International Journal of Biochemistry and Cell Biology 112 (2019) 88–94

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Metformin inhibits β-catenin phosphorylation on Ser-552 through an AMPK/PI3K/Akt pathway in colorectal cancer cells

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ARTICLE INFO

Keywords:
Metformin
β-Catenin
Colorectal cancer
AMPK
PI3K/Akt

ABSTRACT

Several epidemiologic studies have revealed strong inverse associations between metformin use and risk of colorectal cancer development. Nevertheless, the underlying mechanisms are still uncertain. The Wnt/β-catenin pathway, which plays a central role in intestinal homeostasis and sporadic colorectal cancer development, is regulated by phosphorylation cascades that are dependent and independent of Wnt. Here we report that a non-canonical Ser^{552} phosphorylation in β-catenin, which promotes its nuclear accumulation and transcriptional activity, is blocked by metformin via AMPK-mediated PI3K/Akt signaling inhibition.

1. Introduction

Despite advances in early detection, surgery and chemotherapy, colorectal cancer (CRC) remains the second leading cause of cancer-related mortality in the U.S. and other developed countries. Novel targets and agents for therapy and chemoprevention are urgently needed. They most likely will arise from a clear understanding of the signaling pathways involved and the consequent repurposing of currently used drugs.

Considerable evidence has linked metabolic syndrome, obesity and type 2 diabetes mellitus (T2DM), conditions characterized by peripheral insulin resistance and hyperinsulinemia, with an increase in the incidence of multiple malignancies, including CRC (Gonzalez et al., 2017). Consequently, antidiabetic therapies have emerged as possible new strategies in the prevention and treatment of different types of cancer such as CRC (Chang et al., 2018; Gonzalez et al., 2017; Ikhlas and Ahmad, 2017; Jackson and Garcia-Albeniz, 2018; Klil-Drori et al., 2017; Kobiela et al., 2018). In epidemiological studies, the administration of the biguanide metformin, the most commonly prescribed anti-diabetic agent (He and Wondisford, 2015; Rena et al., 2013), has been associated with lower CRC incidence and mortality in T2DM patients (Zhang et al., 2011). Furthermore, a recent double-blind placebo-controlled, randomized trial that showed that low-dose metformin significantly decreased colorectal adenoma recurrence (Higurashi et al., 2016). Despite its potential clinical importance, the cellular and molecular mechanism(s) by which metformin acts as a preventive agent in CRC and other malignancies remains poorly understood (Li et al., 2018).

It is recognized that aberrant activation of β-catenin signaling plays a central role in the majority of sporadic colorectal tumors (Clevers, 2006; Clevers and Nusse, 2012; McDonald et al., 2006). In most cases, the Wnt/β-catenin pathway undergoes an early deregulation that leads to 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). Phosphorylation cascades that are dependent and independent of Wnt signaling play a critical role in the control of β-catenin stability, intracellular distribution and transcriptional activity (Clevers, 2006; Fang et al., 2007; He et al., 2007; Taurin et al., 2006, 2008; Vermeulen et al., 2010). For example, the canonical sequential phosphorylation of β-catenin in its N-terminal domain targets β-catenin for proteosomal degradation (Clevers and Nusse, 2012) whereas its non-canonical phosphorylation at Ser^{552} and Ser^{675} promotes its nuclear translocation and transcriptional activity (Fang et al., 2007; Rey et al., 2012; Taurin et al., 2006). We hypothesized that phosphorylation of β-catenin at Ser^{552} in response to insulin and IGF-1

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https://doi.org/10.1016/j.biocel.2019.05.004
Received 11 March 2019; Received in revised form 6 May 2019; Accepted 8 May 2019
Available online 10 May 2019

provides a plausible mechanism by which T2DM and metabolic syndrome potentiates the promotion of CRC. As a corollary of this hypothesis, we considered that metformin targets this pathway, thus inhibiting β-catenin signaling and progression of CRC. In an effort to test these hypotheses, we examined whether metformin affects β-catenin phosphorylation in human colorectal adenocarcinoma-derived cells stimulated with insulin or IGF-1. Our results show that metformin inhibits β-catenin Ser^{552} phosphorylation by a mechanism that involves 5′ AMP-activated protein kinase (AMPK)-mediated phosphoinositide 3-kinase (PI3K)/Akt signaling inhibition.

2. Materials and methods

2.1. Cell culture and transfections

The human colorectal adenocarcinoma-derived cell lines SW-480 and HT-29 were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and maintained as recommended. Transfections were performed with Lipofectamine Plus (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as previously described (Rey et al., 2001). Dulbecco's Modified Eagle's medium (DMEM) containing physiological (5.0 mM) concentration of glucose was employed for all the experimental procedures.

2.2. Western blot analysis

The collected cells were directly solubilized by boiling in 2X Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) sample buffer and resolved in SDS-PAGE, transferred to Immobilon-P membranes (Millipore Corp., Burlington, Massachusetts, USA) and processed for Western blot as previously described (Rey et al., 2001) using horseradish peroxidase-conjugated IgGs as secondary antibody and enhanced chemiluminescence ECL reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Images were obtained with a GeneGnome XRQ chemiluminescence imaging system (Syngene, Cambridge, UK) and the intensity of the detected bands quantified with the GeneTools software (Syngene, Cambridge, UK). The Western blots results displayed in the figures are representative of at least three independent experiments.

2.3. Immunocytochemistry

Cell cultures prepared for immunocytochemistry as previously described (Rey et al., 2001) were examined with a Nikon Eclipse Ti-E microscope (Nikon Instruments Inc., Tokyo, Japan) and fluorescence images captured with an Andor Neo 5.5 sCMOS camera (Oxford Instruments, Oxfordshire, UK) driven by NIS-Elements AR v 4.30.01 software (Nikon Instrument, Inc., Tokyo, Japan). The cells displayed in the figures are representative of 90% of the population of cells.

2.4. Live cell imaging

To acquire time-lapse images of living cells transiently expressing the PtdIns(3,4,5)P_3 sensor pcDNA3-Akt-PH-GFP (Kwon et al., 2007), which was obtained from Addgene, Watertown, Massachusetts, USA (Cat. #18836), we employed a Delta T Culture Dish System (Bioptechs, Butler, Philadelphia, USA) mounted on a Nikon stage adapter (Bioptechs, Butler, Philadelphia, USA) connected to a Delta T temperature controller (Bioptechs, Butler, Philadelphia, USA) in order to maintain the cultures at 37 °C. Six hours after transfection, the supernantants were removed and the cultures were further incubated for 16 h in DMEM plus 10% fetal bovine serum (FBS). Subsequently, the cultures were incubated in DMEM supplemented with 2.0 mM or 0.5 mM metformin for 16 h before stimulation. Alternatively, after the 16 h incubation with complete DMEM the transfected cultures were further incubated in DMEM without metformin during 14 h and then for 4 h with the AMPK allosteric activator ZLN 024 (80 μM) or for 30 min with Wortmannin (100 nM) before stimulation. Stimulation was performed by perfusing the cultures with Hanks Balanced Salt Solution (138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5 mM KCl, 0.3 mM KH_2PO_4, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5.6 mM D-glucose and 20 mM HEPES, pH: 7.4) supplemented with 10 ng/ml of insulin or 10 ng/ml of IGF-1 without metformin plus ZLN 024 (80 μM) or Wortmannin (100 nM) or with metformin (2 mM or 0.5 mM). The cells were visualized with a Nikon Eclipse Ti-E motorized microscope with integrated Perfect Focus System (Nikon Instruments Inc., Tokyo, Japan) and images captured at 10 s intervals during 20 min starting 2.5 min before IGF-1 stimulation with an Andor Neo 5.5 sCMOS camera (Oxford Instruments, Oxfordshire, UK) driven by NIS-Elements AR v 4.30.01 software (Nikon Instruments Inc., Tokyo, Japan). Analysis of PH-GFP translocation of equivalent regions of the plasma membrane was performed with NIS-Elements AR v 4.30.01 software (Nikon Instruments Inc., Tokyo, Japan). No least than thirty individual cells were examined per condition in three independent experiments. The cells displayed in the appropriate figures are representative of 90% of the transfected cells.

2.5. Materials

Antibodies were obtained from: *Thermo Fisher Scientific* (Waltham, Massachusetts, USA): anti-Akt, anti-phospho Akt Ser^{473}, anti-phospho GSK3β Ser^{9}; Abcam Inc: anti-α-tubulin, Alexa Fluor 488 conjugated anti-mouse IgGs; *Cell Signaling Technology* (Danvers, Massachusetts, USA): anti-phospho β-catenin, Ser^{33}/Ser^{37}/Thr^{41}, Ser^{552}, Thr^{41}/Ser^{45}, Ser^{675}, anti-acetyl CoA carboxylase, anti-phospho acetyl CoA carboxylase Ser^{79}; *GE Healthcare* (Little Chalfont, Buckinghamshire, UK): horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs. IGF-1, insulin and metformin were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The PI3K inhibitor Wortmannin, the Akt inhibitor GSK 690693, and the AMPK allosteric activator ZLN 024 were obtained from Tocris Bioscience (Bristol, UK). All other reagents were of the highest grade commercially available.

3. Results and discussion

3.1. Metformin inhibits β-catenin Ser^{552} phosphorylation induced by insulin and IGF-1 in human colorectal cell lines

The phosphorylation of β-catenin on Ser^{552} has been linked to non-canonical positive regulation of β-catenin intracellular distribution and transcriptional activity (Fang et al., 2007; Rey et al., 2012; Taurin et al., 2006). Consequently, we initially determined whether insulin and IGF-1, which promote CRC development (Aleman et al., 2014; Cohen and LeRoith, 2012; Kant and Hull, 2011), have any impact on phosphorylation of β-catenin on Ser^{552} in CRC-derived cells. Stimulation of human SW-480 CRC cells with either insulin or IGF-1 (10 ng/ml) for 15 min induced a rapid and striking (15-20-fold) increase in the phosphorylation β-catenin at Ser^{552} (Fig. 1A). In contrast, we did not detect any change in the phosphorylation of β-catenin at Ser^{33}/Ser^{37}/Thr^{41}, Thr^{41}/Ser^{45} or Ser^{675} in response to insulin, IGF-1 or metformin (data not shown). The salient feature of the results shown in Fig. 1A is that prior exposure of SW-480 cells to metformin suppressed the increase in β-catenin phosphorylation on Ser^{552} induced by insulin or IGF-1.

To substantiate these results, we examined the effects of insulin and IGF-1 on β-catenin phosphorylation on Ser^{552} with or without prior exposure to metformin in HT-29 cells, a CRC-derived cell line with a genetic background different from SW-480 cells (Ahmed et al., 2013; Gayet et al., 2001). As shown in Fig. 1B, stimulation with insulin or IGF-1 also promoted rapid phosphorylation of β-catenin at Ser^{552} in HT-29 cells, an effect markedly inhibited by prior exposure to metformin. These findings show, for the first time, that exposure to metformin inhibits β-catenin phosphorylation on Ser^{552} induced by insulin or IGF-1

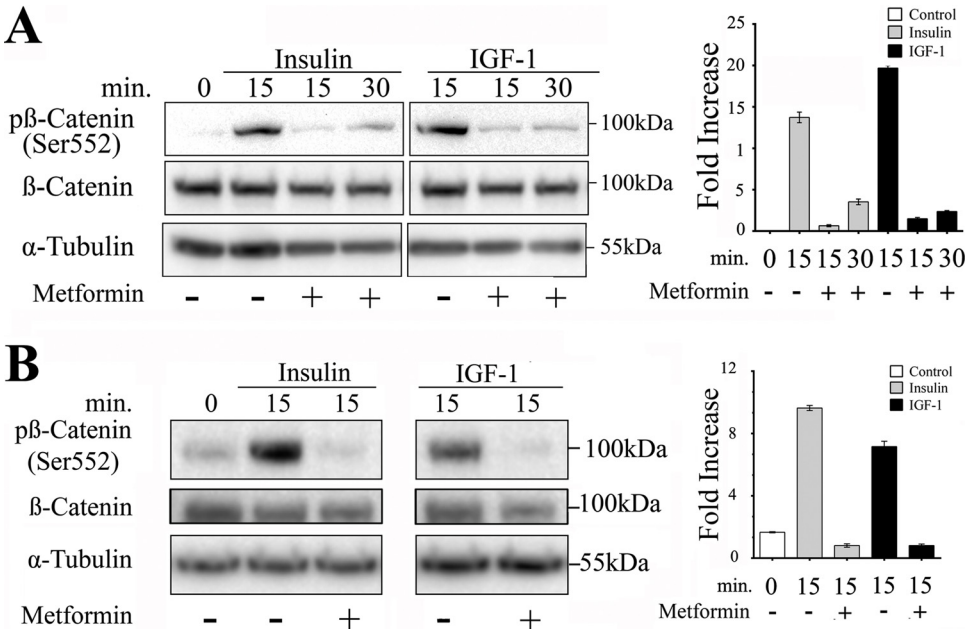

Fig. 1. Metformin inhibits β-catenin Ser^{552} phosphorylation. SW-480 (A) or HT-29 clls (B SW-480 (A) or HT-29 cells (B) preincubated for 16 h with 2.0 mM metformin and challenged with 10 ng/ml insulin or 10 ng/ml IGF-1 were lysed at the indicated times and analyzed by Western blot using a rabbit antibody against β-catenin phospho-Ser^{552} (pβ-Catenin Ser552) and mouse monoclonal antibodies against β-catenin and α-tubulin. Signals were detected with a chemiluminescence imaging system and the intensity of the detected bands quantified as described under Materials and Methods. Bars represent the mean ± SE (n = 4) fold increase in β-catenin Ser^{552} phosphorylation normalized by total β-catenin.

in human colorectal adenocarcinoma-derived cell lines.

3.2. Metformin promotes the plasma membrane translocation of β-catenin in human colorectal cell lines

Because metformin inhibits the phosphorylation of Ser^{552} in β-catenin, a post-translational modification that promotes its nuclear accumulation and transcriptional activity (Fang et al., 2007; Rey et al., 2012), we examined whether metformin treatment also affected its intracellular distribution. As shown in Fig. 2, whereas β-catenin was present throughout the untreated cells, insulin and IGF-1 enhanced its nuclear localization. In contrast, metformin treatment promoted a redistribution of β-catenin characterized by its translocation to the plasma membrane and by a significant reduction in its cytosolic and nuclear localization.

3.3. Metformin inhibits β-catenin phosphorylation on Ser^{552} via Akt

We next examined the mechanism(s) by which metformin inhibits β−catenin phosphorylation on Ser^{552}. This residue is a consensus site for Akt (Fang et al., 2007; Rey et al., 2012), though it can be also phosphorylated by other serine/threonine protein kinases (Verheyen and Gottardi, 2010). As shown in Fig. 3A, insulin and IGF-1 promoted a sustained and dose-dependent increase in the phosphorylation of Akt on Ser^{473} in SW-480 or HT-29 cells. Crucially, exposure to metformin completely abrogated Akt phosphorylation at Ser^{473} induced by insulin or IGF-1 (Fig. 3B), implying that metformin prevents Akt activation in response to insulin and IGF-1 and thereby inhibits β-catenin phosphorylation on Ser^{552}

Akt activation is a multi-step process that involves its plasma membrane recruitment via the binding of its pleckstrin homology (PH) domain to phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5)P_3) produced by PI3K at the inner leaflet of the plasma membrane (James et al., 1996; Naguib, 2016). In order to corroborate that Akt

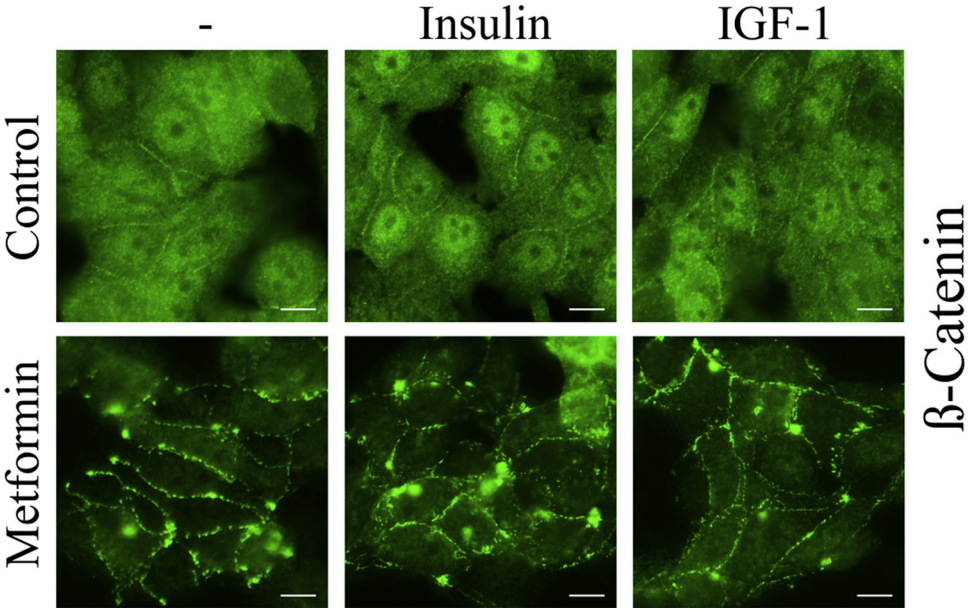

Fig. 2. Metformin promotes the plasma membrane translocation of β-catenin. SW-480 cells preincubated for 16 h with 2.0 mM metformin and challenged with 10 ng/ml IGF-1 were fixed 30 min later and processed for immunofluorescence analysis as described under Materials and Methods using a mouse monoclonal antibody against β-catenin and Alexa Fluor 488 conjugated anti-mouse IgGs. Bar: 10 μm.

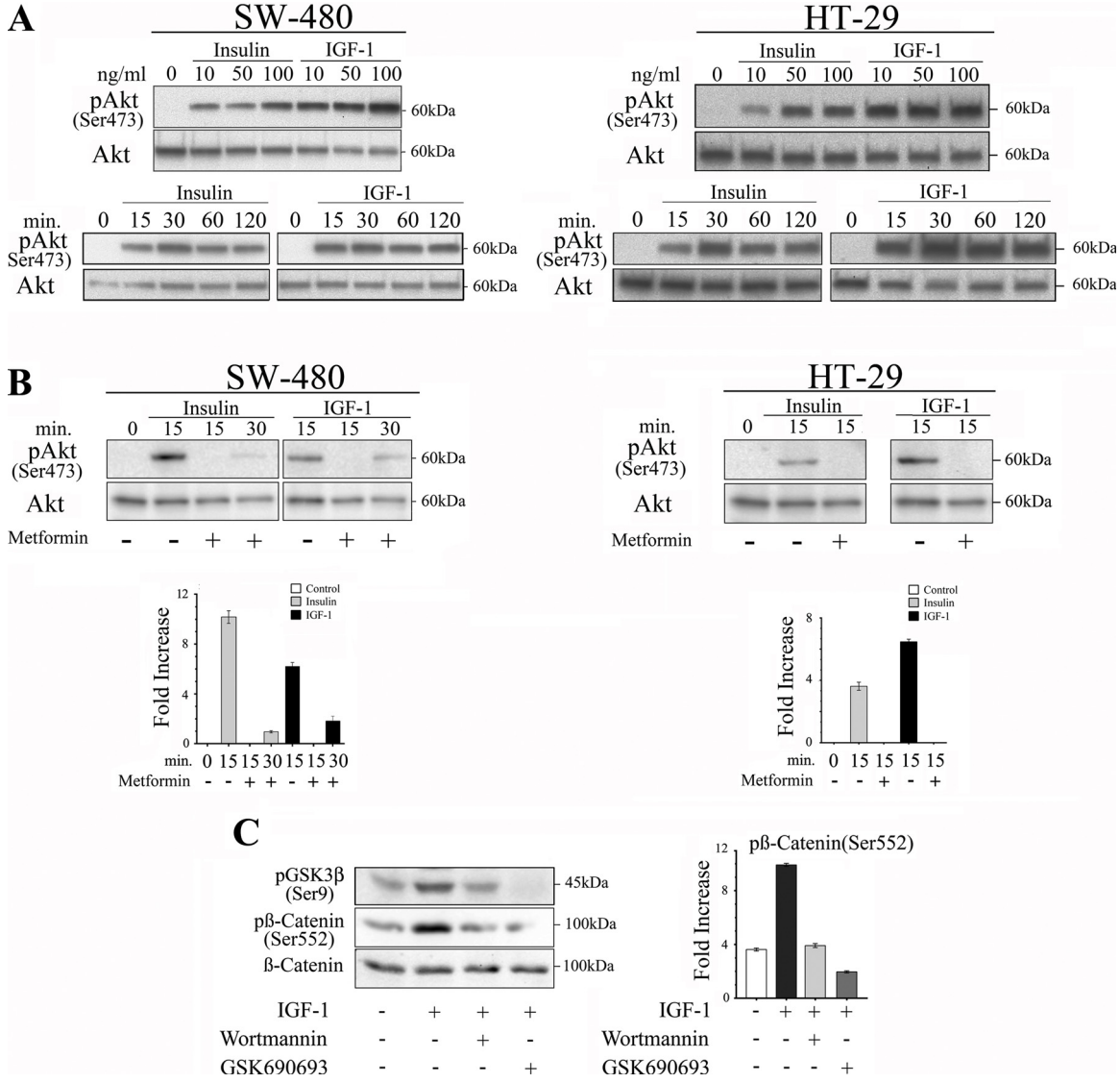

Fig. 3. Insulin and IGF-1 promotes Akt Ser^{473} phosphorylation (A) SW-480 and HT-29 cells challenged for 15 min with the indicated concentrations of insulin or IGF-1 (top panel) or with 10 ng/ml of insulin or 10 ng/ml IGF-1 for the indicated times (lower panel) were lysed and analyzed by Western blot using rabbit antibodies against Akt phospho-Ser^{473} (pAkt Ser473) and Akt (Akt). Signals were detected with a chemiluminescence imaging system. Images are representative of 3 independent experiments ($n = 3$). Metformin inhibits Akt Ser^{473} phosphorylation (B) SW-480 and HT-29 cells preincubated for 16 h with 2.0 mM metformin were challenged with 10 ng/ml insulin or 10 ng/ml IGF-1, lysed at the indicated times and analyzed by Western blot using rabbit antibodies against Akt phospho-Ser^{473} and Akt. Signals were detected and quantified as described in Fig. 1. Bars represent the mean ± SE ($n = 4$) fold increase in Akt Ser^{473} phosphorylation normalized by total Akt. PI3K/Akt inhibition prevents β-catenin Ser^{552} phosphorylation (C) SW-480 cells preincubated for 30 min with Wortmannin (100 nM) or 1 h with the Akt inhibitor GSK 690693 (2.5 μM) were challenged for 15 min with 10 ng/ml of IGF-1 and analyzed by Western blot using a rabbit antibodies against GSK3β phospho-Ser^{9} (pGSK3β Ser9), β-catenin phospho-Ser^{552} or a mouse monoclonal against β-catenin. Signals were detected and quantified as in Fig. 1. Bars represent the mean ± SE ($n = 4$) fold increase in β-catenin Ser^{552} phosphorylation normalized by total β-catenin.

phosphorylates β-catenin at Ser^{552}, the cultures were treated with the PI3K inhibitor Wortmannin (Arcaro and Wymann, 1993) or the Akt inhibitor GSK 690693 (Heerding et al., 2008) and the activation of Akt and phosphorylation of β-catenin at Ser^{552} in response to IGF-1was assessed. As Fig. 3C shows, both inhibitors blocked the phosphorylation of β-catenin on Ser^{552} and Akt activation, monitored by the inhibition of Ser^{9} phosphorylation in GSK3β, a well-characterized Akt substrate (Beurel et al., 2015; Cohen and Frame, 2001; Cross et al., 1995). Collectively, the results support the conclusion that Akt phosphorylates β-catenin at Ser^{552}. Similar results were obtained when the cells were stimulated with insulin (data not shown).

3.4. Metformin-stimulated AMPK signaling inhibits the PI3K/Akt pathway and β-catenin Ser^{552} phosphorylation

Our next task was to elucidate the role of AMPK in the mechanism by which metformin inhibits Akt and β-catenin phosphorylation. Initially, we examined which concentrations of metformin induce AMPK activation in human colorectal adenocarcinoma-derived cells and next whether direct AMPK activation interferes with Akt phosphorylation. As shown in Fig. 4A, metformin concentrations ranging from 0.1 to 5.0 mM promoted the activation of AMPK as revealed by the phosphorylation of acetyl CoA carboxylase Ser^{79}, a well-established AMPK substrate (Hardie and Pan, 2002). These results indicate that metformin induced AMPK activation at the concentrations that inhibit Akt and β-catenin phosphorylation. Importantly, treatment of the

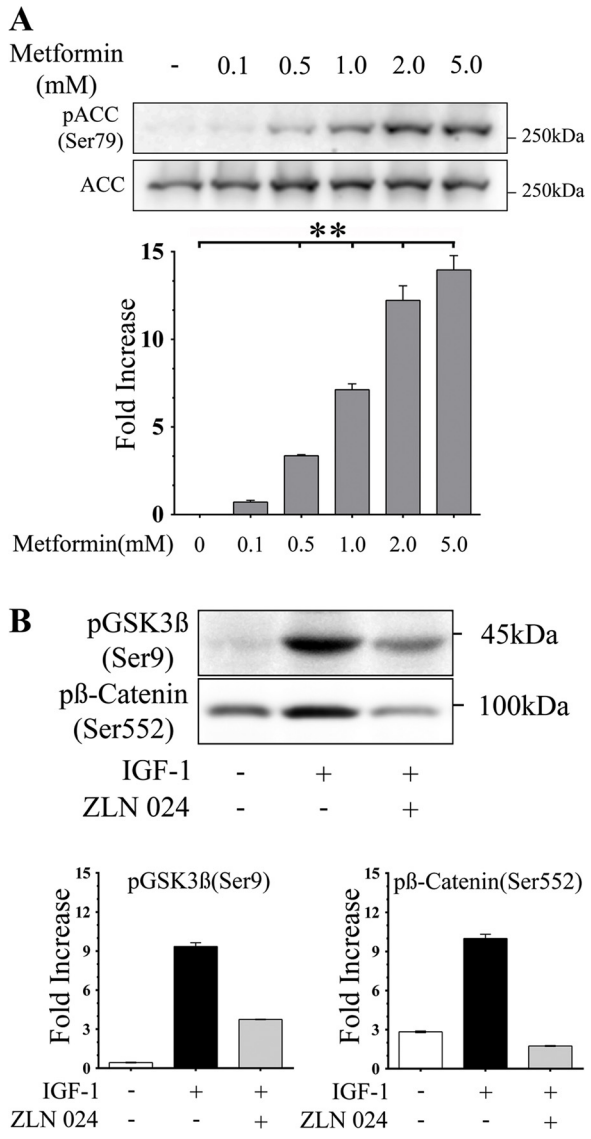

Fig. 4. Metformin promotes the activation of AMPK (A). SW-480 cells incubated for 16 with the indicated concentration of metformin were lysed and analyzed by Western blot using rabbit antibodies against acetyl CoA carboxylase phospho-Ser^{79} (pACC Ser79) and acetyl CoA carboxylase (ACC). Signals were detected and quantified as in Fig. 1 B. Bars represent the mean ± SE (n = 3) fold increase in acetyl CoA carboxylase Ser^{79} phosphorylation normalized by total acetyl CoA carboxylase. The statistical analysis was performed using the Student's t-test (** < 0.001). AMPK activation inhibits Akt and β-catenin Ser^{552} phosphorylation (B). SW-480 cells preincubated for 4 h with the AMPK allosteric activator ZLN 024 (80 μM) and challenged for 15 min with 10 ng/ml IGF-1 were examined by Western blot using rabbit antibodies against β-catenin phospho-Ser^{552} and GSK3β phospho-Ser^{9}. Signals were detected and quantified as in Fig. 1 B. Bars represent the mean ± SE (n = 3) fold increase in β-catenin Ser^{552} phosphorylation and GSK3β Ser^{9} phosphorylation normalized by α-tubulin.

cultures with the novel AMPK allosteric activator ZLN 024 (Zhang et al., 2013) blocked the phosphorylation of β-catenin Ser^{552} and of Akt at Ser^{473} (Fig. 4B).

In order to further understand the mechanism mediating metformin-mediated Akt inhibition, we determined whether metformin interfered with the plasma membrane translocation of Akt, a necessary step in its activation (Manning and Toker, 2017). We examined by real-time imaging the localization of a reporter protein consisting of the Akt-derived PH domain fused to green fluorescent protein (Akt-PH-GFP) (Kwon et al., 2007). As illustrated in Fig. 5, the PH-GFP expressed in non-stimulated SW-480 cells was distributed throughout the cell. Time-lapsed microscopy showed that IGF-1 stimulation promoted rapid translocation of PH-GFP to the plasma membrane as revealed by a noticeable increment of the fluorescent signal at the cell periphery (see also supplementary video 1). Similar results were obtained with insulin (data not shown). Crucially, prior exposure to metformin (0.5–2.0 mM) prevented the plasma membrane translocation of PH-GFP, implying that metformin abrogated PtdIns (3,4,5)P_3 accumulation at the plasma membrane (Fig. 5 and supplementary video 2).

Because the results indicated that metformin inhibited the plasma membrane translocation of the PtdIns (3,4,5)P_3 sensor at concentrations that activate AMPK, we determined whether exposure to an allosteric activation of AMPK also inhibits PI3K. Cultures were treated with the AMPK allosteric activator ZLN 024 and the distribution of PH-GFP examined by time-lapsed microscopy. In agreement with the notion that AMPK inhibits PI3K activation induced by IGF-1 in CRC-derived cells, ZLN 024 prevented the plasma membrane translocation of PH-GFP (Fig. 5 and supplementary video 3). As a control, we verified that inhibition of PI3K with Wortmannin also inhibited the plasma membrane translocation of PH-GFP (Fig. 5 and supplementary video 4). In addition, we confirmed that SW-480 cells transfected with a plasmid encoding GFP instead of Akt-PH-GFP, showed that GFP was present throughout the cell and that its distribution was not affected by IGF-1, insulin, metformin or ZLN 024 (data not shown). Overall, these results support the notion that metformin-mediated AMPK activation interferes with PI3K/Akt signaling via inhibition of PtdIns (3,4,5)P_3 accumulation at the plasma membrane.

4. Concluding remarks

It is well established that over 90% of CRCs are promoted by activation of the Wnt/β-catenin pathway leading to robust β-catenin nuclear accumulation (Walther et al., 2009). Consequently, we hypothesized that the chemopreventive properties of the antidiabetic agent metformin are mediated, at least in part, by direct interference with β-catenin signaling. In support of this hypothesis, our results indicate that metformin inhibited β-catenin Ser^{552} phosphorylation and promoted its plasma membrane localization by a mechanism mediated by the activation of AMPK leading to suppression of PI3K/Akt induced by insulin or IGF-1 in colorectal cancer cells, including SW-480 and HT-29 cells. Previous studies in other cancer cells demonstrated that AMPK inhibits mTORC1 activation through stimulation of TSC2 function, leading to accumulation of Rheb-GDP (the inactive form) and by direct phosphorylation of Raptor, leading to dissociation of the mTORC1 complex (Rozengurt et al., 2014). 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}. Our results showing a marked sensitivity of CRC cells to AMPK-mediated inhibition of PI3K/Akt revealed an exploitable vulnerability in CRC cells that can explain the mechanisms by which metformin acts as a chemopreventive agent in bowel cancer.

Several studies exploring the mechanism of action of metformin in cultured cells reported many effects including respiratory chain complex 1 inhibition, Akt phosphorylation inhibition, ATP depletion, AMPK activation and Wnt/β-catenin signaling inhibition (El-Mir et al., 2000; Isoda et al., 2006; King et al., 2006; Melnik et al., 2018; Park et al., 2019; Takatani et al., 2011). However, these results were obtained employing metformin at concentrations ˜100-fold higher than those achieved in target tissues in diabetic patients receiving therapeutic recommended doses. Consequently, the physiological relevance of the conclusion drawn from these in vitro studies has been questioned (He and Wondisford, 2015). Our results are not subject to this important objection because in contrast to other tissues, metformin accumulates in the gut following its oral administration (Bailey et al., 2008; Paleari et al., 2018). Our results in CRC cells, obtained with metformin at concentrations compatible with those detected in human and rodent

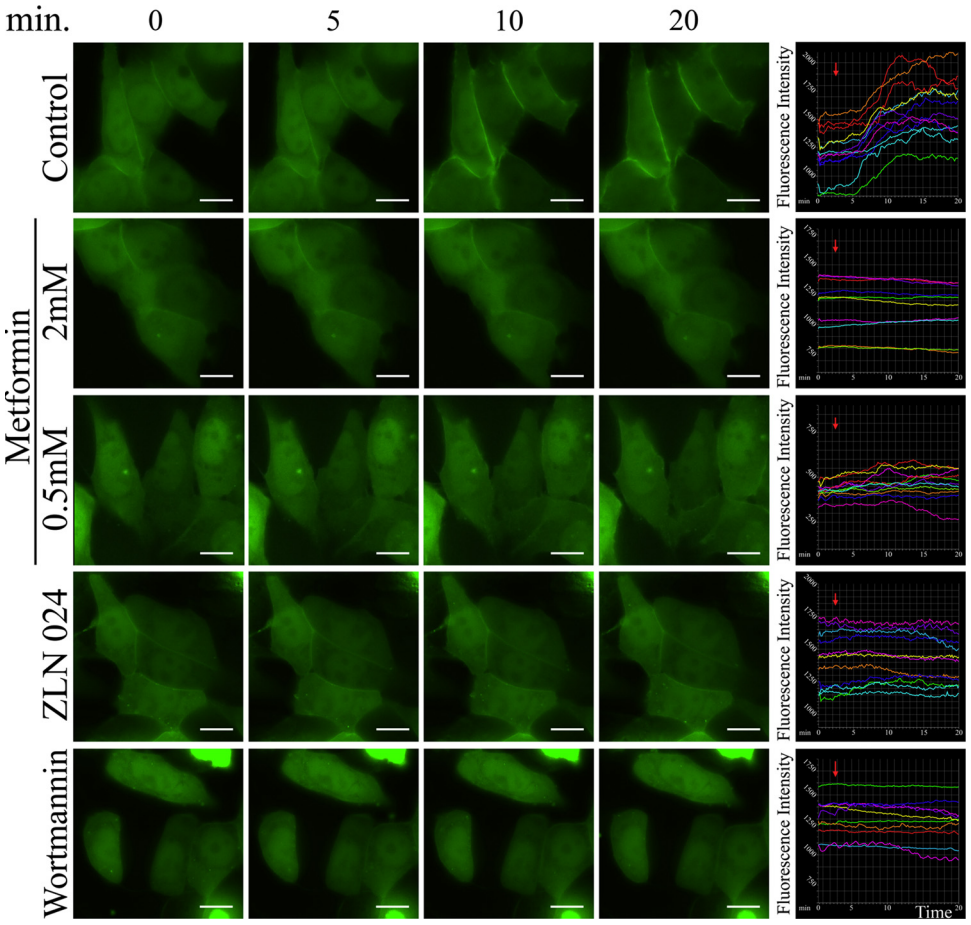

Fig. 5. Metformin impairs PtdIns(3,4,5)P_3 plasma membrane production (A). SW-480 cells expressing a PtdIns(3,4,5)P_3 sensor consisting of the Akt-derived PH domain fused to GFP were incubated for 16 h in DMEM containing 2.0 mM or 0.5 mM metformin before being perfused with Hanks Balanced Salt Solution containing IGF-1 (10 ng/ml). Alternatively, the AMPK allosteric activator ZLN 024 (80 μM) or Wortmannin (100 nM) were added to non-metformin treated cultures 4 h or 30 min, respectively, prior to IGF-1 stimulation. Images of the living cells were acquired by time-lapse microscopy as described under Materials and Methods. Bar: 10 μm. The relative change in plasma membrane fluorescence for each experimental condition is displayed as intensity profiles of equivalent regions of cell plasma membrane as determined with NIS-Elements v 4.30.01 software (Nikon Instrument, Inc.). Red arrow corresponds to IGF-1 perfusion initiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

intestines after oral administration of therapeutic levels of metformin (Bailey et al., 2008; Wilcock and Bailey, 1994), imply that metformin targets CRC development and progression via, at least in part, direct AMPK-mediated PI3K/Akt signaling inhibition leading to abrogation of non-canonical β-catenin-mediated signaling.

Acknowledgements

This work was supported by FONCYT, Secretaría de Ciencia Tecnología e Innovación Productiva, Argentina, PICT 2012-0875 and PICT 2013-0891 to O. R. and PICT 2015-2996 to C. D. E. R. is supported by NIH P30DK041301 and VA I01BX003801. G. A. and E. M. L. are recipients of fellowship awards from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.05.004.

References

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknaes, M., Hektoen, M., Lind, G.E., Lothe, R.A., 2013. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2, e71.
Aleman, J.O., Eusebi, L.H., Ricciardiello, L., Patidar, K., Sanyal, A.J., Holt, P.R., 2014. Mechanisms of obesity-induced gastrointestinal neoplasia. Gastroenterology 146 (2), 357–373.
Arcaro, A., Wymann, M.P., 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. 296 (Pt 2), 297–301.
Bailey, C.J., Wilcock, C., Scarpello, J.H., 2008. Metformin and the intestine. Diabetologia 51 (8), 1552–1553.
Beurel, E., Grieco, S.F., Jope, R.S., 2015. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacol. Ther. 148, 114–131.
Chang, Y.T., Tsai, H.L., Kung, Y.T., Yeh, Y.S., Huang, C.W., Ma, C.J., Chiu, H.C., Wang, J.Y., 2018. Dose-dependent relationship between metformin and colorectal Cancer occurrence among patients with type 2 Diabetes-A nationwide cohort study. Trans. Oncol. 11 (2), 535–541.
Cheah, P.Y., 2009. Recent advances in colorectal cancer genetics and diagnostics. Crit. Rev. Oncol. Hematol. 69 (1), 45–55.
Clevers, H., 2006. Wnt/beta-catenin signaling in development and disease. Cell 127 (3), 469–480.
Clevers, H., Nusse, R., 2012. Wnt/beta-catenin signaling and disease. Cell 149 (6), 1192–1205.
Cohen, P., Frame, S., 2001. The renaissance of GSK3. Nat. Rev. Mol. Cell Biol. 2 (10), 769–776.
Cohen, D.H., LeRoith, D., 2012. Obesity, type 2 diabetes, and cancer: the insulin and IGF connection. Endocr. Relat. Cancer 19 (5), F27–45.
Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A., 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378 (6559), 785–789.
El-Mir, M.Y., Nogueira, V., Fontaine, E., Averet, N., Rigoulet, M., Leverve, X., 2000. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J. Biol. Chem. 275 (1), 223–228.
Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G.B., Kobayashi, R., Hunter, T., Lu, Z., 2007. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J. Biol. Chem. 282 (15), 11221–11229.
Gayet, J., Zhou, X.P., Duval, A., Rolland, S., Hoang, J.M., Cottu, P., Hamelin, R., 2001. Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene 20 (36), 5025–5032.
Gonzalez, N., Prieto, I., Del Puerto-Nevado, L., Portal-Nunez, S., Ardura, J.A., Corton, M., Fernandez-Fernandez, B., Aguilera, O., Gomez-Guerrero, C., Mas, S., Moreno, J.A., Ruiz-Ortega, M., Sanz, A.B., Sanchez-Nino, M.D., Rojo, F., Vivanco, F., Esbrit, P., Ayuso, C., Alvarez-Llamas, G., Egido, J., Garcia-Foncillas, J., Ortiz, A., 2017. 2017 update on the relationship between diabetes and colorectal cancer: epidemiology, potential molecular mechanisms and therapeutic implications. Oncotarget 8 (11), 18456–18485.
Hardie, D.G., Pan, D.A., 2002. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. Biochem. Soc. Trans. 30 (Pt 6), 1064–1070.
He, L., Wondisford, F.E., 2015. Metformin action: concentrations matter. Cell Metab. 21 (2), 159–162.
He, X.C., Yin, T., Grindley, J.C., Tian, Q., Sato, T., Tao, W.A., Dirisina, R., Porter-Westpfahl, K.S., Hembree, M., Johnson, T., Wiedemann, L.M., Barrett, T.A., Hood, L.,

Wu, H., Li, L., 2007. PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat. Gen. 39 (2), 189–198.
Heerding, D.A., Rhodes, N., Leber, J.D., Clark, T.J., Keenan, R.M., Lafrance, L.V., Li, M., Safonov, I.G., Takata, D.T., Venslavsky, J.W., Yamashita, D.S., Choudhry, A.E., Copeland, R.A., Lai, Z., Schaber, M.D., Tummino, P.J., Strum, S.L., Wood, E.R., Duckett, D.R., Eberwein, D., Knick, V.B., Lansing, T.J., McConnell, R.T., Zhang, S., Minthorn, E.A., Concha, N.O., Warren, G.L., Kumar, R., 2008. Identification of 4-(2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-{[(3S)-3-piperidinylmethyl]oxy}-1H- imidazo[4,5-c]pyridin-4-yl)-2-methyl-3-butyn-2-ol (GSK690693), a novel inhibitor of AKT kinase. J. Med. Chem. 51 (18), 5663–5679.
Higurashi, T., Hosono, K., Takahashi, H., Komiya, Y., Umezawa, S., Sakai, E., Uchiyama, T., Taniguchi, L., Hata, Y., Uchiyama, S., Hattori, A., Nagase, H., Kessoku, T., Arimoto, J., Matsuhashi, N., Inayama, Y., Yamanaka, S., Taguri, M., Nakajima, A., 2016. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. Lancet Oncol. 17 (4), 475–483.
Huels, D.J., Ridgway, R.A., Radulescu, S., Leushacke, M., Campbell, A.D., Biswas, S., Leedham, S., Serra, S., Chetty, R., Moreaux, G., Parry, L., Matthews, J., Song, F., Hedley, A., Kalna, G., Ceteci, F., Reed, K.R., Meniel, V.S., Maguire, A., Doyle, B., Soderberg, O., Barker, N., Watson, A., Larue, L., Clarke, A.R., Sansom, O.J., 2015. E-cadherin can limit the transforming properties of activating beta-catenin mutations. EMBO J. 34 (18), 2321–2333.
Ikhlas, S., Ahmad, M., 2017. Metformin: insights into its anticancer potential with special reference to AMPK dependent and independent pathways. Life Sci. 185, 53–62.
Isoda, K., Young, J.L., Zirlik, A., MacFarlane, L.A., Tsuboi, N., Gerdes, N., Schonbeck, U., Libby, P., 2006. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. Arterioscler. Thromb. Vasc. Biol. 26 (3), 611–617.
Jackson, J.W., Garcia-Albeniz, X., 2018. Studying the effects of nonindicated medications on Cancer: etiologic versus action-focused analysis of epidemiologic data. Cancer Epidemiol. Biomark. Prev. 27 (5), 520–524.
James, S.R., Downes, C.P., Gigg, R., Grove, S.J., Holmes, A.B., Alessi, D.R., 1996. Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. Biochem. J. 315, 709–713.
Kant, P., Hull, M.A., 2011. Excess body weight and obesity–the link with gastrointestinal and hepatobiliary cancer. Nat. Rev. Gastroenterol. Hepatol. 8 (4), 224–238.
King, T.D., Song, L., Jope, R.S., 2006. AMP-activated protein kinase (AMPK) activating agents cause dephosphorylation of Akt and glycogen synthase kinase-3. Biochem. Pharmacol. 71 (11), 1637–1647.
Kinzler, K.W., Vogelstein, B., 1996. Lessons from hereditary colorectal cancer. Cell 87 (2), 159–170.
Klil-Drori, A.J., Azoulay, L., Pollak, M.N., 2017. Cancer, obesity, diabetes, and anti-diabetic drugs: is the fog clearing? Nat. Rev. Clin. Oncol. 14 (2), 85–99.
Kobiela, J., Dobrzycka, M., Jedrusik, P., Kobiela, P., Spychalski, P., Sledzinski, Z., Zdrojewski, T., 2018. Metformin and colorectal Cancer - a systematic review. Exp. Clin. Endocrinol. Diabetes: German Soc. Endocrinol. German Diab. Assoc a:0621-8830.
Krausova, M., Korinek, V., 2014. Wnt signaling in adult intestinal stem cells and cancer. Cell. Signal. 26 (3), 570–579.
Kwon, Y., Hofmann, T., Montell, C., 2007. Integration of phosphoinositide- and calmodulin-mediated regulation of TRPC6. Mol. Cell 25 (4), 491–503.
Li, M., Li, X., Zhang, H., Lu, Y., 2018. Molecular mechanisms of metformin for diabetes and Cancer treatment. Front. Physiol. 9, 1039.
Manning, B.D., Toker, A., 2017. AKT/PKB signaling: navigating the network. Cell 169 (3), 381–405.
McDonald, S.A., Preston, S.L., Lovell, M.J., Wright, N.A., Jankowski, J.A., 2006. Mechanisms of disease: from stem cells to colorectal cancer. Nat. Clin. Pract. Gastroenterol. Hepatol. 3 (5), 267–274.
Melnik, S., Dvornikov, D., Muller-Decker, K., Depner, S., Stannek, P., Meister, M., Warth, A., Thomas, M., Muley, T., Risch, A., Plass, C., Klingmuller, U., Niehrs, C., Glinka, A., 2018. Cancer cell specific inhibition of Wnt/beta-catenin signaling by forced intracellular acidification. Cell Discov. 4, 37.
Naguib, A., 2016. Following the trail of lipids: signals initiated by PI3K function at multiple cellular membranes. Sci. Signal. 9 (428), re4.
Paleari, L., Burhenne, J., Weiss, J., Foersch, S., Roth, W., Parodi, A., Gnant, M., Bachleitner-Hofmann, T., Scherer, D., Ulrich, C.M., Stabuc, B., Puntoni, M., Coccia, G., Petrera, M., Haefeli, W.E., DeCensi, A., 2018. High accumulation of metformin in colonic tissue of subjects with diabetes or the metabolic syndrome. Gastroenterology 154 (5), 1543–1545.
Park, S.Y., Kim, D., Kee, S.H., 2019. Metformin-activated AMPK regulates beta-catenin to reduce cell proliferation in colon carcinoma RKO cells. Oncol. Lett. 17 (3), 2695–2702.
Polakis, P., 2012. Wnt signaling in cancer. Cold Spring Harbor Persp. Biol. 4 a008052(5).
Rena, G., Pearson, E.R., Sakamoto, K., 2013. Molecular mechanism of action of metformin: old or new insights? Diabetologia 56 (9), 1898–1906.
Rey, O., Young, S.H., Cantrell, D., Rozengurt, E., 2001. Rapid protein kinase D translocation in response to G protein-coupled receptor activation: dependence on protein kinase Ce. J. Biol. Chem. 276 (35), 32616–32626.
Rey, O., Chang, W., Bikle, D., Rozengurt, N., Young, S.H., Rozengurt, E., 2012. Negative cross-talk between calcium-sensing receptor and beta-catenin signaling systems in colonic epithelium. J. Biol. Chem. 287 (2), 1158–1167.
Rozengurt, E., Soares, H.P., Sinnet-Smith, J., 2014. Suppression of feedback loops mediated by PI3K/mTOR induces multiple overactivation of compensatory pathways: an unintended consequence leading to drug resistance. Mol. Cancer Ther. 13 (11), 2477–2488.
Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., Clarke, A.R., Winton, D.J., 2004. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. 18 (12), 1385–1390.
Takatani, T., Minagawa, M., Takatani, R., Kinoshita, K., Kohno, Y., 2011. AMP-activated protein kinase attenuates Wnt/beta-catenin signaling in human osteoblastic Saos-2 cells. Mol. Cell. Endocrinol. 339 (1-2), 114–119.
Taurin, S., Sandbo, N., Qin, Y., Browning, D., Dulin, N.O., 2006. Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase. J. Biol. Chem. 281 (15), 9971–9976.
Taurin, S., Sandbo, N., Yau, D.M., Sethakorn, N., Dulin, N.O., 2008. Phosphorylation of beta-catenin by PKA promotes ATP-induced proliferation of vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. 294 (5), C1169–1174.
Verheyen, E.M., Gottardi, C.J., 2010. Regulation of Wnt/beta-catenin signaling by protein kinases. Dev. Dyn. 239 (1), 34–44.
Vermeulen, L., De Sousa, E.M.F., van der Heijden, M., Cameron, K., de Jong, J.H., Borovski, T., Tuynman, J.B., Todaro, M., Merz, C., Rodermond, H., Sprick, M.R., Kemper, K., Richel, D.J., Stassi, G., Medema, J.P., 2010. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat. Cell Biol. 12 (5), 468–476.
Walther, A., Johnstone, E., Swanton, C., Midgley, R., Tomlinson, I., Kerr, D., 2009. Genetic prognostic and predictive markers in colorectal cancer. Nat. Rev. Cancer 9 (7), 489–499.
Wilcock, C., Bailey, C.J., 1994. Accumulation of metformin by tissues of the normal and diabetic mouse. Xenobiotica 24 (1), 49–57.
Zhang, Z.J., Zheng, Z.J., Kan, H., Song, Y., Cui, W., Zhao, G., Kip, K.E., 2011. Reduced risk of colorectal cancer with metformin therapy in patients with type 2 diabetes: a meta-analysis. Diabetes Care 34 (10), 2323–2328.
Zhang, L.N., Xu, L., Zhou, H.Y., Wu, L.Y., Li, Y.Y., Pang, T., Xia, C.M., Qiu, B.Y., Gu, M., Dong, T.C., Li, J.Y., Shen, J.K., Li, J., 2013. Novel small-molecule AMP-activated protein kinase allosteric activator with beneficial effects in db/db mice. PLoS One 8 (8), e72092.