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Metformin-inhibition of colorectal cancer cell migration is associated with rebuilt adherens junctions and FAK down-regulation

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Abstract

E-cadherin, a central component of the adherens junction (AJ), is a single-pass transmembrane protein that mediates cell-cell adhesion. The loss of E-cadherin surface expression, and therefore cell-cell adhesion, leads to increased cell migration and invasion. Treatment of colorectal (CRC)-derived cells (SW-480, HT-29) with 2 mM metformin promoted a redistribution of cytosolic E-cadherin to *de novo* formed puncta along the length of the contacting membranes of these cells. Metformin also promoted translocation from the cytosol to the plasma membrane of p120-catenin, another core component of the AJs. Furthermore, E-cadherin and p120-catenin co-localized with β-catenin at cell-cell contacts. Western blot analysis of lysates of CRC-derived cells revealed a substantial metformin-induced increase in the level of p120-catenin as well as E-cadherin phosphorylation on $Ser^{838/840}$, a modification associated to β-catenin/E-cadherin interaction. These modifications in E-cadherin, p120-catenin and β-catenin localization suggest that metformin induces rebuilding of AJs in CRC-derived cells. Those modifications were accompanied by the inhibition of focal adhesion kinase (FAK) as revealed by a significant decrease in the phosphorylation of FAK at Tyr^{397} and paxillin at Tyr^{118}. These changes were

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AUTHOR CONTRIBUTIONS

G. A., E. M-L and O. R. designed the study. G. A., E. M-L., and M. E. P. carried out the experiments. G. A., E. M-L., M. E. P., S. I. N., E. R. and O. R. contributed to the interpretation of the results. E. R. and O. R. wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

associated to a reduction in the numbers, but an increase in the size, of focal adhesions (FAs) and by the inhibition of cell migration. Overall, these observations indicate that metformin targets multiple pathways associated to colorectal cancer development and progression.

1. INTRODUCTION

Despite recent developments in the detection and treatment of colorectal cancer (CRC), this disease is still one of the main causes of cancer-related mortality in the developed world (Ferlay et al., 2013; Siegel, Miller, & Jemal, 2019). Therefore, an understanding of the mechanisms involved in CRC development and progression is needed in order to identify novel targets and therapeutic agents.

Several epidemiological studies indicate that metformin, a widely prescribed antidiabetic drug, exerts a protective effect on different cancers including CRC (Bradley et al., 2018; Chang et al., 2018; Gonzalez et al., 2017; Ikhlas & Ahmad, 2017; Jackson & Garcia-Albeniz, 2018; Klil-Drori, Azoulay, & Pollak, 2017; Kobiela et al., 2018). Importantly, a recent randomized trial showed that low-dose metformin administration to non-diabetic patients markedly decreased metachronous colorectal adenoma/polyp formation (Higurashi et al., 2016). Accordingly, the elucidation of the mechanism(s) by which metformin acts as a chemopreventive agent is of major significance. Although inhibition of the mTORC1 pathway through AMPK-mediated phosphorylation is a prominent mechanism for the anti-proliferative effects of metformin in a variety of cell types (Howell et al., 2017; Rozengurt, Sinnett-Smith, & Kisfalvi, 2010; Soares, Ni, Kisfalvi, Sinnett-Smith, & Rozengurt, 2013) other mechanisms remain unclear, especially those implicated in suppressing cell migration.

Adherens junctions (AJs) are dynamic multiprotein structures that mediate cell-cell adhesions via their binding to the actin cytoskeleton (Bruser & Bogdan, 2017). AJs are also related to CRC growth and distant metastasis (Venhuizen, Jacobs, Span, & Zegers, 2019). The core components of the AJs in epithelial cells are members of the cadherin and catenin families (Daulagala, Bridges, & Kourtidis, 2019). Indeed, expression or surface localization of E-cadherin, a key component of the AJs of the colonic epithelium, is frequently lost or its function disrupted in CRC (Kourtidis, Lu, Pence, & Anastasiadis, 2017; Petrova, Schecterson, & Gumbiner, 2016). β-Catenin, another AJs component that interacts with E-cadherin (Berx & van Roy, 2009; Nelson, 2008), is also frequently absent in the surface of tumor cells, a factor that contributes to diminished cell-cell adhesion and deregulated Wnt signaling (Heuberger & Birchmeier, 2010; Valenta, Hausmann, & Basler, 2012). Another key element of the AJs is p120-catenin, a member of the catenin family that also interacts with E-cadherin and stabilizes the AJs. Recent studies from our laboratory showed that exposure to metformin induce translocation of β-catenin to the plasma membrane (Amable et al., 2019). Accordingly, we examined whether metformin induces re-distribution of E-cadherin and p120-catenin to the plasma membrane in CRC-derived cells.

Focal adhesions (FAs) are integrin-containing multiprotein complexes that connect the cell to the extracellular matrix (ECM). These highly dynamic structures play a key role in cell adhesion, spreading and migration (Berrier & Yamada, 2007). Different lines of evidence support the notion that a crosstalk between AJs and FAs is central to the changes that

mediate cell-cell and cell-ECM interactions (Avizienyte & Frame, 2005; Brunton, MacPherson, & Frame, 2004; Canel, Serrels, Frame, & Brunton, 2013; Cicchini et al., 2008; Serrels, Canel, Brunton, & Frame, 2011). Consequently, we hypothesized that metformin coordinately induces rebuilding AJs and down-regulation FA function in CRC-derived cells, thereby leading to inhibition of cell migration. In support of this hypothesis, the results show that treatment of the human CRC-derived SW-480 and HT-29 cells with metformin promoted robust plasma membrane translocation of E-cadherin, p120-catenin and β-catenin, which co-localized along the surface of contacting cells. These changes were accompanied by metformin-induced inhibition of focal adhesion kinase (FAK) and ERK signaling and reduction in the numbers, but an increase in the size, of focal adhesions (FAs). Finally, we show that treatment with metformin suppressed cell migration.

2. Materials and methods

2.1. Cell culture

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 previously described (Amable et al., 2019). Cells were passed no longer than six months after removal from liquid nitrogen.

2.2. Immunocytochemistry, Western blot, subcellular fractionation and cell imaging

Immunocytochemistry and indirect immunofluorescence were done as previously described (Rey, Young, Cantrell, & Rozengurt, 2001). Western blot analysis was performed as reported (Rey et al., 2001). The immunoblots signals were acquired with a GeneGnome XRQ chemiluminescence imaging system (Syngene) and the intensity of the detected bands quantified with GeneTools software (Syngene)(Martinez-Leon et al., 2019). The Subcellular Protein Fractionation Kit for Cultured Cells from Thermo Scientific (part # 78840), Massachusetts, USA, a widely employed kit to separate cytosolic and membrane fractions - enriched in integral and membrane-associated proteins (Frescas et al., 2017; Y. Kim et al., 2018; Liao et al., 2011; Rosenberg et al., 2018; Roy, Placzek, & Scanlan, 2012; Schernthaner-Reiter, Trivellin, & Stratakis, 2018)- was employed according to the manufacturer's recommendations. α-Tubulin and the plasma membrane Ca^{2+} ATPase (PMCA) were used as cytosolic and membrane markers (Li et al., 2007; Sepulveda, Hidalgo-Sanchez, & Mata, 2004) and as controls for loading normalization of the proteins examined in the cytosolic and membrane fractions, i. e. E-cadherin, β-catenin and p120-catenin. Images analysis to assess co-localization of E-cadherin/β-catenin and E-cadherin/p120-catenin was performed using Mander's overlap coefficient (R) (Manders, Verbeek, & Aten, 1993) as implemented by CoLocalizer Pro v 1.4 (CoLocalization Research Software, Tokyo, Japan). The overlap coefficients have values between 0 and 1; a value of 0 indicates that there is no co-localization, whereas a value of 1.0 means there is complete co-localization. A total of 5 fields, each one containing at least 10 cells, were analyzed per condition as described (Rey, Young, Papazyan, Shapiro, & Rozengurt, 2006). Quantification of FAs number and area (n= 30 cells/condition) was performed as previously described (Horzum, Ozdil, & Pesen-Okvur, 2014).

2.3. Cell wound closure assay

Cells (3×10^5 cells/ml) were seeded into ibidi-Treat μ-Dish$^{35mm,\ high}$ Culture-Insert (Ibidi GmbH, Martinsried, Germany) and 24 h later the culture inserts were detached leaving a cell-free gap of 500 μm. The cultures were washed with pre-warmed DMEM and incubated with DMEM plus 10% Fetal Bovine Serum with or without 2.0 mM metformin. Differential contrast phase images were captured every 24 h during 4 days (SW-480 cells) or 5 days (HT-29 cells). Wound-healing/cell migration was quantified with the Wound Healing Automated Cellular Analysis application (Ibidi GmbH, Martinsried, Germany).

2.5 Statistical analysis

Western blots, FAs numbers and size, and cell wound closure assay values represent the mean ± SE and their statistical analysis was performed with Welch's unequal variances t-tests (Ruxton, 2006; Welch, 1947) applying multiple-testing-correction of the p-values with the Bonferroni's method (Dunn, 1958). To analyse changes in the protein content proportion/percentage in membrane and cytoplasmic fractions we utilized beta regressions (Douma & Weedon, 2019) since they originate from continuous variables, applying multiple-testing-correction of the p-values with the Bonferroni's method (Dunn, 1958).

2.6. Materials

Antibodies were obtained from *Thermo Fisher Scientific* (Waltham, Massachusetts, USA): anti-FAK, anti-phospho FAK Tyr^{397}, anti-phospho FAK Ser^{910}, anti-p120-catenin, anti-paxillin, anti-phospho paxillin Tyr^{118}, Alexa Fluor-conjugated anti-rabbit or anti-mouse IgGs; *Abcam Inc*. (Cambridge, UK): anti-α-tubulin, anti-E-cadherin, anti-phospho E-cadherin Ser$^{838/840}$, anti-Ca^{2+} pan PMCA ATPase; *Cell Signaling Technology* (Danvers, Massachusetts, USA): anti β-catenin, anti-phospho ERK1/2 Thr^{202}/Tyr^{204}; *Santa Cruz Biotech* (Dallas, Texas, USA): anti-E-cadherin, anti-ERK 2; *GE Healthcare* (Little Chalfont, Buckinghamshire, UK): horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs. Metformin was from Sigma-Aldrich (St. Louis, Missouri, USA). All other reagents were of the highest grade commercially available.

3. Results and discussion

3.1. Metformin promotes the plasma membrane translocation and co-localization of E-cadherin, β-catenin and p120-catenin

In order to determine whether metformin induces redistribution of E-cadherin from the cytosol to the surface of CRC-derived cells, SW-480 cells were treated with 2 mM metformin and its localization examined by indirect immunofluorescence. As shown in Fig. 1A, E-cadherin was present in the cytoplasm of control cells in a punctuate pattern as well as in their periphery. Treatment with metformin promoted a noticeable redistribution of cytosolic E-cadherin to *de novo* formed puncta along the length of the contacting membranes. Next, we determined the effect of metformin on the intracellular localization of β-catenin. In agreement with our previous results (Amable et al., 2019), metformin also induced the redistribution of β-catenin to the plasma membrane, specifically at the periphery of contacting cells (Fig. 1A), a localization that coincided with the distribution of E-cadherin

(Fig. 1A, insets). Similar intracellular redistribution of E-cadherin and β-catenin was elicited by metformin in HT-29 cells (Fig. 1A), a human CRC-derived cell line with a genetic background distinct from SW-480 cells (Ahmed et al., 2013; Gayet et al., 2001).

p120-Catenin is a protein that regulates E-cadherin endocytosis by preventing the binding of endocytic adaptors to its juxtamembrane domain (Bruser & Bogdan, 2017; Cadwell, Su, & Kowalczyk, 2016; Kourtidis, Ngok, & Anastasiadis, 2013; Kowalczyk & Nanes, 2012; Nanes et al., 2012). In view of the results presented in Fig. 1A, we examined whether metformin also influenced the intracellular distribution of p120-catenin. As illustrated by the images presented in Fig. 1B, most of p120-catenin in control cells was detected in the cytoplasm with a minor fraction at the cell periphery consistent with plasma membrane localization. In contrast, a marked p120-catenin shift from the cytosol to the plasma membrane was observed in metformin-treated cells where it co-localized with E-cadherin at discrete regions along the plasma membrane (Fig. 1B, insets). Interestingly, we did not find significant changes in the protein expression level of either E-cadherin or β-catenin in metformin-treated cells, but we detected a ~2-fold increment in the level of p120-catenin (Fig. 1C).

In order to assess the level of co-localization of E-cadherin, β-catenin and p120-catenin promoted by metformin, we performed quantitative image analysis of cells incubated with antibodies against E-cadherin/β-catenin or E-cadherin/p120-catenin followed by Alexa Fluor-conjugated anti-rabbit or anti-mouse IgGs as previously described (Rey et al., 2006). E-cadherin and β-catenin displayed overlap coefficients (R) of 0.8742 ± 0.079 (mean ± SD) for metformin-treated (n=50 cells) versus 0.3513 ± 0.042 for untreated cells (n=50 cells) whereas p120-catenin and E-cadherin increased their overlap coefficients from 0.286 ± 0.062 for untreated cells (n= 50) to 0.687 ± 0.093, for metformin-treated cells (n= 50). Collectively, these results support the notion that metformin promotes the plasma membrane redistribution and co-localization of E-cadherin, β-catenin and p120-catenin.

To further support the observation that metformin promoted the redistribution of E-cadherin, β-catenin and p120-catenin to the plasma membrane, SW-480 cells were fractionated and the distribution of these proteins examined in the cytosolic and membrane fractions of non-treated or metformin-treated cultures. In agreement with the results presented in Fig. 1, metformin promoted a marked redistribution of E-cadherin, β-catenin and p120-catenin to the membrane fraction (Fig. 2A).

The phosphorylation of the cytoplasmic tail of E-cadherin at $Ser^{838/840}$ promotes β-catenin binding and interaction stability (Choi, Huber, & Weis, 2006; Dupre-Crochet et al., 2007; Ishiyama & Ikura, 2012; Lickert, Bauer, Kemler, & Stappert, 2000; McEwen, Maher, Mo, & Gottardi, 2014; Pokutta & Weis, 2007; Serres et al., 2000). In view of our results, we examined whether the redistribution of E-cadherin and its co-localization with β-catenin to distinct regions of the plasma membrane in response to metformin was associated with the phosphorylation of E-cadherin cytoplasmic tail. Western blot analysis of cytosolic and membrane fractions and from total cell lysates revealed that E-cadherin phosphorylated at $Ser^{838/840}$ was predominantly present in the membrane fraction (Fig. 2A) and that metformin promoted a substantial increase in E-cadherin $Ser^{838/840}$ phosphorylation (Fig. 2A–B).

Interestingly, the amino acid sequence surrounding $Ser^{838/840}$ conforms to a GSK3β recognition site, a kinase that is activated in human colorectal adenocarcinoma-derived cells as a result of metformin treatment (Amable et al., 2019).

3.2. Metformin inhibits FAK signaling and reorganize the FAs

Since crosstalk between AJs and FAs is central to the changes that mediate cell-cell and cell-ECM interactions (Avizienyte & Frame, 2005; Brunton et al., 2004; Canel et al., 2013; Cicchini et al., 2008; Serrels et al., 2011), we next determined whether metformin treatment also modifies the size, number or function of the FAs. FAK, an integral component of the FAs that regulates their maturation and turnover (Lawson et al., 2012; Tomar, Lawson, Ghassemian, & Schlaepfer, 2012), is frequently activated in different types of cancers including CRC (Ashton et al., 2010; Golubovskaya et al., 2012; Sulzmaier, Jean, & Schlaepfer, 2014a; Tai, Lai, Peng, Ding, & Shen, 2016). Accordingly, we first examined the effect of metformin upon FAK activation by examining Tyr^{397} phosphorylation, an auto-phosphorylation site that regulates downstream signaling (Brunton & Frame, 2008; Jacamo, Jiang, Lunn, & Rozengurt, 2007; Mitra & Schlaepfer, 2006).

Metformin-treated cells showed a significant decrease in FAK Tyr^{397} phosphorylation (Fig. 3A) suggesting that cell exposure to metformin inhibited FAK catalytic activaty. In order to further support this conclusion, we examined Tyr^{118} phosphorylation in paxillin, a direct FAK substrate that also forms part of the FAs (Horton et al., 2015). As Fig. 3B shows, metformin inhibited Tyr^{118} phosphorylation. Moreover, metformin also inhibited FAK Ser^{910} phosphorylation (Fig. 3C), a modification associated to paxillin/FAK interaction, cell spreading and migration (Chu et al., 2011; Luo, Matthews, Robinson, & Jones, 2019; Vincent & Settleman, 1997). Since FAK Ser^{910} phosphorylation is mediated by ERK (Hunger-Glaser, Fan, Perez-Salazar, & Rozengurt, 2004; Hunger-Glaser, Salazar, Sinnett-Smith, & Rozengurt, 2003; Jiang, Sinnett-Smith, & Rozengurt, 2007) and ERK signaling is implicated in promoting cell migration (Samson et al., 2019), we examined whether metformin also inhibits this pathway. In agreement with results reported in other cell types (Ming et al., 2014; Zhou et al., 2016), exposure to metformin effectively blocked ERK activity in CRC-derived cells (Fig. 3D).

FAs increase their size upon FAK inhibition or depletion very likely by a mechanism that interferes with FAK-induced FAs turnover (Ilic et al., 1995; Iwanicki et al., 2008; D. H. Kim & Wirtz, 2013; Plotnikov, Pasapera, Sabass, & Waterman, 2012). Because our results indicated that metformin inhibited FAK signaling, we examined whether its inhibition led to FAs structural changes. Untreated or metformin-treated SW-480 cells processed for indirect immunofluorescence were co-stained with antibodies against FAK and paxillin in order to locate and determine FAs numbers and size. As Fig. 4 shows, numerous and small FAs were present in control cells whereas metformin-treated cells showed fewer and larger FAs. Quantitative images analysis (n=30 cells/condition) corroborated that metformin treatment was associated to FAs numbers reduction and size enlargement. Overall, these results imply that metformin reduces the number of FAs while increases their size by a mechanism that involves FAK signaling inhibition.

3.3. Metformin inhibits cell migration

AJs *de novo* formation and FAK signaling inhibition in response to metformin led us to examine whether these effects would reduce cell migration. In order to examine this possibility, HT-29 and SW-480 cells were seeded into μ-Dish system (Nyegaard, Christensen, & Rasmussen, 2016) and incubated with or without metformin. Differential contrast phase images of the cultures were captured every 24 h during 5 days (HT-29 cells) or 4 days (SW-480 cells). As shown in Fig. 5, the effect of metformin on HT-29 cells started to be evident after 24 h, being greater at 120 h when the wound was completely closed in control cells but remained approximately 40–45% open in metformin-treated cells. A noticeable effect of metformin upon wound closure was evident in SW-480 cells at 72 h reaching a gap of 30–35% at 96 h when the wound was close in control cells further supporting the notion that metformin inhibits CRC-derived cell migration.

4. Concluding remarks

AJs are dynamic multiprotein structures that play a critical role in cell-cell adhesion and are disrupted in inflammation and cancer. E-cadherin, β-catenin and p120 constitute the core elements of AJs. Indeed, several lines of evidence indicate that β-catenin regulates E-cadherin cell surface availability and function (Ishiyama & Ikura, 2012; McEwen et al., 2014; Pokutta & Weis, 2007; Valenta et al., 2012). Here, we report that treatment of CRC-derived cells with metformin induces redistribution of E-cadherin to the plasma membrane that coincided with the translocation of β-catenin to the same compartment. Moreover expression at the plasma membrane was dramatically enhanced by metformin. Importantly, β-catenin, E-cadherin and p120-catenin co-localized along the length of contacting membranes suggesting that metformin stimulated the formation of AJs. Within this context, and considering the inhibitory role of p120-catenin in cadherin endocytosis and recycling (Bruser & Bogdan, 2017), it is plausible that the increase in the level of p120-catenin induced by metformin treatment facilitates the rebuilding of AJs. TJs and desmosomes, together with AJs, form an apical junction complex that control epithelial barrier function, cell-cell adhesion and signaling (Mehta, Nijhuis, Kumagai, Lindsay, & Silver, 2015; Shigetomi & Ikenouchi, 2019). Interestingly, several studies indicate that AMPK signaling exerts a protective effect on intestinal barrier function by a mechanism that stimulates the formation of TJs (Chen et al., 2018; Peng, Li, Green, Holzman, & Lin, 2009; Wu, Wang, Liu, Shan, & Wang, 2018; Zhang, Li, Young, & Caplan, 2006). Since TJs assembly is coupled to AJs formation (Campbell, Maiers, & DeMali, 2017), it is conceivable that the formation of AJs in response to metformin contributes to TJs assembly and intestinal barrier recovery after injury.

FAK occupies a central node in various signaling pathways that control tumor growth and metastasis (Canel et al., 2010; Sulzmaier, Jean, & Schlaepfer, 2014b; Tai, Chen, & Shen, 2015). For example, FAK null mice fibroblasts showed a reduced rate of migration associated to FAs reorganization (Ilic et al., 1995) while FAK deficient cancer cells display large FAs and reduced motility (Chan, Cortesio, & Huttenlocher, 2009; Hsia et al., 2003; Huttenlocher & Horwitz, 2011; Webb et al., 2004). Very little is known about the impact of metformin on FAK regulation. Previous studies reported that metformin inhibited FAK

phosphorylation in ovarian (Erices et al., 2017) and prostatic cancer cells (Yu et al., 2017) but its impact on CRC-derived cells was unknown. Here, we found that metformin markedly reduced FAK autophosphorylation at Tyr^{397}, a site that plays a critical role in FAK signaling. In line with this conclusion, metformin also inhibited the phosphorylation of the FA-associated protein paxillin at Tyr^{118}, a residue targeted by FAK/Src (Zhao & Guan, 2011). Based on our findings and current literature, we propose a scheme model (Fig. 5B) to explain the inhibitory effect of metformin on CRC-derived cells migration. In this simplified model, metformin treatment promotes the up-regulation and plasma membrane translocation of p120-catenin. Once in the plasma membrane, p120-catenin interacts with E-cadherin and inhibits its endocytosis thereby facilitating the redistribution of E-cadherin/β-catenin to the plasma membrane and therefore AJs rebuilding. Inhibition of FAK catalytic activity and Tyr^{397} phosphorylation in response to metformin leads to FAs reorganization very likely through a modification of FAs turnover. All these effects converge in the inhibition of CRC-derived cell migration. Further experiments expressing non- and phosphorylatable forms of E-cadherin and FAK in CRC-derived cells with the corresponding endogenous proteins knocked-out, as well as studies on metformin-mediated p120-catenin up-regulation, are needed to reach a better understanding of the molecular mechanisms mediating metformin effects on AJ and FA.

Previous studies with metformin in cultured cells have used concentrations 10–100-fold higher than those found in the serum of type 2 diabetes mellitus patients receiving the recommended doses of this drug leading to conclusions that remain controversial (He and Wondisford, 2015), a caveat that does not apply to our studies since we employed metformin concentrations within the range of those found in human and rodent intestines after administration of therapeutic doses of metformin (Bailey, Wilcock, & Scarpello, 2008; Paleari et al., 2018; Wilcock & Bailey, 1994). Collectively, the results presented here indicate that metformin targets key pathways associated to CRC development, progression and dissemination.

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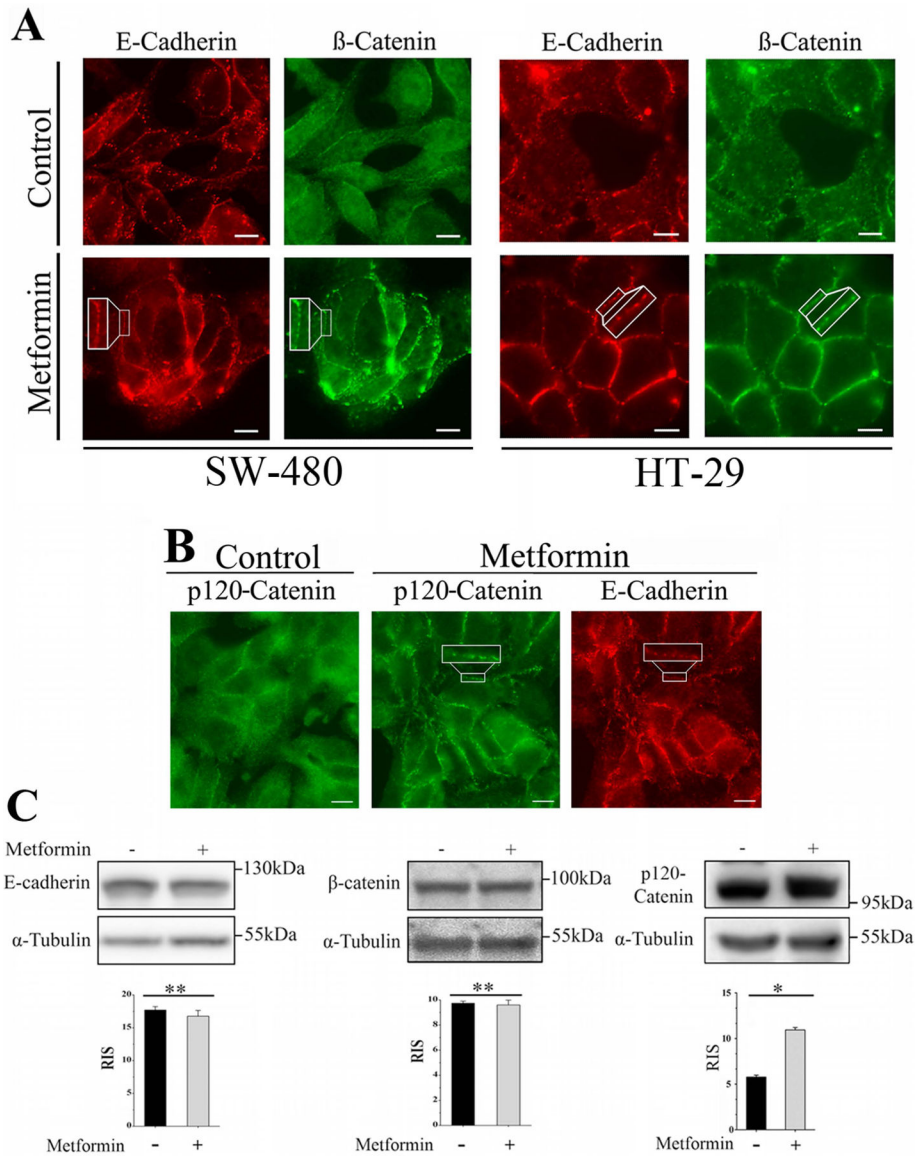

Figure 1.

Metformin promotes the plasma membrane translocation of E-cadherin, β-catenin and p120-catenin. **(A)** SW-480 or HT-29 cells preincubated for 16 h with 2.0 mM metformin were processed for immunofluorescence using a murine monoclonal antibody against E-cadherin and a rabbit polyclonal antibody against β-catenin followed by Alexa Fluor 568 or Alexa Fluor 488 conjugated anti-mouse and anti-rabbit IgGs, respectively. The images are representative of four independent experiments. Bar: 10 μm. (**B**) SW-480 cells preincubated for 16 h with 2.0 mM metformin were processed for immunofluorescence using a murine monoclonal antibody against p120-catenin and a rabbit antibody against E-cadherin followed by Alexa Fluor 488 or Alexa Fluor 568 conjugated anti-mouse and anti-rabbit IgGs, respectively. The images are representative of four independent experiments. Bar: 10 μm. **(C)** Total SW-480 cells lysates incubated for 16 h with 2.0 mM metformin were analyzed by Western blot using antibodies against E-cadherin, β-catenin, p120-catenin and

α-tubulin. Bars represent the mean ± SE relative cellular content E-cadherin (four experiments), β-catenin (three experiments), and p120-catenin (three experiments) normalized by α-tubulin. $p_{(Bonferroni)}$ **< 0.01, *<0.05. RIS= Relative Intensity Signal.

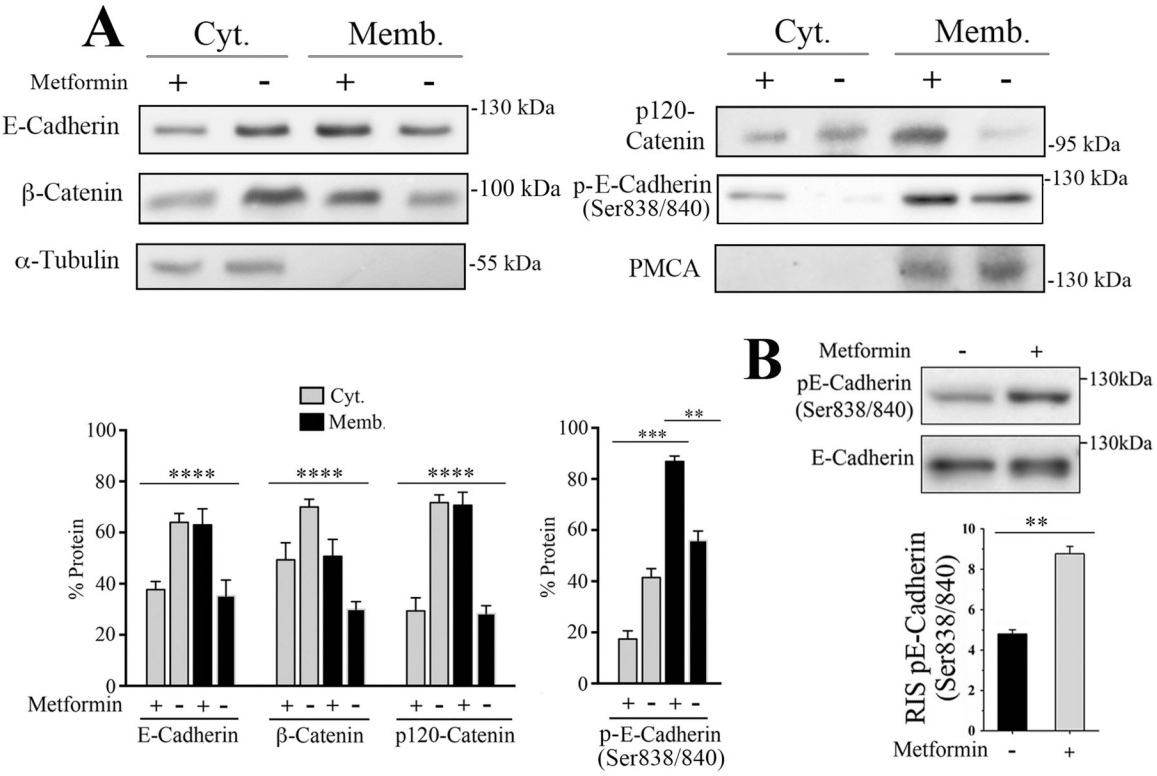

Figure 2.

Metformin promotes the intracellular redistribution of E-cadherin, β-catenin and p120-catenin and E-cadherin Ser$^{838/840}$ phosphorylation. **(A)** SW-480 cells incubated for 16 with 2.0 mM metformin underwent subcellular fractionation and the content of E-cadherin, β-catenin, p120-catenin and E-cadherin phosphorylated at Ser$^{838/840}$ determined in the cytosolic and membrane fractions of three independent experiments by Western blot. Bars represent the mean ± SE as percentage of the total (Cyt. + memb.) of each examined protein, with or without metformin treatment. $p_{(Bonferroni)}$ ****<0.0001, ***< 0.001, **<0.01. α-Tubulin and the plasma membrane Ca^{2+} ATPase (PMCA) were used as cytosolic and membrane markers and as controls for loading normalization. (**B**) Total SW-480 cells lysates incubated for 16 with 2.0 mM metformin were analyzed by Western blot using rabbit antibodies against E-cadherin phospho-Ser$^{838/840}$ (pE-Cadherin Ser838/840) and a murine monoclonal antibody against E-cadherin. Bars represent the mean ± SE relative cellular content of three independent experiments normalized by α-tubulin. $p_{(Bonferroni)}$ **< 0.01. RIS= Relative Intensity Signal.

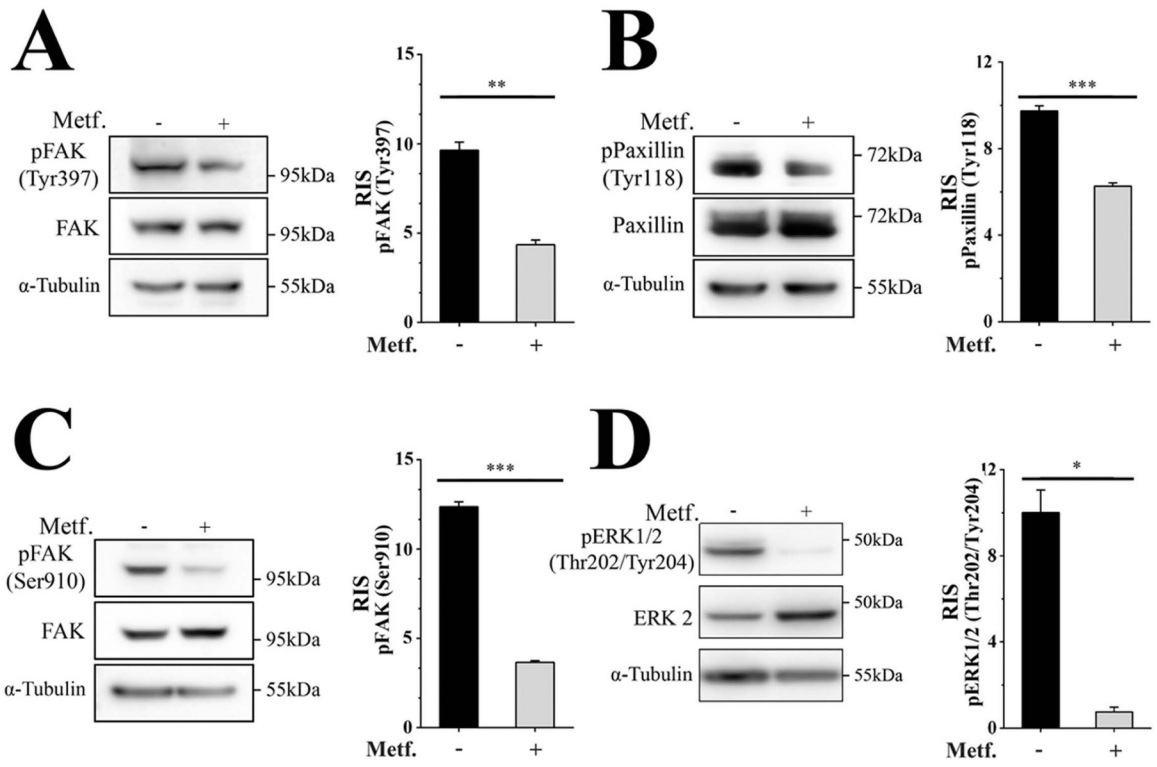

Figure 3.

Metformin inhibits FAK and ERK signaling. SW-480 cells incubated for 16 with 2.0 mM metformin were analyzed in three independent experiments by Western blot using rabbit and murine antibodies against FAK phospho-Tyr^{397}, FAK phospho-Ser^{910}, FAK, paxillin phospho-Tyr^{118}, paxillin, ERK1/2 phospho-Thr^{202}/Tyr^{204}, ERK2 and α-tubulin. Bars represent the mean ± SE for each phosphorylated protein normalized by the total counterpart, i. e. FAK, paxillin and ERK2. $p_{(Bonferroni)}$ ***< 0.001, **<0.01, *<0.05. RIS= Relative Intensity Signal.

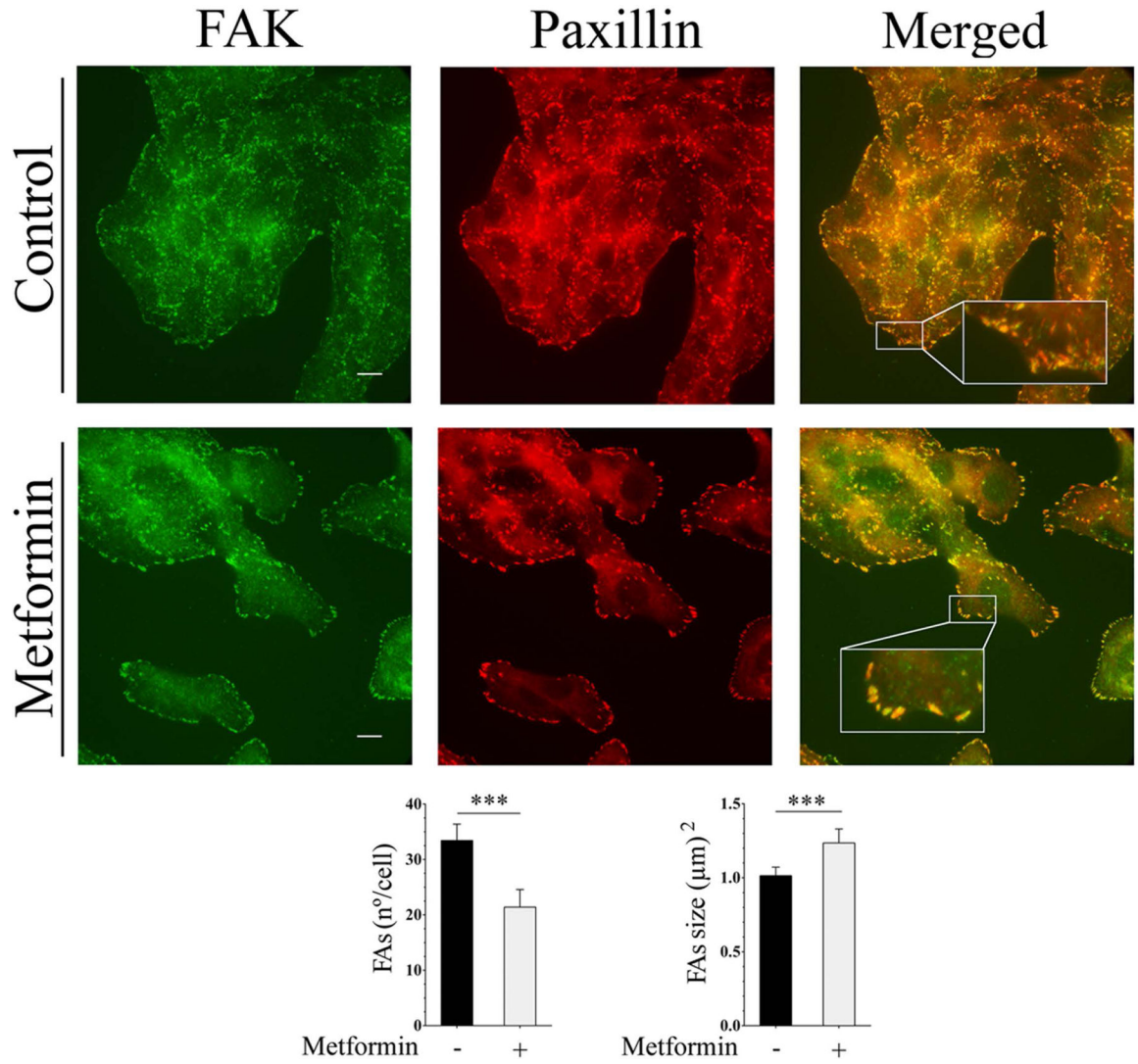

Figure 4.

Metformin stimulates the reorganization of FAs. SW-480 cells preincubated for 16 h with 2.0 mM metformin were processed for immunofluorescence analysis using a murine monoclonal antibody against FAK and a rabbit antibody against paxillin followed by Alexa Fluor 488 or Alexa Fluor 568 conjugated anti-mouse and anti-rabbit IgGs, respectively. Bar: 10 µm. FAs numbers and size quantification was performed as described under Materials and Methods. Bars represent the mean ± SE (n=30 cells/condition). $p_{(Bonferroni)}$ ***≤ 0.001.

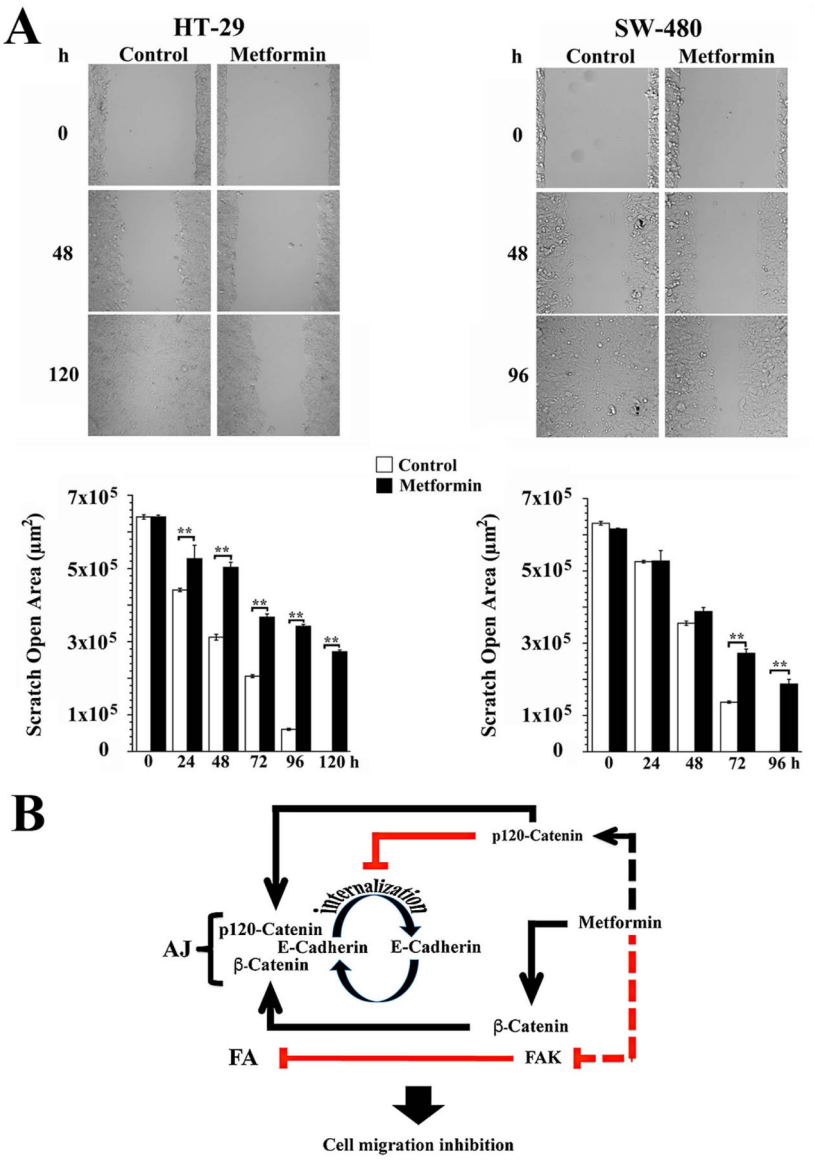

Figure 5.

Metformin inhibits cell migration. **(A)** HT-29 and SW-480 cells were incubated during 120 h and 96 h, respectively, in the presence of 2.0 mM metformin and images acquired every 24 h. Cell migration was quantified using the Wound Healing Automated Cellular Analysis System application (Ibidi GmbH). Values represent the mean ± SE of the scratch open area (μm^2) of three independent experiments. $p_{(Bonferroni)}$ ** <0.01. **(B)** Schematic representation of a simplified model of metformin-associated cell migration inhibition. Metformin treatment promotes the up-regulation and plasma membrane translocation of p120-catenin where interacts with E-cadherin and inhibits its endocytosis thereby facilitating the redistribution of E-cadherin/β-catenin to the plasma membrane and AJs rebuilding. Metformin-mediated inhibition of FAK catalytic activity leads to FAs reorganization very

likely through a modification of FAs turnover. All these effects converge in the inhibition of CRC-derived cell migration.