# Sitagliptin inhibits CD26/DPP4-dependent cell motility and invasion functionalities of colorectal cancer cell lines

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20 # Abstract

21 **Background:** CD26 transmembrane glycoprotein with serine peptidase activity (DPP4)
22 and/or its soluble CD26/DPP4 counterpart expression and/or activity are altered in several
23 cancers. Recently, CD26 was shown to promote metastasis development, probably
24 through the presence of CSCs subsets with high expression of CD26. In fact, DPP4
25 inhibitors already in use for diabetes such as sitagliptin have shown promising anti-
26 metastatic effects in animal models. However, the mechanism of action of these inhibitors
27 in this context is unclear.

28 **Methods:** We used herein a panel of human colorectal cancer cell lines to characterize
29 the roles of CD26 expression and and the effect of DPP4 enzymatic activity inhibitor
30 sitagliptin in malignant-cell features such as cell-cell homotypic aggregation, cancer cell
31 motility and invasion.

32 **Results:** Our data shows that CD26 protein is important to induce invasion, motility, and
33 aggregation of CD26 positive CRC cell lines. However, only invasion and motility
34 assays, which are collagen matrix-dependent, were decreased upon treatment with the
35 DPP4 inhibitor sitagliptin.

36 **Conclusions:** These results confirm the CD26/DPP4 role in the induction of highly
37 malignant features and contributes to the elucidation of the molecular mechanism behind
38 sitagliptin inhibition of metastasis. We conclude that additional tools against CD26 as
39 target might be used or developed for metastasis prevention in addition to sitagliptin. At
40 the same time, the mechanism of sitagliptin may help to define areas of medicine where
41 DPP4 inhibitors might be introduced.

42

43

## Keywords

Colorectal cancer, Metastasis, Sitagliptin, Dipeptidyl peptidase 4, CD26, Collagen, Invasion, Aggregation, Motility

## Background

Colorectal cancer (CRC) is the third type of cancer with the highest incidence in the world [1]. Although surgery, radiotherapy and chemotherapy are available, metastases continue to be the main cause of poor prognosis. In recent years cancer stem cells (CSCs) have raised at the status of core participants in the origin of tumour growth and metastasis [2].

CD26 (dipeptidyl peptidase IV, DPP4, EC 3.3.14.5) is a transmembrane glycoprotein expressed on the cell surface of lymphocytes and many endothelial and epithelial cells [3-5], including those of the gut. Although in the early stages of carcinogenesis cell surface CD26 seems to be tumour suppressor [6-10], before CD26 expression was lost in tumour tissues [11,12], CD26 was recently identified also as a marker of cancer stem cells (CSCs) in several types of cancer [13-20]. In colorectal cancer, it identifies subsets of CSCs that could correspond to metastatic stem cells [17, and in press]. CD26 promotes metastasis development by regulating the expression of epithelial-to-mesenchymal transition (EMT) markers and binding to extracellular matrix components [17,18].

The fact that in CRC, CD26 expression associates with tumour malignant stages, the presence of distant metastasis, and worse prognosis [21,22] agrees with the above results of CSCs. Likewise, sCD26, the soluble form with uncertain function found in

67 several biological fluids including serum [5,23-28], levels are decreased in patients of
68 early stages of CRC [24] and increased in Dukes D stage where sCD26 has been proposed
69 as a good biomarker for early detection of disease recurrence and metastasis [26].
70 Suppression of CD26 expression inhibited metastasis of pancreatic cancer [29],
71 and the flavonoid apigenin restrained metastasis of lung cancer by downregulating CD26
72 [30]. Likewise, a monoclonal antibody directed against CD26 is being tested in advanced
73 CD26-expressing cancers [31]. Interestingly, *in vivo* studies have shown that chronic
74 administration of sitagliptin or vildagliptin, two of the CD26/DPP4 inhibitors used in DM
75 T2 to prolong the active life of incretins [3,5,32,33], prevented colon cancer in rats and
76 mice and lung metastasis in mice, respectively [34-36].
77 In addition to the extracellular domain with DPP4 exo-protease activity capable
78 of hydrolysing N-terminal dipeptides from polypeptides with alanine or proline in the
79 second position such as many chemokines and incretins, CD26 has also non-enzymatic
80 functions: It binds to extracellular matrix proteins such as collagens and fibronectin and
81 anchors the ecto-adenosine deaminase (eADA), with a role in cell-cell adhesion [(rev. in
82 3-5,16,37]. In fact, binding of adenosine to CD26 has been shown to trigger CD26
83 downregulation, resulting in a decrease of colorectal tumour cells binding to cellular
84 fibronectin and migration [38].
85 As a recent work has shown that sitagliptin affects migration ability of cervical
86 carcinoma cells independently of DPP4/CD26 [39], the aim of the present work was to
87 study if sitagliptin influence CRC cell invasion, motility, and aggregation, in order to
88 clarify its importance as a possible therapeutic tool against metastasis.
89
90

91 # Materials and methods

92 ## Maintenance of cell lines

93 Human CRC cell lines, SW1116, SW480, SW620, RK0, HT-29, Caco-2, HCT116
94 and T84 were obtained from the American Type Culture Collection (ATCC). SW1116,
95 Caco-2, HT-29, SW480, SW620 and RK0 were maintained in DMEM, HCT116 was
96 maintained in RPMI and T84 was maintained in DMEM/ Ham's F12. All of them were
97 supplemented with 10% FBS, 1% L- glutamine and 1% penicillin/streptomycin. Cells
98 were cultured at 37ºC in a humidified atmosphere of 5% CO2.

99

100 ## Flow cytometry analysis and cell sorting

101 Human CRC cell lines derived from monolayer cultures were adjusted to a final
102 concentration of 10x $10^6$ cells/mL in sorting buffer (PBS; 2% FBS (v/v); 1 mM EDTA
103 (v/v); 25mM HEPES). Cell suspensions were incubated with anti-CD26-PE (26PE100T,
104 Immunostep), or anti-E-cadherin-PerCP-Cy5.5 (≠563573, BD Biosciences), in the dark
105 at 4ºC for 30 min. Cells were then washed with sorting buffer before analysis or sorting
106 with a FACS Aria IIu analyzer (BD Biosciences) by using the PC FACSDiva software
107 program (BD Biosciences). Gating strategies for the cell lines are explained in detail in
108 (in press). Each characterization was repeated at least three times to validate the results
109 observed. Data were analysed with FlowJo 10 software.

110

111 ## Measurements of DPP4 enzymatic activity and cell viability

112 The procedure routinely used in our laboratory was modified according to Sato el
113 al [40,41]. Cells from the above cultures were cultured at $5x10^5$ cells/mL in 96-well plates
114 in triplicate (100 μL per well) in the same conditions until the cells reached confluence.
115 For the measurement of the DPP4 activity the wells were washed three times with
116 PBS before the addition of 100 μL of the substrate Gly- Pro-p-nitroanilide (Merck Sigma-
117 Aldrich, 2 mM final concentration) in the absence or presence of sitagliptin (sitagliptin
118 phosphate monohydrate, Merck Sigma-Aldrich) at 0.2, 0.5 and 1.2 mM concentrations.
119 The plates were maintained for 1 h at 37ºC in a shaker, the reaction stopped with 100 μL
120 of acetic-acetate buffer 1M, and the total volumes transferred to a different plate to be
121 measured in a iMark Microplate Reader (Bio-Rad, California, USA), at 405 nm
122 wavelength. Catalytic activity was obtained from standard curves with 4-nitroaniline and
123 expressed as mU/cell.
124 For the measurement of cell viability, after the incubations for the different times
125 mentioned in the text according to the experiments, the wells were washed with PBS for
126 three times and the cells trypsinised with 50 μL/well of trypsin-EDTA (Millipore). Then,
127 trypsin was neutralised with 50 μL of complete medium and after washing the cells with
128 the same medium in tubes, trypan blue was added to avoid the cell count of the dead cells
129 (the dyed cells).
130

## 131 Invasion assay

132 Invasion assays were performed using Matrigel Invasion Chambers (Corning)
133 following manufacturer's instructions. Briefly, polyethylene terephthalate (PET)
134 membrane containing-inserts with 8 μm pores and coated with Matrigel basement
135 membrane matrix were placed inside a 24-well plate and let thaw for 10 min. The filters

136 were then rehydrated for 30 min in the cell culture incubator using 500 µL of media in
137 the top and bottom chambers. 500 µL of cell suspension with $10^5$ cells/mL were added
138 to the top of the chambers, whereas 750 µL of complete medium or conditioned media
139 from fibroblasts (also supplemented with 10% FBS) were placed under the insert. This
140 experimental protocol was performed in the presence or absence of sitagliptin (0.5 mM)
141 (sitagliptin phosphate monohydrate, Merck Sigma-Aldrich) to the cell suspension. Cells
142 were maintained for 22 h at 37°C, in a 5% $CO_2$ atmosphere. After the incubation period,
143 the filters were washed in PBS 1x and cells on the upper side of the membrane (non-
144 invasive) were removed by scraping with a cotton bud. Invasive cells on the lower side
145 of the membrane were fixed for 20 min in ice-cold methanol. Inserts were then washed
146 in PBS and the membranes were removed and mounted on a slide in Vectashield
147 Mounting Medium with DAPI (Vector Laboratories). Invasive cells were counted under
148 Leica DM2000 (Leica) fluorescence microscope. This process was independently
149 repeated three times.

150

151 **Motility assay**

152 Motility assay was carried out using an eight-well slide coated with Collagen IV
153 (Ibidi, Martinsried, Germany). CRC lines' cells were seeded at a density of 4000
154 cells/well and maintained at 37°C and 5% $CO_2$ overnight. Cells were imaged at 10
155 different points/well every 10 min over a 24 h period using time-lapse microscopy (Time-
156 lapse Leica DMI6000). The experiments were done by keeping the cells in their regular
157 culture media or, in the case where the effect of CD26 enzymatic activity was being
158 addressed, using media supplemented with 0.5 mM of sitagliptin. Images were captured
159 at 20x magnification, and cell motility was analysed using Lsmib software. Because cells

160 entering mitosis slightly detach from the well and therefore increase their movement,
161 motility analysis was made in cells that did not divide or, in the case of cells that entered
162 mitosis during the imaging period, only the frames before the division were counted.
163 Motility studies were performed in three independent experiments.

164

165 **Slow aggregation assay**

166 Fifty μL of an agar solution (100 mg of Bacto-Agar in 15 mL of PBS 1x) were
167 used to coat 96-well plates [42]. Adherent cells were treated with trypsin and suspended
168 in culture medium to a final concentration of $10^5$ cells/mL. Two hundred μL of this
169 suspension were added to each well. Cells were incubated for 24 and 48 h at 37°C, in a
170 5% CO2 atmosphere in the absence or in the presence of sitagliptin (0.2 mM) diluted in
171 PBS. Aggregation was assessed under an inverted microscope. Three images were
172 captured with a Nikon (Tokyo, Japan) digital camera from at least three biologic
173 replicates of each cell line and for the two different times.

174

175 **Statistical analysis**

176 All graphs and statistical analysis were performed using GraphPad Prism
177 Software v5 (GraphPad-trial version). T-test (paired, non-paired or one sample *t*-test) was
178 used to compare data. Statistical significance was achieved when $P< 0.05$. *$P < 0.05$,
179 **$P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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181

182

# Results

## CD26 expression and DPP4 enzymatic activity in CRC cell lines

Expression levels of CD26 were evaluated in a panel of CRC cell lines derived from tumours at different stages of malignancy (information in Table 1). Flow cytometry analysis of CD26 membrane expression revealed that SW1116 (stage A), HT-29 (stage C), HCT116 (stage D) and T84 (derived from a lung metastasis) cell lines express high levels of CD26 whereas SW480 (stage B), SW620 (stage C) as well as RKO (stage D) express very low levels of CD26 (Figure 1 A and B). These data correlate with the in vivo immunohistochemical data [3-5,11,12,21,22], the loss of CD26 in early stages cells, and retained (or re-expressed) in cells with metastatic status such as T84 or HCT116 [43].

DPP4 enzymatic activity was measured in four representative cell lines. Fig 2 A shows that enzymatic activity of the lines does not totally correlate with their surface expression (compare with Fig 1 A). This may be explained, at least in part, because other proteins of the same family with DPP activity [3-5,39] could be differentially expressed in the lines.

The same fact also may explain why sitagliptin, specific inhibitor of only DPP4, achieves no more than 80% of inhibition when enzymatic activity was measured in the HT-29 living cells adhered to the flask (Fig 2 B).

## Cell viability in the presence of sitagliptin

It was important to measure in this context the cell viability of cultures with sitagliptin at the inhibitory doses and for the culture times required in the following experiments. After 24 h of culture, the viability of the cells is greater than 80% in the

206 presence of 0.2 and 0.5 of sitagliptin. Higher concentrations of the inhibitor compromise
207 the viability of the culture (HT-29 as example, Table 2).
208

## 209 Sitagliptin reduces motility of CRC cell lines

210 We first evaluated motility, in terms of velocity, with respect to CD26 expression
211 levels. Cells of the four selected CRC cell lines were seeded onto collagen type IV-coated
212 plates and cell motility was analysed by time-lapse microscopy during 24 h. The two lines
213 from advanced stages and high CD26 expression show important motility values whereas
214 the stage A line SW1116 in spite of their CD26 expression, and SW620 cells hardly move
215 (see the different range in the y-axe, Fig 3A).
216 Afterwards, HT-29 cells, being almost 100% CD26+, were sorted into CD26high
217 and CD26low subsets. We also used in this experiment HCT116 cells to be sorted into
218 both CD26+ and CD26- cell subsets. Fig 3 B shows that the absence of CD26 in HCT116
219 cells decreases the velocity from 0.76±0.017 (μm/min) to 0.39 ±0.027 (μm/min)
220 ($p<0.0001$), and that the HT-29 CD26high subpopulation also move faster (0.38±0.018
221 μm/min) than its CD26low counterpart (0.21±0.009 μm/min) ($p<0.0001$). These data
222 show that the role of CD26 in cell motility is not essential (HCT116 CD26- cells move to
223 a velocity similar to that of HT-29 CD26high cells) and only present in those cells that
224 had developed this function (SW1116 cells with high levels of CD26 don't move).
225 Then, it was tested if CRC cell motility was affected by CD26 enzymatic activity.
226 Motility assays revealed that in the presence of sitagliptin, all the cell lines with high
227 CD26 expression reduce their motility capacity (**** $P < 0.0001$ for HT-29, HCT116
228 and T84 cells, and **$P < 0.01$ for SW1116) (Fig 4). It is important to note a) that in the
229 SW1116 cell line, although significant, the effect of sitagliptin in motility is minimum;

230 b) in the other lines, although the effect is important (around 50% of the motility measured
231 in its absence), the basal levels of the SW1116 line are not achieved; and c) all cells in
232 each line, the faster and the slower, were affected. These facts altogether support that the
233 mechanism of sitagliptin is not related to the mortality of cells, although a DPP-IV-
234 independent effect of sitagliptin cannot be discarded.
235

236 **Sitagliptin impairs invasion of CRC cell lines**

237 Cancer cell invasion was evaluated using transwell chambers coated with
238 Matrigel. No CRC cell line show significant invasive potential (more than 100 cells) at
239 basal conditions (although an inhibitory effect of sitagliptin was observed in these
240 conditions).

241 However, HCT116 cells were converted to invasive in an experimental system
242 using conditioned media from colonic fibroblasts instead of complete medium [44]. Fig
243 5 shows that the invasive capacity of HCT116 cells is significantly reduced in the
244 presence of sitagliptin.
245

246 **Low levels of CD26 expression but not sitagliptin impair CRC cell**
247 **aggregation**

248 Slow aggregation assay in triplicates was first performed to test a role of CD26
249 on the cell-cell adhesion of CRC cells. All cell lines with a high percentage of CD26
250 positive cells originate compact aggregates after 24 and 48 h of culture (Fig 6 A), although
251 at 24 h compact aggregates are clearly observed only in the T84 and HCT116 aggressive
252 cell lines (data not shown). On the other hand, the cell lines with a very low percentage

253 of CD26 positive cells (SW620 and RKO) did not form those compact aggregates (Fig 6
254 A, some cells are together in a more two-dimensional form).

255 The aggregation assay was repeated after sorting HT-29 cells in CD26high and
256 CD26low subsets. The role of CD26 in CRC cell aggregation or cell-to-cell adhesion was
257 again confirmed because CD26high cells are able to form larger aggregates than the
258 CD26low cells (Fig 6 B).

259 However, we did not observe sitagliptin inhibition of cell aggregation (or
260 adhesion) in any cell line (Fig 6 C, an example with HCT116 cells at 48 h).

261

## 262 Discussion

263 Our results show that CD26 has a role in CRC cell invasion, motility, and
264 aggregation and that the inhibition of its DPP4 activity using sitagliptin results in a
265 decreased cell motility and invasion capacities. Sitagliptin and other DPP4 inhibitors have
266 been used for diabetes therapy since 2006, with high effectiveness and tolerance
267 [13,20,38,45]. Very recently, it has been shown they can prevent colon cancer and lung
268 metastasis in animal models [31,34-36] while a recent epidemiological study has showed
269 that use of incretin-based drugs is not associated with colorectal cancer incidence among
270 patients with type 2 diabetes [45]. Clinical trials for prevention of metastasis in humans
271 might be envisaged if a similar study confirms their preventive effect on metastasis.

272 Many studies had indeed addressed the role of CD26 in promoting cancer cell
273 migration and invasion in several cancer models, including in CRC [13,16,17, 46-48]. We
274 have avoided the models that include the physiological role of DPP4 in chemotaxis
275 (which is also involved in metastasis) [3-5,32], and included a motility assay to focus on
276 the interaction between CD26 and ECM proteins. For example, in cervical cancer cell

277 lines, it was found that sitagliptin enhanced migration capacity (probably by preventing
278 chemokine degradation) whereas it reduced adhesion of cells by a CD26/DPP4-
279 independent (unknown) mechanism [39]. In previous studies related with the CD26
280 function through adhesion to ECM, the adhesion was abrogated by the use of blocking
281 antibodies that do not recognize the catalytic domain of the protein, or by RNA
282 interference. This is the first work showing that motility and invasion, but not
283 aggregation, processes were decreased in the presence of CD26 enzymatic activity
284 inhibitor, sitagliptin, in CRC cell lines.

285 Cell-cell homotypic aggregation was enhanced in the presence of CD26, since
286 sorted cells expressing high levels of CD26 formed more and larger aggregates than cells
287 expressing low levels or none CD26, consistent with a previous study showing that CD26
288 binding to cell surface fibronectin was important to promote cell-cell aggregation [38].
289 An increase in cell-cell aggregation seems contradictory to increased motility and
290 invasive properties. However, the capacity to form homo and heterotypic aggregates is
291 also associated with increased malignant behaviour. In fact, collective cell migration has
292 been described as being more efficient and ideal than single cell migration (cellular
293 responses are better coordinated) [49]. Additionally, it has been shown that highly
294 metastatic cells possess a higher ability to form multicellular homo and heterotypic
295 aggregates [50], a feature associated with resistance to anoikis, adhesion to the
296 endothelium, and adhesion to other cells within the metastatic site.

297 Sitagliptin does not inhibit this fibronectin-dependent property, which is coherent
298 with the fact that fibronectin binds to an epitope of CD26 that does not involve its catalytic
299 site [44,51,52]. It seems plausible that ecto-ADA is participating in linking cells through
300 binding to CD26 and Adenosine Receptors [5,37].

301 On the other side, neither motility nor invasion are dependent of CD26. In the
302 motility assay, collagen type 4 was used, and at least type 1, 3 and 4 collagens are present
303 in Matrigel (used in the invasion assay). Although both collagen and fibronectin bind to
304 similar epitopes in the cystein-rich region of CD26 [44,47,51,52], sitagliptin only
305 inhibited these collagen-dependent assays. This finding cannot be considered unexpected
306 because original studies had already shown that the tripeptide Gly-Pro-Ala, a substrate
307 for DPP4, interfered with initial spreading of hepatocytes on a matrix consisting of
308 fibronectin and denatured collagen [46]. The work of Bauvois, Iwase-Okada et al, and
309 Ghersi et al [53-55] showed that CD26/DPP4 participates in a protease complex with
310 gelatinase/collagenase activity at invadopodia of migratory cells [54].

311 Circulating human CD133+CD26+CD44+, but no CD133+CD26-CD44+ cells,
312 were detected in the portal vein of mice at week 6 after cecal wall injection, demonstrating
313 the invasion of CD26+ cells into the circulation of mice orthotopically implanted [17],
314 and leading to the development of liver metastasis. Those cells displayed higher adhesion
315 to fibronectin and type 1 collagen (a major component of the ECM synthesized by
316 fibroblasts) compared with CD26- cells, and integrin beta1 is involved in this cell
317 adhesion to both [17]. Consequently, differences in the motility values among the cell
318 lines may also be related to CD26+ CSCs enrichment in each line.

319 In coherence with these reports, when CD26 has been suggested as a marker of
320 aggressive disease, as in thyroid cancers, some types of lung adenocarcinomas [56],
321 haematological malignancies [57] or Ewing sarcoma [10], it probably represents the
322 presence of CD26high CSCs and the development of metastasis. This means that
323 colorectal cancer patients may benefit from sitagliptin treatment in those tumour stages

324 where CD26+ cells (i.e. CSCs or MetSC, that went through MET)) are present, as shown
325 by cell lines from advanced stages [13-19,42].

326 However, the cell lines with low CD26 expression of CD26 also showed low E-
327 cadherin expression (and other markers of EMT (in press), resembling the early stages of
328 primary tumours. These findings can explain why this glycoprotein plays distinct roles in
329 different studies, being tumour suppressive in some and oncogenic in others [57]. In
330 studies supporting that CD26 has a tumour suppressive activity, in ovarian and
331 endometrial carcinomas [6,7], melanoma [8] or non-small cell lung cancers [9],
332 suppression of CD26 expression or DPP4 activity promotes cancer cell migration and
333 invasion that are related with the molecular and morphologic features of the EMT [8,9,39]
334 in stages earlier than the development of CD26+ CSCs and metastasis of later stages
335 (probably after a TEM).

336

## 337 Conclusions

338 In summary, our data confirms that CD26 plays a role in the invasion, motility,
339 and aggregation of CRC cell lines and support it as a target for prevention of metastasis.
340 They also show the molecular mechanism behind sitagliptin inhibition of some of these
341 malignant properties while others aren't affected. This finding may also help to define
342 better the sitagliptin use in other areas of medicine where DPP4 inhibitors are showing
343 promising results [58-60].

344

345

346

# Declarations

## Ethics approval and consent to participate

Not applicable

## Consent for publication

The authors declare that they all consented the publication of this manuscript.

As for a written informed consent from patient/participants, this is not applicable to our manuscript.

## Competing interests

The authors declare that they have no conflict of interests.

## Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.


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## Authors' contributions

MRQ, PDC, FM and ASR, Data curation; LVI, Data curation and Formal analysis; MPC, Conceptualization, Funding acquisition and Supervision; SV and OJC, Conceptualization, Formal analysis, Funding acquisition, Writing - original draft; Writing - review & editing.

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619 # Figure legends

620 ## Fig 1. CD26 expression in colorectal cancer cells.

621 **(**A) Flow cytometry analysis of CD26 expression in the cell lines used in this work (N=3
622 Mean ±SD). Cells in suspension derived from monolayer cultures were adjusted to a final
623 concentration of 10x $10^6$ cells/mL in sorting buffer before the addition of antibodies. (B)
624 Representative histograms of CD26 expression on colorectal cancer cell lines. The
625 expression of CD26 on SW1116, HT-29, HCT116, SW480, SW620, RKO and T84 cells
626 was analysed using FlowJo software. Thin black lines represent the control, unstained
627 cells, and thick lines represent the specific binding of CD26-PE antibody to its antigen at
628 the cell surface.

629 ## Fig 2. DPP-IV activity and sitagliptin inhibition curve in colorectal
630 ## cancer cells.

631 **(**A) DPP-IV activity in four representative cell lines according to their CD26 expression
632 (N=3 Mean ±SD). Cells were cultured until the cells reached confluence, and the wells
633 were washed three times with PBS before the addition of 100 μL of the substrate Gly-
634 Pro-p-nitroanilide. (B) Specific activity of Gly-Pro-p-nitroanilide hydrolysis by HT-29
635 living cells adhered to the flask, in the absence (control) or presence of different doses of
636 CD26/ DPP4 inhibitor sitagliptin.

637 ## Fig 3. The effect of CD26 expression levels on the motility capacity of
638 ## colorectal cancer cells.

639 (A) Motility ability of four colorectal cancer cell lines representative of different tumour
640 stages. (B) Expression levels of CD26 affect the motility of colorectal cancer cell lines.
641 HT-29 and HCT116 cell lines and histograms with the sorted subpopulations (CD26high

642 and CD26low from HT-29 cells and $CD26^+$ and $CD26^-$ from HCT116 cells). Data is

643 representative of three experiments.

644

645 **Fig 4. The effect of sitagliptin on the motility capacity of colorectal**

646 **cancer cells.**

647 Treatment with sitagliptin (0.5 mM) reduces the motility ability of four representative

648 colorectal cancer cell lines. Data is representative of three experiments.

649 **Fig 5. The effect of sitagliptin on the invasive capacity of HCT116 cell**

650 **line.**

651 Conditioned media (CM) from fibroblasts was added to the bottom of the transwell.

652 HCT116 cells, non-treated or treated with DPP4 inhibitor sitagliptin (0.5 mM), were

653 seeded in the upper chamber of the transwell plate containing a matrigel coating. Invasive

654 cells were counted 22 h after incubation. Values were normalized to the control (HCT116

655 + CM Fibroblasts). Mean ± SD of three experiments is presented. (** represents a

656 significant difference of $p \leq 0.01$).

657 **Fig 6. Representative pictures of aggregates from colorectal cancer cell**

658 **lines in the absence or presence of sitagliptin.**

659 Different morphology of aggregates formed in 48 hours cell cultures (24 h photographs

660 were also taken, data not shown). (A) Colorectal cancer cell lines SW1116, HT-29,

661 HCT116, SW620, RK0 and T84. (B) Aggregates formed by HT-29 CD26high and

662 CD26low subpopulations. (C) Aggregates formed by HCT116 cells, as example, in the

663 presence or absence of sitagliptin (0.5 mM).



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