Article

Colorectal cancer metastases settle in the hepatic microenvironment through α5β1 integrin†

Chiara Pelillo[1], Alberta Bergamo[1*], Hilaria Mollica[1], Marco Bestagno[2], Gianni Sava[1,3]

[1]Callerio Foundation Onlus, via A. Fleming 22-31, 34127 Trieste, Italy;
[2]International Centre for Genetic Engineering and Biotechnology, AREA Science Park, Padriciano 99, 34102 Trieste, Italy; [3]Dept. of Life Sciences, University of Trieste, via A. Fleming 22, 34127, Trieste, Italy.

***Corresponding author**: Alberta Bergamo
Carlo and Dirce Callerio Foundation Onlus
Via A. Fleming 22-31, 34127 Trieste (Italy)
a.bergamo@callerio.org
Tel: +390405588639

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Abstract

Colorectal cancer (CRC) metastasis dissemination to secondary sites represents the critical point for the patient's survival. The microenvironment is crucial to cancer progression, influencing tumour cell behaviour by modulating the expression and activation of molecules such as integrins, the cell-extracellular matrix interacting proteins participating in different steps of the tumour metastatic process. In this work, we investigated the role of α5β1 integrin and how the microenvironment influences this adhesion molecule, in a model of colon cancer progression to the liver. The culture medium conditioned by the IHH hepatic cell line, and the extracellular matrix (ECM) proteins, modulate the activation of α5β1 integrin in the colon cancer cell line HCT-116 and drives FAK phosphorylation during the process of cell adhesion to fibronectin, one of the main components of liver ECM. In these conditions, α5β1 modulates the expression/activity of another integrin, α2β1, involved in the cell adhesion to collagen I.

These results suggest that α5β1 integrin holds a leading role in HCT-116 colorectal cancer cells adhesion to the ECM through the modulation of the intracellular focal adhesion kinase FAK and the α2β1 integrin activity. The driving role of the tumour microenvironment on CRC dissemination, here detected and described, strengthens and adds new value to the concept that α5β1 integrin can be an appropriate and relevant therapeutic target for the control of CRC metastases.

The microenvironment surrounding cancer cells acts as a functional entity inducing and responding to tumour and host factors [Vidal-Vanaclocha, 2011; Schedin and Keely, 2011], and determining the colonization of invasive cells in particularly favourite organs [Weiss, 1992; Friedl and Wolf, 2003].

Colon cancer frequently metastasizes to the liver. Anatomical/hemodynamic and functional/micro-environmental factors work together to trap circulating cancer cells into this site. The hepatic microenvironment may contribute to cancer cell retention, and facilitates the ability of some of them to grow in the liver as a metastasis. In fact, fenestrated endothelial cells and Kuppfer cells constitutively express a rich profile of surface oligosaccharides [Barbera-Guillem et al., 1991], cell adhesion proteins [Scoazec and Feldman, 1991] and recognition receptors [Szabo et al., 1997] that account for an efficient hepatic uptake and clearance of cancer cells. Therefore, the liver induces colon cancer cells to express pro-metastatic receptors, and to secrete soluble factors which, in turn, cause remote activation of hepatic cells and pre-metastatic niches [Kaplan et al., 2006].

The extracellular matrix (ECM) constitutes the non-cellular part of the microenvironment, and its components regulate cell growth, migration and survival. At the same time, tumour cells need to remodel the ECM to create a nutrient rich milieu permitting their survival and growth.

Cells use integrins to take advantage of the great amount of biological information coming from the ECM [Hynes, 1992; Ruoslahti and Reed, 1994; Huhtala et al., 1995]. These cell surface adhesion receptors play a critical role also in tumour progression and metastasis, and alterations in their expression and/or activity influence the metastatic potential of tumour cells. β1 integrin is related to the malignant phenotype in breast cancer [Weaver et al., 1997; Jia et al., 2004], mediates drug resistance and stimulates metastasis in gastric, ovarian, and lung cancer [White and Muller, 2007; Shibue and Weinberg, 2009]. α1β1 and α2β1 integrins are key regulators of hepatocarcinoma cell invasion [Yang et al., 2003], and several integrins are up- (α2β1, αvβ3, α4β1, and αIIbβ3) or down-regulated (α6β1, αvβ5) in melanoma [Kuphal et al., 2005]. α5β1 integrin, the fibronectin receptor, is involved in the malignant behaviour of colon carcinoma cells. Highly invasive colon cell lines, such as HCT-116 cells, express higher levels of α5β1 integrin than poorly invasive cells [Janouskova et al., 2013].

Integrins activate many different intracellular pathways through the formation of protein-protein signalling complexes [DeMali et al., 2003; Mitra and Schlaepfer, 2006; Hood and Cheresh, 2002]. One of the early mediators of the integrin pathway is the intracellular focal adhesion kinase FAK. FAK activates downstream signalling cascades, such as the PI3K/Akt and MAPK/Erk pathways, implicated in the regulation of tumour cell proliferation, invasion, migration and survival [Ridley et al., 2003; Sieg et al., 2000]. FAK also mediates α5β1 integrin activation during the early stages of cell adhesion to ECM, inducing integrin clustering and increasing the number of bound integrin molecules [Michael et al., 2009; Sieg et al., 1999; Zeng et al., 2006].

This study sets up an *in vitro* CRC model that, overcoming the bias implicit in the single cell cultures, aims to resemble the complex *in vivo* milieu run into by tumour invasive cells. In particular, it focuses on the behaviour of the highly invasive colon cancer cells HCT-116 inserted in the context of the liver microenvironment, here reproduced by both its structural components (i.e. ECM proteins) and the soluble factors secreted by the hepatocytes. The results demonstrate the straightforward effect of the microenvironment of the liver on cancer progression to metastases, in particular on the tumour cell adhesion and settlement process. These metastatic steps are favoured by the hepatic microenvironment through the modulation of the α5β1 integrin and the activation of its downstream signalling pathway molecule FAK.

The ECM proteins synergize with the soluble factors secreted by the hepatocytes, driving the molecular events determinant for the adhesion of HCT-116 colorectal cancer cells to their favourite metastasis target organ.

2. Materials and Methods

2.1 Cell cultures

The adenocarcinoma colon cancer cell line HCT-116 was kindly supplied by the group of dr. C. Gaiddon, University of Strasbourg, France. The cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Euroclone, Ltd., UK) supplemented with 10% foetal bovine serum (FBS; Gibco-Invitrogen Corp., UK), 2 mM L-glutamine (Euroclone Ltd., UK), 100 IU/ml penicillin, 100 µg/ml streptomycin solution (Euroclone Ltd., UK).

The human immortalized hepatocytes cell line IHH was kindly supplied by the group of prof. C. Tiribelli, CSF, University of Trieste, Italy. The cell line was cultured in 1:1 DMEM/HAM's F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin solution, dexamethasone 10^{-4} M (SIGMA-Aldrich®, MO, USA), pancreatic bovine insulin 10^{-8} M (SIGMA-Aldrich®, MO, USA), Hepes Buffer 1 M (SIGMA-Aldrich®, MO, USA).

The human kidney cell line HK-2, a proximal tubular cell (PTC) line derived from normal kidney, was supplied by prof. R. Bulla (Department of Life Sciences, University of Trieste). The cell line was cultured in 1:1 DMEM/HAM's F12 medium supplemented with de-complemented FBS 5%, antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin), 2 mM L-glutamine, 5 mg/ml insulin from bovine pancreas, 5 mg/ml holo-transferrin, 5 ng/ml sodium selenite, 5 ng/ml hydrocortisone, ng/ml EGF 10, 5 pg/ml T3, and 5 pg/ml PGE1 (SIGMA-Aldrich®, MO, USA).

The cell lines were grown in a CO_2 incubator with 5% CO_2 and 100% relative humidity at 37°C. Once 90% of confluence was reached, the cell lines were detached with trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA in PBS, SIGMA-Aldrich®, MO, USA). Cell viability was determined by the trypan blue dye exclusion test.

2.2 IHH (H-CM) and HK-2 (HK-CM) conditioned medium preparation

The H-CM medium was obtained culturing IHH cells at approximately 70 - 80% of confluence in their standard medium for 24 h, afterwards it was collected and stored at -20°C until use.

The same procedure was followed to prepare HK-CM medium from HK-2 cell line.

2.3 Invasion Assay

Invasion assay was performed using 8.0 µm pore size Transwell® inserts (Costar, Cambridge MA, USA) coated with Matrigel™ (600 mg/ml) at room temperature overnight. HCT-116 cells were incubated with serum-free medium 24 h before seeding them on inserts (50,000 cells/insert). HCT-116 (ST-M), IHH (H-M) and IHH conditioned medium (H-CM) were added in the lower chambers of the plate. The basal invasion rate, i.e. not induced by the presence of FBS in the attracting medium, was measured.

After 96 h at 37°C, cells remained on the upper side of the membrane were removed using cotton swabs, while the cells that migrated to the underside were fixed in glutaraldehyde 1.1% (v/v) at room temperature for 15 minutes, and then stained with crystal violet solution 0.1% (w/v) for 20 minutes; after washing to remove unreacted reagent, the dye was solubilized in acetic acid 10% (v/v) [Kueng et al., 1989]. The absorbance units were read at 590 nm and related to cell invasion rate. Each assay was done in triplicate and data presented as mean with the standard deviation.

2.4 Adhesion assay

A 96-well plate was pre-coated with fibronectin, collagen I, collagen IV, poly-L-lysine (SIGMA-Aldrich®, MO, USA,), laminin (SIGMA-Aldrich®, MO, USA) or Matrigel™ (20 mg/ml) for 4 h at 37°C or at 4°C overnight and subsequently blocked with PBS-BSA 0.1% (w/v) for 15 min at 37°C. Sub-confluent tumour cells were incubated 24 h in ST-M, H-M and H-CM medium, and then in serum-free medium for additional 24 h. The same procedure was followed to perform the adhesion assay in the presence of medium conditioned by the human renal cell line HK-2 (HK-M and HK-CM).
Cells were trypsinized with 1 mM EDTA, re-suspended in serum free medium with 0.1% BSA for 30 minutes at room temperature to ensure re-expression of adhesion receptors on the cell surface and seeded in the plate (20,000 cells/well). HCT-116 were allowed to attach to each substrate for 1 h at 37°C and the non-adherent cells removed by washing. The adherent cells were fixed with trichloro-acetic acid 10% (v/v) for 1 h at 4°C and stained with sulphorodamine B 0.4% (w/v) according to the method of Skehan et al. [Skehan et al., 1990]. The absorbance units were read at 570 nm and related to the adhesion rate.
For the adhesion assay in the presence of α5β1 function blocking antibody, tumour cells were re-suspended in PBS with or without 5 μg/ml of the antibody JBS5, (Millipore, MA, USA) and then plated as described above. The adhesion assays were repeated for each condition in quadruplicate for three times. The results shown represent the mean with the standard error of one representative experiment out of the three performed.

2.5 Integrin profile analyses

Sub-confluent tumour cells were incubated 24 h in ST-M, H-M and H-CM medium, and then detached with 1mM EDTA, re-suspended in serum free medium with 0.1% BSA for 30 minutes at room temperature to ensure re-expression of adhesion receptors on the cell surface. Integrin expression levels were analyzed using an integrin mediated cell adhesion fluorimetric array (Chemicon International, ECM 535). Integrin array kit recognizes extracellular epitopes of human α chains (α1, α2, α3, α4, α5, αV, and αVβ3) and β chains (β1, β2, β3, β4, β6, αVβ5, α5β1) of integrins. In this kit, the wells of a 96 well plate contain immobilized monoclonal human integrin antibodies used to capture cells expressing integrins on their surface. Cells were re-suspended in DMEM medium and left adhere to the plates 1 h at 37°C and 5% CO_2. Unbound cells were washed off, cells adherent on the plate were lysed with the cell lysis buffer included in the kit, and integrins were detected using the molecular probe CyQuant GR dye. Fluorescence was determined at 540-570 nm on a microtiter plate reader (FluoroCount™, Packard). The experiment was repeated for each condition in triplicate, two times. The results shown represent the mean with the standard error of the two experiments performed.

2.6 Flow Cytometric Analyses of α5β1 cell surface expression

Sub-confluent tumour cells were incubated 24 h in ST-M, H-M and H-CM medium, and then in serum-free medium for additional 24 h. Cells were trypsinized with 1 mM EDTA and re-suspended in serum free medium with 0.1% BSA for 30 minutes at room temperature. Cells (500,000/sample) were then centrifuged and incubated with an anti-α5β1 primary antibody (clone HA5 5 μg/ml, Millipore, MA, USA) in PBS for 30 minutes at 4°C. Cells were then centrifuged and incubated with a FITC-conjugated goat anti mouse GAM-antibody (diluted 1:100, SIGMA-Aldrich®, MO, USA) in PBS for 30 minutes at 4°C. After centrifugation cells were re-suspended in PBS-0.1% BSA (w/v)- 0.1% NaN_3 (w/v)

and analysed at flow cytometer (FACS Calibur cell analyser, Becton Dickinson, Milano, Italy). For each sample 10,000 events were recorded.

Data obtained were processed using WinMDI 2.9 software.

The same analyses were performed on HCT-116 cells pre-adhered on fibronectin (20 mg/ml) for 1 h at 37°C before α5β1 integrin expression detection.

The analyses were repeated for each condition in triplicate, three times. The results shown represent the mean with the standard deviation of the three analyses performed.

2.7 Western blot analyses of FAK activation

Sub-confluent HCT-116 cells were incubated 24 h in ST-M, H-M and H-CM medium and then in serum-free medium for additional 24 h; cells were then detached with PBS-EDTA 1 mM, incubated in serum free medium with 0.1% BSA for 30 minutes at room temperature and seeded on a 6-well plate ($1x10^6$/well) pre-coated or not with fibronectin (20 mg/ml).

Cells were allowed to adhere for 1 h at 37°C, then detached with Trypsin/EDTA and lysed with the lysis buffer (0.5% Na-deoxycholate, 1% Igepal, 50 mM HEPES, 150 mM NaCl, 1 mM Na-orthovanadate, 50 mM NaF, 20 mM β-glicerophosphate, 0.1 μM okadaic acid, 1 mM PMSF, 50 μg/ml leupeptine, 20 μg/ml aprotinin, 10 mM pepstatin). Total protein extract was quantified following the method of Bradford [Bradford, 1976].

Proteins in the total cell lysate (10 μg of extract for FAK detection and 30 μg for P-FAK detection) were separated on 8% polyacrylamide SDS-PAGE and electro-transferred to a nitrocellulose membrane (Bio-Rad, Milano, Italy). Afterwards, the blot was blocked in a solution of 5% skimmed milk, 0.1% tween in TBS for FAK detection, or in a solution of 3% BSA, 0.1% Tween in TBS for P-FAK detection for 1 h at room temperature. Membrane-bound proteins were probed with an anti-FAK primary antibody (#3285 Cell Signalling, Milano, Italy, 1:1000) and an anti-P-Y397-FAK primary antibody (#3283 Cell Signalling, Milano, Italy, 1:1000) over night at 4°C and then washed before incubation with a horseradish peroxidase-conjugated secondary antibody (#7074 Cell Signalling, Milano, Italy, 1:1000) for 1 h at room temperature in agitation. Proteins were detected incubating membranes with horseradish substrate (Cell Signalling, Milano, Italy) and developed with appropriate reagents and Kodak autoradiography film (Amersham Pharmacia Biotech, NJ, USA).

2.8 Western blot analyses of α5β1 - α2β1 cross-talk

Sub-confluent HCT-116 cells were incubated 24 h in ST-M, H-M and H-CM medium and then in serum-free medium for additional 24 h; cells were then detached with PBS-EDTA 1 mM, incubated in serum free medium with 0.1% BSA for 30 minutes at room temperature and seeded on a 6-well plate ($1x10^6$/well) pre-coated with collagen I (20 mg/ml).

Cells were allowed to adhere for 1 h at 37°C, then detached with Trypsin/EDTA and lysed with the lysis buffer (0.5% Na-deoxycholate, 1% Igepal, 50 mM HEPES, 150 mM NaCl, 1mM Na-orthovanadate, 50 mM NaF, 20 mM β-glicerophosphate, 0.1 μM okadaic acid, 1 mM PMSF, 50 μg/ml leupeptine, 20 mg/ml aprotinin, 10 μM pepstatin). Total proteins extract was quantified and western blot analyses (40 μg of total protein extract) performed as previously described.

For α2β1 integrin identification an anti-α2 primary antibody was used (AB1936 Millipore, 1:1000).

2.9 Real Time PCR for α5, β1, and FAK expression

The differential expression of the α5, β1, and FAK genes in HCT-116 cells grown 24 h in ST-M, in H-M, and in H-CM was analysed by real time PCR. RNAs were reverse-transcribed with a qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA) according to the manufacturer's protocol. Expression levels were determined by real time PCR on a CFX96 system (Bio-Rad, Hercules, CA, USA). The 15 mL reaction mix included 7.5 mL of 2x SsoAdvanced™ SYBR Green® Supermix (Bio-Rad), 0.3 mL of each 10 mM primer and 2 ml of a 1:20 cDNA dilution. The following thermal profile was used: an initial 30" denaturation step at 95°C, followed by 40 cycles at 95° for 5" and 58° for 30". Amplification products were analyzed with a 65°/95°C melting curve. The following α5, β1, and FAK primer pairs were designed using the MIT Prime3 online primer design protocol from the relative gene sequence*:* for *FAK* 5'-TGA CCC CAA CTT GAA TCA CA-3' (forward) and 5'-CCA GGT GGT TGG CTC ACT AT-3' (reverse); for *ITGA5* (α5-integrin subunit) 5'-GTG GGC CAA CAA AGA ACA CT-3' (forward) and 5'-TGA GTT CTG ATT CCC CTT GG-3' (reverse); for *ITGB1* (β1-integrin subunit) 5'-CCC TTG CAC AAG TGA ACA GA-3' (forward) and 5'-CCA CCT TCT GGA GAA TCC AA-3' (reverse).

RPL5, a highly stable housekeeping gene, was chosen for normalization, and amplified using 5′-GCACACGAACTGCCAAAATA-3′ (forward) and 5′-TTCATCACCAGTCACCTCCA-3′ (reverse) primers. The expression levels of the selected transcripts were determined using the comparative Ct method (2-DD Ct method) [Livak and Schmittgen, 2001]. Ct values used for quantification were corrected based on PCR efficiencies using LinRegPCR [Ramakers et al., 2003]. Results are given as the mean with standard deviation of three technical replicates.

2.10 Statistical analysis

Results obtained were processed using InstatGraph3 software and presented as mean ± standard deviation. The group means were compared using a Two-Way Analysis of Variance (ANOVA) followed by t-test, or Tukey-Kramer post-test and considered significant when $p < 0.05$.

3. Results

3.1 The hepatic microenvironment affects HCT-116 cell invasion

The effects of the hepatic microenvironment soluble factors on the basic invasion potential of HCT-116 colorectal cancer cells were analysed using a medium conditioned by the IHH hepatocyte cell line (H-CM), in comparison to the same fresh medium, not exposed to the IHH hepatocytes (H-M), and to the standard culture medium of HCT-116 cells (ST-M) (**Fig.1**).

When cultured in the presence of ST-M, HCT-116 cells showed a basic, i.e. not FBS-induced, efficiency to invade the Matrigel™ barrier. When the stimulus was given by the H-M medium, the invasive ability of HCT-116 cells was comparable to that observed under the standard condition of ST-M. On the contrary, the hepatocyte conditioned medium H-CM significantly increased the invasion rate of these cells (+ 41% vs H-M and + 64% vs ST-M, respectively), suggesting that the soluble factors secreted by hepatocytes can act as invasion inductors.

3.2 The hepatic microenvironment affects HCT-116 cell adhesion

The HCT-116 cells showed a significantly increased adhesion potential when they were grown *in vitro* on fibronectin and laminin, for 24 h in H-M medium in comparison to the standard medium ST-M (**Fig.2a**). The adhesion rate to fibronectin and collagen I, two proteins largely represented in the liver ECM, further increased when tumour cells were

grown in the H-CM medium (+ 37% ± 4 and + 35% ± 11 *versus* ST-M, respectively for fibronectin and collagen I), suggesting the synergy between the structural components and the soluble factors of the hepatic microenvironment (H-M versus H-CM on both substrates: $p < 0.05$).

The adhesion of HCT-116 to ECM substrates was also tested in the presence of a medium (HK-M) conditioned for 24 h by the human renal cell line HK-2 (HK-CM). HK-M increased the adhesion of HCT-116 cells to all ECM proteins examined, likely because of the presence in this medium of many factors promoting cell growth, metabolism and functions. The consumption of the same factors, during the conditioning by HK-2 cells, is supposed to be the reason of the lack of a similar effect of HK-CM on the HCT-116 cells (**Fig.2b**). These data allow us to credit the liver microenvironment with a specific pro-adhesive effect, and particularly the soluble factors released by the hepatocytes during this step of the metastatic dissemination of CRC cells.

3.3 The hepatic microenvironment modulates HCT-116 integrin profile

Considering the changes of cell adhesion potential influenced by the microenvironment, the integrin expression profile of HCT-116 cells cultured in ST-M, H-M and H-CM mediums, was investigated measuring the cell membrane expression of several alpha and beta chains as well as of some fully assembled integrins. The integrin profile of HCT-116 cells grown in standard medium was in line with the adhesion potential shown in the previous experiment: the high expression of the alpha 2, 3 and 5 chains together with the beta 1 chain (**Fig.3**) reflected the preferred adhesion of HCT-116 cells respectively to collagen I, laminin and fibronectin. In fact, α2β1, α3β1 and α5β1 are the main integrins mediating the cell interactions to the ECM substrates mentioned above. Interestingly, we detected the absence of the αvβ3 integrin that, together with α5β1, usually manages cell-fibronectin interactions [Schaffner et al., 2013]. The results showed that the medium conditioned by the hepatocytes significantly reduced the expression of the α1, α2 and α3 chains in the HCT-116 cells. These changes of the integrin profile cannot explain the increased adhesion of HCT-116 to ECM proteins measured when cells are cultured in H-CM medium. These data highlight the fundamental role of the ECM, modulating cancer cell features and inducing the expression of specific integrins, the levels and/or activity of which can influence tumour cell behaviour during the disease progression.

3.4 The hepatic microenvironment affects α5β1 activation in HCT-116 cells

The relevance of α5β1 integrin for HCT-116 cell adhesion to fibronectin, was confirmed with the use of a α5β1 function blocking antibody. As expected, HCT-116 colon carcinoma cells drastically reduced (by 60–80%) their adhesion to this substrate, independently of the culture medium used (compare **Fig 4 Fibronectin**, absence (-) to presence (+) of blocking antibody). Interestingly, this assay unveiled also the basic role of α5β1 integrin for HCT-116 cell adhesion to collagen I and IV in the hepatic microenvironment. The anti-α5β1 integrin blocking antibody significantly reduced the adhesion rate of HCT-116 cells on both substrates, depending on the contemporary presence of the structural components of the ECM and the soluble factors released by the hepatocytes cultured in the conditioned medium (H-CM (+) *versus* H-CM (-) on collagen I and IV: $p < 0.001$).

The soluble factors secreted by IHH hepatocytes in the conditioned medium and fibronectin did not affect the density of α5β1 integrin receptors on the surface of HCT-116 cells (Mean Fluorescence Intensity, MFI) (**Fig.5a**), or the percentage of cells positive to the receptor (data not shown).

Western blot analyses supported the hypothesis that FAK regulates cell adhesion strengthening via integrin activation. Comparing FAK levels of HCT-116 cells grown in H-CM medium to those of its Y397-phosphorylated form, the rate

of the phosphorylated FAK is increased in comparison to the same test performed using ST-M (+ 126% ± 19, $p < 0.001$) and H-M (+ 97% ± 27, $p < 0.05$) (**Fig.5b**). This result was obtained when cells were allowed to adhere to fibronectin-coated wells, emphasizing the need of the presence of this ECM protein, particularly abundant in the liver matrix, for the settlement of CRC cells in the metastasis target organ.

3.5 The activation of α5β1 and FAK is regulated by the hepatic microenvironment at the post-translational level

The quantitation of the expression levels of the gene *FAK*, by real time PCR experiments, in HCT-116 cells grown in different media and adhered to fibronectin, suggested an up-regulation of the gene induced by the H-M medium respect to the ST-M medium. The activity of H-CM medium compared to that of the H-M medium was reduced, even though it increased the expression of *FAK*. This effect did not reflect what happens at the protein level, where the activation of the protein FAK was higher in HCT-116 cells grown in H-CM compared to the other media tested, and supported the hypothesis of the post-translational modulation guided by the hepatic microenvironment, rather than of a direct effect at the transcriptional level (**Fig.6**). No relevant differences in the expression levels of *α5* and *β1* genes, induced by the different media tested, were detected (data not shown).

3.6 The hepatic microenvironment stimulates the cross-talk between α5β1 and α2β1 integrins

When α5β1 activity of HCT-116 cells was blocked by a specific antibody, western blot analyses revealed a remarkable reduction of the α2β1 protein level caused by the combined action of soluble (H-CM) and structural (collagen I) constituents of the hepatic microenvironment (**Fig.7**).

4. Discussion

The progression of colorectal cancer to secondary tumours represents the critical point in patient's survival [www.nlm.nih.gov/medlineplus/cancers.html]. It is increasingly recognized that disease progression is influenced by the bi-directional communication between tumour cells and their microenvironment, whose interactions determine disease initiation and progression, and consequently patient's prognosis [Joyce and Pollard, 2009]. This also means that cancer is a heterogeneous disease also by virtue of the composition and content and/or activation state, of the microenvironment-derived stromal cells [Balkwill and Mantovani, 2001; Hanahan and Coussens, 2012].

We report here on the events involved in the liver colonization by CRC cells, focusing on the role of the microenvironment, represented by the structural components (ECM proteins) and by the soluble factors secreted by the hepatocytes, on the step of cell settlement and adhesion, a crucial event for metastasis formation. The aim was to set up an *in vitro* model of CRC metastasis depicting, more closely than a conventional cell culture, the *in vivo* hepatic metastatic niche that tumour cells face during their liver colonization.

Under the influence of the medium enriched of soluble factors secreted by the hepatocytes, the HCT-116 cells increase their adhesion to fibronectin and collagen I, two ECM proteins particularly abundant in the liver matrix during pathological processes such as fibrosis and cancer [Cutroneo et al, 2006; Kershenobich Stalnikowitz and Weissbrod, 2003]. The absence of similar effects of the medium conditioned by renal cells, as well as of the medium conditioned by the HCT-116 cells themselves on the adhesion profile of HCT-116 cells to ECM (data not shown), allow us to attribute to the soluble factors released by the hepatic cells, and associated with specific ECM proteins, the increased adhesion of CRC cells. This result also stresses the fundamental contribution of the hepatic microenvironment to colon cancer

progression in this cellular model. Preliminary analyses, performed on the medium conditioned by the human hepatocytes IHH, revealed the increased presence of growth factors such as VEGF and of chemokines such as MCP-1, as some of the main components released by the cells in this medium (Chambery A et al., SUN, Italy, personal communication).

It is known that integrins can interact with many different factors of the tumour microenvironment, determining important consequences on a number of intracellular processes, among which the adhesion of tumour cells to ECM, thus significantly affecting the invasive and metastatic tumour cell potential. Since integrins are linked to the cytoskeleton structures, their engagement triggers crucial signals for tumour cell migration and metastasis formation [Desgrosellier and Cheresh, 2010; Friedl and Alexander, 2011]. The fibronectin-cell interaction through α5β1 integrin triggers the auto-phosphorylation at Y397 site of the intracellular focal adhesion kinase FAK [Michael et al., 2009]. Quantitative relationships among integrin-ligand binding, adhesion, and signalling via FAK are also documented [Asthagiri et al., 1999]. The presently reported results indicate that the IHH hepatocytes release molecules reinforcing the fibronectin-induced FAK auto-phosphorylation and, consequently, trigger the activation of this pathway, therefore inducing HCT-116 cells to adhere more avidly to fibronectin via α5β1 integrin.

Our results are in line with other reports showing the need of integrin α5β1 tethering for Y397-FAK auto-phosphorylation during the integrin signalling activation [Shi and Boettiger, 2003]. Our data also add a significant role to the hepatic microenvironment for the adhesion process of colorectal cancer cells (a fundamental step during metastasis formation), and show that the soluble factors of the metastatic niche are necessary to get a synergistic interplay with the ECM structural proteins. These findings add further knowledge in the mechanisms of CRC metastasis biology and confer reliance to the further development of this experimental model system.

α5β1 integrin is the leading actor during HCT-116 colorectal cancer cell dissemination to the liver, since cells use almost exclusively this molecule to adhere to fibronectin. In fact, αvβ3 the other integrin mainly involved in cell-fibronectin adhesion [Schaffner et al., 2013], is not significantly expressed on the surface of the HCT-116 cells. Based on the presented data, we observed that the microenvironment stimulates the activation of α5β1 integrin signalling pathway rather than modulates the expression of the protein or of its coding genes (no changes detected on the *α5* and *β1* chains coding genes, data not shown). The role of the hepatic microenvironment seems to intervene on FAK at the post-translational level, since changes of its gene expression levels in the HCT-116 cells grown in H-CM medium and on fibronectin, are not in agreement with the increased activation (i.e. phosphorylation) of the protein FAK measured in WB. Furthermore, the reduced effect of the H-CM medium and fibronectin on *FAK* gene expression, compared to the level of the up-regulation induced by the H-M medium on the cells cultured on the same substrate, could be attributed to specific hepatic factors released in the H-CM medium which interfere and reduce the stimulating effect of the hepatic non conditioned medium (H-M medium) on the transcription of the *FAK* gene.

The impact of the metastatic microenvironment on the role of α5β1 integrin in the settlement of HCT-116 CRC cells in the liver seems to be even more important if we consider the results obtained with collagen I, the other main ECM protein present in the liver matrix. The synergistic interplay of H-CM and collagen I on the adhesion of HCT-116 cells resembles that detected on fibronectin and, similarly, it is strongly reduced in the presence of an anti-α5β1 integrin blocking antibody. In the present CRC model, the hepatic microenvironment seems to affect α5β1 integrin behaviour inducing an integrin crosstalk event, where the binding of one integrin type (transducer) alters the behaviour of a different integrin type on the same cell by coordinating its activation and availability [Friedrichs et al., 2010]. This event influences the adhesion of HCT-116 colorectal cancer cells to other ECM substrates beside fibronectin. The α2β1 is the main integrin designated for the cell-collagen I interaction [Leitinger, 2011]. Although the role of α2β1 integrin in CRC

is still controversial, it was already proved that the interactions of α5β1 with fibronectin, and that of α2β1 with type I collagen showed a combined effect on the downstream cellular signals [Reyes et al., 2008]. In our model, the engagement of α5β1 integrin seems to trigger α2β1 integrin, since the functional block of the former leads to the complete suppression of the expression of the second. Therefore α5β1 and α2β1 integrins cooperate together to sustain CRC adhesion and progression to the liver. In line with the oncogenic role of α2β1 integrin, the block of its activity inhibits CRC migration [Robertson et al., 2009]; moreover, van der Bij et al. reported that blocking α2 integrins prevented liver metastases outgrowth of a rat colon carcinoma model [van der Bij et al., 2008].

Due to their implication in several cellular functions, including the initiation, progression and metastasis of solid tumours, integrins are emerging as an appealing target for the setting up of anti-tumour and anti-metastatic strategies [Millard et al., 2011]. The α5β1 integrin is claimed to be a pertinent therapeutic target in solid tumours [Schaffner et al., 2013], and to control the progression of experimental lung cancer [Roman et al., 2010]. Many authors propose α5β1 integrin as a therapeutic target also for CRC and, in particular, for the treatment of liver metastases [Kuphal et al., 2005; Robertson et al., 2008]. Novel strategies are also directed to molecules of the signalling pathways engaged by α5β1 integrin, such as FAK. The FAK inhibitor PND-1186 blocks Y-397-FAK phosphorylation *in vivo* and decreases tumour growth and spontaneous breast to lung metastasis in pre-clinical models [Walsh et al., 2010]. Several other ATP-competitive small molecule inhibitors of FAK have been developed and proved to be active in diverse tumour types, colon cancer included [Slack-Davis et al., 2007; Roberts et al., 2008; Shi et al., 2007; Liu et al., 2007; Slack-Davis et al., 2009].

5. Conclusions

This study shows how the microenvironment behaves as a crucial actor in the CRC metastatic process and describes an *in vitro* model system reproducing the hepatic metastatic niche, thus depicting, more closely than a simple cell culture, the *in vivo* milieu that tumour cells face during their liver colonization. The hepatic microenvironment drives the settlement of CRC cells into the liver by modulating the activity of α5β1 integrin and its signalling pathway, which master role in this process is significantly enhanced by the soluble components and by the structural proteins of the liver microenvironment. In addition, this study indirectly suggests the potential impact of the targeting of α5β1 integrin for other oncogenic integrins, such as α2β1 and for their role in liver cell invasion and dissemination. In short, these data highlight the importance of evaluating the influence of the hepatic microenvironment on these pathways to develop new drugs directed to the selective control of CRC liver metastases.

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Conflict of interests

The authors declare no conflicts of interest.

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Legends

Figure 1. Effects of the hepatic microenvironment on basal HCT-116 cell invasion. HCT-116 cells were sown on the top compartment of invasion inserts coated with Matrigel™ (600 µg/ml), and left to invade against stimuli represented by the standard culture medium (ST-M), the IHH hepatocytes culture medium (H-M), and the IHH hepatocytes conditioned culture medium (H-CM). Data represent cells that had completely passed through the Matrigel™-coated barrier after 96 h. Data are mean ± SD of Absorbance Units of three independent experiments, each with triplicate sets. Statistical analysis: ANOVA and Tukey-Kramer post-test: ** $p < 0.01$ *versus* ST-M; ° $p < 0.05$ *versus* H-M.

Figure 2. Influence of different media on the adhesion of HCT-116 cells to ECM components. a) HCT-116 cells grown for 24 h in ST-M, H-M and H-CM, were seeded into 96-well plates pre-coated with different ECM components, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were then fixed and stained with sulphorodamine B. The dye was then solubilized and the absorbance units read at 570 nm. **b)** HCT-116 cells grown for 24 h in ST-M, HK-M and HK-CM, were seeded into 96-well plates pre-coated with different ECM components and treated as described above. Data are mean ± SE of Absorbance Units of one experiment representative of the three independent experiments performed, each with quadruplicate sets. Statistical analysis: ANOVA and Tukey-Kramer post-test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *versus* ST-M on the same substrate; ° $p < 0.05$, °° $p < 0.01$, and °°° $p < 0.001$ *versus* H-M (a) or HK-M (b) on the same substrate.

Figure 3. Integrin profile of HCT-116 cells.
Integrin profile of HCT-116 cells grown for 24 h in ST-M, H-M and H-CM medium. Cells were then detached with 1mM EDTA, re-suspended in serum-free medium with 0.1% BSA for 30 minutes at room temperature to ensure re-expression of adhesion receptors on the cell surface prior to seed them on the functionalized 96 well plates. The Integrin array kit recognizes extracellular epitopes of human α (α1, α2, α3, α4, α5, αV, and αVβ3) and β (β1, β2, β3, β4, β6, αVβ5, α5β1) integrins. Cells were left adhere to the plates 1 h at 37°C 5% CO_2. Unbound cells were washed off; adherent cells on the plate were lysed with cell lysis buffer, and integrins were detected using the molecular probe CyQuant GR dye. Fluorescence was determined at 540-570 nm on a microtiter plate reader. The results represent the mean ± SE of the Relative Fluorescence Units of the two experiments performed. The dashed line around 40000 RFU indicates the fluorescence of the negative control in the assay. Statistical analysis: ANOVA and Tukey-Kramer post-test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *versus* ST-M; ° $p < 0.05$, and °° $p < 0.01$ *versus* H-M.

Figure 4. α5β1 integrin dependence of HCT-116 cell adhesion to ECM components. HCT-116 cells grown 24 h in ST-M, H-M and H-CM were seeded into 96-well plates pre-coated with different ECM substrates without (-) or with (+) an anti-α5β1 integrin blocking antibody, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were then fixed and stained with sulphorodamine B. The dye was then solubilized and the absorbance units read at 570 nm. Data are mean ± SD of the Absorbance Units of three independent experiments, each with quadruplicate sets. Statistical analysis: ANOVA and Tukey-Kramer post-test: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ *versus* ST-M and ° $p < 0.05$, °° $p < 0.01$, and °°° $p < 0.001$ *versus* H-M, within groups without (-) or with (+) anti-α5β1 integrin blocking antibody; §§§ $p < 0.001$ versus the same medium without (-) anti-α5β1 integrin blocking antibody.

Figure 5. Influence of the hepatic microenvironment on α5β1 integrin expression and on FAK activation in HCT-116 cells. a) HCT-116 cells grown 24 h in ST-M, H-M and H-CM were seeded into 6-well plates pre-coated or not with fibronectin, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were harvested, incubated with an anti-α5β1 primary antibody, followed by incubation with a FITC-labelled secondary antibody, and then analysed by flow cytometry. Data are mean ± SEM of the Mean Fluorescence Units of two independent experiments, each with triplicate sets. **b)** HCT-116 cells grown 24 h in ST-M, H-M and H-CM were seeded into 6-well plates pre-coated or not with fibronectin, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were harvested, and their total protein content extracted. Equal amounts of total proteins from each sample were subjected to immune-blotting and then probed with antibodies against FAK and P-Y397-FAK. Western blots analyses and quantitation of Western blot lanes, by using ImageJ software, of one representative experiment out of three, are displayed. Data shown in (b) lower panel are the ratio between P-Y397-FAK and FAK levels, after normalization to β-actin amount in the same sample. This ratio calculated for HCT-116 cells grown in ST-M in each substrate was set equal to 1. Statistical analysis: ANOVA and Tukey-Kramer post-test: ** $p < 0.01$ *versus* ST-M; ° $p < 0.05$ *versus* H-M on the same substrate.

Figure 6. Influence of the hepatic microenvironment on the expression level of the *FAK* gene in HCT-116. HCT-116 cells grown 24 h in ST-M, H-M and H-CM were seeded into 6-well plates pre-coated or not with fibronectin, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were harvested, and their total RNA content extracted. Then, a

copy of cDNA was obtained by retro-transcription and subjected to real time RT-PCR. The expression levels of the gene *FAK* were normalised to the expression of the housekeeping gene RPL5. Data represent the mean ± SD of three independent experiments, and are the ratio between the basal expression of mRNA in HCT-116 grown in H-M, or in H-CM medium, to the basal expression of mRNA in HCT-116 grown in ST-M. This ratio calculated in HCT-116 cells grown in H-M in absence of fibronectin was set equal to 1. Statistical analysis: ANOVA and Tukey-Kramer post-test: *** $p < 0.001$ *versus* ST-M; °°° $p < 0.001$ *versus* H-M on the same substrate.

Figure 7. Induction of a cross-talk between α5β1- and α2β1-integrins by the hepatic microenvironment. HCT-116 cells grown 24 h in ST-M, H-M and H-CM were seeded into 6-well plates pre-coated or not with collagen I with (treated) or without (untreated) an anti-α5β1 integrin blocking antibody, and allowed to adhere 1 h at 37°C 5% CO_2. Adherent cells were harvested, and their total protein content extracted. Equal amounts of total proteins from each sample were subjected to immune-blotting and then probed with an antibody against α2β1 integrin. Western blots analyses (a), and quantitation of Western blot lanes by using ImageJ software (b), of one representative experiment out of three, are displayed. Data shown in panel (b) are normalized for β-actin amount in the same sample. The ratio between α2β1 and β-actin expression in HCT-116 in ST-M was set equal to 1. Statistical analysis: ANOVA and Tukey-Kramer post-test: * $p < 0.05$ *versus* ST-M; ° $p < 0.05$ *versus* H-M + blocking antibody; §§ $p < 0.01$ *versus* H-M without blocking antibody.

Figure 1

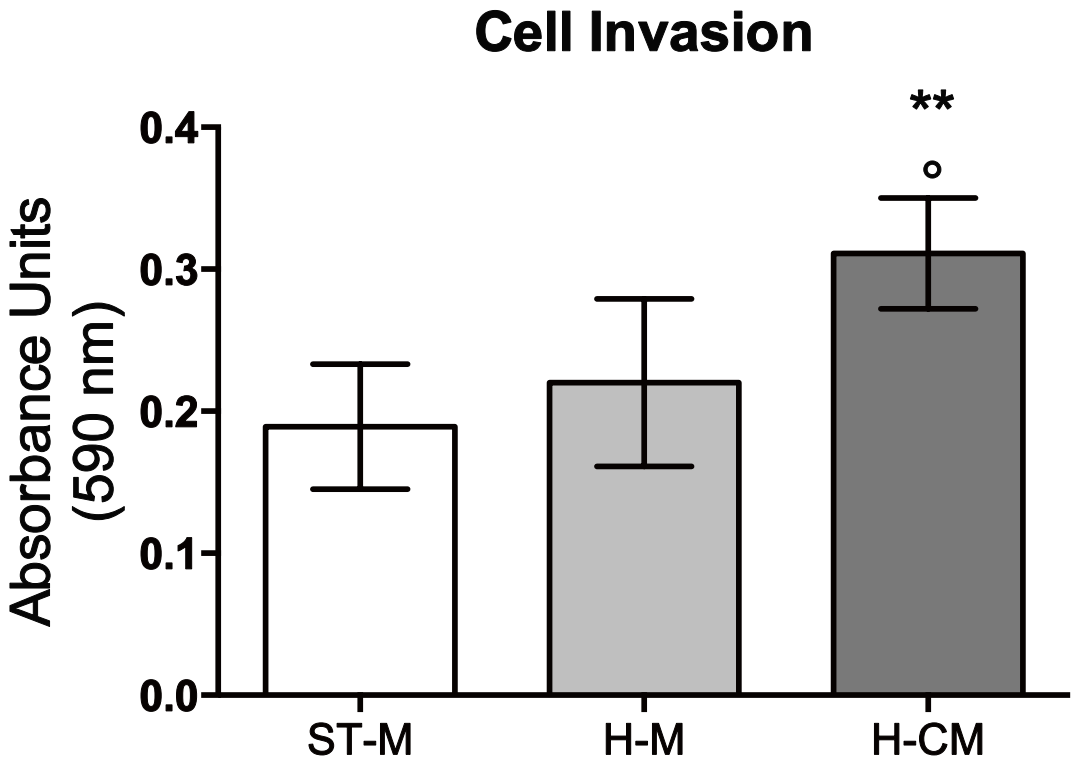

Figure 2

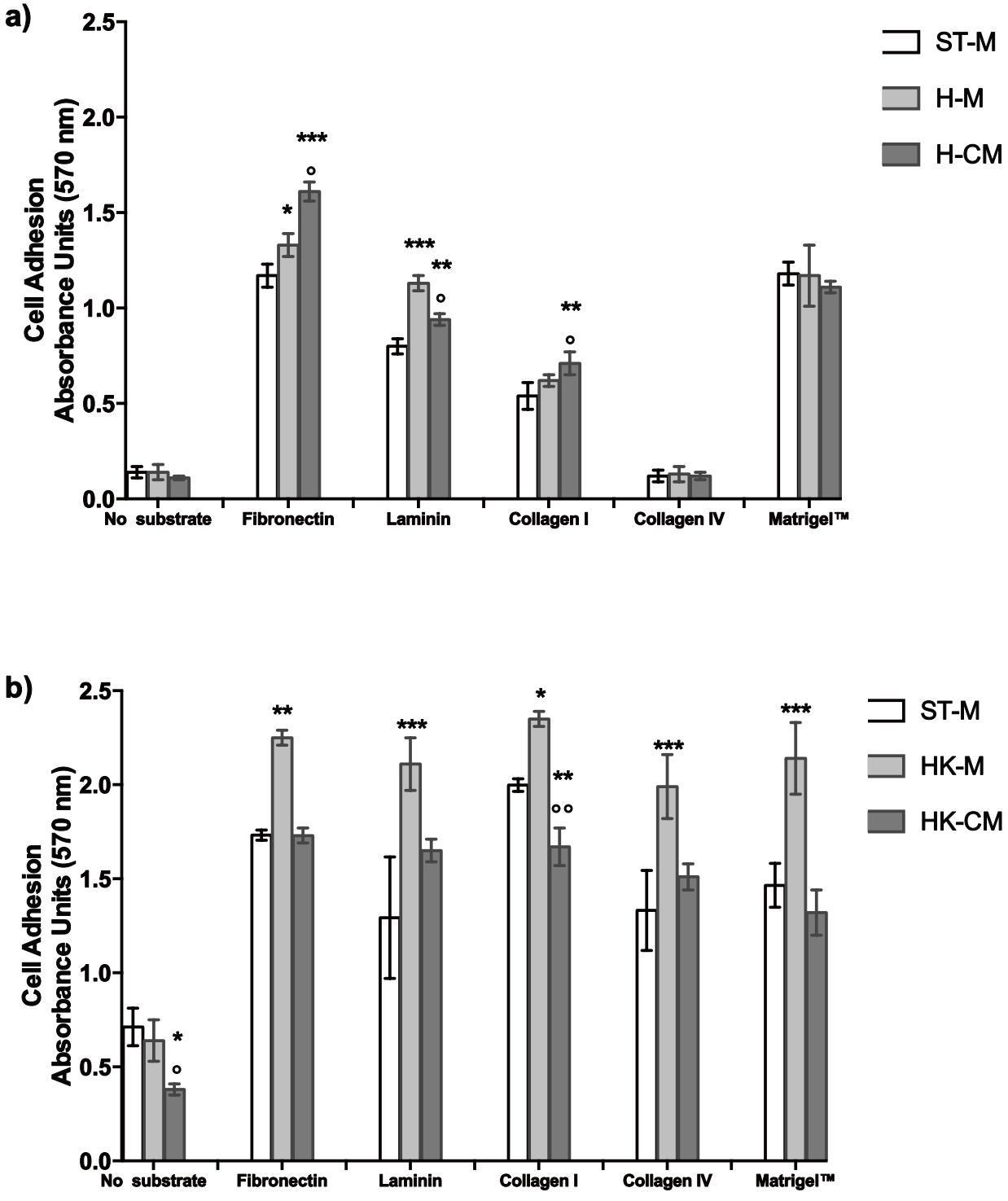

Figure 3

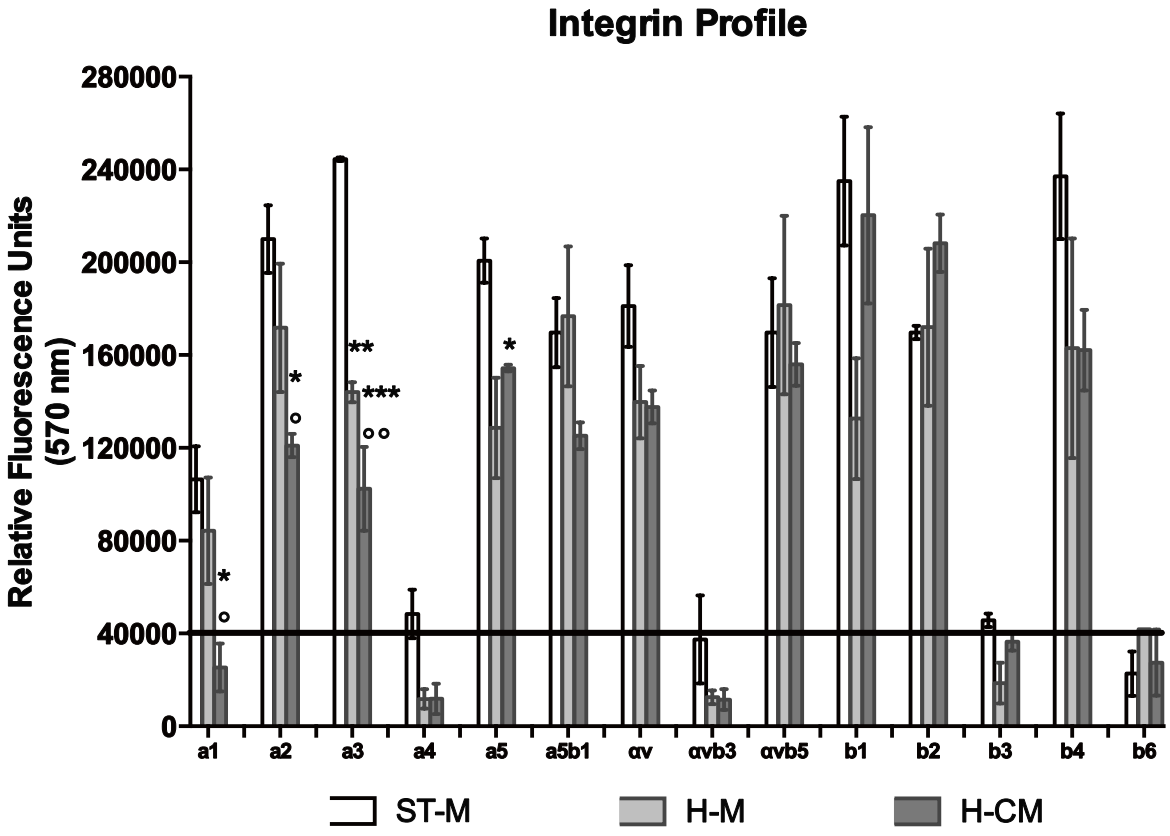

Figure 4

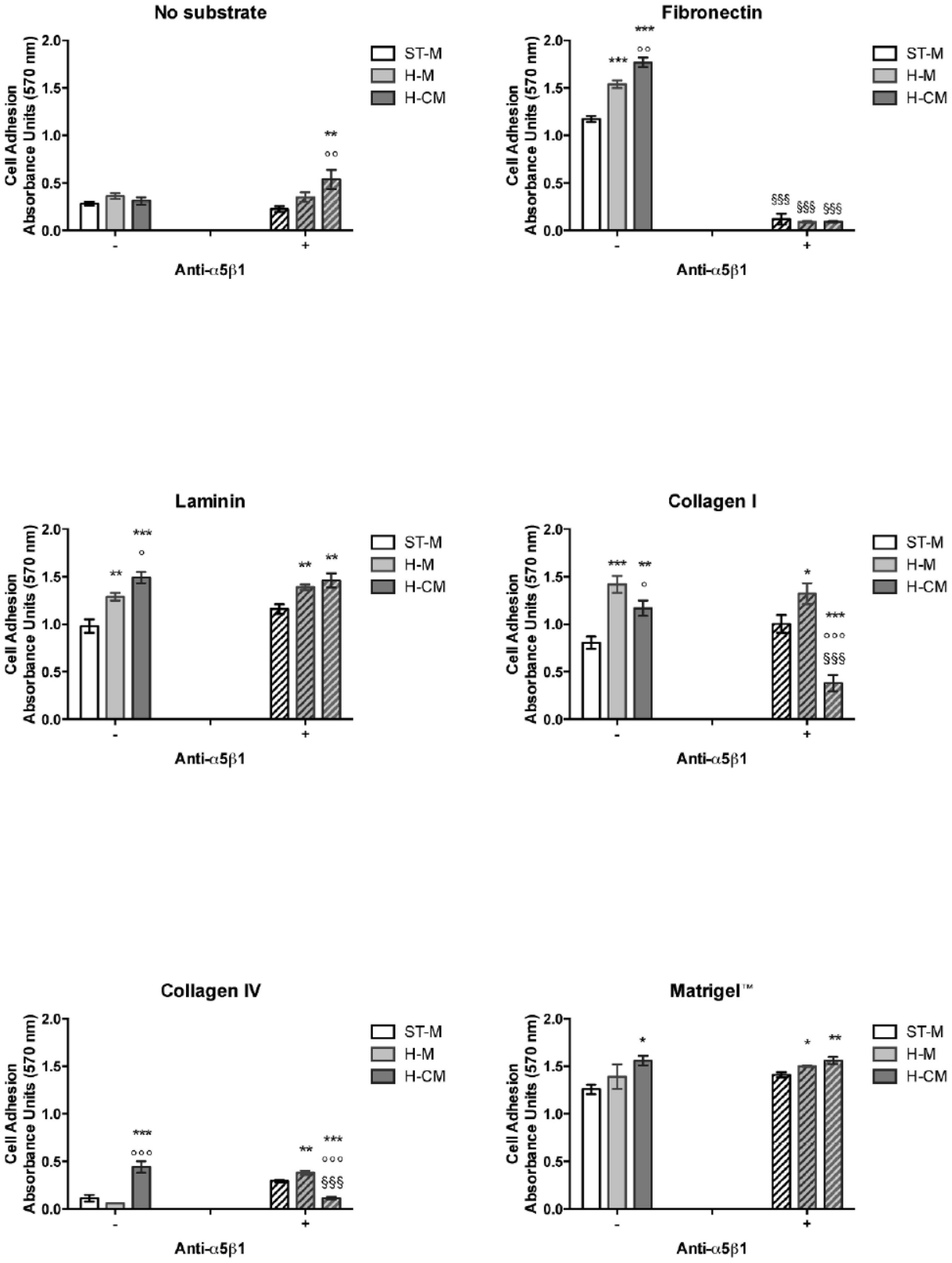

Figure 5

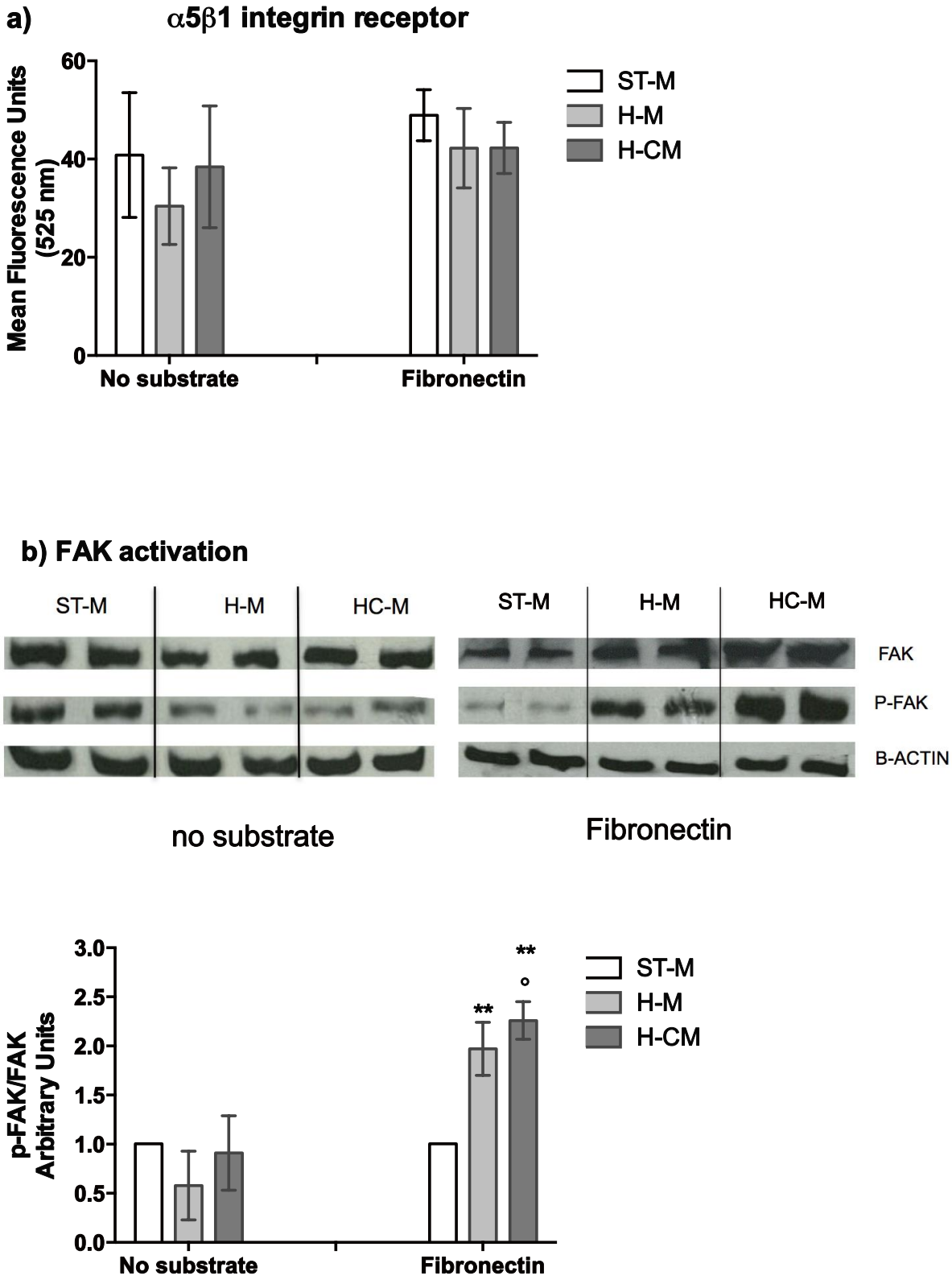

Figure 6

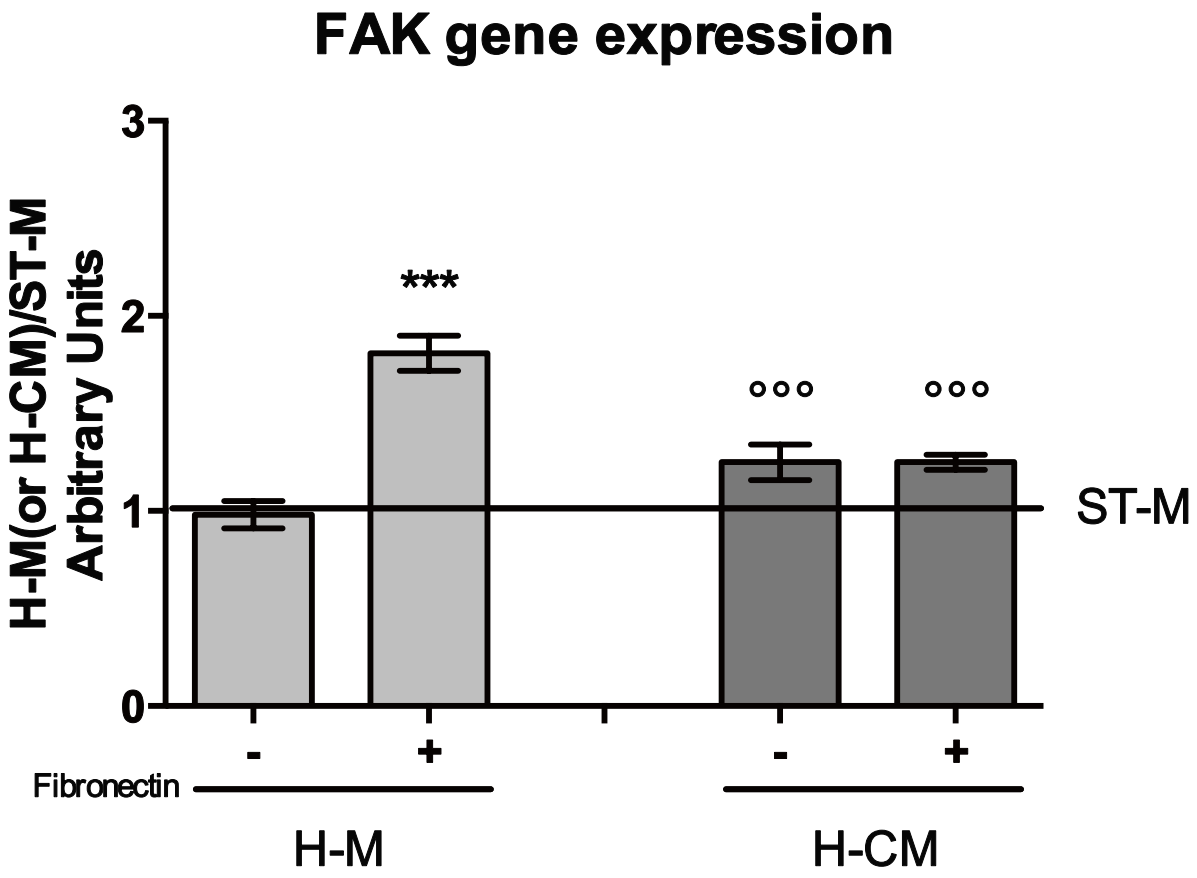

Figure 7