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Metformin and colorectal cancer

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Abstract: Colorectal cancer (CRC) is one of the main causes of cancer-related mortality in the developed world despite recent developments in detection and treatment. Several epidemiological studies indicate that metformin, a widely prescribed antidiabetic drug, exerts a protective effect on different cancers including CRC. Furthermore, a recent double-blind placebo-controlled, randomized trial showed that metformin significantly decreased colorectal adenoma recurrence. Studies exploring the mechanism of action of metformin in cells derived from different types of cancers reported many effects including respiratory chain complex 1 inhibition, Akt phosphorylation inhibition, ATP depletion, PKA activation and Wnt signaling inhibition. However, many of these results were obtained employing metformin at concentrations several fold higher than those achieved in target tissues in diabetic patients receiving therapeutic recommended doses of metformin. In contrast, recent studies obtained with metformin at concentrations compatible with those detected in human intestines revealed that metformin elicit responses that target β-catenin, PI3K/Akt, E-cadherin, p120-catenin and focal adhesion kinase which are key molecules and signaling pathways associated to colorectal cancer development. This brief review revisit several know aspects as well as novel ones on the effects of metformin on cancer cells.

Introduction

Cancer is a leading and growing cause of morbidity and mortality worldwide (Bray *et al.*, 2018). Risk factors associated to cancer development include non-modifiable factors such as age and genetic background along with modifiable factors that include limited physical activity, poor dietary habits, obesity, metabolic syndrome and type II diabetes mellitus (T2DM) (Aleman *et al.*, 2014; Gonzalez *et al.*, 2017). T2DM, a chronic disease that will affect by 2040 up to ≈ 642 million people worldwide (Unnikrishnan *et al.*, 2017), is distinguished by hyperglycemia, hyperinsulinemia, insulin resistance and by an increase in the bioavailability of insulin-like growth factor-1 (IGF-1) and overexpression of the insulin receptor (IR). The binding of insulin and IGF to their receptors, or hybrid IR/IGF receptors, activate the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling pathways promoting diverse cellular responses including proliferation (Cohen and LeRoith, 2012; Gallagher and LeRoith, 2011).

Metformin (1,1-dimethylbiguanide hydrochloride) is the drug most commonly prescribed to treat hyperglycemia in T2DM patients. After oral administration of therapeutic doses (1,000–2,250 mg/day), metformin is absorbed by intestinal enterocytes reaching the liver through the portal vein. In the kidney, metformin is absorbed from the circulation and excreted into the urine. The concentration of metformin in portal vein can reach 40–70 μM whereas in systemic plasma fluctuates between 10–40 μM (He and Wondisford, 2015). In contrast, metformin can reach in intestinal tissue concentrations up to 150 fold higher than in plasma (Paleari *et al.*, 2018).

Metformin reduces blood glucose levels by inhibiting hepatic gluconeogenesis via activation of the serine–threonine liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK), a conserved regulator of the cellular response to low energy that is activated when ATP concentrations decrease and 5'-AMP concentrations increase in response to nutrient deprivation, hypoxia and metformin administration (Cusi *et al.*, 1996; He *et al.*, 2009; Hundal *et al.*, 2000; Shaw *et al.*, 2005; Zhou *et al.*, 2001). There are other proposed mechanisms by which metformin suppresses gluconeogenesis independent of AMPK like, for example, by decreasing ATP and increasing

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AMP levels which leads to adenylate cyclase inhibition (Foretz *et al.*, 2010; Johanns *et al.*, 2016; Miller *et al.*, 2013). Other studies indicated that metformin inhibits the respiratory chain complex 1, proinflammatory responses, cellular proliferation and that interferes with mechanisms associated to autoimmune diseases, such as the T helper 17/regulatory T cell balance, germinal centers formation, autoantibodies production, macrophage polarization and cytokine synthesis (El-Mir *et al.*, 2000; Isoda *et al.*, 2006; Park *et al.*, 2019; Marcucci *et al.*, 2020; Ursini *et al.*, 2018). Other effects of metformin include suppression of cancer stem cells in some cancers, Akt phosphorylation and β-catenin-mediated signaling (King *et al.*, 2006; Melnik *et al.*, 2018; Takatani *et al.*, 2011; Saini and Yang, 2018). Regarding the effects of metformin upon β-catenin, several reports indicate that metformin down-regulates its expression in different cell types including endometrial cancer cells, osteoblast-like Saos-2 cells and colon carcinoma RKO cells as well as the transcriptional activity of c-MYC and β-catenin/TCF-Lef reporters in epithelial ovarian cancer cells (Conza *et al.*, 2021; Park *et al.*, 2019; Takatani *et al.*, 2011; Garrido *et al.*, 2020). Several studies also indicate that metformin halt the conversion of oral premalignant lesions into head and neck squamous cell carcinoma, inhibits pancreatic cancer induction, DNA damage by the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, that attenuates the increase in reactive oxygen species (ROS) and that promotes anti- and pro-angiogenic effects in different cell contexts (Algire *et al.*, 2012; Dallaglio *et al.*, 2014; Memmott *et al.*, 2010; Schneider *et al.*, 2001; Vitale-Cross *et al.*, 2012; Zolali *et al.*, 2019). Furthermore, epidemiological studies suggest that metformin exerts a protective effect on different types of cancer including sporadic colorectal cancer (CRC) (Chang *et al.*, 2018; Klil-Drori *et al.*, 2017; Kobiela *et al.*, 2018), observations recently reinforced by a double-blind placebo-controlled/randomized trial demonstrating that metformin decreased up to 40% colorectal adenoma recurrence (Higurashi *et al.*, 2016). An important caveat regarding the implications of many of the above mentioned *in vitro* studies is that the employed metformin concentrations on some cases were up to ≈ 100–150 fold higher than those achieved in the target tissues after oral administration of therapeutic doses of metformin (Foretz *et al.*, 2019; He and Wondisford, 2015). Nevertheless, the growing interest in metformin is evident by the number of worldwide ongoing clinical trials (337) examining its effects upon several pathologies including different cancers, fragile X syndrome, glaucoma, amyotrophic lateral sclerosis, cerebral palsy and HIV/AIDS (for a list of ongoing clinical trials see: https://clinicaltrials.gov/ct2/results?term=metformin&Search=Apply&recrs=a&recrs=d&age_v=&gndr=&type=&rslt=).

β-Catenin and Metformin

CRC development is associated with the sequential accumulation of mutations and/or deletions of tumor suppressor and oncogenes along with alterations in genetic stability. In the current model of sporadic colon cancer, the initial event that sets the stage for intestinal adenoma formation is the deregulation Wnt/β-catenin signaling, an event that promotes the nuclear accumulation of β-catenin and the constitutive activation of its target genes (Cheah, 2009; Huels *et al.*, 2015; Kinzler and Vogelstein, 1996; Krausova and Korinek, 2014; Polakis, 2012; Sansom *et al.*, 2004; Walther *et al.*, 2009). In most cases, the mechanism mediating the aberrant nuclear accumulation of β-catenin involves mutations in the *Adenomatous Polyposis Coli* (*APC*) tumor suppressor gene and/or β-catenin (Bienz and Clevers, 2000; Clevers, 2006; Iwao *et al.*, 1998; Phelps *et al.*, 2009). In normal colonocytes, APC is part of a destruction complex that includes axis inhibition protein (Axin), glycogen synthase kinase 3 β (GSK3β and casein kinase 1a (CK1α). The interaction of β-catenin with the destruction complex leads to its sequential phosphorylation in Ser^{45} by CK1 and $Thr^{41}/Ser^{37}/Ser^{33}$ by GSK3β (Polakis, 2002). Phosphorylated β-catenin is then targeted for ubiquitination and later degradation by the proteasome (Clevers, 2006). Wnt binding to its receptor Frizzled, and co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP 5/6), leads to the disassembly of the destruction complex, β-catenin $Ser^{45}/Thr^{41}/Ser^{37}/Ser^{33}$ phosphorylation inhibition and nuclear entry. Once in the nuclei, β-catenin interacts with the T-cell factor/lymphoid enhancer-binding factor (LEF/TCF) promoting the transcription of genes associated with proliferation, differentiation, adhesion and cellular migration (Clevers and Nusse, 2012; Nusse and Clevers, 2017; Valenta *et al.*, 2012). In the case of proliferation, CYCLIN D1, one of the first reported transcriptional genes targeted in CRC by β-catenin (Niehrs and Acebron, 2012), and cMYC promote G1 phase advancement whereas cMYC induces the S phase (Lecarpentier *et al.*, 2019). Accordingly, abnormal nuclear accumulation of β-catenin promotes CyclinD1 and cMyc overexpression and hyper-proliferation. *Lgr5* and *Axin 2*, which are components of the Wnt pathway, are also stem cell specific genes targeted Wnt/β-catenin (Nusse and Clevers, 2017). Other genes targeted by β-catenin include *Tcf1*, PDK, *fibronectin*, MMP7, *Claudin* and *cJun* between others (for a list of genes regulated by β-catenin see: https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes).

Wnt-independent phosphorylation cascades also play a central role in the control of β-catenin stability, intracellular distribution and transcriptional activity (Daugherty and Gottardi, 2007; He *et al.*, 2007; Kriz and Korinek, 2018). For example, the phosphorylation of β-catenin at Ser^{552} and Ser^{675} by Akt or protein kinase A (PKA) promotes its nuclear translocation and transcriptional activity (Fang *et al.*, 2007; Rey *et al.*, 2012; Taurin *et al.*, 2006; Taurin *et al.*, 2008). Because T2DM is associated with chronic PI3K/Akt signaling (Hopkins *et al.*, 2020; Lien *et al.*, 2017), Akt-mediated chronic Ser^{552} β-catenin phosphorylation provides a plausible mechanism by which T2DM could potentiate CRC development. Within this framework, metformin, at concentrations found in the colon (1.5–3.5 mM) after oral administration of therapeutic doses (Paleari *et al.*, 2018), inhibited Akt Ser^{473} phosphorylation and catalytic activity in CRC-derived cell lines challenged with insulin or IGF-1 (Amable *et al.*, 2019).

Previous studies in other cancer cells demonstrated that AMPK inhibits mTORC1 activation through a mechanism that involves stimulation of TSC2 function, accumulation of Rheb-GDP (the inactive form) and direct phosphorylation

of Raptor, (Gwinn *et al.*, 2008; Inoki *et al.*, 2006; Rozengurt *et al.*, 2014). Because mTORC1 is involved in metabolism, growth and differentiation of cancer cells, it has been proposed that its inhibition by metformin is associated to metformin anticancer properties. Furthermore, a few studies indicate that metformin-mediated mTORC1 inhibition also promotes autophagy in cells derived from different tumors including myeloma, pancreatic ductal adenocarcinoma, T-cell acute lymphoblastic leukemia and hepatocellular carcinoma (Candido *et al.*, 2018; Gao *et al.*, 2020; Grimaldi *et al.*, 2012; Ling *et al.*, 2017; Wang *et al.*, 2018b). In contrast, there is little information concerning the impact of metformin/AMPK on mTORC2, the molecular complex responsible for the phosphorylation of Akt at Ser^{473} and Thr^{479}, PKC classical and novel family members and glucocorticoid-induced kinase 1 (Baffi *et al.*, 2021; Fu and Hall, 2020). Within this context, recent results revealed a marked sensitivity of CRC cells to metformin-mediated inhibition of Akt Ser^{473} phosphorylation (Amable *et al.*, 2019), an exploitable vulnerability in CRC cells that can further explain the mechanisms by which metformin acts as a chemopreventive agent in bowel cancer.

Amable *et al.* (2019) studie also revealed that PI3K/Akt signaling suppression was mediated by AMPK and occurred upstream of Akt, very likely due to a defect in phosphatidylinositol 3,4,5-triphosphate generation. Regarding the possible mechanisms by which metformin can interfere with PI3K/Akt signaling, previous studies suggest that AMPK activity can promote a displacement of PI3K from its site of action. For example, AMPK-mediated Ser^{794} phosphorylation of the insulin receptor substrate 1 (IRS-1) inhibited the binding and activation of PI3K (Tzatsos and Tsichlis, 2007) while AMPK signaling shifted PI3K from its site of action at the neurite tip (Amato *et al.*, 2011). Whether the defect observed in CRC-derived cells in response to metformin treatment was due to a block in PI3K plasma membrane translocation, inhibition of its catalytic activity or enhanced phosphatases activity needs further scrutiny.

Amable *et al.* (2019), studies also showed that metformin-associated PI3K/Akt signaling inhibition prevented β-catenin Ser^{552} phosphorylation and β-catenin-mediated transcription while promoting its plasma membrane localization. Although β-catenin does not contain nuclear localization or export signals, it shuttles between the cytoplasm and the nucleus by interacting with a variety of partners including Chibby, Axin, APC, Mucin 1, LEF-1 and BCL9 (Anthony *et al.*, 2020; Jamieson *et al.*, 2014; Sharma *et al.*, 2014). Additional studies are required to elucidate how Ser^{552} phosphorylation inhibition affects β-catenin nucleo-cytoplasmic distribution and shuttling. Nevertheless, it is tempting to speculate that Ser^{552} phosphorylation enhances the interaction of β-catenin with a binding partner that favors its nuclear import and/or anchor.

Metformin E-Cadherin, Fak and Metformin

E-cadherin, a tumor suppressor, is a core component of the epithelial adherens junctions (AJ) that interacts *via* its cytoplasmic tail with catenin family members α, β, and p120 while its extracellular domain interacts with E-cadherin present in neighboring cells (Daulagala *et al.*, 2019). In contrast to the continuous degradation of cytoplasmic β-catenin, AJs-associated β-catenin is highly stable and associated to the regulation of E-cadherin availability at the cell surface (Ishiyama and Ikura, 2012; Mendonsa *et al.*, 2018; Pokutta and Weis, 2007), a function shared with p120-catenin which regulates E-cadherin endocytosis (Cadwell *et al.*, 2016; Kowalczyk and Nanes, 2012; Nanes *et al.*, 2012). E-cadherin expression or surface localization is frequently lost or its function disrupted in many epithelial-derived cancer cells including CRC (Kourtidis *et al.*, 2017; Petrova *et al.*, 2016). The loss of E-cadherin diminish cell-cell adhesion and deregulates Wnt signaling (Heuberger and Birchmeier, 2010; Valenta *et al.*, 2012).

N-cadherin, another member of the cadherin family of proteins, is expressed in mesenchymal cells which are characterized by displaying a major motility and a less polarized phenotype than normal epithelial cells. N-cadherin is also found in some epithelia-derived cancer cells, a factor that contributes to their enhanced motility and invasive phenotype (Gul *et al.*, 2017). Within this context, the transdifferentiation of epithelial cells into motile mesenchymal cells, a process known as epithelial-mesenchymal transition (EMT), play a central role in several normal and pathological processes including development, wound healing, stem cell behavior and cancer progression (Lamouille *et al.*, 2014). Hallmarks of the EMT include destabilization of adherens junctions, tight junctions and desmosomes, critical structures necessary to maintain epithelial integrity, as well as up regulation of vimentin and α-smooth muscle actin (Lamouille *et al.*, 2014). Recent studies indicated that metformin inhibits EMT in cells derived from different types of cancer including gastric, colon, thyroid, breast, oral and prostate (Esparza-Lopez *et al.*, 2019; Han *et al.*, 2015; Valaee *et al.*, 2017; Wang *et al.*, 2018a; Yin *et al.*, 2021; Zhang and Wang, 2019; Zhang *et al.*, 2014). Several mechanisms had been proposed to explain the inhibitory effect of metformin upon EMT such as down-regulation of transcription factors (SNAIL, TWIST and ZEB), inhibition of PI3K/AKT/mTOR, MAPK, TGFβ, IL-6 and IL-8 signaling and up regulation of miR-381 and miR-200c (Chen *et al.*, 2020). Such variety of mechanisms could be related to the distinct origin of the cancer cells or to off-target effects since most experimental models use concentrations of metformin that exceed the levels reached in target tissues with the doses recommended to treat T2DM patients.

Matrix metalloproteinases (MMPs), a family of endopeptidases that promote the degradation of proteins in the extracellular matrix, are associated to cell proliferation, migration, and differentiation (Cui *et al.*, 2017). In the tumor microenvironment, MMPs facilitate invasion and metastasis, two key processes associated to EMT transition. Indeed, MMPs are involved in the process that lead to the spread of metastatic cancers such as bladder, breast, colon, kidney, melanoma and sarcoma as well as various cancers including hepatocellular carcinoma, pancreatic ductal adenocarcinoma and bone (Paolillo and Schinelli, 2019; Scheau *et al.*, 2019). Several studies indicate that *MMP-2* and MMP-9, two key MMPs that promote tumor cell invasion and metastasis, are down-regulated in their expression and activity by metformin in cells derived from breast cancer, renal carcinoma, esophageal squamous cancer and human ovarian granulosa cancer (Chen *et al.*, 2019;

Fang *et al.*, 2014; Jang *et al.*, 2014; Liang *et al.*, 2018). In several cases the down-regulation of these MMP2/9, as a results of metformin treatment, coincided with the inhibition of cell growth and migration.

Recent studies employing metformin concentrations compatible with the ones in the colon after oral administration of therapeutic doses of this drug indicate that metformin not only promoted the plasma membrane localization of β-catenin and E-cadherin but also their colocalization to *de novo* formed puncta along the length of CRC-derived cells contacting membranes (Amable *et al.*, 2020). The plasma membrane redistribution of E-cadherin in response to metformin treatment was accompanied by its phosphorylation at $Ser^{838/840}$, modifications associated to E-cadherin/β-catenin binding and increased interaction stability between both proteins (McEwen *et al.*, 2014). E-cadherin $Ser^{838/840}$ conforms to a GSK3β recognition site, a kinase activated in CRC-derived cells in response to metformin (Amable *et al.*, 2019). Metformin treatment was also associated with an increase in the intracellular levels of p120-catenin, a result consistent with the observation that β-catenin drives the transcription of forkhead/winged-helix transcription factors (Savage *et al.*, 2010), which in turn down-regulate p120-catenin transcription (Mortazavi *et al.*, 2010; Pham *et al.*, 2017). In addition, metformin promoted the redistribution of p120-catenin to the plasma membrane where co-localized with E-cadherin/β-catenin, suggesting that metformin promotes the *novo* formation of AJs (Amable *et al.*, 2020). Nevertheless, Amable *et al.* (2020), did not examine whether N-cadherin, which is expressed in the cell lines SW-480 and HT-29 employed in those studies (Yan *et al.*, 2015; Ye *et al.*, 2017) was down regulated in response to metformin.

AJs, desmosomes and tight junctions (TJs) form the apical junction complex that regulates epithelial barrier function and signaling (Mehta *et al.*, 2015; Shigetomi and Ikenouchi, 2019). Previous studies showed that AMPK exerts a protective effect on the intestinal barrier function by stimulating the formation of TJs (Chen *et al.*, 2018; Peng *et al.*, 2009; Wu *et al.*, 2018; Zhang *et al.*, 2006). Because TJs assembly is coupled to AJs formation (Campbell *et al.*, 2017), it is plausible that AJs formation in response to metformin contributes to TJs assembly and intestinal barrier recovery after injury.

Focal adhesions (FAs) are integrin-containing structures that connect the cell to the extracellular matrix. These highly dynamic multiprotein complexes include focal adhesion kinase (FAK), a tyrosine kinase that regulates several signaling pathways associated with cell adhesion, spreading and migration (Berrier and Yamada, 2007) as well as tumor growth and metastasis (Canel *et al.*, 2010; Sulzmaier *et al.*, 2014; Tai *et al.*, 2015). For example, FAK null mice fibroblasts showed a reduced rate of migration associated with FAs reorganization (Ilic *et al.*, 1995) while FAK deficient cancer cells display large FAs and reduced motility (Chan *et al.*, 2009; Hsia *et al.*, 2003; Huttenlocher and Horwitz, 2011; Webb *et al.*, 2004). Former reports indicated that metformin inhibited FAK phosphorylation in ovarian (Erices *et al.*, 2017) and prostatic cancer cells (Yu *et al.*, 2017) whereas a more recent study showed that, in CRC-derived cells, metformin inhibited FAK catalytic activity and ERK-dependent FAK Ser^{910} phosphorylation (Hunger-Glaser *et al.*, 2003; Hunger-Glaser *et al.*, 2004; Jiang *et al.*, 2007), a modification associated with paxillin/FAK interaction, cell spreading and migration (Chu *et al.*, 2011; Luo *et al.*, 2019; Vincent and Settleman, 1997). Metformin-mediated inhibition of FAK led to FAs structural changes including a reduction in their numbers and increase in their size (Amable *et al.*, 2020), very likely through a modification of FAs turnover (Ilic *et al.*, 1995; Iwanicki *et al.*, 2008; Kim and Wirtz, 2013; Plotnikov *et al.*, 2012), changes that were followed by cellular migration inhibition (Amable *et al.*, 2020).

Concluding Remarks

In summary (Fig. 1), the most recent studies described here (Amable *et al.*, 2019, Amable *et al.*, 2020), indicate that metformin, at concentrations within the range of those found in human intestines after administration of therapeutic doses of this drug, targets key molecules and

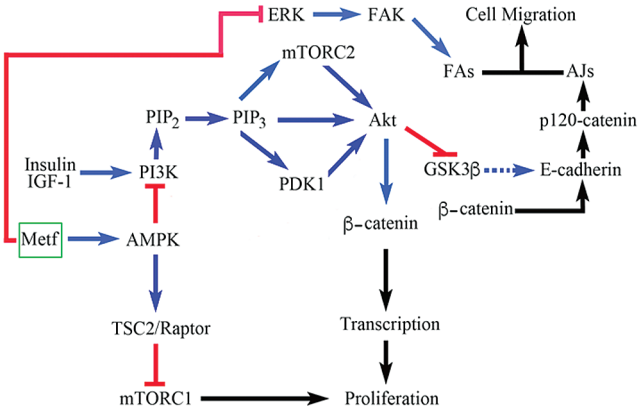

FIGURE 1. Simplified model of novel metformin targets associated to CRC development and progression. The binding of insulin and IGF-1 to their receptors triggers the activity of phosphoinositide-3 kinase (PI3K) that catalyzes the phosphorylation of PtdIns (4,5) P_2 (PIP_2) to produce PtdIns (3,4,5) P_3 (PIP_3), a second messenger that binds and recruits proteins containing a pleckstrin-homology (PH) domain such as Akt, PDK1-that phosphorylates Akt at Thr^{308}- and mSIN1 -a component of mTORC2 (Fu and Hall, 2020), a complex that mediates Akt Ser^{473} phosphorylation. Activated Akt phosphorylates β-catenin at Ser^{552} promoting its nuclear localization and transcription of its target genes. Metformin-mediated AMPK signaling inhibits mTORC1 activation by stimulating TSC2 -which leads to the accumulation of the inactive form Rheb-GDP- and by direct phosphorylation of Raptor -which promotes the dissociation of the mTORC1 complex. AMPK also interferes with the plasma membrane accumulation of PIP_3, which leads to Akt Ser^{473} phosphorylation inhibition. Inhibition of Akt prevents β-catenin Ser^{552} phosphorylation inhibition promoting its plasma membrane localization. Akt inhibition also mediates the activation of GSK3β the phosphorylation of E-cadherin at $Ser^{838/840}$ and its plasma membrane recruitment where co-localizes with β and p120 catenins in the *novo* formed AJs. Metformin treatment also inhibited ERK and FAK catalytic activities, results that were accompanied by a reduction in the number and increase in the size of FAs along with cellular migration inhibition. Red Lines: inhibitory effects; blue arrows: phosphorylation/signaling cascades; black arrows: effects like redistribution of proteins/transcription/proliferation; dotted blue line: putative phosphorylation.

signaling pathways associated with CRC development and progression. Further studies are needed in order to refine our understanding of the underlying mechanisms.

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