Inhibition of the GTPase Rac1 mediates the anti-migratory effects of metformin in prostate cancer cells

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ABSTRACT

Cell migration is a critical step in the progression of prostate cancer to the metastatic state, the lethal form of the disease. The anti-diabetic drug metformin has been shown to display anti-tumoral properties in prostate cancer cell and animal models, however, its role in the formation of metastases remains poorly documented. Here, we show that metformin reduces the formation of metastases to fewer solid organs in an orthotopic metastatic prostate cancer cell model established in nude mice. As predicted, metformin hampers cell motility in PC3 and DU145 prostate cancer cells and triggers a radical reorganization of the cell cytoskeleton. The small GTPase Rac1 is a master regulator of cytoskeleton organization and cell migration. We report that metformin leads to a major inhibition of Rac1 GTPase activity by interfering with some of its multiple upstream signaling pathways, namely P-Rex1 (a Guanine nucleotide exchange factor and activator of Rac1), cAMP and CXCL12/CXCR4, resulting in decreased migration of prostate cancer cells. Importantly, overexpression of a constitutively active form of Rac1, or P-Rex as well as the inhibition of the adenylate cyclase were able to reverse the anti-migratory effects of metformin. These results establish a novel mechanism of action for metformin and highlight its potential anti-metastatic properties in prostate cancer.
INTRODUCTION

Metformin is an antidiabetic drug used by more than 120 million people worldwide. In agreement with retrospective epidemiological studies in which diabetic patients on metformin display decreased cancer incidence and cancer related mortality, (1-3) metformin has been shown to inhibit cancer cell proliferation and decrease tumor growth in many animal models (4-7). Prostate cancer is the second leading cause of death by cancer in men and most prostate cancer-related deaths are due to metastasis, a process which requires cancer cell migration. This migration is a complex biological process orchestrated by environmental factors, signal transduction and cytoskeletal rearrangement. Several studies demonstrated that metformin exerts an anti-migratory effect on cancer cells, however, its mechanism of action remains largely unknown (8-13). In addition, how metformin interferes with the small GTPase Rac1, one of a master regulator of cell migration, is not known.

Rac1 belongs to the family of the Rho GTPases which play a central role in the control of cytoskeleton organization and cell motility. The best characterized family members are: Rho, involved in stress fibers and focal adhesion formation, together with Rac and Cdc42, respectively involved in lamellipodia and filipodia formation (14). Rho GTPases switch from a GTP-bound active form to a GDP-bound inactive form. The exchange of GDP to GTP is regulated by guanine nucleotide exchange factors (GEF) and the inactivation of Rho GTPases is controlled by GTPase activating enzymes (GAP). The Rac subclass (or subfamily) of RhoGTPases includes Rac1, Rac2 and Rac3. Rac1 is required for lamellipodium extension induced by growth factors, cytokines and extracellular matrix components (15). Rac1 is overexpressed in cancers including prostate cancer, where its expression is significantly increased in aggressive tumors (16). The PIP3 Phosphatidylinositol (3,4,5)-triphosphate-
dependent Rac Exchanger 1 (P-Rex1), a Rac selective GEF, plays an important role in actin remodeling and cell migration. Importantly, upregulation of P-Rex1 promotes metastasis whereas its downregulation inhibits cell migration in prostate cancer cells (17).

Rac1 activity is regulated by numerous biological signals such as cAMP and cytokines. Recent studies have highlighted an important role for cAMP metabolism in the migration of carcinoma cells (18) and the regulation of Rac1 activity (19). For example, cAMP specific phosphodiesterase (PDEs) facilitate cell migration as well as lamellae formation by lowering cAMP levels. In addition, Chen et al. have shown that increased cAMP levels correlated with the inhibition of cell migration in both Mouse Embryonic Fibroblasts and 4T1a breast tumors cells by interfering with the formation of lamellipodia (20).

Chemokines are also important regulators of Rac1, one of them, CXCL12 (also known as SDF-1α) activates Rac1, decreases cAMP levels and favors prostate cancer cells migration (21-23). In addition, CXCR4, the CXCL12 receptor, is frequently overexpressed in malignant epithelial cells and the CXCL12/CXCR4 axis plays a pivotal role in directing the metastasis of CXCR4 positive tumor cells to organs that express CXCL12, such as lungs, liver and bones (24, 25).

Here, we investigated the effects of metformin on Rac1 GTPase activity and determined if it interferes with some of Rac1 multiple upstream signaling pathways, namely P-Rex1, cAMP and CXCL12/CXCR4.

We demonstrate that metformin inhibits the migration of prostate cancer cells and limits the formation of metastasis to fewer solid organs in an orthotopic xenograft model using PC3 cells. In addition, we show that metformin strongly modifies actin cytoskeletal organization. Reversal of the decreased Rac1GTPase activity through the expression of constitutively active Rac1GTP or P-Rex1 overturned the anti-migratory effects of metformin. Similarly,
blocking the metformin-induced cAMP increase with an adenylate cyclase inhibitor hampered the effects of metformin on migration. We also show that metformin inhibits CXCL12 chemotactism and counteracts the increase of Rac1GTP by CXCL12. Our study reveals a novel mechanism of action for metformin, in which it targets Rac1GTPase and cytoskeletal organization.
Materials and Methods

Orthotopic implantation of PC3-GFP prostate cancer cells and analysis of metastasis

Intraprostatic human prostate cancer xenografts were established in nude mice by surgical orthotopic implantation as originally described (26). Briefly, mice were anesthetized by isoflurane inhalation and placed in the supine position. A lower midline abdominal incision was made and a tumor cell suspension (1 x 10^6 cells/20µl) was injected into the dorsal lobe of the prostate using a 30-gauge needle and glass syringe (Hamilton, Massy, France). After implantation, the surgical wound was closed in two layers with 4-0 Dexon interrupted sutures. All procedures were performed with a dissecting microscope. Autopsy and in vivo fluorescence imaging were conducted as previously detailed. The measurement were performed blinded. Animal use and care was approved by the local Animal Care committee according to the European Legislation.

Cell culture and transfection

The human PC3 and DU145 cancer cell lines were obtained from ATCC and authenticated by ATCC, the experiments performed in this work were performed during the year and half following the reception of the cells. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100U/ml penicillin and 50µg/ml streptomycin. GFP expressing PC3 cells were generated as described previously (26). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, and the media were replaced every 2-3 days. In all experiments cells were treated for 4h with 5mM Metformin. Cells were transiently transfected with HA-Prex1 expressing vector (kind gift of Dr C. Mitchell from Monash University, Victoria, Australia) (27), or the Rac1 mutant expression vectors: RacQ61L and RacV12 expression vector using Lipofectamine 2000 (Invitrogen).
Chemicals

Metformin, the adenylate cyclase inhibitor (SQ22536), dibutyryl-cAMP (dbcAMP) and fibronectin were purchased from Sigma Aldrich. The Rac inhibitor and AMD3100 were from Merck Chemicals. CXCL12 was purchased from Peprotech.

Boyden chamber assay

Boyden chambers with filter inserts coated with fibronectin (10µg/ml) and 8-µm pores (BD Bioscience) were used to quantify cell migration. In order to respond better to the chemoattractant cells were serum starved overnight, 12 x 10⁴ cells were seeded in the upper chamber in serum free DMEM medium in presence or absence of 5mM metformin. The lower chamber contained complete DMEM, 10% FBS, or DMEM with CXCL12 at the indicated concentration. Cell migration was determined after 4h by counting all cells in five randomly selected counting areas at the lower surface of the filter. Cells on the upper surface were removed with a cotton swab; filters were fixed and stained with blue toluidin. Each experiment was repeated at least three times. For invasion experiments, the inserts were coated with 25µg/µl of Matrigel (Beckton Dickinson) and invading cells were counted after a 24h incubation with metformin.

Spheroid migration assays in three-dimensional (3D) matrigel matrices.

Prostate spheroids were generated using the liquid overlay technique. Briefly, 24-well culture plates were coated with 1.5% agarose prepared in sterile water. Cells from a single-cell suspension were added at 10,000 cells per well. The plates were gently swirled and incubated at 37°C in 5% CO₂ atmosphere until spheroid aggregates were formed. Then,
spheroids were included in a matrigel matrix and images of invasion were obtained 24h later.

**Cell migration observation with video microscopy.**

Cell migration was monitored in duplicate experiments by time lapse digital microscopy. Cells were seeded on a 6-well plate at low density. Computer-assisted cell tracking of 20-30 randomly selected cells was performed. Briefly, the x- and y-coordinates were collected from the center of the cell with a step interval of 5 min and reconstructed either as path at orthotopic position or as migration speed over time.

**Immunofluorescence and fluorescence microscopy.**

Cells grown on coverslips were fixed in 3.7% paraformaldehyde, permeabilized in 0.2% Triton X-100 for 20 min, blocked with 2% bovine serum albumin for 1 h (all reagents were diluted in phosphate-buffered saline) and then incubated with Texas red Phalloidin and anti-HA antibodies (Covance). Cells were simultaneously stained with Hoescht. Images were recorded with a Leica scanning microscopy system DM5500B. Image acquisition and image analysis were performed on the C3M (or MicorBio) Cell Imaging Facility.

**Western blot analysis**

40µg of total cell lysate protein was separated by SDS PAGE, transferred on a PVDF membrane (Millipore) and incubated with the antibodies against Rac1 (BD Biosciences); ERK and HSP90 (Santa Cruz Biotechnology); HA (Covance).

**Pull down assay for the measurement of RAC1 GTP activity**
The assay was performed as previously described (28). DU145 and PC3 were seeded in DMEM medium with 10% FBS. After a 4h treatment with 5mM Metformin, cells were washed once with ice-cold PBS and immediately lysed in 25mM Tris buffer, pH 7.5, 150mM NaCl, 5mM MgCl₂, 0.5% Triton X100, 4% glycerol, 10mM Sodium Fluoride, 2mM sodium orthovanadate, 5mM DTT, 1mM PMSF. Cleared extracts were mixed with 20µg of GST-PAK in the presence of glutathione-agarose beads (Sigma Aldrich). After a 40 min incubation at 4°C, beads were pelleted by centrifugation and washed three times in lysis buffer, and the proteins were eluted in SDS-PAGE sample buffer for analysis by Western blot using a monoclonal antibody to Rac1 (BD Bioscience).

cAMP concentration.

cAMP levels were assessed using a commercially available fluorimetric kit (Arbor Assays). In brief, DU145 or PC3 were seeded in 6-well plates and half the wells were treated with 5mM of Metformin for 4h before cAMP measurement performed according to the manufacturer’s protocol.

Flow cytometry

Cells were harvested after 4hrs of Metformin treatment (5mM) M. Cells were labelled with anti-CRCR4-APC conjugated antibody (R&D) and fixed in PAF 3.7% for 10min. Labelling was carried out in ice for 2hrs. Cells were then washed in PBS-0.5% BSA at 1100rpm for 5minutes and resuspended in 400µL of PBS. For each tube, 10,000 events were acquired. Samples were analysed using FACSCanto II cytometer (Beckton Dickinson).

CRE-luciferase reporter gene assay.

PC3 and DU145 cells were transiently transfected using lipofectamine 2000 with 1µg of a plasmid encoding for the cyclic AMP responsive element (CRE) coupled to the luciferase
gene (CRE-Luc) and 1µg of pRL Renilla Luciferase Control vector. Two days post-transfection, the culture medium was discarded and cells were treated with DMEM supplemented with 10% FCS ± 5mM Metformin. After a 4h incubation at 37°C, the stimulation medium was discarded and the Luciferase activity was determined using the Dual Luciferase reporter Assay System (Promega).

**Statistical analysis**

The statistical significance of differences between the means of 2 groups was evaluated using Student's t test.
Results

Metformin inhibits tumor growth and reduces metastasis in an orthotopic model of PC3 cells

We first investigated the effects of metformin on the formation of metastases using an orthotopic model of PC3 cells overexpressing GFP. In these experimental conditions, cells grow in their native environment and the primary tumor forms distant metastasis (26, 29). Tumor growth and metastasis dissemination were analysed five weeks after the injection of PC3-GFP cells into the prostate. Metformin (100 mg/kg/day) was given in drinking water for five weeks (Met 5w) starting three days after cell injection or only two final weeks (Met 2w). A group was injected i.p with Docetaxel (20mg/kg) for the last two weeks. Metformin had no toxic effect on mice, it did not affect animal weight and insulinemia (Fig.S1). A whole-body open imaging of the animals revealed the fluorescence of primary tumors and metastases, including periaortic and periadrenal lymph nodes, liver, pancreas, lungs and mesentery indicating a disseminating disease as described previously (29). A representative picture of the GFP-positive tumors is shown in figure 1A. As expected the tumors were significantly smaller in the docetaxel-treated group and metformin induced a strong anti-tumoral effect. Indeed, it significantly reduced by more than 50% the growth of the primary tumor when given for 5 weeks (Fig. 1 A,B). However, when administrated only during the last two weeks like docetaxel, metformin did not have any impact on tumor growth (Fig. 1 A,B). Our findings show that metformin has a preventative effect on primary tumor growth but yet does not manifest a curative effect when the tumor is already established. Interestingly, the dissemination pattern of metastases showed that all mice had metastases regardless of the treatment except 2 mice in the “metformin 5 weeks” group (Table 1). Among the three mice without solid metastasis two of them had primary tumors bigger than the average tumor
volume of the metformin 5 weeks group (162.51 and 295.64 vs 135.53 mm$^3$ for the average tumor volume) suggesting that the absence of metastasis is not associated with small tumors. Only docetaxel significantly reduced the formation of retroperitoneal lymph nodes as well as liver, pancreas, lung and mesentary metastases (Table 1). Nevertheless, mice from the “metformin 5 weeks” arm exhibited statistically less metastasis ($p=0.04$) suggesting that metformin may hinder the metastatic dissemination.

**Metformin inhibits the migration and the invasive properties of PC3 and DU145 prostate cancer cell lines**

Since metastasis requires cancer cell migration, we investigated the effects of metformin on human prostate cancer cell migration using Boyden chamber assay. According to our previous studies (4, 30) and a dose response experiment (data not shown) we treated the cells with 5mM metformin. In order to exclude any action of metformin on cell proliferation, PC3 and DU145 were treated with metformin for 4h during the migration towards culture medium supplemented with foetal bovine serum (chemoattractant medium). We monitored cell viability and apoptosis in the same conditions. As expected, viabilities in all cell cultures treated for 4 hours with metformin exceeded 95% (data not shown) and markers of apoptosis were negative (Fig. S2). Interestingly, a significant inhibitory effect of metformin of 50% on the migration of PC3 and DU145 cells was revealed (Fig. 2A). In contrary, metformin did not alter the migration of normal epithelial prostate cells (P69 cells) (Fig. S3). Next, we determined the impact of metformin on invasion. Cells were treated 4h with metformin prior to assessing 2D-invasion in matrigel using Boyden chambers, as described in the materials and methods section. As shown in Figure 2B, metformin strongly inhibited the invasive properties of PC3 and DU145 cells. To further explore whether metformin reduces
invasion, we performed a spheroid assay with DU145 cells. Untreated DU145 cells were able to invade the adjacent matrigel matrix in a collective migration/invasion pattern. Spheroids treated with metformin remained compact with almost no cells migrating out (Fig. S4). We then tracked individual cell migration over a period of 12 h using time lapse video microscopy. Untreated PC3 cells moved in several directions over an extended area compared to those treated with metformin (Fig. S5). The total accumulated distance covered by the untreated cells was 1329.1 µm ± 369.2 versus 9.30 µm ± 7.74 for those treated with metformin, and the mean euclidean distance (shortest linear distance between point A and point B) was 89.02 µm ± 56.73 versus 6.47 µm ± 4.22. Metformin also affected cell velocity because untreated cells migrated at a 1.84 µm/min ± 0.51 vs 0.012 ± 0.01 µm/min for metformin treated cells. Our results establish that metformin inhibits all movement parameters of prostate cancer cells with a major inhibitory impact on their invasive properties.

Metformin induces the reorganization of actin cytoskeleton

Because cells coordinate their migration through the regulation of actin dynamics (31), we studied the effect of metformin on α actin, β actin and fascin expression, three important proteins implicated in cell migration. We did not observe any change in the expression these proteins except a slight decrease of fascin expression in PC3 cells only (Fig. S6). More importantly, we analyzed actin cytoskeleton organization, PC3 and DU145 cells were seeded on fibronectin coated wells and fluorescence microscopy was used to analyze F-actin. In the control (untreated) conditions, elongated cells forming lamellipodia extensions rich in F-actin and stress fibers and ruffle formations were visible (Fig. 2C). Four hours of treatment with 5 mM metformin induced a drastic change of cell morphology, with cells reorganizing their
actin cytoskeleton, becoming circular, displaying less lamellipodia (Fig. 2C). The shape of the PC3 and DU145 cells confirmed that metformin treatment significantly decreased invasive morphology (Fig. 2C).

*Metformin decreases Rac1 GTPase activity*

The known role of the small GTPase Rac1 as a major driver of cell motility (32, 33) prompted us to assess Rac1 activity, using GST-Pak pulldown assay, as described (34). Interestingly, this series of measurements revealed a significant decrease in Rac1-GTP levels in PC3 and DU145 cells treated with 5mM metformin for 4 h (Fig. 3A). Rho activity was not affected by metformin, thereby pointing to a specific decrease in Rac1 activity (Fig. S7). To establish the link between the inhibition of migration and Rac1GTPase activity triggered by metformin treatment, we used a Rac1 inhibitor that specifically and reversibly inhibits Rac1 GDP/GTP exchange activity, while exhibiting no effect on Cdc42 or RhoA (35). We found that treatment of PC3 and DU145 cells phenocopied the effects of metformin on cell migration (Fig. 3B) and induced a circular cell morphology (Fig. S8). To further gain insight in the relationship between metformin and Rac1GTPase, constitutively active forms of Rac1 (HA-Rac1-Q61L or HA-Rac1-V12) were overexpressed in PC3 and DU145 cells. In the presence of metformin, cells expressing the active form of Rac1 no longer displayed the “rounded shape” phenotype that could be observed in non-transfected cells (Fig. 3C). Furthermore, we found that the expression of the constitutive forms of Rac1 slightly but significantly inhibits basal cell migration (Fig 3D). Importantly, the inhibitory effect of metformin on control PC3 and DU145 cell migration was abolished in cells expressing the constitutive forms of Rac1: Rac1-Q61L or Rac1-V12 (Fig. 3D). This reveals that constitutive activation of Rac1 overrides the effects of metformin on actin cytoskeleton reorganization and cancer cell migration.
P-Rex1 overexpression reverses the anti-migratory action of metformin

P-Rex1 is a Guanidine Exchange Factor (GEF) which modulates cellular Rac1-GTP levels. It is implicated in cytoskeleton remodeling (36) and facilitates prostate cancer metastasis (17). We asked whether P-Rex1 overexpression (HA-P-Rex1 wt) reversed metformin effects on cell migration. HA-P-Rex1 expression did not affect basal Rac1 GTP levels but restored Rac1 GTP levels in cancer cells treated with metformin (Fig. 4A). Accordingly, the overexpression of wild-type P-Rex1 does not affect cell migration but reversed the anti-migratory effects of metformin (Fig. 4B). Altogether, our results support the idea that the forced activation of Rac1 alleviates the metformin-mediated inhibition of cancer cell migration.

Metformin increases cAMP levels in prostate cancer cells

Because cyclic AMP inhibits Rac1 activity (37), we investigated whether cAMP acts as a potential mediator by which metformin modulates migration of prostate tumor cells. Accordingly, we measured cAMP content in cells treated with metformin. We did not detect any change in cAMP concentration after 4h of treatment with metformin in PC3 cells (Fig. S9). On contrary, metformin induced a slight but significant increase in cAMP levels in DU145 (Fig. 5A), which was associated with the augmentation of luciferase activity in cells transfected with the CRE-Luc construct (to monitor cAMP increase through the activation of CREB, the cAMP response element binding protein) (Fig. 5B) and increased CREB phosphorylation (Fig. S10).

In order to firmly establish that increased cAMP is directly implicated in the anti-migratory effects of metformin, we treated DU145 cells with SQ22536, an inhibitor of adenylate cyclase. Treatment with 100µM SQ22536 prevented the increase of cAMP (Fig. 5A) as well as the decrease in cell migration (Fig. 5C) induced by metformin, while leaving basal cAMP
concentration and basal cell migration unaffected (Fig. 5A,C). To directly observe the effects of elevated cAMP on cell migration, we treated DU145 cells with 500µM of dibutyryl cyclic AMP (db-cAMP), a cell-permeable cAMP analog. A 4h treatment with db-cAMP inhibited the migration of DU145 cells (Fig. 5D) and decreased Rac1 activity (Fig. S11). Importantly, the overexpression of a constitutively active Rac1 in DU145 cells overcame the anti-migratory effects of db-cAMP (Fig. 5E). These results suggest that the anti-migratory effect of metformin requires increased cAMP levels.

Metformin inhibits CXCL12 chemotactism in prostate cancer cells

Regardless of its chemoattractive properties, CXCL12 was recently shown to regulate Rac1 (38). Therefore, CXCL12 was used as a chemoattractant in a cell migration assay; where it significantly promoted DU145 migration (Fig. 6A). Importantly, we found that addition of metformin prevented CXCL12 pro-migratory effects (Fig. 6A). CXCL12 binds to the chemokine receptor 4 (CXCR4) to affect cell migration. In order to validate the role of CXCL12/CXCR4 signaling in prostate cancer cell migration, we treated cells with AMD3100, a well characterized and specific antagonist of CXCR4, which inhibits the binding and function of CXCL12 (39). In the presence of CXCL12, AMD3100 significantly inhibited DU145 cell migration showing that the CXCL12/CXCR4 axis plays an important role in the migration of prostate cancer cells (Fig. 6B). Flow cytometry analysis to monitor expression of CXCR4 at the cell surface revealed a decrease upon metformin treatment (Fig. 6C and Fig. S12). We measured Rac1-GTP levels and found that CXCL12 increased Rac1 activity in a metformin-sensitive manner (Fig. 6D). In conclusion, our results show that metformin interferes with CXCL12 signaling through the regulation of CXCR4 and Rac1 to inhibit prostate cancer cell migration.
DISCUSSION

Prostate cancer can be very aggressive in advanced stages and commonly metastasizes to bone and lymph nodes, more rarely to the liver and lung and. Cell migration, which is required for metastasis, is a complex biological process regulated by environmental factors, signaling pathways and cytoskeletal rearrangement. Here, we report that the anti-diabetic drug metformin reduces the formation of metastasis to fewer solid organs in an orthotopic mouse model and affects cell cytoskeleton organization, which drastically inhibits prostate cancer cell migration through decreased Rac1 activity. Since our previous studies showed that metformin inhibits cancer cell proliferation and blocks cell cycle in G0/G1 within 24h (4), all cell migration assays were performed within 4h of treatment to exclude any effects due to cell cycle arrest.

Metformin inhibits the migration of glioblastoma, ovarian and pancreatic cancer cells (8, 12, 13). However, the cellular and molecular mechanisms responsible for this inhibition are poorly documented. In melanoma, metformin does not affect cell migration but inhibits invasion by reducing the activity of matrix metalloproteinases (9). Similarly, two studies reported that metformin inhibits the activity of matrix metalloproteinase (MMP-9) and therefore, blocks cancer cell invasion in endothelial and fibrosarcoma cells (10, 40). Bao et al. correlated the anti-migratory effects of metformin with the decreased expression of let-7b, miR-26a and miR-200b (8). In glioma cell lines, metformin suppresses matrix metalloproteinase-2 expression and affects cell adhesion through the diminution of fibulin-3 a secreted glycoprotein that associates to the extracellular matrix (41). Here, we show that metformin induces drastic changes in cell morphology with a marked reduction of lamellipodia. These modifications are not associated with changes in α actin or β actin.
expression (Fig. S6). However, we observed a slight decrease of fascin upon metformin treatment. Fascin downstream of Rac contributes to cancer cell migration and the formation of metastasis (42-44). Further investigations are required to determine how metformin interferes with lamellipodia formation and if fascin is implicated in its anti-migratory effect.

The drastic change in prostate cancer cell morphology is associated with a decrease in the active form of Rac1, a master regulator of actin polymerization (14). Expression of a constitutively active form of Rac1 inhibited the anti-migratory effects of metformin and restored the formation of lamellipodia in cancer cells. Conflicting reports have been published regarding the role Rac1 in cell migration (45-49). For instance, the RacGEF Tiam1 inhibits cell migration of melanoma cells (48), in accordance, we also observe a slight inhibition of cell migration when we express Rac61L and RacV12 in DU145. On the other hand, the GEF P-Rex1 promotes cell migration and its downregulation with siRNA inhibits PC3 cell migration (17). In the present study, the expression of P-Rex1 reversed the anti-migratory effects of metformin, supporting the notion that metformin acts in a Rac1-dependent manner. Metformin could therefore act as a GEF inhibitor. Indeed, P-Rex1 activity is enhanced by PIP3 and Gβγ proteins, which is inhibited by cAMP through the phosphorylation of P-Rex1 by PKA (50). As a result, the increased cAMP levels induced by metformin could inhibit P-Rex1 through PKA and downregulate Rac1. In line with this hypothesis, increased intracellular cAMP levels and PKA activity following morphine treatment lead to inhibition of Rac1GTPase and p38 MAPK, cause attenuation of actin polymerization, and decrease bacterial phagocytosis (51).

cAMP plays an important and sometimes controversial role in apoptosis (52, 53) but cAMP is also a well-established inhibitor of cell migration (20) and a regulator of cytoskeleton organization (54). It was demonstrated that cAMP inhibits the Rho family small GTPases via
PKA. For example, prostaglandin E2 inhibits IGF-1 induced cell migration and inhibited Rac1 activity through a mechanism involving cAMP. In addition, cAMP has been shown to regulate Rac1 and breast cancer cell migration via PKA. We showed that metformin increases cAMP levels in DU145, but not in PC3. The cellular cAMP level depends on the activity of two enzymes, the adenylyl cyclases (AC) that produce cAMP and the phosphodiesterases (PDE) that hydrolyze cAMP. This discrepancy between the cell lines may be related to a different action of metformin on AC or/and PDE depending on the cell lines. Indeed, a study demonstrates that metformin decreases Phosphodiesterase 3B mRNA levels in breast cancer biopsies after the treatment (55). More recently, a work performed in primary hepatocytes showed that a pre-treatment with metformin inhibited glucagon-induced accumulation of cAMP, but did not affect basal cAMP levels (56). Indeed, metformin induced an increase in the AMP levels, possibly due to the decreased ATP concentration, which inhibits the activity of adenylate cyclase. We and others have shown that metformin inhibits the activity of the mitochondrial complex 1, and decreases the intracellular concentration of ATP, resulting in the increase of AMP within 8h (30, 57).

We established that CXCL12 increases Rac1 activity as previously shown in endothelial cells (58) and that metformin inhibits CXCL12-induced Rac1 activation. Our work suggests that metformin hampers the pro-migratory effects of CXCL12 by affecting Rac1 GTPase activity. Interestingly, the CXCL12/CXCR4 pathway was recently associated with Rac activation and metastasis (38). Therapeutic approaches target this pathway by either blocking CXCL12 with antibodies or acting on CXCR4 by preventing CXCL12 binding. We anticipate that metformin may represent a novel and alternative way of inhibiting this pathway known to play a major role in prostate cancer metastasis.
Regarding prostate cancer therapy, we demonstrated in an orthotopic metastatic model that metformin reduces the formation of metastasis to fewer organs in addition to its inhibitory effect on the growth of primary tumors. Several studies have shown in different mouse xenograft models and transgenic mice that metformin inhibits tumor growth (see review: (59), but few works analyzed metastasis dissemination. Our data are encouraging for a potential use of metformin in the treatment of advanced metastatic prostate cancer. However, one of the limitation of our *in vivo* model is the injection of exogenous cancer cells in the mouse prostate. Therefore, we are aware that we need to confirm the effect of metformin on the formation of metastasis in another mouse model. Thus, it would be interesting to test the effects of metformin in the "RapidCaP" model recently described by Cho et al. (60). In this new model, unlike our study, mice develop metastasis from mouse prostate tumors. Rattan *et al.* demonstrated that metformin significantly reduces the growth of metastatic nodules of ovarian cancer cells in nude mice (11). They also indicated that metformin potentiates cisplatin-induced toxicity. To this regard, it would be interesting to determine if metformin can improve the efficiency of docetaxel, the standard treatment for prostate cancer patients who are refractory to hormonal manipulations.

Collectively, our results shed light on a new mechanism of action of metformin and novel properties of this drug in prostate cancer.
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Table 1: pattern of metastatic dissemination

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</tr>
<tr>
<td>No. of metastases</td>
<td>24/28 (3.5 per animal)</td>
<td>21/32 (2.6 per animal) NS</td>
<td>21/24 (3.5 per animal) NS</td>
<td>15/28 (2.1 per animal) *</td>
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<td>Pancreas</td>
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<td>Lung</td>
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<td>Mesentery</td>
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<tr>
<td>No. of metastases</td>
<td>26/28 (3.7 per animal)</td>
<td>16/32 (2.0 per animal) †</td>
<td>19/24 (3.2 per animal) NS</td>
<td>10/28 (1.4 per animal) #</td>
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</tbody>
</table>

* P = 0.049 compared with sham-treated animals (t test)
† P = 0.040 compared with sham-treated animals (t test)
‡ P = 0.002 compared with sham-treated animals (t test)

For retroperitoneal lymph nodes, the numbers 0, 1, or 2 represent the quantity of invaded lymph nodes. For other organs, 0 means no presence of metastasis; 1 means a metastatic organ (regardless of the intensity of metastasis dissemination in this organ).
Figure legends

Figure 1: Effects of metformin on growth of established human fluorescent PC-3 orthotopic xenografts in nude mice

The day of the orthotopic implantations with 1x10^6 PC-3 cells, mice were randomized into four groups. Animals were given drinking water (control, C) or 100mg/kg Metformin (Met 5w) in drinking water for five weeks. Alternatively, three weeks after implantation, animals were treated for the final 2 weeks with Metformin in drinking water (Met 2w) or weekly i.p. 20 mg/kg docetaxel A, representative photos of the primary tumors (magnification x0.8). The tumors are in green and the bladder appears in orange (autofluorescence) on the picture. B, tumor volume calculated as described in Materials and Methods. Columns, mean from 6 to 8 animals; bars, SEM. Statistical analysis were performed using the Student's t test *, P < 0.05.

Figure 2: Metformin inhibits prostate cancer cell migration and invasion. (A) PC3 and DU145 cells were seeded in Boyden chambers and metformin (5mM) was added during the migration for 4h. Graphs are expressed as a percentage of cells migrating across the Boyden chamber relatively to the control conditions (100%) and the inserts represent picture of the counted fields. (B) Quantification of the invasion assay performed in Boyden chambers during 24h in presence of 5mM metformin. (C) Immunofluorescence performed with Texas red Phalloidin in PC3 and DU145 treated with 5mM metformin for 4h. The graph represents the circularity of the cells expressed in arbitrary units. Columns are the means of four independent experiments and bars are SEM. The Statistical analysis were performed using the Student's t test *, P < 0.05; and **, P < 0.01.
Figure 3: Metformin inhibits Rac 1 activity and constitutively active Rac 1 restores cell migration. (A) Immunoblot of Rac1 performed with DU145 and PC3 prostate cancer cells treated with 5mM metformin for 4h as described in Material and Methods. The graph represents the quantification of the ratio of Rac1 GTP/Total Rac1. (B) Quantification of the migration assay in Boyden chambers of cells treated for 4h with the Rac inhibitor (50µM). (C) DU145 transfected with HA-RacQ61L and treated with 5mM Metformin? were analysed by immunofluorescence using Texas red Phalloidin (red) and HA (green). (D) PC3 and DU145 were transfected with empty vector, active forms of Rac1:RacQ61L or RacV12 and treated with 5mM metformin for 4h during the migration assay. Columns are the mean of five independent experiments; bars are SEM. Statistical analysis were performed using the Student's t test *, P < 0.05 ; and **, P < 0.01.

Figure 4: Overexpression of P-Rex1 counteracts the anti-migratory effects of metformin. (A) Immunoblot analysis of Rac1GTP and total Rac1 in DU145 cells transfected with the indicated vectors and treated for 4h with 5mM metformin. Expression of the HA-tagged proteins in the cell lysates was revealed by an anti-HA immunoblot. Similar results were obtained in 3 independent experiments. (B) DU145 transfected with control vector (empty vector) or P-Rex1 expression vector (HA-Prex1 wt) were assayed for migration during 4 h in the presence or absence of 5mM metformin. The graph represents the average of three independent migration assays. Statistical analysis were performed using the Student's t test *, P < 0.05.

Figure 5: Metformin increases cAMP levels and inhibition of adenylate cyclase reverses the anti-migratory effects of metformin. (A) cAMP concentration in DU145 cells treated with 5mM metformin for 4h in the presence or absence of 100µM SQ22536 (Adenylate cyclase
inhibitor). (B) Luciferase activity of the CRE promoter element in DU145 cells transfected with CRE-Luc vector and treated with 5mM metformin for 4h. (C) Quantification of a migration assay performed in Boyden chambers with DU145 cells treated with both 5mM metformin and 100µM SQ22536 (Adenylate cyclase inhibitor) for 4h. (D) Quantification of a migration assay performed in Boyden chambers with DU145 cells treated with 5mM metformin (M) or 100µM of the cell permeant cAMP analogue dibutyryl cAMP (dbcAMP) for 4h. (E) DU145 cells were transfected with empty vector, active forms of Rac1: RacQ61L or RacV12 and treated with dbcAMP for 4h during the migration assay. The graphs represent the quantification of at least three experiments performed independently. Statistical analysis were performed using the Student's t test. The differences are significant with $p<0.05$ (*) and $p<0.01$ (**).

**Figure 6: Metformin inhibits the pro-migratory effects of CXCL12.** (A) Quantification of a migration assay performed with DU145 cells treated with 250ng/ml of CXCL12 in absence (C) or presence of 5mM metformin for 4h. (0) corresponds to the untreated condition. (B) Quantification of migration assay with DU145 cells incubated with 250ng/ml CXCL12 for 4h in the presence or absence of 25µg/ml AMD3100 (a CXCR4 antagonist). (C) Relative expression of CXCR4, determined by Flow cytometry analysis, in DU145 cells treated with 5mM metformin for 4h. (D) Immunoblot of Rac1GTP in DU145 cells treated with CXCL12 in presence of 5mM metformin for 4h. The graphs represent the quantification of at least three independent experiments performed independently. Statistical analysis were performed using the Student’s t test. The differences are significant with $p<0.05$ (*) and $p<0.01$ (**).
Figure 1
Figure 2

A

PC3

MIGRATION

DU145

Relative migration (% of control)

C  M

PC3

INVASION

DU145

Relative invasion (% of control)

C  M

B

C

M

C

M

PC3

DU145

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Figure 3

A

PC3

-  +

Metformin

Rac61L

RacV12

DU145

-  +

Relative migration (% of control)

PC3

C  Rac Inh

DU145

C  Rac Inh

B

C  F-actin

HA-RacQ61L Merge

C

D

PC3  Control  Metformin

DU145

empty  Rac61L  RacV12

**  **

**  **

Relative migration (% of control)
Figure 4
Figure 5
Figure 6

Part A: Graph showing relative migration (% of control) for different conditions.

Part B: Graph showing relative migration (% of control) for different treatments.

Part C: Graph showing CXCR4 relative expression for different conditions.

Part D: Western blot analysis for Rac1 GTP, Rac1 total, and ERK.

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Inhibition of the GTPase Rac1 mediates the anti-migratory effects of metformin in prostate cancer cells
Beatrice Dirat, Isabelle Ader, Muriel Golzio, et al.
Mol Cancer Ther Published OnlineFirst December 19, 2014.