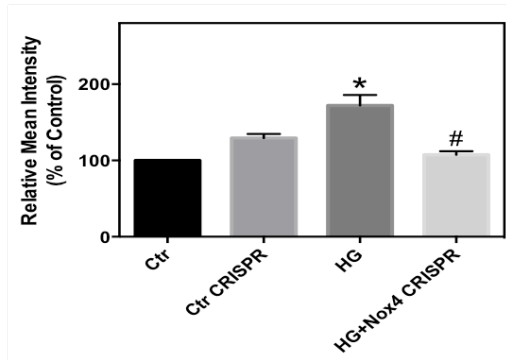
Figure 14a. Nox4 mediates HG-induced ROS production and cancer cell injury. HT-29 cells were seeded at low confluence and subsequently transfected with either control CRISPR or Nox4 CRISPR treated with or without HG (25 mM). No significant difference was observed between the non-treated cells (control) and the cells transfected with control CRISPR. (A) Representative figures of DHE staining along with the corresponding histogram (B) showing a reduction in ROS production upon Nox4 knockdown. (C) NADPH oxidase activity assay showing a reduction in Noxs activity upon transfection with Nox4 CRISPR. (D) Representative figures of the

migration assay showing a decrease in migration distance of HG treated cells upon knockdown of Nox4 gene expression. (E) Histogram of the migration assay. (F) Invasion assay showing a decrease in cancer invasion upon Nox4 knockdown. (G) Histogram showing relative mRNA levels of Nox4; cells treated with HG+Nox4 CRISPR showed a decrease in Nox4 gene expression as compared to the HG treated cells. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

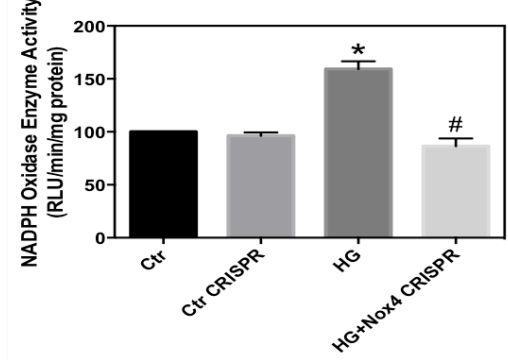
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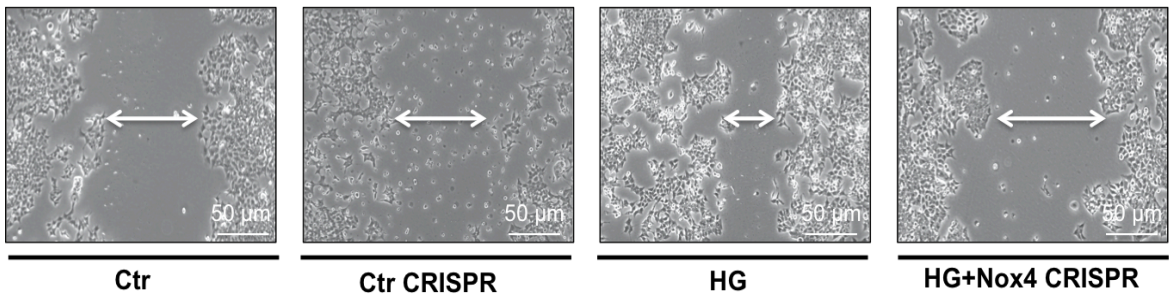
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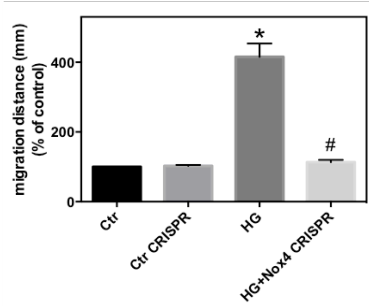
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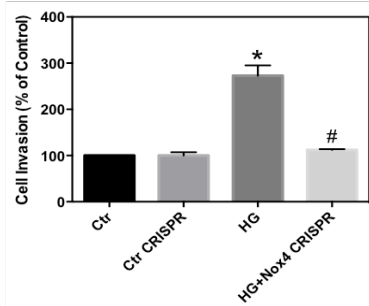
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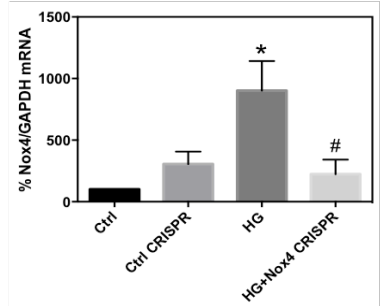


Figure 14b. Nox4 mediates HG-induced ROS production and cancer cell injury. Caco2 cells were seeded at low confluence and subsequently transfected with either control CRISPR or Nox4 CRISPR treated with or without HG (25 mM). No significant difference was observed between the non-treated cells (control) and the cells transfected with control CRISPR. (A) Representative figures of DHE staining along with the corresponding histogram (B) showing a reduction in ROS production upon Nox4 knockdown. (C) NADPH oxidase activity assay showing a reduction in Noxs activity upon transfection with Nox4 CRISPR. (D) Representative figures of the migration assay showing a decrease in migration distance of HG treated cells upon knockdown of Nox4 gene expression. (E) Histogram of the migration assay. (F) Invasion assay showing a decrease in cancer invasion upon Nox4 knockdown. (G) Histogram showing relative mRNA levels of Nox4; cells treated with HG+Nox4 CRISPR showed a decrease in Nox4 gene expression as compared to the HG treated cells. All values are the mean \pm S.E. from at least three independent experiments. *, $p < 0.05$ versus the control. #, $p < 0.05$ versus HG.

F. Nox4 mediates its influence on the AMPK/mTOR pathway in APC and C57 mice

Extensive data from our group have implicated mTORC1/Nox4 axis as a key player in the onset and development of diabetic nephropathy (Eid et al., 2013; Eid et al., 2016) as well as neuropathy (Eid et al., unpublished data) and heart injury (Zhao et al., 2015). In line with these observations, we set out to explore the cross talk between AMPK/mTORC1 and Nox4 *in vivo* and pinpoint any signaling alterations that maybe eliciting the diabetes-induced cancer-cell aggressiveness seen *in vitro*. Subsequently, we evaluated our *in vitro* findings in a set of *in vivo* experiments (**Table 2 and Fig. 15&16**) by employing the C57BL/6-Apc^{tm1Tyj}/J mice (The Jackson Laboratory, Bar Harbor, ME). APC mice possess a genotype similar to Apc^{Min} mice due to an induced germ-line heterozygous deletion of the Apc gene and thus serve as a good prototype for colon cancer (The Jackson Laboratory, Bar Harbor, ME). The APC mice along with the control C57 mice were rendered type 1 diabetic by STZ injections and were either left untreated or treated with metformin (50 mg/Kg), the AMPK activator or a pharmacologically low dose of rapamycin (0.5 mg/Kg), the mTORC1 inhibitor (**table 2**) for six weeks after diabetes onset.

The following groups of mice were used in our study:

-Group 1: Control C57BL/6J mice

-Group 2: Control C57BL/6J mice rendered diabetic by STZ injections

-Group 3: Genetically-induced CRC mice or APC mice

-Group 4: APC mice rendered diabetic by STZ injections

In parallel experiments, additional groups of diabetic mice (both C57 and APC) were treated with either metformin or rapamycin.

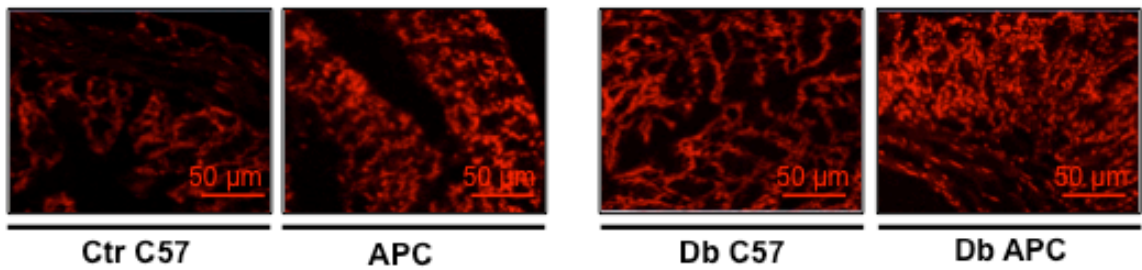
The following table displays the metabolic characteristics of the mice groups:

Table 1. Glucose level and body weight in APC and C57 control mice: -STZ induced type 1 diabetic mice -STZ induced type 1 diabetic mice treated with metformin (150 mg/Kg) or rapamycin (0.5 mg/Kg) for 6 weeks after the onset of the disease Values are the means \pm S.E. from 4 -6 animals for each group.			
Group	n	Blood Glucose (mg/dl)	Body weight (g)
Control (citrate)	5	151.8 \pm 11	28.9 \pm 0.6
STZ induced type 1 diabetic mice	5	522 \pm 40*	24.5 \pm 0.4*
STZ induced type 1 diabetic mice + Metformin	5	391 \pm 38*	24.4 \pm 0.5*
STZ induced type 1 diabetic mice + Rapamycin	6	519 \pm 36*	23.3 \pm 0.7*
APC mice	5	140 \pm 18	24.9 \pm 1.3
STZ induced type 1 diabetic APC mice	4	365 \pm 24 [#]	19.8 \pm 1 [#]
STZ induced type 1 diabetic APC mice + Metformin	4	362 \pm 47 [#]	20.3 \pm 0.6 [#]
STZ induced type 1 diabetic APC mice + Rapamycin	6	435 \pm 36 [#]	17.6 \pm 0.4 [#]
*p<0.05 versus control mice			
[#] p<0.05 versus APC mice			

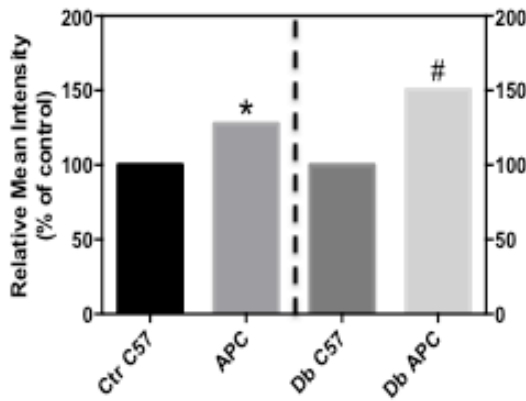
Table 2. Metabolic characteristics of the animal models. Type 1 diabetic mice exhibited significantly lower body weights and glucose levels > 250 mg/dl.

Interestingly, colons of the APC mice and the diabetic APC mice exhibited higher ROS levels as assessed by DHE staining (**Fig. 15A&B**) as well as increased NADPH oxidase activity (**Fig. 15C**) compared to the control C57BL/6J mice and the C57 diabetic mice. In particular, Nox4 relative mRNA expressions were upregulated in APC and diabetic APC colon tissues (**Fig. 15D**) reflecting its role in cancer as well as diabetes-induced ROS production.

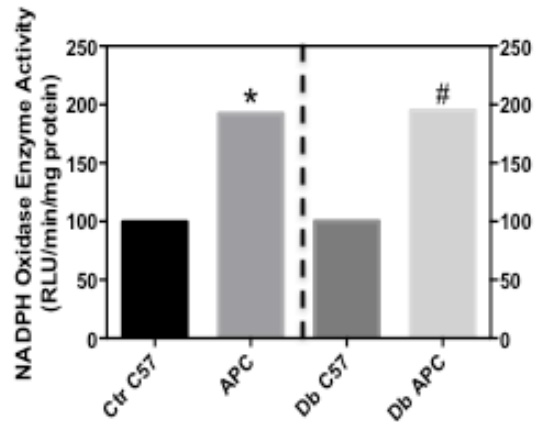
A



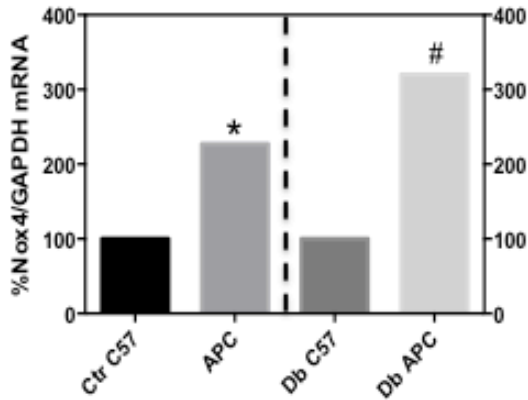
B



C



D



E

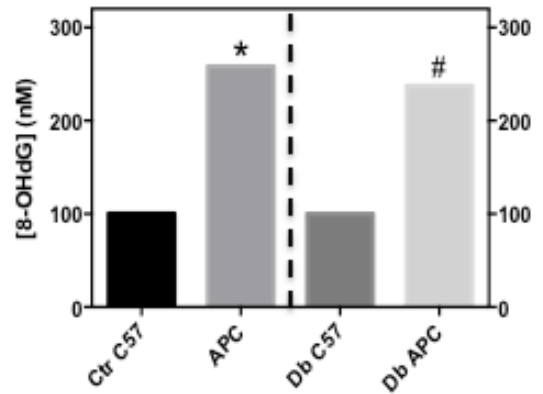


Figure 15. Colon cancer induces ROS production, Nox4 mRNA expression and 8-oxodG concentrations in control and diabetic mice. APC and C57 mice received either a vehicle or STZ injections to induce type 1 diabetes. (A) DHE staining of colon sections from APC and C57 mice. Fresh colon tissue sections (4-5 μ m thick) from the colons of APC and C57 mice were incubated for 1 hour at 37°C with a 15 μ M DHE stain. Images were taken directly using a laser scanning confocal microscope. The results displaying the relative mean intensity (B) show an increase in ROS production in the colons of APC mice compared to C57 mice. (C) Relative mRNA levels of Nox4 in the colons of APC and C57 mice. (D) Histogram displaying the concentration of 8-oxo-dG in the colons of APC and C57 mice; DNA was extracted from the extracted colons using the high salt method and calculation of the 8-oxodG concentration was done in reference to an 8-OHdG standard curve. All values are the mean \pm S.E. from 4 mice per group. *, $p < 0.05$ versus the control C57 mice. #, $p < 0.05$ versus the diabetic C57 mice.

As for ROS-induced mutagenesis, the concentrations of 8-oxodG adducts were found to be higher in DNA extracted from colon tissues of the APC mice compared to that of the C57 mice as well as the diabetic APC mice compared to the diabetic mice (**Fig. 15E**).

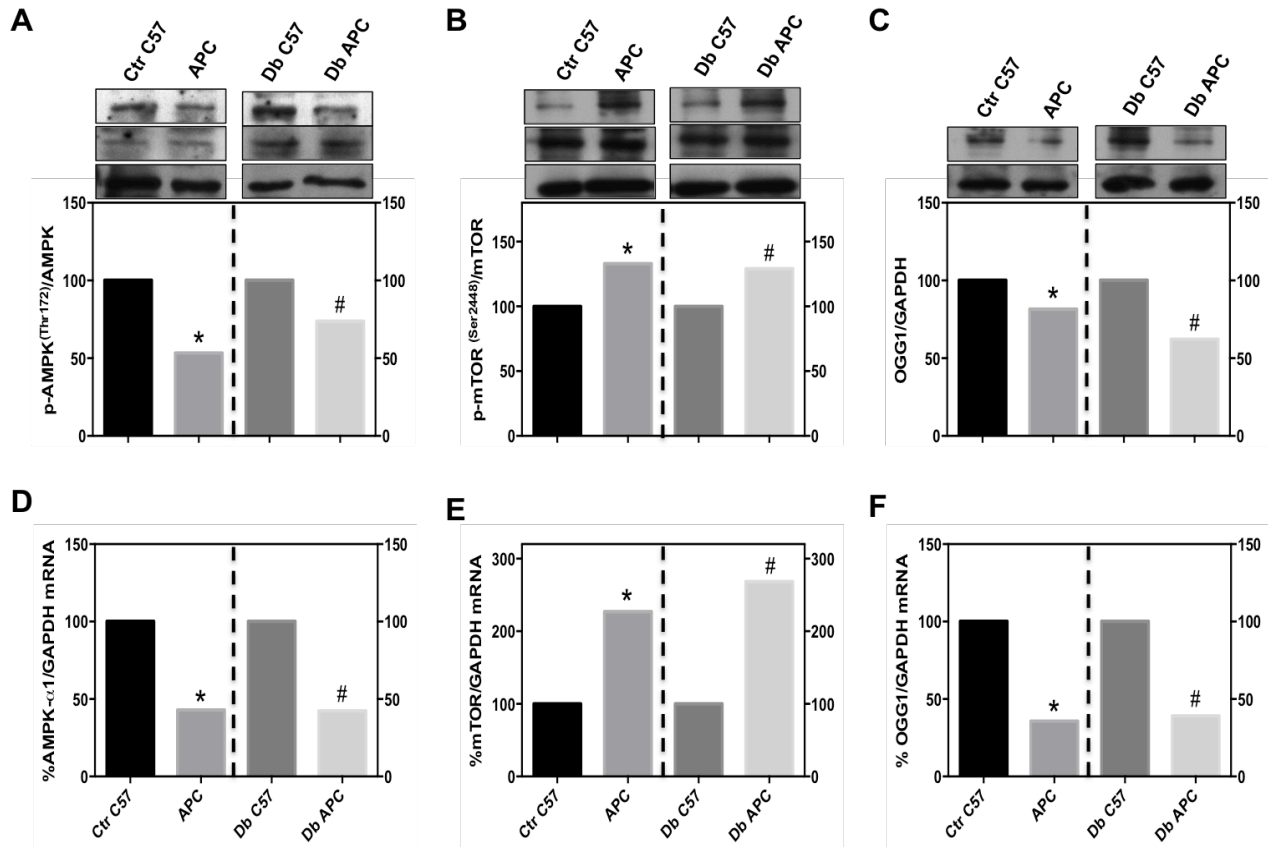


Figure 16. Colon cancer induces mTOR expression and downregulates AMPK and OGG1 expressions in both control and diabetic APC mice. (A)-(C) Representative western blots of phospho-Th172 AMPK and AMPK (A), phospho-Ser2448 mTOR and mTOR (B), and OGG1 (C) with their corresponding densitometric analysis in control and diabetic APC mice compared to control and diabetic C57 mice respectively. (D-F) Relative mRNA levels of AMPK (D), mTOR (E), and OGG1 (F) in control and diabetic APC mice compared to control and diabetic C57 mice

respectively. All values are the mean \pm S.E. from 4 mice per group. *, $p < 0.05$ versus the control C57 mice. #, $p < 0.05$ versus the diabetic C57 mice.

Our side-to-side comparison between the control and diabetic C57 colons versus APC colons extended to include the protein and gene expressions of AMPK, mTOR and OGG1 as key effectors/modulators in the Nox4-influenced CRC progression. Indeed colon tissues of both the APC and diabetic APC mice displayed reduced AMPK phosphorylation/activation at the Thr¹⁷² residue found on the α -subunit as well as mRNA levels when compared to their respective controls; control C57 and diabetic C57 mice (**Fig. 16**). As expected, mTOR protein and mRNA expressions received a giant boost, in response to attenuation of AMPK expression, in the colons of APC control and diabetic mice as compared to the C57 mice of the same group (**Fig. 16**). OGG1 levels were also attenuated in the colons of mice with CRC compared to those of the normal mice (**Fig. 16**).

Yet, comparing APC mice with the diabetic APC mice, our findings revealed increased ROS production, NADPH oxidase activity, as well as Nox4 mRNA levels in the colons of the APC diabetic mice compared to those of the non-diabetic mice (**Fig. 18**). Diabetes-induced overproduction of 8-oxodG adducts was also found in the colons of APC diabetic mice compared to the colons of the APC mice (**Fig. 19**). This was paralleled with a similar dysregulation in the AMPK/mTOR pathway. More specifically, colons of APC diabetic mice displayed a downregulation in AMPK and OGG1 expressions/activity and an upregulation of mTOR expression/activity (**Fig. 19 & Fig. 20**). Similar results were also seen in the colons of the C57 diabetic mice as compared to the C57 control mice (**Fig. 18 & Fig. 19**). These findings stress the role of diabetes in exacerbating Nox4-induced ROS production and colon mutagenesis through a perturbation in the AMPK/mTOR pathway in colons of both normal and CRC mice.

Collectively, these results identify a novel role for Nox4 in the generation of colon cancer and diabetes-induced ROS as well as pushing the AMPK/mTOR signaling pathway in the direction that favors tumorigenesis, in particular, OGG1 silencing. Our findings also highlight the involvement of the AMPK/mTOR/Nox4 axis as a common signaling pathway in diabetes and cancer.

G. Metformin and Rapamycin treatments attenuate polyp number in the colons of APC mice.

We next wanted to assess whether the activation of AMPK or the inhibition of mTOR reverses the aggressiveness of CRC in diabetic APC mice when compared to APC mice. Our results show that the number of polyps in the colons of the APC diabetic mice was significantly higher than that in the non-diabetic APC mice. Moreover, the administration of metformin or rapamycin to the diabetic mice with colorectal cancer was able to reduce polyp number to almost baseline levels as seen in the non-diabetic APC group (**Fig. 17A&B**).

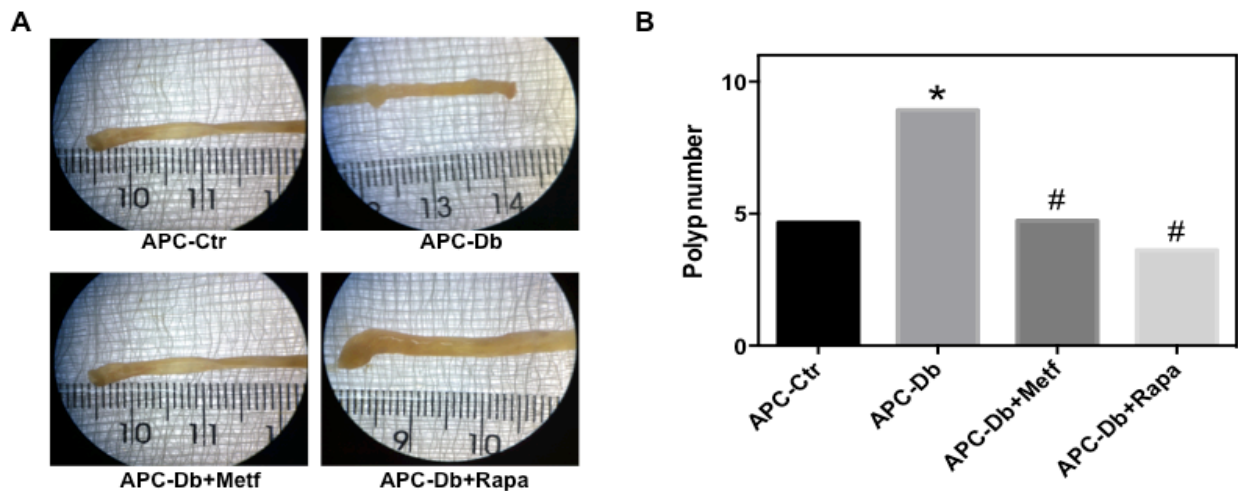


Figure 17. Number of polyps found in the colons of APC mice. STZ-induced type 1 diabetic APC mice were treated with either vehicle, metformin (150 mg/kg/day), or rapamycin (0.5 mg/kg/day) administered by intraperitoneal injection for 6 weeks while mice in the control groups (Ctr) received the vehicle. The colons were isolated, cleaned with saline and the polyps were counted under a dissecting microscope. The diabetic mice showed a higher number of polyps than the controls while metformin and rapamycin treatments reduced polyp number to almost control levels. (A) Representative colon images and the corresponding histogram (B) showing average polyp number in control, STZ-induced type 1 diabetic, and STZ-induced type 1 diabetic mice treated with metformin or

rapamycin. *, $p < 0.05$ versus the vehicle-treated APC mice. #, $p < 0.05$ versus the vehicle-treated STZ-induced type 1 diabetic APC mice.

H. Metformin and Rapamycin treatments reverse diabetes induced Nox4-mediated colon tumorigenesis via AMPK/mTORC1 pathway.

Our previous results clearly demonstrate a pioneering role for Nox4 in formulating the proper settings for cancer cell injury through increased ROS production, downregulation of AMPK activity, and upregulation of mTOR activity. Yet, little is known about the involvement of Nox4 in diabetes and colon cancer and the crosstalk between the AMPK/mTOR pathway and Nox4. Our studies show that intracellular ROS and NADPH oxidase activity were enhanced in the colon tissues of the diabetic APC (**Fig. 18A-C**) and C57 mice (**Fig. 18E-G**) as compared to the vehicle treated control of each strain. Once again, Nox4, whose mRNA expression shot upwards in the diabetic colons (**Fig. 18D&H**), pled guilty for its implication in diabetes and cancer induced ROS generation. In the same context, metformin and rapamycin treatments reversed diabetes-induced ROS production (**Fig. 18A&B, E&F**) and NADPH oxidase activity (**Fig. 18C&G**) including Nox4 mRNA expression (**Fig. 18D&H**).

Treatment with metformin and rapamycin assumes an activation of AMPK and inhibition of mTOR expression; as it is the case in our *in vitro* and *in vivo* findings (**Fig. 19C&D, G&H**). A more interesting out-turn was the diabetes-induced inhibition of AMPK expression and upregulation of mTOR expression in the colons of APC (**Fig. 19C&D**) and C57 (**Fig. 19G&H**) mice.

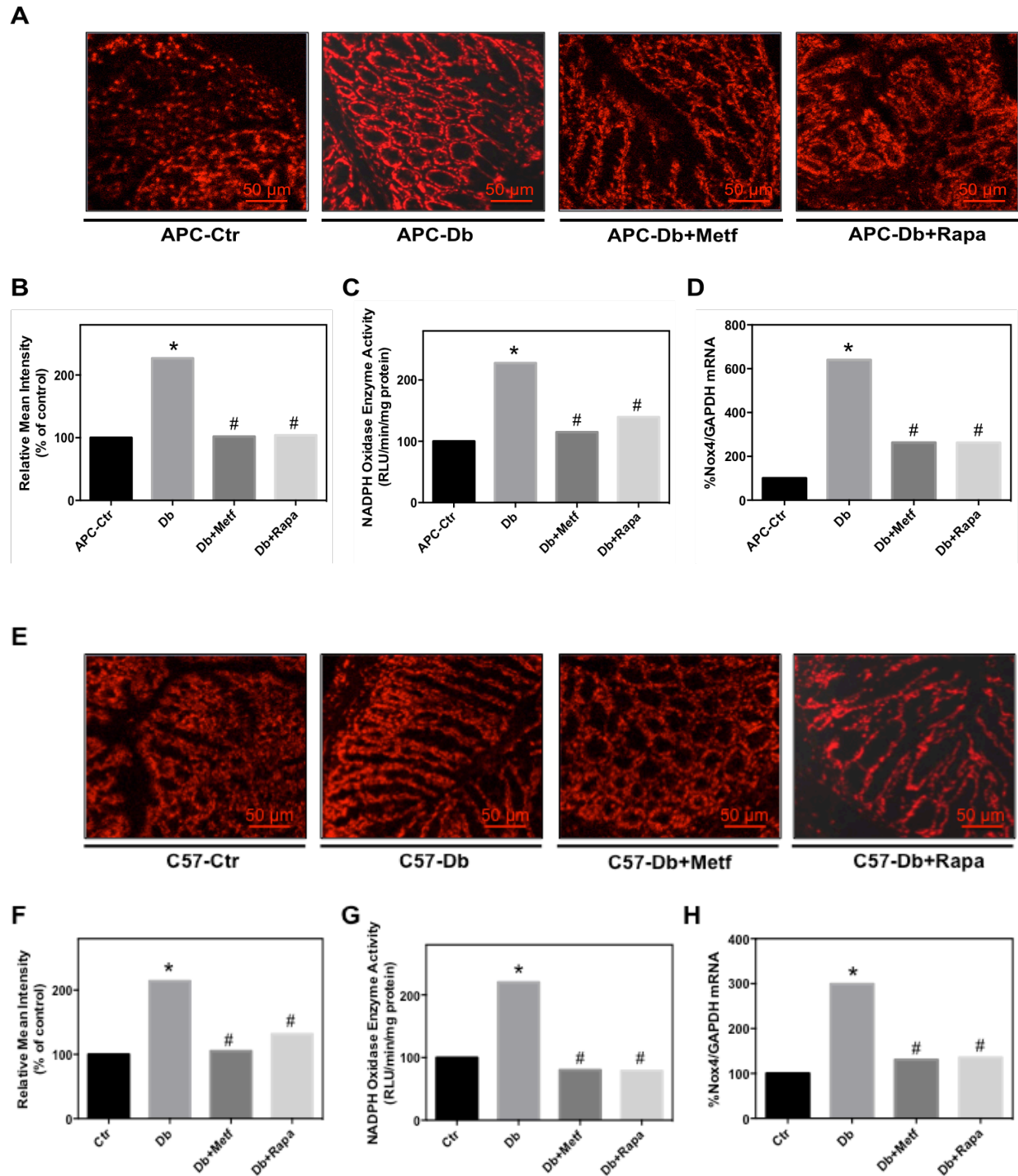


Figure 18. Metformin and rapamycin treatments reverse Nox4-induced ROS production in the colons of APC and C57 mice. STZ-induced type 1 diabetic APC and C57 mice were treated with either vehicle, metformin (150 mg/kg/day), or rapamycin (0.5 mg/kg/day) administered by intraperitoneal injection for 6 weeks while mice in the control groups (Ctr) received the vehicle. Fresh colon tissue sections (4-5 μ m thick) from the colons of APC and C57 mice were incubated for 1 hour at 37°C with a 15 μ M DHE stain. Images were taken directly using a laser scanning confocal microscope. Representative images of DHE stains of colon tissues of APC (A) and C57 (E) mice.

Histograms showing the result of the DHE stain from APC (B) and C57 (F) mice. NADPH oxidase activity assay from colons of APC mice (C) and C57 mice (G). Relative mRNA levels of Nox4 from colons of APC mice (D) and C57 mice (H). All values are the mean \pm S.E. from 4 mice per group. *, $p < 0.05$ versus the vehicle-treated APC or C57 mice. #, $p < 0.05$ versus the vehicle-treated STZ-induced type 1 diabetic APC or C57 mice.

Metformin treatment was additionally able to reverse diabetes-induced effect on mTOR expression (**Fig. 19D&H**) while rapamycin treatment had no effect on AMPK expression (**Fig. 19C&G**) adding an extra vote to the benefit of previous findings by our group placing AMPK in the front row as a regulator of the mTOR/p70S6K pathway in renal podocytes through TSC expertise (Eid et al., 2013).

Besides the mTORC1 pathway, Nox4-dependent ROS facilitates CRC tumorigenesis through the silencing of OGG1-DNA repair skills. In fact, 8-oxodG concentrations were attenuated in the colon tissues of the diabetic mice through AMPK activation by metformin (**Fig. 19A&E**) after a dramatic rise that occurred, rather conveniently, subsequent to a drop in OGG1 expression (**Fig. 19B&F**). These outcomes highlight once more our *in vitro* findings thus culminating in a definitive crosstalk between Nox4 and AMPK/mTOR pathway in diabetic mice either with or without colorectal cancer.

A final but indispensable sequel to our novel series of findings recapitulates the previous alterations in the described signaling pathways, only this time, at the protein level. Our *in vitro* findings bracket Nox4, AMPK, mTOR, and OGG1 as vital mediators of HG-induced colon cancer cell aggressiveness. Similarly, in colon tissues of APC diabetic mice, the phosphorylation of AMPK at its activation site was reduced (**Fig. 20A**) whereas phosphorylation of mTOR on Ser²⁴⁴⁸ (**Fig. 20B**), along with its downstream effector p70-S6K^{Thr389} (**Fig. 20C**), was clearly increased. Additionally, the colons of APC mice with type 1 diabetes elicited spiked Laminin protein expressions (**Fig. 20F**) and turned down OGG1 expressions (**Fig. 20E**).

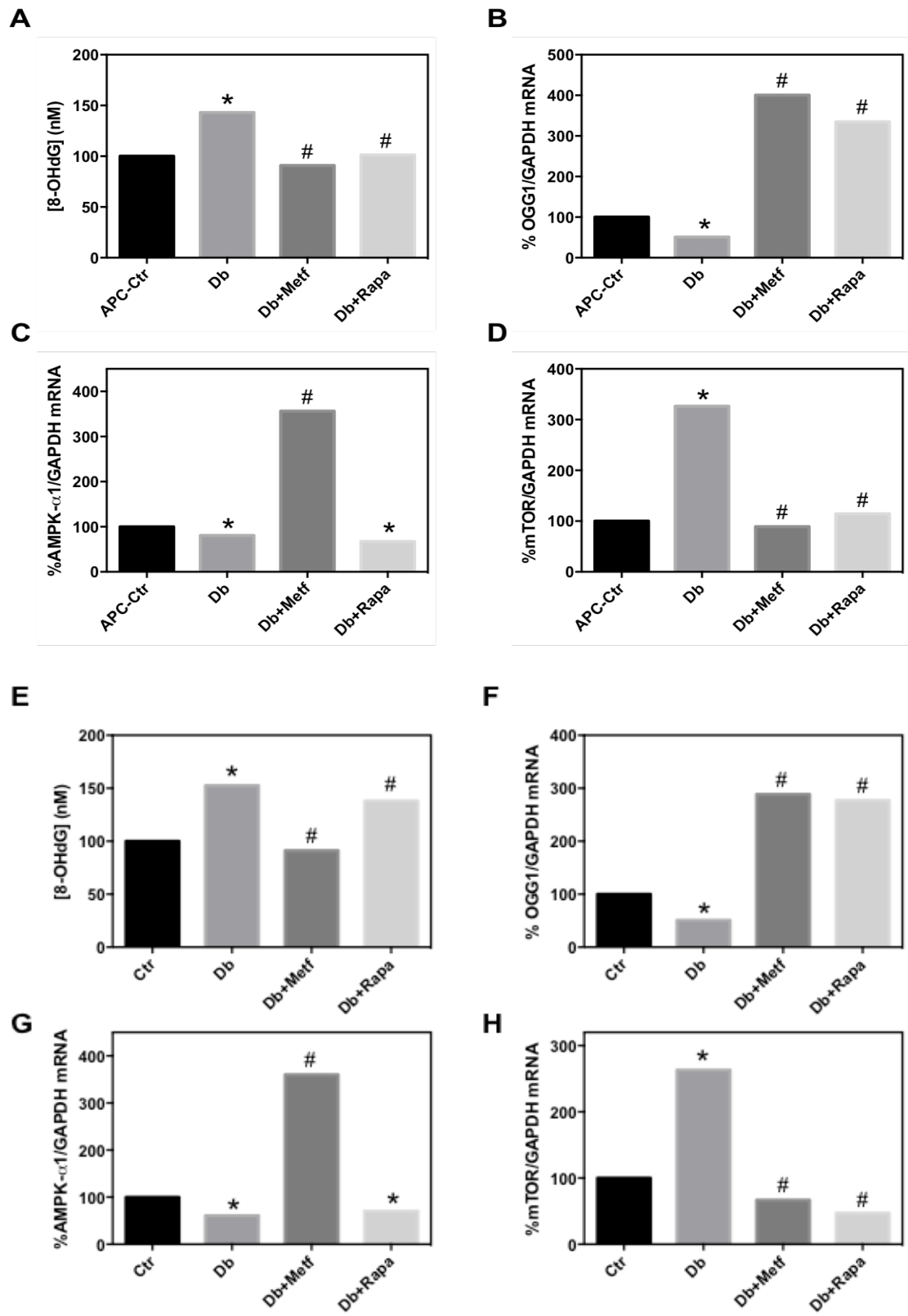


Figure 19. Metformin and rapamycin treatments reduce 8-oxodG adducts and boost OGG1 expression in the colons of APC and C57 mice. STZ-induced type 1 diabetic APC and C57 mice were treated with either vehicle,

metformin (150 mg/kg/day), or rapamycin (0.5 mg/kg/day) administered by intraperitoneal injection for 6 weeks while mice in the control groups (Ctr) received the vehicle. 8-oxodG concentration significantly decreases in the colon tissues of APC mice (A) and C57 mice (E) treated with metformin or rapamycin as compared to the diabetic and diabetic APC mice. Relative mRNA levels of OGG1 in colons of APC (B) and C57 (F) mice. Relative mRNA levels of AMPK- α 1 in colons of APC (C) and C57 mice (G) as well as that of mTOR in colons of APC mice (D) and C57 mice (H). All values are the mean \pm S.E. from 4 mice per group. *, $p < 0.05$ versus the vehicle-treated APC or control C57 mice. #, $p < 0.05$ versus the vehicle-treated STZ-induced type 1 diabetic C57 or diabetic APC mice.

Activation of AMPK through metformin treatment and inhibition of the mTORC1 pathway through the administration of rapamycin, reversed the observed modifications on the above signaling pathway and therefore restored OGG1 levels (**Fig. 20E**) and attenuated Laminin protein expressions (**Fig. 20F**) in diabetic APC colon tissues. On a side note, AMPK activation exhibited an inhibitory effect on mTOR phosphorylation/activation (**Fig. 20B**) whereas mTOR inhibition elicited no change in AMPK phosphorylation/activation (**Fig. 20A**) thus confirming once again that AMPK is located upstream of the mTOR/p70S6K pathway. Nevertheless, we cannot fail to acknowledge the significant boost in Nox4 expression, in the colons of diabetic mice, which was attenuated upon treatment with metformin or rapamycin (**Fig. 20D**) placing it under the spotlight each time diabetic complications are scrutinized and in particular diabetes-induced CRC progression.

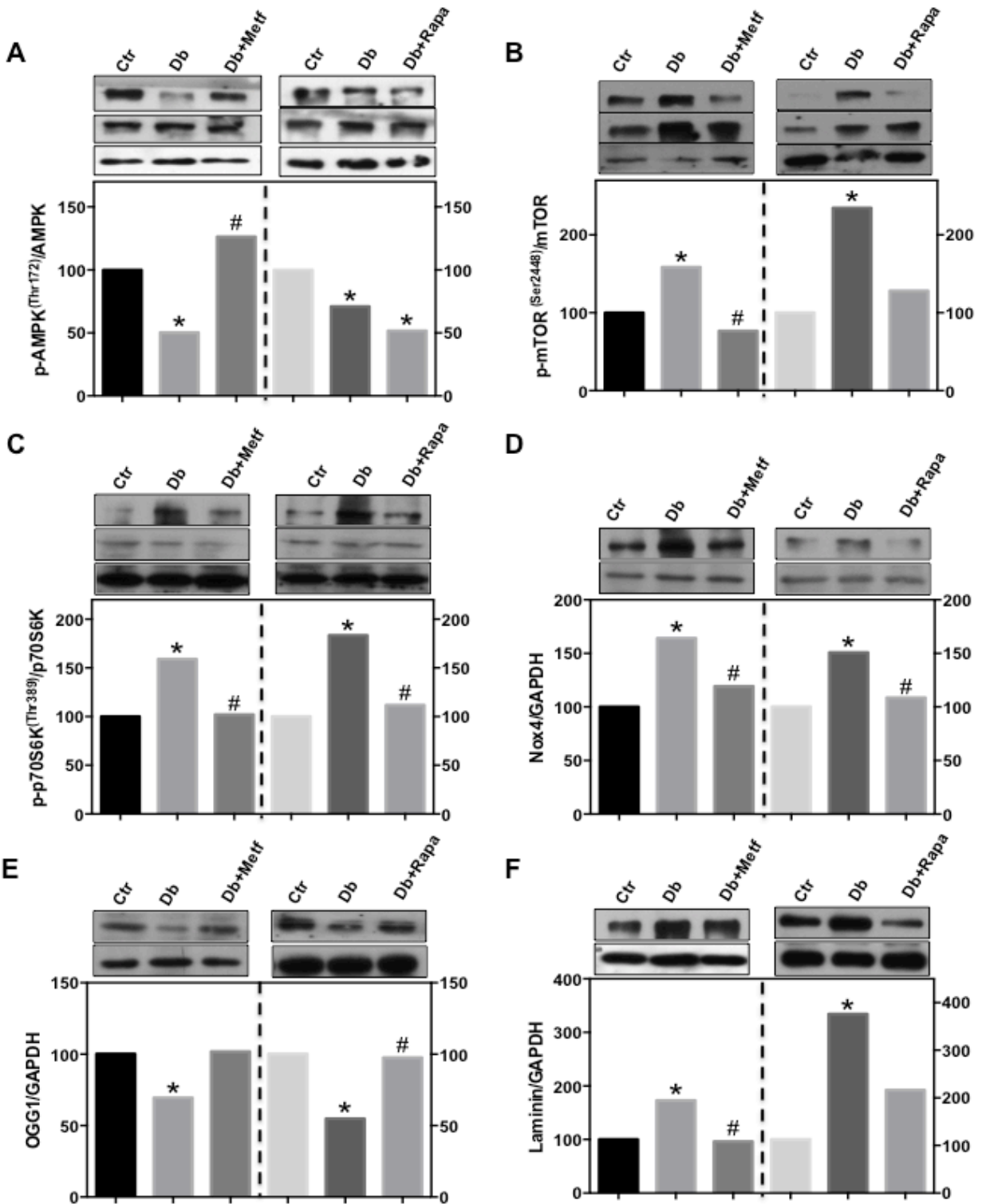


Figure 20. Metformin and rapamycin treatments reverse diabetes effect in APC mice. STZ-induced type 1 diabetic APC mice were treated with either vehicle, metformin (150 mg/kg/day), or rapamycin (0.5 mg/kg/day) administered by intraperitoneal injection for 6 weeks while mice in the control groups (Ctr) received the vehicle. Representative Western Blots of phosphor-Th172 AMPK and AMPK (A) phosphor-Ser2448 mTOR and mTOR (B),

phosphor-Thr389 p70S6 Kinase and p70S6 Kinase (C), Nox4 (D), OGG1 (E), Laminin (F) and GAPDH with the respective densitometric quantifications in colons of APC mice, STZ-induced type 1 diabetic APC mice, and STZ-induced type 1 diabetic APC mice treated with metformin or rapamycin. All values are the mean \pm S.E. from 4 mice per group. *, $p < 0.05$ versus the vehicle-treated control APC mice. #, $p < 0.05$ versus the vehicle-treated STZ-induced type 1 diabetic APC mice.

Collectively, these results indicate that in response to diabetes, the upregulation of Nox4 as well as the activation of the mTORC1/p70S6K axis and inhibition of AMPK pathway play a critical role in CRC induced progression and aggressiveness in APC mice.

DISCUSSION

Diabetes Mellitus (DM) is a chronic, systemic malfunction that is characterized by elevated blood glucose levels (Neville, R.F. & Sidawy, A.N. 2012) affecting currently over 415 million people worldwide (International Diabetes Federation, 2015). Both types of diabetes are characterized by a persistent hyperglycemia that never fails to inflict damage to the diverse organs and organ systems leading to drastic life threatening or disabling illnesses (DCCT, 1993; Ali et al., 2013). In recent years, one of the complications that has been increasingly shown to be associated with diabetes is cancer (De Bruijn et al., 2013; Shikata et al., 2013; Shi & Hu., 2014). In fact, various cancers have been correlated with diabetes progression such as pancreatic, gastric, leukemic, hepatocellular, skin, and kidney cancers (Shu et al., 2010; Vigneri et al., 2009; Suh & Kim, 2011; Johnson et al., 2012; Larsson & Wolk., 2011; Ge et al., 2011).

Recently, colorectal cancer (CRC) has been added to the list of cancers associated with diabetes. In fact, CRC has been reported to rank second for men and third for women in cancer-related deaths worldwide (American Cancer Society, 2017). More importantly, it has been estimated that 75% of diabetic subjects experience GI dysfunction as a result of numerous etiologies (Bytzer et al, 2001; Samsom et al, 2003). Previous work has described the diabetogenic colon to undergo morphometric and morphomechanical alterations in type 2 diabetes, especially mediated by a source of reactive oxygen species (Jingbo Zhao et al., 2013). In type 1 diabetes, injury to colonic tissue was also pronounced in rat models marked by inflated actin, myosin, and collagen expression (Siegman et al., 2015). Consequently, in our study, we aimed to examine the effect of diabetes on another parameter of colonic injury, within the context of CRC. For that, we initiated our search for a possible explanation that might describe

the mechanism by which cancer progression occurs in diabetes as well as identifying therapeutic targets for diabetes-associated CRC.

In this study, we demonstrated that the colons of diabetic Adenomatous Polyposis Coli (APC) mice exhibited a larger polyp number than their non-diabetic counterparts. In fact, APC mice, with single ($APC^{+/-}$) or double silencing ($APC^{-/-}$) or mutation of the APC tumor suppressor gene, develop polyps in the intestine and colon around 4-6 weeks after birth (The Jackson Laboratory, Bar Harbor, ME). APC mice serve as good models that mimic human colorectal cancer development since mutations in the APC gene are found in more than 80% of CRCs (Cheung et al., 2010), and hence have been extensively used to further explore this disease (Qiao et al., 2017, Huang et al. 2017, He et al., 2017, Hull et al., 2017, Fujishita et al., 2017). For our investigation, we chose APC mice with a germline heterozygous mutation rendering them similar to APC^{Min} animals (JAX stock #009045, Jackson Laboratory, Bar Harbor, ME) since the level of expression of the APC gene affects the number, size and allocation of the polyps in the intestine and colon; hence, more polyps are expected to form in the $APC^{-/-}$ mice as compared to the $APC^{+/-}$ mice (Cheung et al., 2010, Nieuwenhuis & Vasen et al., 2007, Soravia et al., 1998). Moreover, treatment doses were chosen based on previous studies performed by our group (Eid et al., 2013).

Hyperglycemia and/or Hyperinsulinemia: No Fairytale ending

Hyperglycemia and/or hyperinsulinemia resulting from either type 1 or type 2 diabetes have been continuously linked to colorectal cancer progression. Our study shows that polyp number is increased in the colons of diabetic mice with colorectal cancer as compared to the non-diabetic ones. Looking at diabetes-induced hyperglycemia, an association seems to exist between elevated glucose levels or glycated hemoglobin levels and the predisposition to CRC

malignancies (Yu et al., 2016; Suchanek et al., 2016; Asterholm et al., 2016; Lee et al., 2017; Vasconcelos-dos-Santos et al., 2017). Indeed, clinical studies reported that patients with poorly controlled Type 2 DM have more right sided and advanced CRCs, a younger age of presentation, greater use of exogenous insulin, and a poorer 5-year survival (Siddiqui et al., 2008). As for hyperinsulinemia, whether it is exaggerated endogenous insulin resulting from cell resistance or exogenous insulin administered as treatment, a fair share of data support the fact that insulin levels are positively correlated with CRC risk (Yang et al., 2004, Giouleme et al., 2011, Nagel & Göke, 2006).

In our *in vitro* studies, HT-29 and Caco2 cells treated with either high glucose or a combination of high glucose and insulin for 72 hours exhibited higher proliferation rates and were able to migrate faster and invade the matrigel in higher numbers. However, insulin alone failed to reproduce the glucose-induced increase in cancer cell aggressiveness. On that note, knowing that Insulin-like growth factor (IGF) receptors are known to be expressed on the surface of most cancerous cells, it was shown that the downregulation of IGF receptor expression in breast cancer cells elevated their sensitivity to insulin, and reduced the malignancy, proliferation and metastatic potential (Zhang et al., 2007). In light of these findings, our data are indicative of a similar state that is observed in colon adenocarcinoma cells. However, additional testing is essential to better explore this phenomenon to validate insulin resistance *in vitro*. For instance, immunohistological studies maybe beneficial in this case to be able to have a qualitative as well as a quantitative assessment of insulin and IGF receptor expression on the surface of colon cancer cells.

Taken together, these findings support the claim that hyperglycemia promotes colon cancer progression and aggressiveness *in vitro* and *in vivo*. Nevertheless, the metabolic and

mitogenic mechanisms by which glucose signaling induces neoplastic growth and malignancies have not been clearly understood.

Diabetes-Induced Nox4 Electric Glitch

We further examined the role of oxidative stress in CRC. Oxidative stress is a common theory unifying the progression of diabetic complications. Our laboratory, as well as other groups, has shown the role of overly produced ROS in diabetic nephropathy, neuropathy, retinopathy and cardiomyopathy. Moreover, oxidative stress by cause of oxidative injury at the level of proteins, lipids and genetic material is believed to mediate carcinogenesis. In our study, we aimed to investigate oxidative stress as an overlying mechanism to the etiologies of cancer and diabetes, and trace the mechanism in diabetic CRC. Our data demonstrate an increase in ROS production via an NADPH-dependent mechanism, reflected through elevated superoxide anion generation in the colons of the APC mice as compared to those of the C57 mice. This proves an increase in ROS production when cancer is manifested regardless of diabetes. In fact, several studies have shown that oxidative stress maybe the major player behind the carcinogenesis scheme after being caught red handed plotting several cancers (Prasad et al., 2017; Li et al., 2011; Li et al., 2013; Deep et al., 2016; Auyeung & Ko, 2017) either by enhancing genomic instability (Dizdaroglu & Jaruga, 2012; Cadet & Wagner, 2014) or inducing DNA mutagenesis (Shibutani et al., 1991; Ogrunc et al., 2014; Ishikawa et al., 2015). Additionally, ROS production was also increased in the colons of the diabetic mice (either C57 or APC) as compared to their non-diabetic counterparts, paralleled by a rise in NADPH oxidase activity. These data corroborate and reinforce the involvement and over-production of ROS in the pathogenic states triggered by diabetes in multiple organs (Lambeth Krause, K. H., & Clark, R. A., 2008; Naziroglu et al., 2012; Eid et al., 2009; Eid et al., 2010; Eid et al., 2013a,b,c;

Nayernia et al., 2014; Kowluru, A. R., & Mishra, M. 2015; Filla, A. L., & Edwards, L. J. 2016; Eid et al., 2016) further impeding on the debilitating role of ROS overproduction in the pathogenesis of almost all diabetic complications including cancer.

These data were further verified at the molecular level whereby an increase in a hallmark of oxidative injury at the genetic level was exhibited. 8-oxodG adduct formation was evident in the colons of the APC mice compared to those of the C57 mice. The results also showed significantly increased 8-oxodG concentrations in the diabetic versus non-diabetic colons of both APC and C57 mice. These results lend support to studies previously performed reporting the contributions of ROS over-production to the risk of genetic mutations in genes involved in cell cycle, cell death and metabolic regulation (Bhatt et al., 2010; Reuter et al., 2010; Chew, S. H., & Toyokuni, S. 2015; Zhong et al., 2017). Rodriguez et al provided one explanation to ROS-dependent DNA damage by stating that it inflicts DNA via misincorporation of DNA bases, for example, due to the presence of unrepaired DNA adducts, or by slippage of DNA polymerase during replicative by-pass (Rodriguez et al., 2013). The latter was confirmed by our work when levels of OGG1, the enzyme that recognizes and excises 8-oxodG adducts, were investigated. In the diabetic state, either in APC or C57 colon tissues or in colon cancer cells, OGG1 mRNA and protein expressions were downregulated further explaining the increase in 8-oxodG concentrations. Oxidative stress by virtue of diabetes has been shown by kakimoto et al to influence 8-oxodG adducts production in addition to mitochondrial injury at the genetic levels further exacerbating renal injury *in vivo* in experimental animal models (Kakimoto et al., 2002). Similarly, in a recent study, Cividini et al demonstrated the effect of OGG1 mutations on mitochondrial DNA repair mechanisms in cardiovascular complications of diabetes (Cividini et al., 2016). These data support the role of diabetes in amplifying cellular malfunction through

mitochondrial damage which was also shown to fuel cancer bioenergetics (Wallace, D., 2012). Consequently, diabetes seems to inflict lethal alterations to both nuclear and mitochondrial environments that could exacerbate cancer progression. In our study, the results emphasize the mechanistic approach of diabetes to CRC progression, at least partially through insults to genomic DNA. However, additional studies are necessary to further unveil any potential effects on mitochondrial DNA.

The elevated ROS production demonstrated was shown to be concurrent with a significant increase in mRNA and protein expressions of Nox4, a key member of the Nox family, in the colons of both diabetic and APC diabetic mice indicating that it is responsible, at least in part, for the overload in the generated ROS. These findings provide an extension to our group's work describing the role of Nox4 in diabetic neuropathy, nephropathy and podocyte depletion in diabetic experimental animal models (Eid et al., 2010, Eid et al., 2013, Eid et al., 2016, Eid et al., unpublished data) as well as that described by others in several chronic diseases, such as cardiovascular disorders (Pedruzzi et al., 2004), cancer (Vaquero et al., 2004) and kidney disease (Gorin et al., 2003). Findings have also correlated Nox4 overexpression with a poorer prognosis in CRC patients (Lin et al., 2017) and a higher likelihood of metastatic growth (Zhang et al., 2014), angiogenicity (Helfinger et al., 2016) and apoptotic death (Zhu et al., 2013). In fact, Nox4-mediated ROS is reported to prevent apoptosis and promote tumor cell growth in pancreatic cancer cells (Mochizuki et al., 2006; Vaquero et al., 2004), melanoma cancer cell proliferation and growth (Yamaura et al., 2009), breast cancer cells (Tobar et al., 2010), glioblastoma cancer cells (Mondol et al., 2014) and colon cancer cells (Bauer et al., 2013), and currently, lending support to our data on Nox4 in HT-29 and Caco2 colon cancer cells. An increase in Nox4 protein expression, accompanied by an increase in ROS production and

NADPH oxidase activity was seen in cancer cells compared to normal epithelial colon cells and in cancer cells treated with high glucose, high insulin, or a combination of both compared to the non-treated cancer cells. These findings reinforce our *in vivo* work indicating that cancer cells, like other non-malignant cells, produce ROS and those placed in a diabetic milieu generate even more ROS which is shown by our given data to be highly dependent on Nox4.

To further confirm the implementation of Nox4 in the over production of ROS in cancer and cancer-cell aggressiveness, colon cancer cells were transfected with a Nox4 CRISPR plasmid that knocks down Nox4 gene expression. Our results revealed that Nox4 knockdown did not only reduce NADPH oxidase activity and ROS production in HT-29 and Caco2 cells, but also normalized migratory and extracellular matrix invasion capabilities of the cancer cells as well as the elevated levels of 8-oxodG adducts. These results corroborate with earlier findings allowing Nox4 to gain power in the pathophysiology of CRC by modulating cytoskeletal-regulating proteins (Bauer et al., 2014). Nox4 also gained popularity in the CRC field through the regulation of cellular motility via a TGF- β -activated protein tyrosin phosphatases axis where it unites with Nox1 in a mission initiated by the latter in the early stage adenoma and progressed through Nox4 actions to accomplish the malignant transition (Zhang et al., 2015). These studies combined with our own depict a major role for Nox4-induced ROS production in CRC progression and further extend what is available concerning various sources of intracellular ROS in modulating the colonic environment. For instance, studies performed by Chen et al identified AGEs as potential sources of oxidative stress that lead to diabetes-induced colonic malfunction (Chen et., 2012). These studies come alongside Nox1 and Nox2 (Banskota et al., 2015) in addition to cyclooxygenase enzymes (COXs) (Hwang et al., 2007) as additional sources of ROS that can affect colon physiology but more specifically these were linked to colon cancer

pathogenesis. To our knowledge, this study is the first to investigate the implications of Nox4 in colon pathophysiology induced by diabetes especially within the context of CRC.

The Master Switch and the Cellular Commander: AMPK and mTOR

The current study set out to also investigate the link between NADPH-induced ROS production and pivotal pathways for cellular survival: the AMPK and mTORC1 pathways. Data from this study show that in colon tissues of type 1 diabetic mice or colon cancer cells placed in a diabetic milieu, the AMPK signaling pathway was down regulated in parallel with increased ROS production. In fact, AMPK is a cellular energy sensor that is activated through phosphorylation on its threonine residue Thr¹⁷² allowing it to act as a master regulator of glucose and lipid metabolism (Hardie & Carling, 1997; Hardie et al., 1994; Kemp et al., 1999; Mitchelhill et al., 1994; Tsuboi et al., 2003; Carling, 2004; Hardie, 2004; Eid et al., 2010; Ronnett et al., 2009). Additionally, it has been recently reported that under high glucose concentrations, AMPK may ‘switch off’ in several cell types contributing to the pathogenesis of several disorders including our very own, diabetes and cancer (Eid et al., 2010; Chowdhury et al., 2011; Tsuboi et al., 2003; Mountjoy & Rutter, 2007, Jeon, S., 2015). Keeping that in mind, our group has provided evidence that AMPK activation largely influences podocyte renal function through a reversal of hyperglycemia-induced elevations in ROS production (Eid et al., 2010). Countless other studies provide strong evidence that the AMPK pathway is involved in tumorigenesis such as in melanomas and lymphomas, in addition to gastric, pancreatic, thyroid and colorectal cancers (Arbiser et al., 2002, Park et al., 2006; Kim et al., 2012; Hardie, D. G., 2013; Yue et al., 2014; Sui et al., 2014; Faubert et al., 2015) mediating at least part of its influence through cell cycle arrest and activation of tumor suppressors (Kim et al., 2010, Rattan et al., 2005, Xiang et al., 2004, Imamuro et al., 2004). On that note, our group has identified

Tuberin (TSC2) as a downstream target of LKB1/AMPK. What is even more interesting is that the LKB1/AMPK/TSC2 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). Alternatively, AMPK directly phosphorylates mTOR at Thr²⁴⁴⁶ to reduce S6K1 phosphorylation by insulin, suggesting the inhibition of mTOR action (Cheng *et al.* 2004).

In the same spirit, our results also show that the mTORC1/p70S6K pathway was significantly activated in parallel to Nox4 upregulation and AMPK inhibition. Treatment with metformin, an AMPK activator, or rapamycin, an mTORC1 inhibitor, reduced ROS production, Nox4 expression, and NADPH oxidase activity. OGG1 levels were restored while 8-oxodG concentrations shied away upon administration of the above treatments both *in vivo* and *in vitro*. In line with these observations, extensive data from our group have implicated mTORC1/Nox4 axis as a key player in the onset and development of diabetic nephropathy and neuropathy (Eid et al., 2013; Eid et al., 2016, Eid et al., unpublished data). In fact, numerous studies have suggested that inhibition of the mTORC1 pathway with Rapamycin significantly reduces NADPH-oxidase dependent oxidative stress which may have protective effects on the diabetic kidney in both type 1 and type 2 diabetic animal models (Eid et al., 2013; Lloberas et al., 2006; Yang et al., 2007). Rapamycin treatment has also been shown to attenuate oxidative stress and hamper cardiac dysfunction in type 2 diabetic mice (Das et al., 2014). In regards to cancer, numerous studies have reported an inhibition of the AMPK pathway and activation of the mTOR pathway in several types of cancer (Schneidar et al., 2008, Chiang et al., 2010, Tam et al., 2009, Shaw RJ, et al. 2004) and CRC in particular (Sugiyama et al., 2009, Faubert et al., 2014, Farhat et al., 2012). Interestingly, both Aspirin and Adiponectin have been reported to aid in the attenuation of CRC

and cancer cell growth via the AMPK and mTOR duo (Farhat et al., 2012, Sugiyama et al., 2009). Taken together, these findings highlight the role for the AMPK/mTORC1 pathway in CRC progression and tumorigenesis in colon cancer cells (HT-29 and Caco2) and APC mice keeping in mind that both HG and insulin have been extensively linked to the AMPK/TSC2 pathway. In fact, AMPK/TSC2 activation is altered by HG-induced ROS production (Eid et al., 2010). Additionally, insulin is currently emerging to be involved in the AMPK pathway through a mediating pathway, the PI3K/AKT pathway (Hawley et al., 2014), knowing that AMPK activation has been shown to enhance insulin uptake by the muscles and endothelial cells and reverse oxidative injury (Ruderman et al., 2003). Extensive studies have in fact, convicted AMPK at least partially responsible for tumorigenesis of several cancers (Park et al., 2006; Kim et al., 2012; Hardie, D. G., 2013; Yue et al., 2014; Sui et al., 2014; Faubert et al., 2015) through its ability to mediate cell survival and tumorigenesis, via mTORC1 and TSC2 (Eid et al., 2013). We emphasize the mechanism of action to encompass the NADPH-induced ROS, and specifically Nox4-induced ROS (Eid et al., 2010, Eid et al., 2013; Eid et al., 2016, Eid et al., unpublished data).

Briefly, our findings uncover a novel role for Nox4-induced ROS in modifying DNA damage/repair pathway as well as cancer cell behavior through the AMPK/mTORC1 pathway which results in the accumulation of 8-oxodG and exacerbates cancer cell aggressiveness that altogether contribute to the genomic instability and predisposition to cancer in patients with diabetes. A study conducted by Sui et al suggested a joint therapy of AICAR, an AMPK activator, and 5-Flourouracil (5-FU), a chemotherapeutic agent, for CRC due to the fact that AICAR treatment exacerbated the anti-cancer potential of 5-FU in colon cancer cells (Sui et al., 2014). Hence, perhaps a drug combination of Nox4 inhibitors, and/or AMPK activators along

with the standard anti-cancer agent may be of therapeutic relevance in addition to glycemic control to alleviate cancer progression in type 1 diabetes. Along these lines, another therapeutic strategy that maybe particularly beneficial deduced from this study is the combination of mTORC1 and Nox inhibitors and AMPK activators. An interesting approach worth combining is the inclusion of poly-ADP ribose polymerase (PARP) inhibitors. Briefly, PARP is known to be a mechanism involved in DNA repair. Studies have proven its role in mediating carcinogenesis however; this contribution is dependent on factors such as inflammation or genetic instability. With regards to diabetes, and having established the biological impact of hyperglycemia-induced CRC aggressivity on OGG1 DNA repair activity, PARP seems to be another aspect of genetic material injury worthy of investigation. In fact, studies in CRC-prone genetically modified animals showed an elevated risk of tumorigenesis concurrent with PARP activity double knock-out, by contrast to a reduced burden in knock-down PARP heterozygote animals (Tarhuni et al., 2016). Thus, this is suggestive of a protective contribution of PARP to the genetic etiology of CRC. Speculating from this standpoint, our type 1 diabetic animal models mimic a CRC-prone genotype. Thus, the administration of PARP inhibitors is expected to reduce the diabetes-induced CRC aggressivity. More importantly, inflammation-mediated CRC onset was demonstrated to occur via PARP overexpression (Tarhuni et al., 2016). Thus, PARP inhibition in type 2 diabetic animals may also be a possible therapeutic strategy. As a closing remark, we can deduce that diabetes and CRC are the mechanistic troubling duo with complex interactions from both players. Additional studies are a necessity to better comprehend their interplay and to devise therapeutic targets of promise.

Perspectives

This study opens future perspectives that include a series of rigorous testing on cells, animals and human samples.

Human Colon Biopsy Analysis

We have recently established a collaborative effort with our associates at AUB-MC; Prof. Walid Faraj and his team, who have been collecting human colon tissue biopsies of patients (diabetic or non-diabetic) with CRC for the past 4 years. Examining NADPH oxidases, Nox4, AMPK and mTOR alteration in colon tissues taken from diabetic patients at different stages and severity of the disease and comparing them to those of non-diabetic patients is a critical step to progress further in this project. Therefore, in order to assess the relevance of our *in vivo* data in humans, we will analyze the expression levels of Nox4, AMPK and mTOR and compare our findings with our previously attained *in vivo* results. Second, we will perform, along with Prof. Ghassan Abou Alfa's team in Memorial Hospital (NY), full genomic and proteomic testing on the attained tissues, combined with biopsies from their own tissue bank to possibly pinpoint a predictor of CRC progression in type 1 and type 2 diabetic patients that may be used as a clinical biomarker for early detection of the disease.

Pharmacological In Vivo Investigations

It would be of great interest to investigate the effect of GKT137831, a specific Nox1/ Nox4 inhibitor, *in vivo* in type 1 as well as type 2 diabetic mice to contemplate its therapeutic potential and the possibility of a complication-reversal benefit. In fact, this dual Nox1/ Nox4 inhibitor has gained considerable attention because of its ability to alleviate disease severity in diabetic peripheral neuropathy, as previously shown by our group (Eid et al., unpublished work), as well

as in pre-clinical settings (Gorin et al., 2015; Sedeek et al., 2013; Jiang et al., 2012; Wiesel et al., 2012). The utilization of GKT as a specific Nox1/Nox4 inhibitor will allow us to examine the extent to which Nox4/AMPK/mTOR pathway affects biochemical and morphological defects brought about by diabetes in colon cancer.

AMPK, Nox4 and mTORC1 in Type 2 Diabetes

It is now widely accepted that oxidative stress is the common feature to colon cancer progression and pathogenesis in type 1 and type 2 diabetes. In order to test if the signals' alterations observed in type 1 diabetes-induced CRC progression follow "one size fits all" approach, we aim to reproduce our previous findings in type 2 diabetic models. In fact, our group is currently working on the cross breeding of APC mice with MKR non obese type 2 diabetic mice to generate pure breeds of a type 2 diabetic mouse model with colorectal cancer. An examination of Nox4 alteration and its crosstalk with AMPK and mTORC1 in the colons of these mice is of major importance. Moreover, it is well known that metabolic perturbations are more complex and pronounced in female animal models of diabetes due to the complex hormonal and endocrinological influence on female metabolisms. In fact, our preliminary data showed that the colons of female APC mice exhibited a higher polyp number relative to the male littermates. Consequently, we aim to extend our investigation within this context to better comprehend diabetes-induced cancer progression in the distinct female model.

Ex Vivo Studies

Due to the prominent role of Tuberin (TSC) in downregulating the mTOR/p70S6K pathway, it would be of great interest to replicate our results in primary cultures of colon epithelial cells isolated from colons of AMPK 1/2 knock out mice or their control littermates and in colon

epithelial cells isolated from colons of TSC2^{+/-} or their control TSC2^{+/+}. This will help validate the cross talk between AMPK/TSC/mTOR pathways in colon cancer as previously validated in diabetic nephropathy.

Thus the listed initiatives are essential to fully fathom diabetes-induced cancer tumorigenesis.

CONCLUSION

Although the contribution of oxidative stress to diabetic complications is established, antioxidant treatment alone does not result in complete protection against diabetes induced-cancer progression and the total blockade of ROS can be in some cases deleterious (De Zeeuw et al., 2013). Subsequently, one of the major aims of this thesis project was to identify the specific cellular sources of ROS that are upregulated in diabetes-induced colorectal cancer progression and determine the involved signaling pathways culminating in exacerbated colorectal cancer aggressiveness.

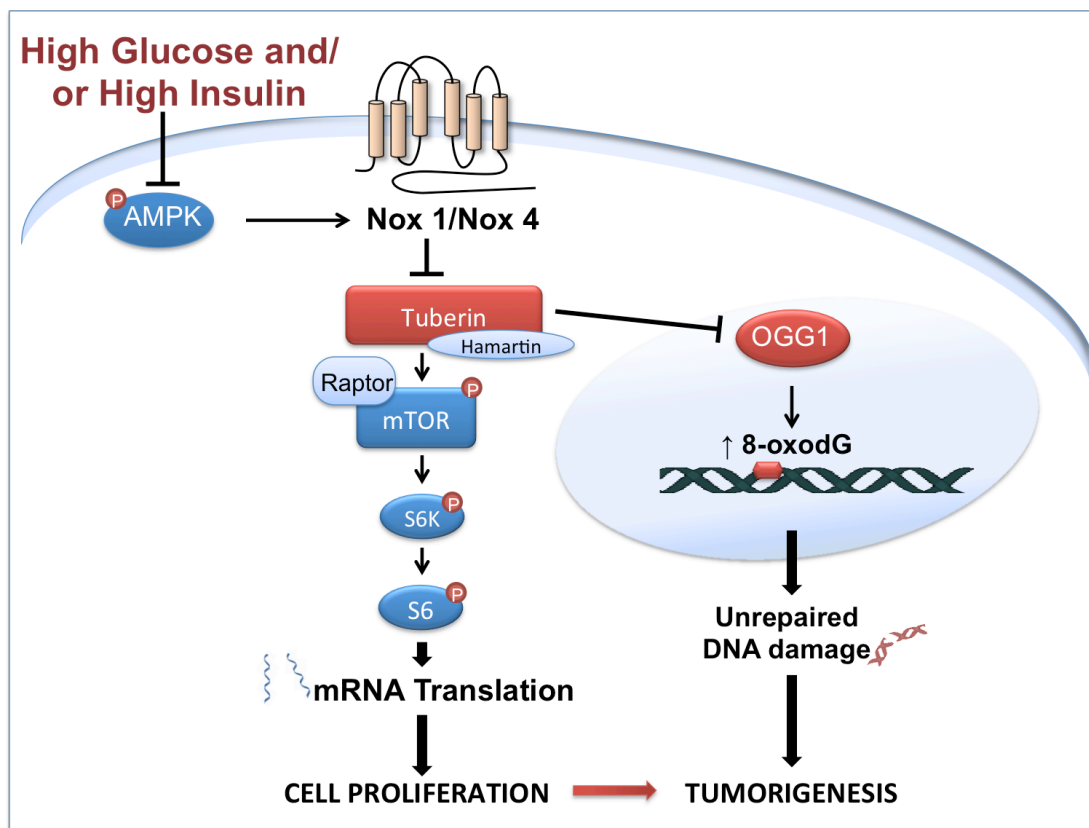


Figure 21. Proposed mechanism of diabetes-induced colon cancer progression. Diabetes leads to an upregulation of Nox4 through downregulation of AMPK activity. This leads to an inhibition of Tuberin and thus an activation of the mTORC1/p70S6K pathway resulting in increased cellular proliferation and aggressiveness. Tuberin inactivation also inhibits OGG1 activity leading to accumulation of DNA damage further contributing to tumorigenesis.

To our knowledge, in this study, we identify for the first time a previously unrecognized direct cellular target, Nox4, as a potential therapeutic target for colon adenocarcinoma cell-aggressiveness. Moreover, the progression of the injury was shown to involve AMPK and mTOR pathways suggesting that AMPK activators, or mTORC1 inhibitors may be therapeutically promising, in addition to glycemic control, to alleviate diabetes-induced CRC progression in type 1 diabetes. These findings expand our understanding of the molecular and cellular mechanisms brought about by diabetes-induced CRC progression and pave the way for future investigation toward a complete understanding of the disease.

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PUBLICATIONS

1. **Mohsen FA**, Boutary S, Eid AH, and Eid AA. Colorectal Cancer Risk in Diabetes: Double The Trouble In Metabolic Diseases. Minor Revision Re-Submitted to Oncogenesis. November 18th, 2017.

Abstract of the work

Diabetes is a chronic systemic malfunction characterized by persistent metabolic disturbances that culminate in a high rate of micro- and macrovascular events to which cancer was recently annexed. In fact, diabetes inflates colorectal cancer (CRC) risk by 1.2-1.5 folds leaving the patients with increased aggressiveness and poorer 5-year survival. The mechanisms that contribute to the onset and development of these complications are still poorly defined even though they are known to orbit reactive oxygen species (ROS) as a common origin. CRC progress has been linked to hyperglycemia and hyperinsulinemia. However, the cellular and molecular pathways involved in diabetes-induced CRC progression are not well understood. In this study, we assessed cancer cell aggressiveness in human colon epithelial adenocarcinoma cells placed in a diabetic milieu and CRC progression in STZ- induced type 1 diabetic APC mice. Our results show that AMPK/mTORC1 pathway is deregulated in both diabetes and CRC. This was paralleled by an elevation in the expression of the NADPH oxidase Nox4 leading to an increase in ROS production. Furthermore, our results show that oxidative stress, secondary to alteration in the level and activity of the NADPH oxidase Nox4 is augmented in diabetes and contributes to the progression of CRC. The resulting oxidative stress further led to an alteration in the signaling of the AMPK/mTORC1 pathways culminating in an exacerbated aggressiveness in cancer cell behavior and colon polyp formation. From a fundamental perspective, our project allows the identification of novel molecular mechanisms involved in diabetes-induced CRC progression and from the clinical angle it sets the pace for development of effective therapeutic strategies to reverse or slow the progression of CRC in diabetic patients.

2. Bouhadir KH, Koubeissi A, **Mohsen FA**, El-Harakeh MD, Cheaib R, Younes J, Azzi G, Eid AA. Novel carbocyclic nucleoside analogs suppress glomerular mesangial cells proliferation and matrix protein accumulation through ROS-dependent mechanism in the diabetic milieu. II. Acylhydrazone-functionalized pyrimidines. *Bioorg Med Chem Lett.* 1;26(3):1020-4, 2016.

Abstract of the work

We report herein the synthesis of a novel series of carbocyclic acylhydrazone derivatives of uracil, thymine and cytosine from the corresponding nucleic bases and their biological activity to treat diabetic nephropathy. Intriguingly, five derivatives significantly reduced high-glucose induced glomerular mesangial cells proliferation and matrix protein accumulation in vitro. The

anti-oxidative effects displayed by these molecules suggest that their activity might involve a ROS-dependent mechanism.

Résumé

Le diabète est une dérégulation systémique chronique caractérisée par des perturbations métaboliques permanentes à l'origine de nombreuses complications, y compris le cancer. Le diabète augmente le risque du cancer colorectal (CRC) de 1,2 à 1,5 fois. Cependant, les voies moléculaires et cellulaires en jeu ne sont pas assez élucidées. Nos résultats témoignent d'une dérégulation de la voie AMPK/mTORC1 dans le diabète et le CRC avec une surexpression de la NADPH oxydase Nox4, augmentant ainsi la production de ROS. Ceci provoque un stress oxydatif qui s'élève en cas de diabète et contribue à la progression du CRC. De plus, nos résultats montrent que ce stress induit une altération de la voie de signalisation AMPK/mTORC1, aboutissant à une agressivité accrue du comportement des cellules cancéreuses du côlon et de la formation de polypes. Notre projet permet, d'une part, d'identifier de nouveaux mécanismes moléculaires impliqués dans la progression du CRC induite par le diabète et d'autre part, de développer des stratégies thérapeutiques efficaces pour inverser la progression du CRC chez les patients diabétiques.

Mots clés : Diabète, CRC, ROS, AMPK

Résumé en anglais

Diabetes is a chronic systemic malfunction characterized by persistent metabolic disturbances that culminate in a high rate of complications to which cancer was recently annexed. In fact, diabetes inflates colorectal cancer (CRC) risk by 1.2-1.5 folds. However, the cellular and molecular pathways involved are not well understood. Our results show that AMPK/mTORC1 pathway is deregulated in both diabetes and CRC. This was paralleled by an elevation in the expression of the NADPH oxidase Nox4 leading to an increase in ROS production. Furthermore, our results show that oxidative stress, secondary to alteration in the level and activity of Nox4 is augmented in diabetes and contributes to the progression of CRC. The resulting oxidative stress further led to an alteration in the signaling of the AMPK/mTORC1 pathways culminating in an exacerbated aggressiveness in cancer cell behavior and colon polyp formation. Our

project allows the identification of novel molecular mechanisms involved in diabetes-induced CRC progression and development of effective therapeutic strategies to reverse the progression of CRC in diabetic patients.

Keywords : Diabetes, CRC, ROS, AMPK