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Unmasking the interplay between mTOR and Nox4: novel insights into the mechanism connecting diabetes and cancer

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ABSTRACT: Cancer was recently annexed to diabetic complications. Furthermore, recent studies suggest that cancer can increase the risk of diabetes. Consequently, diabetes and cancer share many risk factors, but the cellular and molecular pathways correlating diabetes and colon and rectal cancer (CRC) remain far from understood. In this study, we assess the effect of hyperglycemia on cancer cell aggressiveness in human colon epithelial adenocarcinoma cells *in vitro* and in an experimental animal model of CRC. Our results show that Nox (NADPH oxidase enzyme) 4–induced reactive oxygen species (ROS) production is deregulated in both diabetes and CRC. This is paralleled by inactivation of the AMPK and activation of the mammalian target of rapamycin (mTOR) C1 signaling pathways, resulting in 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) accumulation, induction of DNA damage, and exacerbation of cancer cell aggressiveness, thus contributing to the genomic instability and predisposition to increased tumorigenesis in the diabetic milieu. Pharmacologic activation of AMPK, inhibition of mTORC1, or blockade of Nox4 reduce ROS production, restore the homeostatic signaling of 8-oxoguanine DNA glycosylase/8-oxodG, and lessen the progression of CRC malignancy in a diabetic milieu. Taken together, our results identify the AMPK/mTORC1/Nox4 signaling axis as a molecular switch correlating diabetes and CRC. Modulating this pathway may be a strategic target of therapeutic potential aimed at reversing or slowing the progression of CRC in patients with or without diabetes.—Mroueh, F. M., Noureldein, M., Zeidan, Y. H., Boutary, S., Irani, S. A. M., Eid, S., Haddad, M., Barakat, R., Harb, F., Costantine, J., Kanj, R., Sauleau, E.-A., Ouhtit, A., Azar, S. T., Eid, A. H., Eid, A. A. Unmasking the interplay between mTOR and Nox4: novel insights into the mechanism connecting diabetes and cancer. FASEB J. 33, 14051–14066 (2019). www.fasebj.org

KEY WORDS: colorectal cancer · NADPH oxidases · mTORC1 · DNA damage

Diabetes and cancer are prevalent diseases whose incidence rates are increasing worldwide owing to poor lifestyle practices. Epidemiologic studies show that subjects with diabetes are at a significantly higher risk of developing many forms of cancer (1–6). In addition to the development of pancreatic and breast cancer, the incidence of colon and rectal cancers (CRCs) (5, 7, 8) is increased in patients with diabetes.

ABBREVIATIONS: 8-oxo-dG, 8-oxo-2′-deoxyguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; APC, C57BL/6-*Apc*^{tm1Tyj}/J; Cas9, CRISPR-associated protein 9; CRC, colon and rectal cancer; CRISPR, clustered regularly interspaced short palindromic repeats; DHE, dihydroethidium; GKT, 2-(2-chlorophenyl)-4-[3-(dimethylamino)phenyl]-5-methyl-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione; HG, hyperglycemia; mTOR, mammalian target of rapamycin; NG, normal glucose; Nox, NADPH oxidase enzyme; OGG1, 8-oxoguanine DNA glycosylase; p70S6K, ribosomal protein S6 kinase-β1; ROS, reactive oxygen species; STZ, streptozotocin; T1DM, type 1 diabetes mellitus

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Although diabetes (and especially type 2 diabetes) and cancer share many risk factors, the biologic links between the 2 diseases are poorly characterized. Diabetes may influence the neoplastic process through multiple mechanisms, including hyperglycemia (HG), insulin resistance, and hyperinsulinemia. Although most of the studies highlight the role of insulin as a risk factor for cancer progression in diabetes (9–11), little is known about the role and the mechanisms by which HG increases the risk of oncogenesis in diabetes. Several reports associate HG with the onset and progression of CRC (12). In fact, increased levels of glycated hemoglobin were described to be an independent predictor of CRC aggressiveness in patients with diabetes (12). Furthermore, it is described that elevated glucose levels in unfed conditions and diabetes are independent risk factors for the development of cancer in several tissues, including colon cancer (13). However, in these studies the mechanisms correlating HG to CRC onset and development were not described.

Chronic comorbidities, including diabetes, are important characteristics that affect patients with cancer. Despite the limited body of evidence, several studies suggest that cancer can increase the risk of the onset and development of diabetes (14–21). Patients with CRC showed a higher incidence of subsequent diabetes than did individuals without CRC for up to 5 yr after diagnosis (14). Furthermore, in survivors of breast cancer, there was an increase in the incidence of diabetes among women who were postmenopausal that varied over time. In most women the risk began to increase 2 yr after cancer diagnosis (20). Along the same lines, a recent study performed in a Korean general population cohort of 524,089 men and women who were observed for up to 10 yr and developed cancer showed a clear increase in the subsequent risk of diabetes independent of traditional diabetes risk factors (15).

Taken together, these studies point to a high association between diabetes and cancer. However, our understanding of the interplay between the 2 diseases is still fundamental.

Reactive oxygen species (ROS) have evolved as major players in health and diseases (22–27). It is proposed that ROS play a crucial role in the progression and severity of diabetes and are involved in the etiology and progression of multistage carcinogenesis (22–34). Alongside the mitochondria, NADPH oxidase enzymes (Noxes) represent a leading source of ROS (31, 32). NADPH oxidases influence pathways involved in cell and tissue growth, cell signaling, autophagy, and apoptosis (31–33). However, our understanding of the roles of the Noxes in the interplay between diabetes and cancer and in regulating several signaling pathways is limited.

One of the ultimate effects of ROS production is increased DNA damage. ROS induce 8-hydroxylation of guanine bases, resulting in the production of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). The latter reflects the intracellular effects of ROS and is considered the major indicator of DNA oxidation because of its high mutagenic potential (35). In the context of CRC, lower levels of 8-oxodG were described to be associated with longer survival (36). Similarly, lower levels of 8-oxodG in urine were reported to be a good prognostic marker in patients undergoing radiotherapy (37).

The enzyme that recognizes and excises 8-oxodG from cellular genetic material is 8-oxoguanine DNA glycosylase (OGG1); however, there is little information on the cellular and molecular mechanisms of OGG1 regulation (38). Loss of heterozygosity at the *OGG1* allele leads to loss of *OGG1* function, which in turn contributes to tumorigenesis (39). Actually, the *OGG1* gene is found somatically mutated in certain cancers (39–43) and is highly polymorphic among humans. However, the impact of *OGG1* polymorphisms remains poorly investigated.

Our group and others have previously determined that the energy sensor AMPK is a novel upstream regulator of Noxes protein expression and activity (23, 26, 44, 45). AMPK is a known target for the management of metabolic syndrome, yet recent roles have emerged for it as a possible treatment for diabetic complications (23, 46, 47), a metabolic tumor suppressor, and a target for cancer prevention and treatment (47, 48). These functions are achieved by restoring the homeostatic function of several biologic pathways, like autophagy, cellular proliferation, cell survival, and others (23, 46–48). Despite the scientific advances in understanding AMPK signaling, the direct relationship between AMPK activation, Nox4 alteration in diabetes-induced tumorigenesis, or in cancer stimulating the onset of diabetes in particular has yet to be established.

The AMPK pathway has been reported to negatively regulate the mammalian target of rapamycin (mTOR), which plays a pivotal role in cell growth, cell proliferation, cell motility, cell survival, and protein synthesis as well as transcription in response to hormones, growth factors, nutrients, energy, and stress signals (49, 50). mTOR is a serine/threonine protein kinase existing in 2 multiprotein distinct complexes, mTORC1 and mTORC2 (50). mTORC1 activity is negatively regulated by the heterodimeric complex consisting of tuberin and hamartin, which in turn are regulated by the AMPK signaling pathway (50). Our work and that of others demonstrate that deregulated mTORC1 signaling is implicated in the progression of cancer and diabetes (49, 50). Although many inputs to mTORC1 have now been characterized, the role of mTORC1 in connecting colon tumorigenesis and diabetes remains largely unclear.

This study was undertaken to determine the underlying mechanisms that associate diabetes to CRC. The effect of HG in promoting CRC malignancy in diabetes is investigated in addition to the interplay between Nox4, AMPK, and the mTORC1 pathway to identify potential targets that may reduce or inhibit the onset and progression of CRC in a high-glucose or diabetic milieu.

MATERIALS AND METHODS

Animal studies

All animal procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA) and were approved by the Institutional Animal Care and Use Committee at the American University of Beirut. C57BL/6-*Apc*^{tm1Tyj}/J (APC) 6–8-wk-old mice, the model of spontaneous colon cancer (51) (The Jackson Laboratory, Bar Harbor, ME, USA), and C57BL/6J male mice (control) weighing around 23–25 g were divided as follows: C57BL/6J mice (control), C57BL/6J mice

that received up to 5 consecutive 50 mg/kg body weight intraperitoneal injections of streptozotocin (STZ) (Diabetic Complications Consortium protocol; *www.diacomp.org*) (52), and APC mice that received up to 5 consecutive 50 mg/kg body weight intraperitoneal injections of STZ (APC-diabetic). The C57BL/6J and APC mice received similar injections of citrate buffer. Glucose measurement was performed *via* tail vein punctures using an Accucheck glucometer (Roche, Basel, Switzerland). Mice with fasting blood glucose ≥250 mg/dl were considered diabetic. After the onset of diabetes, animals were either intraperitoneally given metformin (150 mg/kg) or rapamycin (0.5 mg/kg) daily or treated by oral gavage with 40 mg/kg of 2-(2-chlorophenyl)-4-[3-(dimethylamino)phenyl]-5-methyl-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione (GKT), an orally bioavailable low toxicity compound acting as an inhibitor of NADPH oxidase isoforms 1 and 4 (Cayman Chemicals, Ann Arbor, MI, USA). All treatments were administered for 8 wk. After euthanasia, colons were collected and cleaned, and the numbers and size of the polyps were assessed. Furthermore, part of the colon was either fixed or frozen for histologic molecular and biochemical assessment.

Cell culture and transfection

The human colon carcinoma cell lines HT-29 and CaCo2 were cultured in 5 mM glucose [normal glucose (NG)] or treated with 25 mM glucose (HG) for 72 h in the presence or absence of 5 mM metformin or 25 nM rapamycin or their combination. In parallel experiments, CaCo2 cells cultured in 25 mM of glucose were treated with the direct AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (1 mM) (53). For the *in vitro* knockout experiments, a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system for Nox4 was used (Santa Cruz Biotechnology, Dallas, TX, USA). CRISPR (0.1 μg/μl) was introduced into the cells using Lipofectamine CRISPRMax Cas9 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Control CRISPR (nontargeting CRISPR, 0.1 μg/μl) was used as a negative control.

mRNA analysis

mRNA was analyzed by real-time RT-PCR as previously described in refs. 22–25 and 34, and human and mice RT^2 quantitative PCR primers (Qiagen, Germantown, MD, USA) of the corresponding gene of interest were used.

Western blot analysis

Homogenates from cells and colon tissues were prepared, and Western blotting was performed and analyzed as previously described in refs. 22–25 and 34. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), except for the Nox4 (Santa Cruz Biotechnology).

NADPH oxidase activity

NADPH oxidase activity was measured in cells grown in serum-free medium or in colon tissues homogenates as previously described in refs. 22–25 and 34.

ROS detection

Cellular H_2O_2 in colorectal tissues was assessed by dihydroethidium (DHE) fluorescent intensity and visualized on confocal laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and by HPLC analysis (54).

Cellular proliferation

Cellular proliferation was assessed by MTT Cell Proliferation Kit I (Roche Life Science, Penzberg, Germany) according to the manufacturer's protocol.

Wound healing assay

CaCO-2 or HT-29 cells were seeded in 60-mm dishes (Corning, Corning, NY, USA). Once a confluent monolayer formed, a scratch was made with a yellow tip (200 μl). After changing the medium, images of cell movement at different magnifications were captured using a digital camera mounted on a light microscope (Carl Zeiss) before and after the 72-h treatment (55).

Cell invasion assay

Invasion assay was performed using the Corning BioCoat Matrigel Invasion Chamber with Corning Matrigel Matrix following the manufacturer's instructions.

8-Oxo-2′-deoxyguanosine concentration assay

An HT 8-oxo-2′-deoxyguanosine (8-oxo-dG) ELISA Kit II (Trevigen, Gaithersburg, MD, USA) was used to quantify the level of 8-hydroxy-2′-deoxyguanosine in DNA following the manufacturer's instructions.

Statistical analysis

Results are expressed as means or percentages of the control ± SE. Statistical significance was assessed by 1-way ANOVA, followed by Tukey's posttest when more than 2 variables were analyzed. Two group comparisons were performed by a Student's t test. Statistical significance was determined as $P < 0.05$.

RESULTS

HG-induced Nox4-dependent ROS production alters CaCo2 and HT-29 phenotypes through an AMPK/mTOR signaling pathway

We have previously highlighted that diabetes or cancer induces oxidative stress through a Nox4-dependent mechanism (22–24, 56). We investigated the impact of HG on CaCo2 and HT-29 cell migration, proliferation, invasion, and matrix accumulation (**Fig. 1*A*–*E*** and Supplemental Fig. S1*A*–*E*). Our data show that the adenocarcinomas cell lines increase their migratory phenotype when exposed to 72 h of HG (Fig. 1*A*, *B*, and Supplemental Fig. S1*A*, *B*). Additionally, HG increases cellular proliferation rate (Fig. 1*C* and Supplemental Fig. S1*C*) and the potential of these cells to invade the reconstituted basement membrane (Fig. 1*D* and Supplemental Fig. S1*D*). This was also accompanied by an increase in fibronectin expression known to reflect the extent of migration, invasion, and metastasis of cancer cells, including CRC cells (57) (Fig. 1*E* and Supplemental Fig. S1*E*). Notably, our results show that HG treatment induces AMPK inactivation (decreases in the AMPK phosphorylation on

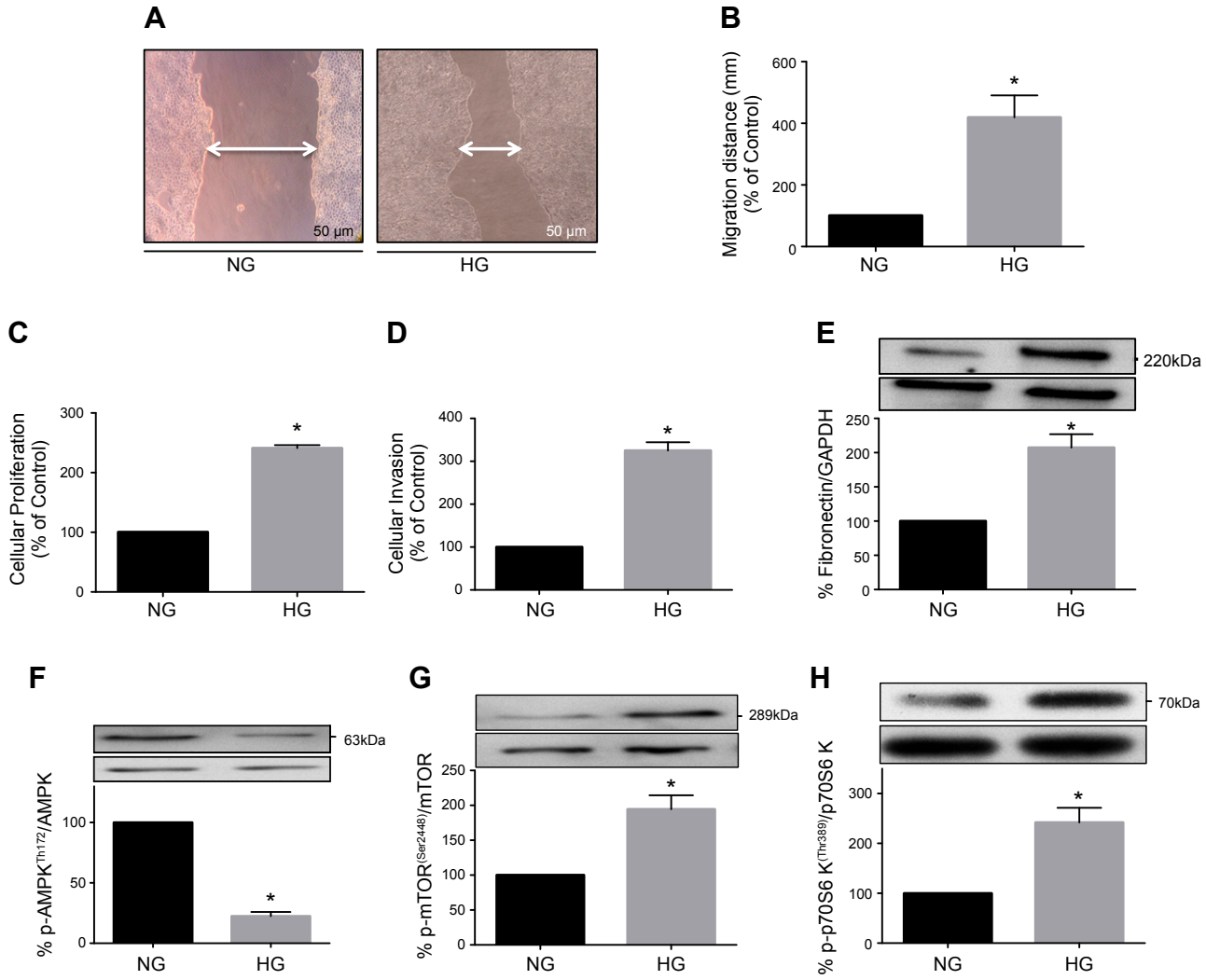

Figure 1. High glucose enhances colon cancer cell migration, proliferation, invasion, and extracellular matrix accumulation. CaCo2 cells were exposed to NG (5 mM) or HG (25 mM) for 72 h. *A*) Representative figures of the migration assay performed on CaCo2 cells. *B*) Bar graph of the migration assay for CaCo2. *C*) MTT proliferation assay for CaCo2 cells. *D*) Cellular invasion assay assessing the number of CaCo2 cells that invade a porous membrane. *E*) Bar graph showing quantitation of fibronectin/GAPDH (%). *F*) Bar graph showing quantitation of AMPK^{Thr172}/AMPK (%). *G*) Bar graph showing quantitation of p-mTORC1^{Ser2448}/mTORC1 (%). *H*) Bar graph showing quantitation of p-p70S6K^{Thr389}/p70S6K (%). All values are the means ± SEM from at least 4 independent experiments. *$P < 0.05$ *vs.* NG.

Thr^{172}) in CaCo2 and HT-29 (Fig. 1*F* and Supplemental Fig. S1*F*) and activates the mTORC1/p70S6 kinase pathway evident by the increased phosphorylation on Ser^{2448} and Thr^{389}, respectively (Fig. 1*G*, *H* and Supplemental Fig. S1*G*, *H*). In all our culture experiments, mannitol was used as an osmotic control and did not show any effect.

Activation of AMPK or inhibition of mTOR alleviates HG-induced epithelial adenocarcinoma cells proliferation, migration, and invasion and reverses DNA damage in CaCo2 and HT-29

In order to investigate if the AMPK/mTOR deregulation correlates with HG-induced epithelial adenocarcinoma cells proliferation, migration, and invasion, CaCo2 and HT-29 were treated with HG in the presence or absence of the AMPK activator, metformin, and the mTORC1 inhibitor, rapamycin, or their combination. Our results show that in both cell lines, the dose of metformin used was able to activate the AMPK pathway, as assessed by the increased phosphorylation on phosphorylated (p)-AMPK^{Thr172} (**Fig. 2*A***and Supplemental Fig. S2*A*), and inhibited the mTORC1/ribosomal protein S6 kinase-β1 (p70S6K) pathway, as assessed by the decreased phosphorylation of p-mTORC1^{Ser2448} and p-p70S6K^{Thr389} (Fig. 2*B*, *C* and Supplemental Fig. S2*B*, *C*). Rapamycin treatment had no effect on AMPK phosphorylation (Fig. 2*A* and Supplemental Fig. S2*A*) but reduced HG-induced p-mTORC1^{Ser2448} and p-p70S6K^{Thr389} expression in CaCo2 and HT-29 cells (Fig. 2*B*, *C* and Supplemental Fig. 2*B*, *C*), suggesting that mTOR acts downstream of AMPK.

Furthermore, our results show that treatment with metformin and rapamycin, significantly alleviates HG-induced migration, proliferation, and invasion of CaCo2 and HT-29 (Fig. 2*D*–*H* and Supplemental Fig.

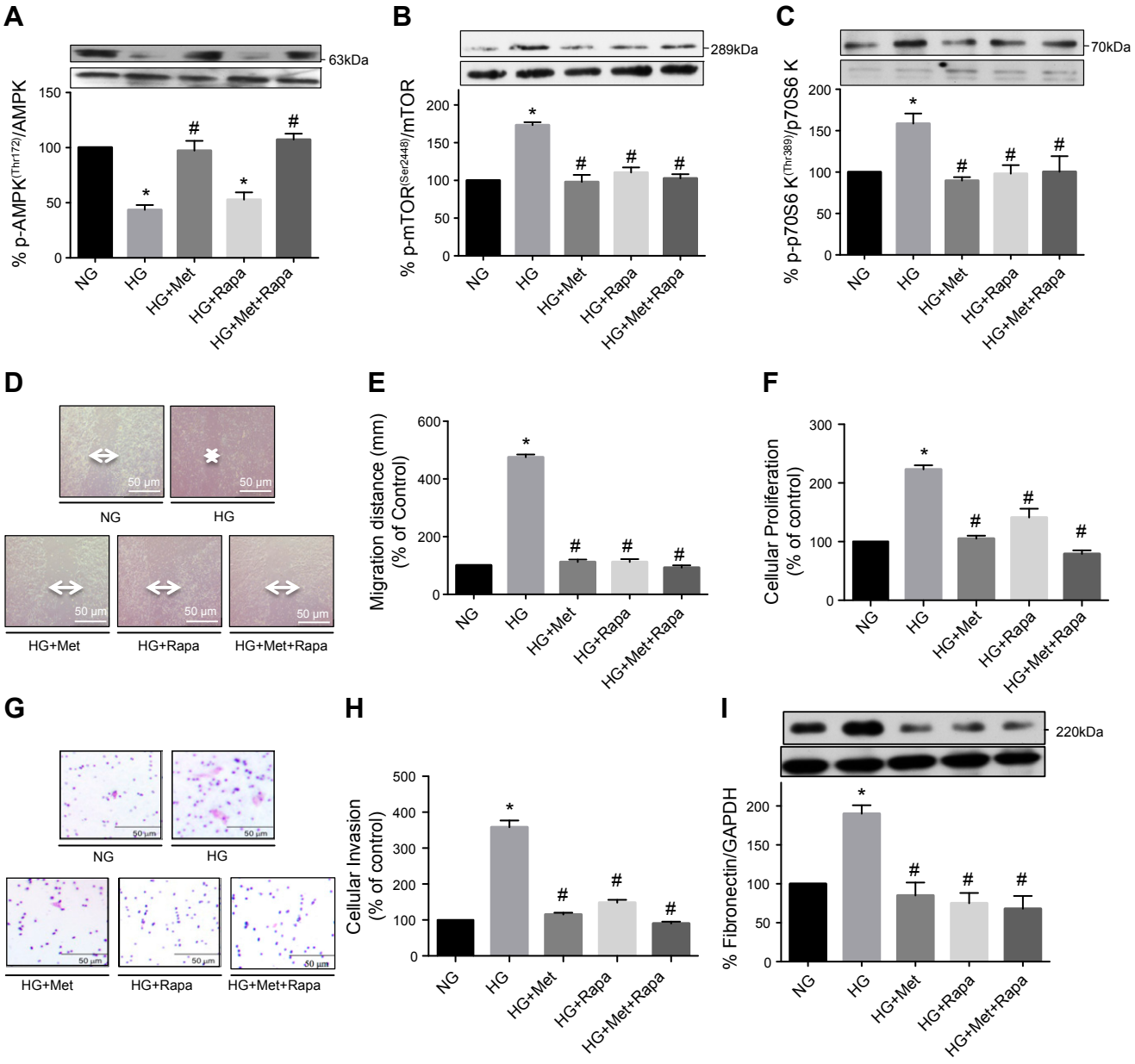

Figure 2. Activation of AMPK using metformin and inhibition of mTOR reduce cancer cell injury and 8-oxodG production. CaCo2 cells were treated with HG (25 mM) alone or with AMPK activators, metformin (5 mM) or rapamycin (25 nM) or a combination of both drugs for 72 h. *A*) Bar graph showing quantitation of $AMPK^{Thr172}$/AMPK (%). *B*) Bar graph showing quantitation of p-$mTORC1^{Ser2448}$/mTORC1 (%). *C*) Bar graph showing quantitation of p-$p70S6K^{Thr389}$/p70S6K (%). *D*) Representative figures of the migration assay performed on CaCo2 cells. *E*) Bar graph of the migration assay for CaCo2. *F*) MTT proliferation assay for CaCo2 cells. *G*) Representative figures of the Matrigel invasion assay performed on CaCo2 cells. *H*) Cellular invasion assay assessing the number of CaCo2 cells that invade a porous membrane. *I*) Bar graph showing quantitation of fibronectin/GAPDH (%). *J*) Bar graph showing quantitation of OGG1/GAPDH (%). *K*) Bar graph showing 8-oxo-dG accumulation. *L*) Superoxide anion production measured using HPLC. *M*) NADPH oxidase activity assay in CaCo2 cells measured using the Lucigenin assay. *N*) Relative mRNA amount of Nox4/GAPDH (%), (*O*) Bar graph showing quantitation of NOX4/GAPDH (%). EOH, 2-hydroxyethidium; Met, metformin; Rapa, rapamycin; RLU, relative light unit. All values are the means ± SEM from at least 4 independent experiments. $^{*}P < 0.05$ *vs.* NG, $^{\#}P < 0.05$ *vs.* HG.

S2*D–H*) concomitant with a decline in fibronectin expression (Fig. 2*I* and Supplemental Fig. S2*I*), restitution of OGG1 protein expression, and attenuation in 8-oxodG concentrations in both cell lines (Fig. 2*J, K* and Supplemental Fig. S2*J, K*). However, neither additive nor synergistic effects are seen alongside the use of the combination treatment (Fig. 2*A–K* and Supplemental Fig. S2*A–K*).

Next, we assessed whether AMPK/mTOR signaling impacted the adenocarcinoma epithelial cells–enhanced malignancy through Nox4 in the diabetic milieu. Our results show that activation of AMPK or inhibition of mTOR reduces HG-induced ROS production in CaCo2 and HT-29 cells as well as NAPDH oxidase enzyme activity and Nox4 mRNA levels and protein expression (Fig. 2*L–O* and Supplemental Fig. S2*L–O*). Metformin activates

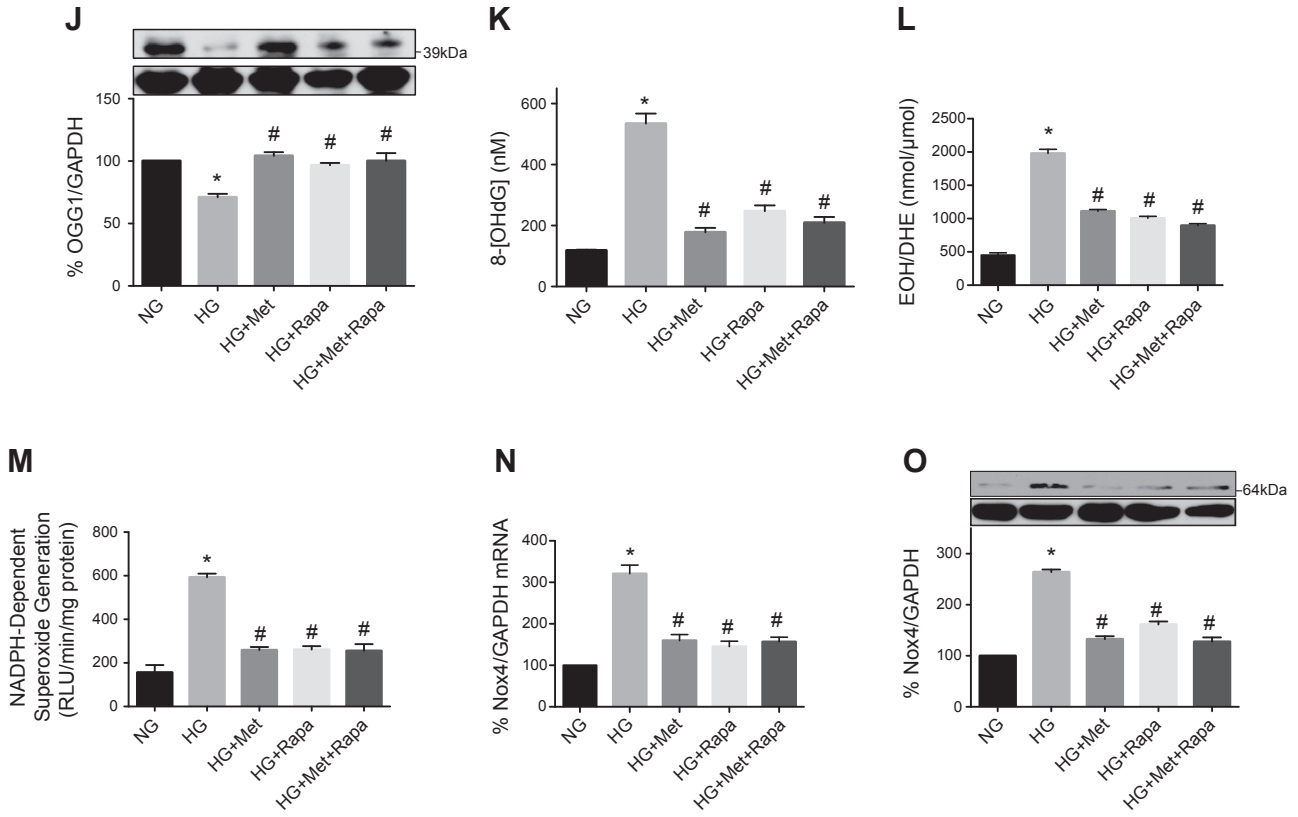

Figure 2. (*Continued*)

AMPK through a direct or indirect manner either by increasing the net phosphorylation of the AMPK catalytic α subunit at Thr^{172} with subsequent activation of AMPK activity or by inhibiting the complex 1 of the mitochondrial respiratory chain, which results in an increase in the AMP/ATP or ADP/ATP ratio, leading to AMPK activation through the binding of either AMP or ADP to AMPK (58). However, the role of a direct AMPK activator was not investigated. To that end, we used AICAR, which is a cell-permeable direct activator of AMPK. AICAR causes accumulation of 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate and consequent phosphorylation and activation of AMPK without affecting the cellular ATP/ADP or ATP/AMP ratios (53). Our results show that the use of AICAR in CaCo2 cells incubated with HG results in similar effects as metformin (**Fig. 3*A*–*M***).

Nox4 stimulate cancer cell malignancy in a diabetic milieu

To further delineate the role of Nox4 in inducing the malignant phenotype in diabetes, we transfected CaCo2 and HT-29 cells with Nox4 CRISPR/Cas9 knockout plasmid. Control CRISPR/Cas9 was also used to evaluate the specificity of the transfection. Cells transfected with the Nox4 CRISPR exhibited a reduction in HG-induced ROS production in CaCo2 and HT-29 (**Fig. 4*A***) and Supplemental Fig. S3*A*), which was paralleled with a decrease in NADPH oxidase activity and the relative mRNA levels and protein expression of Nox4 (Fig. 4*B*–*D* and Supplemental Fig. S3*B*–*D*). Importantly, knockdown of Nox4 significantly attenuated the HG-induced proliferative, migratory, and invasive phenotypes as well as the HG-induced matrix protein accumulation of CaCo2 and HT-29 (Fig. 4*E*–*H* and Supplemental Fig. S3*E*–*I*). Interestingly, Nox4 inhibition significantly reduced several observed biochemical and pathologic changes to levels lower than those observed in adenocarcinoma cells grown in an NG medium (Figs. 3 and 4, and Supplemental Figs. 2 and 3). Taken together, these findings underline the role of Nox4 as the final common signaling pathway playing a key role in promoting cancer cell malignancy in NG or HG.

Diabetes promotes an aggressive malignant CRC phenotype by altering the AMPK/mTOR/Nox4 signaling pathway

To confirm our *in vitro* findings, we used the APC mice and their control littermates, the C57BL/6J mice. The C57BL/6J and APC mice were rendered diabetic by STZ injections [type 1 diabetes mellitus (T1DM) and T1DM/APC, respectively]. The metabolic characteristics of the mice are highlighted in **Table 1**. Our results reveal that the colons of the APC and T1DM/APC mice have augmented ROS production, NADPH oxidase activity, and Nox4 mRNA levels and protein expression when compared with the nondiabetic and T1DM mice (**Fig. 5*A*–*F***). As expected, the T1DM mice showed

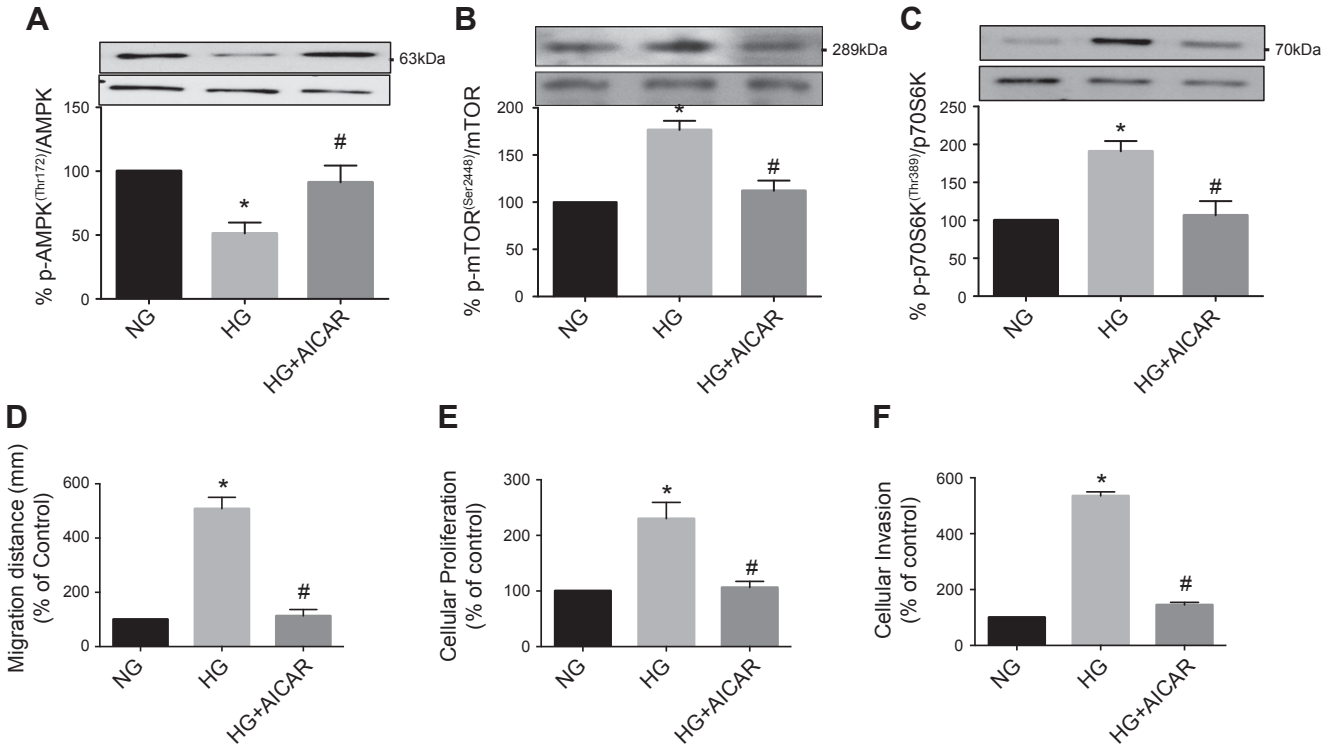

Figure 3. Activation of AMPK using AICAR reduces cancer cell injury and 8-oxodG production**.** CaCo2 cells were treated with HG (25 mM) alone or with AMPK activators AICAR (1 mM) for 72 h. *A*) Bar graph showing quantitation of $AMPK^{Thr172}$/AMPK (%). *B*) Bar graph showing quantitation of p-$mTORC1^{Ser2448}$/mTORC1 (%). *C*) Bar graph showing quantitation of p-$p70S6K^{Thr389}$/p70S6K (%). *D*) Bar graph of the migration assay for CaCo2. *E*) MTT proliferation assay for CaCo2 cells. *F*) Cellular invasion assay assessing the number of CaCo2 cells that invade a porous membrane. *G*) Bar graph showing quantitation of fibronectin/GAPDH (%). *H*) Bar graph showing quantitation of OGG1/GAPDH (%). *I*) Bar graph showing 8-oxo-dG accumulation. *J*) Superoxide anion production measured using HPLC. *K*) NADPH oxidase activity assay in CaCo2 cells measured using the Lucigenin assay. *L*) Relative mRNA amount of Nox4/GAPDH (%). *M*) Bar graph showing quantitation of NOX4/GAPDH (%). EOH, 2-hydroxyethidium; RLU, relative light unit. All values are the means ± SEM from at least 4 independent experiments. $^{*}P < 0.05$ *vs.* NG, $^{\#}P < 0.05$ *vs.* HG.

significantly higher levels of ROS production, NADPH activity, and NOX4 mRNA levels and protein expression when compared with the control mice (Fig. 5*A–F*). Furthermore, OGG1 protein expression and 8-oxodG concentrations were found to be significantly altered in colon tissues of the APC, T1DM, and T1DM/APC mice compared with those of the nondiabetic mice, with more statistically significant alteration in the T1DM/APC mice (Fig. 5*G, H*). These observations were paralleled by inactivation of AMPK (Fig. 5*I, J*) and activation of mTORC1 pathways (Fig. 5*K, L*) when compared with the nondiabetic mice. All of these observations were significantly more pronounced in the T1DM/APC mice when compared with both APC and T1DM mice (Fig. 5*A–L*). Remarkably, these alterations were concomitant with an increase in the proliferation of the polyps' number, an increase in the polyp size, and an increase in fibronectin expression in the colon polyps of the APC/T1DM mice when compared with APC mice (**Fig. 6*A–C***). These changes were paralleled by a significant increase in CD44 mRNA levels. CD44 mRNA levels were previously described in refs. 59 and 60 to be linked to the aggressiveness of CRC and shown to have the ability to differentiate CRC from other intestinal tumors.

Up-regulation of Nox4, inactivation of AMPK, and activation of the mTORC1 fuel the progression and aggressiveness of the CRC malignant phenotype

To validate the role of Nox4 and its crosstalk with AMPK/mTORC1 in provoking a more pronounced malignant CRC phenotype in diabetes, the T1DM/APC mice were either left untreated or treated with metformin, rapamycin, or GKT, and the results were compared with the APC nondiabetic mice. The metabolic characteristics of the mice are highlighted in Table 1. Our results show that diabetes induces polyps number proliferation, has a tendency to increase polyp sizes, induces CD44 mRNA levels, and increases fibronectin protein expression (Fig. 6*A–D*). These anatomic, histologic, molecular, and biochemical changes were reversed by metformin, rapamycin, or GKT treatment (Fig. 6*A–D*). Qualitative analysis of colon tissue sections by hematoxylin and eosin staining revealed that colons of T1DM/APC mice displayed microadenoma toward the surface with a stratification of prominent nuclei migrating from the basement membrane (Fig. 6*E*). This was accompanied by a decrease in goblet cells and an increase in number of crypts per surface area, which denoted the presence of microadenomateous polyps (Fig. 6*E*). All of

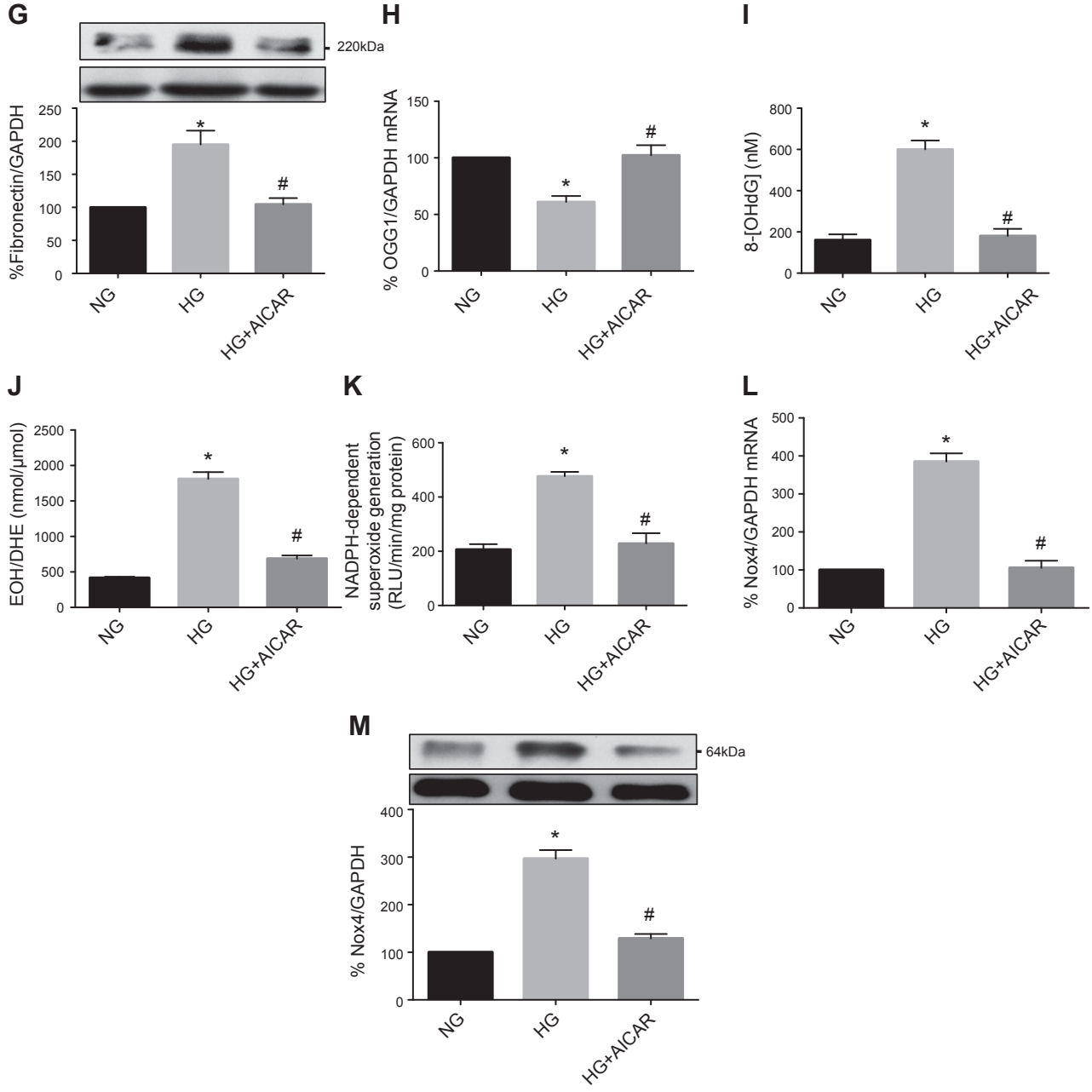

Figure 3. (*Continued*)

these changes were not observed in colon sections of the treated mice (Fig. 6*E*). Moreover, our results show that T1DM-induced ROS production, NADPH oxidase activity, and Nox4 mRNA levels and protein expression are significantly reduced with metformin, rapamycin, or GKT treatment when compared with APC mice (Fig. 6*F–K*). Interestingly, the use of GKT significantly reduced polyp number, decreased polyp size, abolished diabetes-induced CD44 mRNA levels, obliterated fibronectin expression, and lowered Nox4-dependent ROS production in the colon of the T1DM/APC mice to levels significantly lower than the baseline levels of the APC mice (Fig. 6*A–K*).

Next we investigated whether CRC tumorigenesis is mediated in part by silencing of the OGG1-DNA repair activity. In fact, OGG1 mRNA expression was rescued with metformin, rapamycin, and GKT treatment, and 8-oxodG concentrations were attenuated in the colon of T1DM/APC mice when compared with APC (Fig. 6*L, M*). Remarkably, GKT treatment had a pronounced effect in restoring the observed changes (Fig. 6*L, M*).

Collectively, our results indicate that in response to diabetes, the up-regulation of Nox4, the inactivation of the AMPK, and the activation of the mTORC1 play a critical role in the progression and aggressiveness of the colorectal malignant phenotype (**Fig. 7**).

DISCUSSION

Diabetes and cancer are prevalent diseases in the modern era. Many epidemiologic studies have established frequent cooccurrence of diabetes and cancer, suggesting an

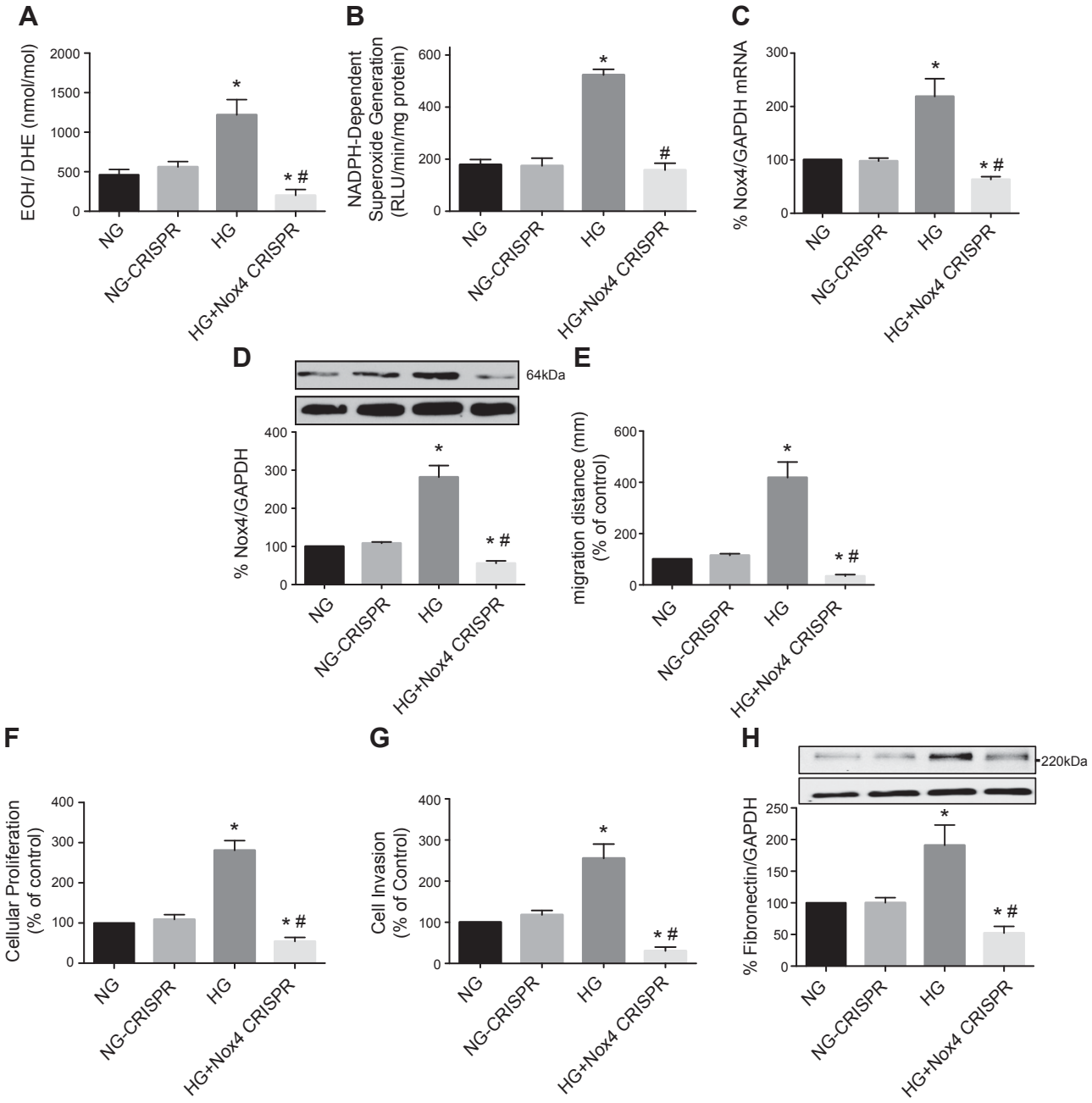

Figure 4. Nox4 mediates HG-induced ROS production and cancer cell injury. CaCo2 cells were seeded at low confluence and subsequently transfected with either control CRISPR or Nox4 CRISPR in the presence or absence of HG (25 mM). *A*) Superoxide anion production measured using HPLC. *B*) NADPH oxidase activity assay on CaCo2 cells. *C*) Relative mRNA levels of Nox4/GAPDH (%). *D*) Bar graph showing quantitation of Nox4/GAPDH (%). *E*) Bar graph of the migration assay for CaCo2 cells. *F*) MTT proliferation assay for CaCo2 cells. *G*) Invasion assay representing CaCo2 cells' invasion upon Nox4 knockdown. *H*) Bar graph showing quantitation of fibronectin/GAPDH (%). EOH, 2-hydroxyethidium; RLU, relative light unit. All values are the means ± SEM from at least 4 independent experiments. $^{*}P < 0.05$ *vs.* NG, $^{\#}P < 0.05$ *vs.* HG.

association of diabetes with specific types of solid tumors, including CRC tumors (34). In fact, patients with type 2 diabetes have a 20–40% increased risk for colorectal cancer *vs.* the general population (28). Likewise, recent studies revealed that cancer, especially CRC, can increase the risk of diabetes. Patients with CRC showed an increased risk to develop diabetes than individuals without CRC for up to 5 yr after the diagnosis (14–21).

While diabetes and cancer share many risk factors, the molecular and cellular mechanisms implicated in their association are poorly characterized. In this study, we uncover a novel biologic pathway that links diabetes and CRC tumorigenesis.

Because mutations in the APC gene are found in more than 80% of human CRCs, we chose APC mice with a germline heterozygous mutation that mimics human CRC development that is extensively used in investigating basic and translational aspects pertaining to CRC (61–63).

It is established that there is an association between elevated glucose or glycated hemoglobin and the

TABLE 1. *Metabolic characteristics*

Group	*n*	Blood glucose (mg/dl)	Body weight (g)
Control (citrate)	5	151.8 ± 11	28.9 ± 0.6
STZ-induced T1DM mice	5	522 ± 40*	26.5 ± 0.2*
STZ-induced T1DM mice + metformin	5	491 ± 38*	26.4 ± 0.5*
STZ-induced T1DM mice + rapamycin	6	519 ± 36*	25.3 ± 0.7*
APC mice	5	140 ± 18	24.9 ± 0.3*
STZ-induced T1DM/APC mice	4	365 ± 24$^{\#}$	22.8 ± 0.5*,$^{\#}$
STZ-induced T1DM/APC mice + metformin	4	362 ± 47$^{\#}$	22.3 ± 0.4*,$^{\#}$
STZ-induced T1DM/APC mice + rapamycin	6	435 ± 36$^{\#}$	21.9 ± 0.4$^{\#}$
STZ-induced T1DM/APC mice + GKT	5	353 ± 34$^{\#}$	22.2 ± 0.8$^{\#}$

Glucose level and body weight in APC and C57BL/6J control mice: STZ-induced T1DM mice. STZ-induced T1DM mice treated with metformin (150 mg/kg) or rapamycin (0.5 mg/kg) or GKT for 8 wk after the onset of the disease. Values are the means ± SEM from 4 to 6 animals for each group. *$P < 0.05$ *vs.* control mice. $^{\#}P < 0.05$ *vs.* APC mice.

predisposition to CRC malignancies (64, 65). Clinical studies reported that patients with poorly controlled type 2 diabetes have more advanced CRCs, a younger age of presentation, greater use of exogenous insulin, and worse 5-yr survival (66). However, the signaling pathways correlating diabetes and CRC are not established. In our studies, CaCo2 and HT-29 cell lines treated with HG exhibited higher proliferative, migratory, and invasive aptitudes, and that was attributed to Nox-produced ROS. Studies from our group and others showed the role of ROS in diabetic nephropathy, neuropathy, retinopathy, and cardiomyopathy (22–26, 67–69). Here, we provide exciting evidence of increased ROS production, which is demonstrated by elevated O_2^- generation, in colons of APC mice. This increase appears to be mediated *via* a NADPH-dependent mechanism, particularly Nox4. This is in line with several studies that show that oxidative stress is involved in carcinogenesis either by enhancing genomic instability (70, 71) or inducing DNA mutagenesis (72). More importantly, and for the first time to our knowledge, we provide evidence for a further increase in ROS production in the colon of the diabetic APC mice (T1DM/APC) compared with the nondiabetic APC. These data corroborate and reinforce the involvement of ROS in the pathogenic states triggered by diabetes in multiple organs (22–25, 73).

Furthermore, it is established that ROS induce 8-oxodG production. Here, 8-oxodG adduct formation was amplified in colons of the APC mice when compared with the control mice. Remarkably, we noted a more significant rise in the 8-oxodG levels in the colons of the T1DM/APC mice when compared with the APC mice. These results support the studies reporting the contribution of ROS overproduction to the risk of genetic mutations in genes involved in cellular dysfunction (74). Indeed, ROS-induced DNA damage may cause misincorporation of DNA bases due to the presence of unrepaired DNA adducts, for example (75). This explains our results that in the diabetic state, both in APC and control colon tissues as well as in the adenocarcinoma cell lines, OGG1 mRNA and protein expressions were down-regulated.

Interestingly, the elevated ROS reported in our study were concomitant with a significant increase in Nox4 mRNA levels and protein expression in T1DM mice colons and more pronounced in the T1DM/APC mice. This suggests that Nox4 is responsible, at least in part, for the generated ROS. In fact, Nox4-mediated ROS are reported to prevent apoptosis and promote tumor cell growth in pancreatic and colon cancer cells (10, 76), further supporting our hypothesis. Additionally, these findings provide an extension to our group's work describing the role of Nox4 in diabetic kidney diseases (22–24) and diabetic cardiovascular disorders (77) and to the research outcomes of others on the role of Nox4 in diabetic complications (78–80).

To further confirm the role of Nox4 in cancer cell aggressiveness, CaCo2 and HT-29 cells were transfected with a Nox4 CRISPR plasmid. Our results show that Nox4 knockdown did not only reduce Nox4 mRNA levels and protein expression, NADPH oxidase activity, and ROS production but also significantly abrogated the migratory and invasive malignant means of cancer cells that were paralleled by a decrease in the elevated 8-oxodG adducts. These observations were also confirmed in T1DM/APC mice, in which pharmacologic inhibition of Nox4 by GKT mitigated the aggressiveness of the malignant phenotype of the CRC in diabetes. These results are in line with earlier findings implicating Nox4 in the pathophysiology of CRC by virtue of its ability to modulate cytoskeletal-regulating proteins (81) and TGF-β–activated protein tyrosine phosphatases (82). To our knowledge, this study is the first to report the implication of Nox4 in colon tumorigenesis induced by diabetes. Moreover, in this study we investigated the link between Nox4 and pivotal pathways of cellular survival, namely the AMPK and mTORC1 pathways. Our findings suggest that AMPK was inactivated in HG in parallel with the Nox4-increased ROS production. It has been recently reported that under HG, AMPK may "switch off" in several cell types, thus contributing to the pathogenesis of several disorders, including diabetes and cancer (24, 83). Contextually, our group previously provided evidence that in diabetes, AMPK activation largely influences kidney function through reversal of NADPH oxidases–dependent ROS production (24). Several other studies provide strong evidence that AMPK is involved in tumorigenesis of different types of cancer (48, 84–86). These effects are likely due to AMPK effects, such as cell cycle arrest and activation of tumor suppressors (87–89). Besides this, our results show that mTORC1 was significantly activated in the T1DM/APC mice as well as in the cultured CaCo2 and HT-29 cells exposed to HG and that metformin or

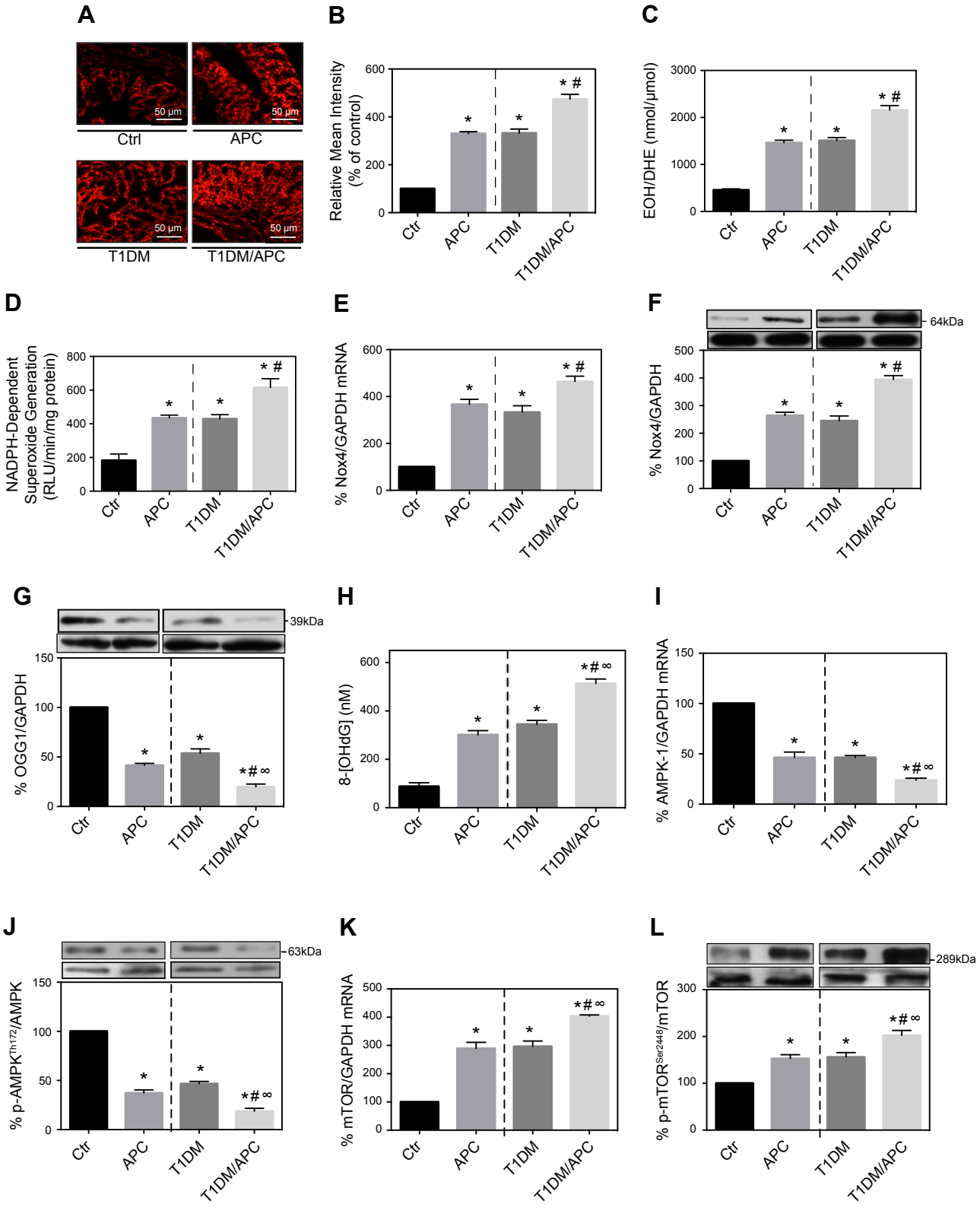

Figure 5. Diabetes promotes an aggressive malignant phenotype in APC mice. *A*) Representative figures of DHE staining of colon tissues of different mice groups. *B*) Bar graph representing ROS production from DHE staining of colon tissues as assessed by confocal microscopy. *C*) Superoxide anion production measured using HPLC. *D*) NADPH oxidase activity assay on colon tissues. *E*) Relative mRNA levels of Nox4/GAPDH (%) in colon tissue. *F*) Bar graph showing quantitation of Nox4/GAPDH (%) in colon tissue. *G*) Bar graph showing quantitation of OGG1/GAPDH (%) in colon tissue. *H*) Bar graph showing 8-oxo-dG accumulation. *I*) Relative mRNA levels of AMPK/GAPDH (%) in colons of different mice groups. *J*) Bar graph showing quantitation of p-AMPK^{Thr172}/AMPK resulting from colon tissue lysates. *K*) Relative mRNA levels of mTOR/GAPDH in colons of different mice groups. *L*) Bar graph showing quantitation of p-mTORC1^{Ser2448}/mTORC1 resulting from colon tissue lysates. Ctr, control; EOH, 2-hydroxyethidium; RLU, relative light unit. All values are the means ± SEM from 4 to 5 mice/group. *$P < 0.05$ *vs.* vehicle-treated controls. $^{\#}P < 0.05$ *vs.* vehicle-treated APC mice, $^{\infty}P < 0.05$ *vs.* STZ-induced T1DM mice.

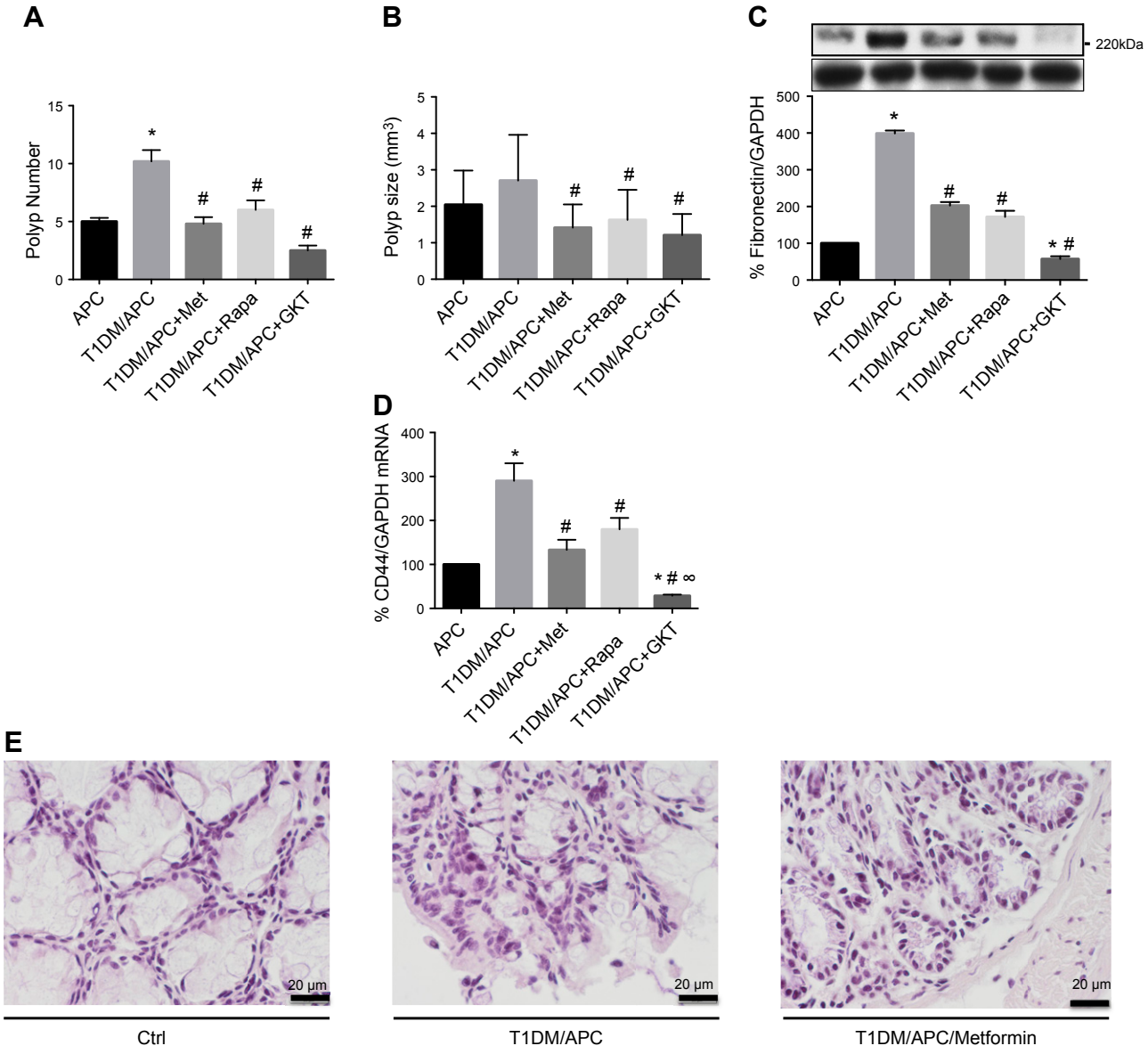

Figure 6. Metformin, rapamycin, and GKT treatments reduce diabetes-induced CRC malignancy, attenuate Nox4-produced ROS, and reverse 8-oxodG adducts in the colons of APC mice. STZ-induced type 1 diabetic APC mice (T1DM/APC) were treated with either vehicle, metformin (150 mg/kg/d), rapamycin (0.5 mg/kg/d), or GKT (40 mg/kg/d). *A*) Bar graph showing the percentage of the mean polyp number in T1DM/APC mice; T1DM/APC mice treated with metformin, rapamycin, or GKT compared with nondiabetic APC mice. *B*) Bar graph showing polyp size mean (mm^3) in T1DM/APC mice; T1DM/APC mice treated with metformin, rapamycin, or GKT compared with nondiabetic APC mice. *C*) Bar graph showing quantitation of fibronectin/GAPDH (%) in colon tissue of the different mice groups. *D*) Relative mRNA levels of CD44/GAPDH in colons of the different mice groups (%). *E*) Representative hematoxylin and eosin–stained sections of colons of control (Ctr), T1DM/APC mice and T1DM/APC mice treated with metformin. *F*) Representative images of DHE stains of colon tissues of different mice groups. *G*) Bar graph showing the quantitation of the DHE stain assessed by confocal microscopy. *H*) Superoxide anion production measured using HPLC. *I*) NADPH oxidase activity assay from colon tissues. *J*) Relative mRNA levels of Nox4/GAPDH (%) in colon tissues of the different mice groups. *K*) Bar graph showing quantitation of Nox4/GAPDH (%) in colon tissues of the different mice groups. *L*) Relative mRNA levels of OGG1/GAPDH (%) in colon tissues of the different mice groups. *M*) Bar graph showing 8-oxo-dG accumulation in colon tissues of the different mice groups. EOH, 2-hydroxyethidium; Met, metformin; Rapa, rapamycin; RLU, relative light unit. All values are the means ± SEM from 4 mice/group. $^{*}P < 0.05$ *vs.* the vehicle-treated STZ-induced type 1 diabetic APC mice. All values are the means ± SEM from 4–5 mice/group. $^{\#}P < 0.05$ *vs.* vehicle-treated T1DM/APC mice, $^{\#}P < 0.05$ *vs.* the metformin- or rapamycin-treated T1DM/APC mice.

rapamycin reduced ROS overproduction, NADPH oxidase activity, and Nox4 mRNA and protein levels.

In parallel, the AMPK/mTOR/Nox4 signaling axis involves OGG1, where OGG1 levels were restored, whereas 8-oxodG concentrations shied away upon administration of the above treatments *in vivo* and *in vitro*. In line with these observations, extensive data from our group have implicated the AMPK/mTORC1 axis as a key player in the onset and development of diabetic kidney diseases (22, 23). In fact, it is suggested that inhibition of the mTORC1 pathway with rapamycin significantly reduces NADPH oxidase–dependent oxidative stress,

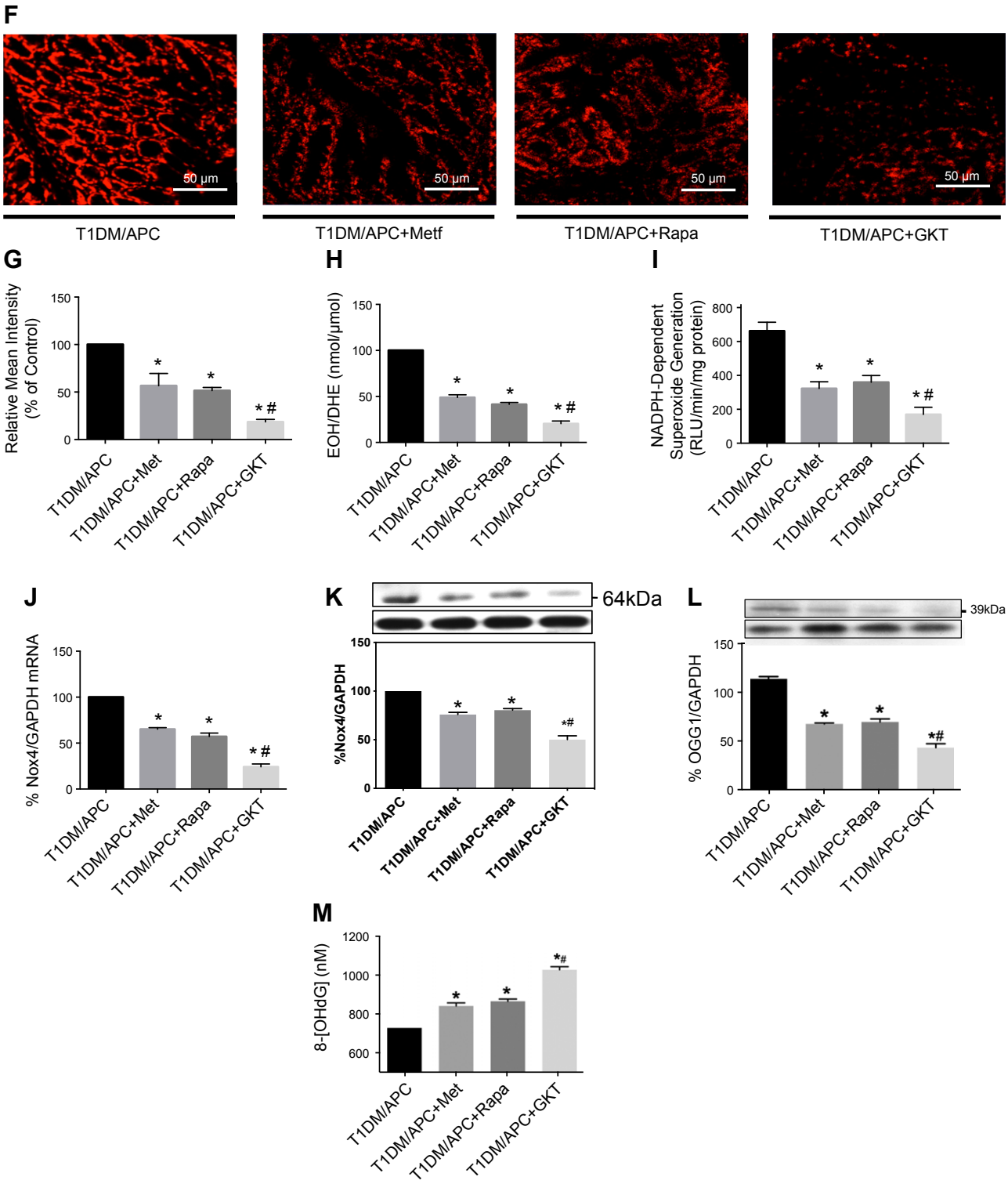

Figure 6. (*Continued*)

which may have protective effects on the diabetic kidney (23, 90, 91). Interestingly, inhibition of AMPK and activation of mTOR in several types of cancer have also been reported (92, 93). Taken together, our findings highlight a contributing role for the AMPK/mTORC1 pathway in CRC progression and tumorigenesis.

In summary, our findings uncover a novel role for Nox4-induced ROS in promoting DNA damage and exacerbating tumorigenesis. This is elicited through alteration in the AMPK/mTORC1 pathway, resulting in the accumulation of 8-oxodG and exacerbating cancer cell aggressiveness that altogether contribute to the genomic instability and predisposition to cancer. Consequently, perhaps a drug combination of Nox4 inhibitors or AMPK activators and mTORC1 inhibitors along with the standard anticancer agents may be of therapeutic relevance to

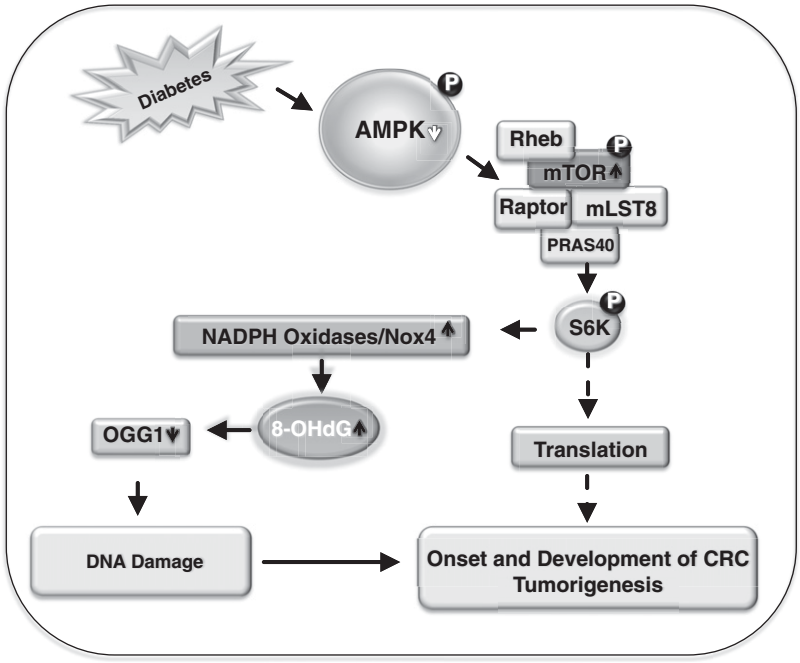

Figure 7. Proposed mechanism linking diabetes to CRC.

alleviate cancer progression in diabetes or to inhibit the increased risk of diabetes in patients with cancer. FJ

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AUTHOR CONTRIBUTIONS

A. A. Eid conceived and designed the experiments of the study and supervised the work; A. H. Eid helped in designing the experiments and overseeing the work; F. M. Mroueh performed the experiments and wrote the manuscript; M. Noureldein, S. Eid, M. Haddad, and R. Barakat helped in some of the experiments of the study; Y. H. Zeidan, F. Harb, J. Costantine, and R. Kanj participated to the discussion of the results; Y. H. Zeidan, A. Ouhtit, and S. T. Azar provided critical scientific input to the experiments and participated to the discussion; E.-A. Sauleau provided critical scientific input to the experiments, helped in the statistical analysis, and participated to the discussion; and all authors reviewed the results, provided essential reviews of the manuscript, and approved the final version of the manuscript.

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