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Exosomes secreted from human colon cancer cells influence the adhesion of neighboring metastatic cells: Role of microRNA-210

Elisabetta Bigagli[a], Cristina Luceri[a], Daniele Guasti[b], and Lorenzo Cinci[a]

[a]Department of Neuroscience, Psychology, Drug Research and Child Health – NEUROFARBA – Section of Pharmacology and Toxicology, University of Florence, Florence, Italy; [b]Department of Experimental and Clinical Medicine – Research Unit of Histology and Embryology, University of Florence, Florence, Italy

ABSTRACT

Cancer-secreted exosomes influence tumor microenvironment and support cancer growth and metastasis. MiR-210 is frequently up-regulated in colorectal cancer tissues and correlates with metastatic disease. We investigated whether exosomes are actively released by HCT-8 colon cancer cells, the role of exosomal miR-210 in the cross-talk between primary cancer cells and neighboring metastatic cells and its contribution in regulating epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET).

After 7 d of culture, a subpopulation of viable HCT-8 cells detached the monolayer and started to grow in suspension, suggesting anoikis resistance and a metastatic potential. The expression of key proteins of EMT revealed that these cells were E-cadherin negative and vimentin positive further confirming their metastatic phenotype and the acquisition of anoikis resistance. Metastatic cells, in the presence of adherently growing HCT-8, continued to grow in suspension whereas only if seeded in cell-free wells, were able to adhere again and to form E-cadherin positive and vimentin negative new colonies, suggesting the occurrence of MET.

The chemosensitivity to 5 fluorouracil and to FOLFOX-like treatment of metastatic cells was significantly diminished compared to adherent HCT-8 cells. Of note, adherent new colonies undergoing MET, were insensitive to both chemotherapeutic strategies. Electron microscopy analysis demonstrated that adherently growing HCT-8, actually secreted exosomes and that exosomes in turn were taken up by metastatic cells. When exosomes secreted by adherently growing HCT-8 were administered to metastatic cells, MET was significantly inhibited. miR-210 was significantly upregulated in exosomes compared to its intracellular levels in adherently growing HCT-8 cells and correlated to anoikis resistance and EMT markers. Exosomes containing miR-210 might be considered as EMT promoting signals that preserve the local cancer-growth permissive milieu and also guide metastatic cells to free, new sites of dissemination.

Introduction

A continuous cross-talk between cancer cells and local/distant environments is required for effective tumor growth and systemic dissemination.[1] Intercellular communication can occur through cell-cell direct contact mediated by gap junctions as well as by extracellular vesicles.[2] Exosomes, are 30–100 nm phospholipid bilayer-enclosed vesicles surrounding a small cytosol which are increasingly recognized as key autocrine and paracrine mediators of cell-cell communication through the transport of bioactive molecules such as mRNAs, proteins and microRNAs (miRNAs).[3] Exosomes from breast cancer cells transfer miRNAs to normal cells, stimulate them to become cancerous[4] and promote cell invasion.[5] It has been suggested that the formation of the "pre-metastatic niches" depends on tumor-derived exosomes but the contribution of miRNAs has not been addressed.[6] The elegant study of Lieberman and her group also clearly demonstrated that the metastatic capability

can be transferred between metastatic and non-metastatic cancer cells via exosomes containing miR-200.[7]

Epithelial–mesenchymal transition (EMT), characterized by the loss of epithelial traits and the acquisition of a mesenchymal phenotype is involved in cancer progression and metastasis.[8] If EMT is critical to the initial transformation from benign to invasive carcinoma, its reverse process, the mesenchymal-to-epithelial transition (MET), is central for the latter stages of the metastatic process.[8] Several miRNAs have been reported to control multiple aspects of EMT and MET and to support tumor progression and metastasis.[9,10]

MiR-210 is frequently up-regulated in colorectal cancer tissues and its expression correlates with aggressive disease and metastasis.[11] A recent study demonstrated that the circulating levels of miR-210 are significantly elevated in colorectal cancer patients and correlate to the occurrence of distant metastasis.[12] Although these studies suggest that mir-210 is associated to

colon cancer metastasis they did not show whether colon cancer cells indeed secrete exosomes containing miR-210 and their relevance in the paracrine communication with nearby disseminated cancer cells.

On these basis, by using an *in vitro* model of spontaneous metastasis of colon cancer cells, we aimed at investigating whether exosomes are actively released by cancer cells to modify the local tumor environment, the role of exosomal miR-210 in the cross-talk between primary cancer cells and metastasis and its contribution in regulating EMT and MET.

Materials and methods

Cell line and metastatic cells

HCT-8 cells (gift from Dr. Coronnello, University of Florence) were maintained in DMEM (Invitrogen, Life technologies, Carlsbad, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin-streptomycin, 1% l-glutamine (200 mmol/L), 4.5 g/L glucose and grown in 5% CO2. After 48 hours from seeding, several cells able to survive in suspension, spontaneously appeared in the culture medium. These cells were interpreted as potentially metastatic and their number was assessed at different time points from seeding (48 h, 72 h, 96 h; 7 d, 10 d, 15 d). Cell viability was measured by trypan blue (Invitrogen).

Immunocytochemistry

To characterize the phenotype of cells growing in suspension, the expression of E-cadherin and vimentin was assessed by immunocytochemistry. Cells were prepared as follows: a) HCT-8 cells seeded on histological slides, b) potentially metastatic cells placed on histological slides by 500xg centrifugation for 5 minutes, c) potentially metastatic cells seeded in HCT-8 free-wells and collected as soon as they formed adherent visible colonies. Samples were fixed in 4% formaldehyde pH 7.4 for 10 min and pre-incubated in 0.5% triton and 1.5% bovine serum albumin (BSA) (Sigma Aldrich, Milan, Italy) for 15 min at room temperature. Immunostaining was performed by incubation for 24 h at 4°C with mouse monoclonal anti E-cadherin at final dilution of 1:50 (Millipore, Billerica, MA, USA) or goat-polyclonal anti-Vimentin at final dilution 1:40 (Sigma Aldrich) followed by Alexa Fluor 488 Goat anti mouse (1:333) or Alexa Fluor 568 Donkey anti Goat (1:333) (Invitrogen).

Image acquisition and analysis

Microscopic analysis was performed with a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan) connected to a CCD camera. Ten photomicrographs (∼100 cells/microscopic field) were randomly taken for each sample and fluorescence was measured using ImageJ 1.33 image analysis software (http://rsb.info.nih.gov/ij). Results were expressed in arbitrary units.

Exosomes isolation

Exosomes were obtained from HCT-8 culture media at day 7 post seeding by using the Exosome Precipitation Solution (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

Transmission electron microscopy

HCT-8 (10^6 cells) were fixed in 4% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 8 h at 4°C. Cells were then embedded in epoxy resin and routinely processed. In order to evaluate the interaction between metastatic cells and exosomes, 10^5 metastatic cells were incubated for 30 min at 37°C with 10% exosomes suspension obtained as described above. At the end of the incubation, cells were fixed and embedded for transmission electron microscopy. Samples were cut in sections about 70 nm thick and examined with a Jeol 1010 transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV.

RNA extraction from exosomes and from adherent HCT-8 cells

Total RNA from exosomes was extracted using the NucleoSpin miRNAs Plasma kit (Macherey-Nagel, Düren, Germany). Total RNA from adherent HCT-8 cells was isolated using the miR-Neasy Mini Kit (Qiagen Duesseldorf, Germany). Reverse transcription of RNA was performed using the miScript Reverse Transcription Kit according to manufacturer's instructions (Qiagen). In the case of exosomal miRNA, cDNAs were preamplified with MiScript Pre-AMP (Qiagen).

mir-210 expression in exosomes and in adherent HCT-8 cells

Quantitative real-time RT-PCR was performed in 7900HT Fast Real-Time PCR System (Applied Biosystems, California, USA) using miScript SYBR Green PCR Kit and miScript Primer Assay according to manufacturer's instructions (Qiagen). Cel-miR-39 was used for the normalization of both exosomal and intracellular miRNAs levels according to Turchinovich et al.[13] After amplification, melting curve analysis was performed to ensure the specificity of the products. The expression levels of miR-210 were calculated as $2^{-\Delta\Delta CT}$.

Colonies formation

Metastatic cells were isolated from HCT-8 culture media by 500xg centrifugation for 5 min. 4×10^3 cells were seeded in 24 well plates and maintained for 72 h in DMEM or in DMEM containing 5% or 10% of exosomes obtained as described above. Adherent new colonies were then counted.

Chemotherapy sensitivity

The chemosensitivity of the 3 cell clones was assessed. Adherent HCT-8 cells, metastatic cells and adherent new colonies were treated for 72 h with 5-fluorouracil (5FU) 10^{-6} M [14] and with a FOLFOX-like treatment (5FU 10^{-6} M + Oxaliplatin 2×10^{-7} M) (Sigma Aldrich, Milan, Italy). Cells viability was assessed by MTS assay (Promega Corporation, WI). The optical density of the chromogenic product was measured at 490 nm.

Statistical analysis

Data were expressed as means ± SEM of 3 independent experiments. Statistical analysis was performed by one-way analysis of variance, followed by the Student–Newman–Keuls multiple comparison post hoc test or by Mann-Whitney test. Calculations were done using a GraphPad Prism 4.0 (GraphPad software, San Diego, CA).

Results

A subpopulation of viable HCT-8 cells spontaneously goes in suspension

After 48h post-seeding, a number of HCT-8 cells spontaneously began to disassociate, migrated away from the cell monolayer and remained fully viable in the culture media. The number of these cells significantly increased from the seventh to the fifteenth day post-seeding (Fig. 1).

The key epithelial-mesenchymal transition (EMT) markers Vimentin and E-cadherin, are differentially expressed in adherent HCT-8 and in those in suspension

At day 7 from seeding, immunofluorescent staining for E-cadherin and Vimentin considerably differed when we compared to adherently growing HCT-8 cells with those in suspension. Suspended viable HCT-8 cells were E-cadherin negative (Fig. 2, panel C) and vimentin positive (Fig. 2, panel D) whereas the adherent originating HCT-8 cells, were E-cadherin positive (Fig. 2, Panel A) and vimentin negative (Fig. 2, panel B). Densitometric analysis further supported these results; in fact, suspended HCT-8 cells (namely metastatic cells) displayed significantly lower levels of E-cadherin and significantly higher levels of vimentin compared to the adherent originating HCT-8 cells (p < 0.01) (Fig. 2, panel G and panel H). Interestingly, these metastatic cells, only if seeded in cell-free wells, formed new, adherent, E-cadherin positive and vimentin negative colonies (Fig. 2, panel E and panel F), suggesting the occurrence of MET. Densitometric analysis confirmed that the adherent new colonies expressed significantly lower levels of vimentin and higher E-cadherin levels compared to metastatic cells (p < 0.01) (Fig. 2, panel G and panel H).
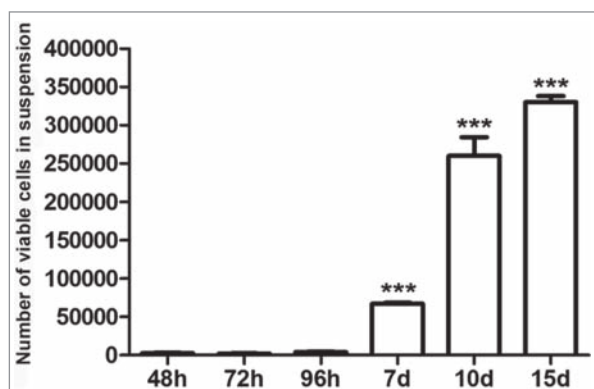

Figure 1. At different time points post seeding, a subpopulation of viable HCT-8 cells spontaneously goes in suspension. Data are expressed as means ± SEM of 3 independent experiments. ***p < 0.001 vs 48h.

HCT-8 secrete exosomes which in turn interact with neighboring metastatic cells

In the cytoplasm of HCT-8 cells, transmission electron microscopy (TEM) highlighted several intracellular multi-vesicle bodies (MVBs) carrying bi-lipidic membrane-bound exosome-like vesicles (Fig. 3, panel A). Metastatic cells did not show any MVBs in their cytoplasm but several round bodies compatible with exosome-like vesicles, were identified closely to their membrane. In some cases these round bodies appeared fused with the metastatic cells membrane (Fig. 3, panel B and C).

The adhesion dynamic of metastatic cells is influenced by miR-210

To test directly if exosomes are indeed able to influence the adhesion of metastatic cells, 5 or 10 % of exosomes from HCT-8 was administered to metastatic cells. In the presence of exosomes, the number of adherent, new colonies expressing E-cadherin, was significantly decreased compared to control culture conditions, further supporting the role of exosomes also in the MET process (Fig. 3, panel D). Interestingly, we found that miR-210 was significantly upregulated in exosomes compared to adherently growing HCT-8 from which they arise (Fig. 3, panel E) and that these exosomes in turn were taken up by nearby disseminated cells (Fig. 3, panel B and C).

The three cell clones show different sensitivity to standard chemotherapy

As expected, adherent HCT-8 cells were sensitive to standard chemotherapy with a percentage of cell death of 30 % upon 5FU and of 50 % upon FOLFOX- like treatment. This sensitivity was significantly diminished in metastatic cells compared to adherent HCT-8 cells. Of note, adherent new colonies undergoing MET, were completely insensitive to both 5FU and FOLFOX- like treatment (Fig. 4).

Discussion

The metastatic process remains one of the most unknown aspects of tumor biology and accounts for 90% of cancer mortality. The identification of the key molecular mechanisms regulating metastasis is therefore of paramount importance for the discovery of novel therapeutic approaches.[15]

Our results demonstrate that a sub-population of HCT-8 cells spontaneously disassociated, migrated away from the cell monolayer and remained viable in suspension. It might be argued that this phenomenon could be due to a physiological transition between adherent and suspended cells: if so, the number of cells in suspension would likely remains constant over time. Our findings instead demonstrated that this is not the case since the number of fully viable cells in suspension increased over time in culture medium, suggesting that HCT-8 actually and progressively underwent a phenotypic transition from an adhesive epithelial type to a subpopulation of cells with a metastatic-like phenotype. Interestingly, a similar behavior was reported by Tang et al. (2014) who discovered that HCT-8 cells can exhibit a dissociative, metastasis-like
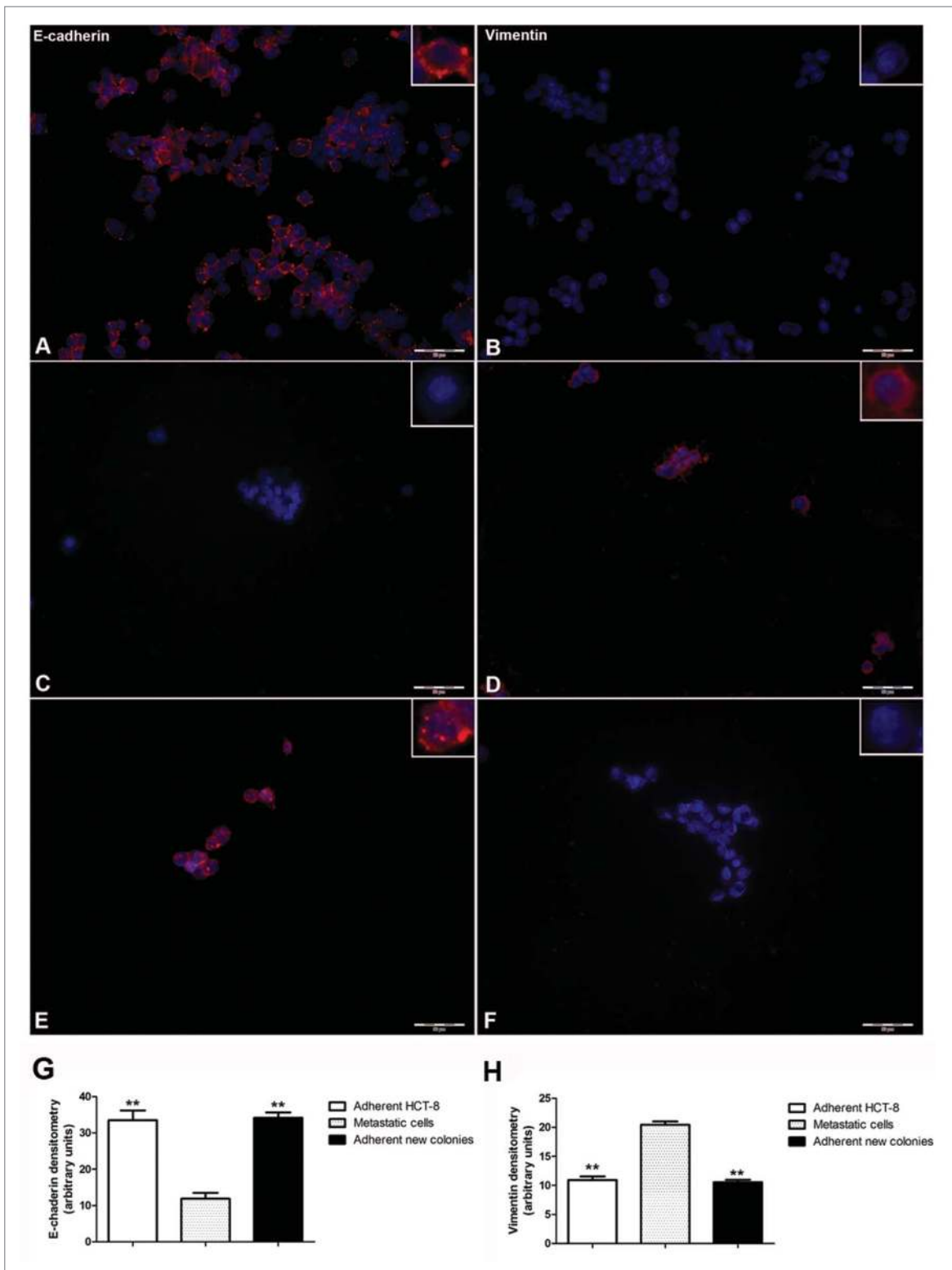
Figure 2. Immunocytochemical evaluation of the expression of E-cadherin (panel A) and vimentin (panel B) in adherently growing HCT-8. Expression of E-cadherin (panel C) and vimentin (panel D) in metastatic cells growing in suspension. Expression of E-cadherin (panel E) and vimentin (panel F) in adherent new colonies obtained by seeding metastatic cells in cell-free wells (20x magnification; Barr 100 μm objective). Panel G: Densitometric analysis of E-cadherin in adherently growing HCT-8, in metastatic cells growing in suspension and in adherent new colonies obtained by seeding metastatic cells in cell-free wells. Panel H: Densitometric analysis of vimentin in adherently growing HCT-8, in metastatic cells growing in suspension and in adherent new colonies obtained by seeding metastatic cells in cell-free wells. **p < 0.01 vs metastatic cells growing in suspension.

phenotype *in vitro*.[16] Moreover, as barrier to metastases, cells normally undergo an apoptotic cell death called "anoikis" after they lose contact with their neighboring cells.[17] In our model, a subpopulation of HCT-8 cells likely acquired resistance to anoikis and were thus able to survive after detachment from the "primary colonies."

Increased invasive and metastatic potential has been associated to the epithelial-mesenchymal transition (EMT). EMT is
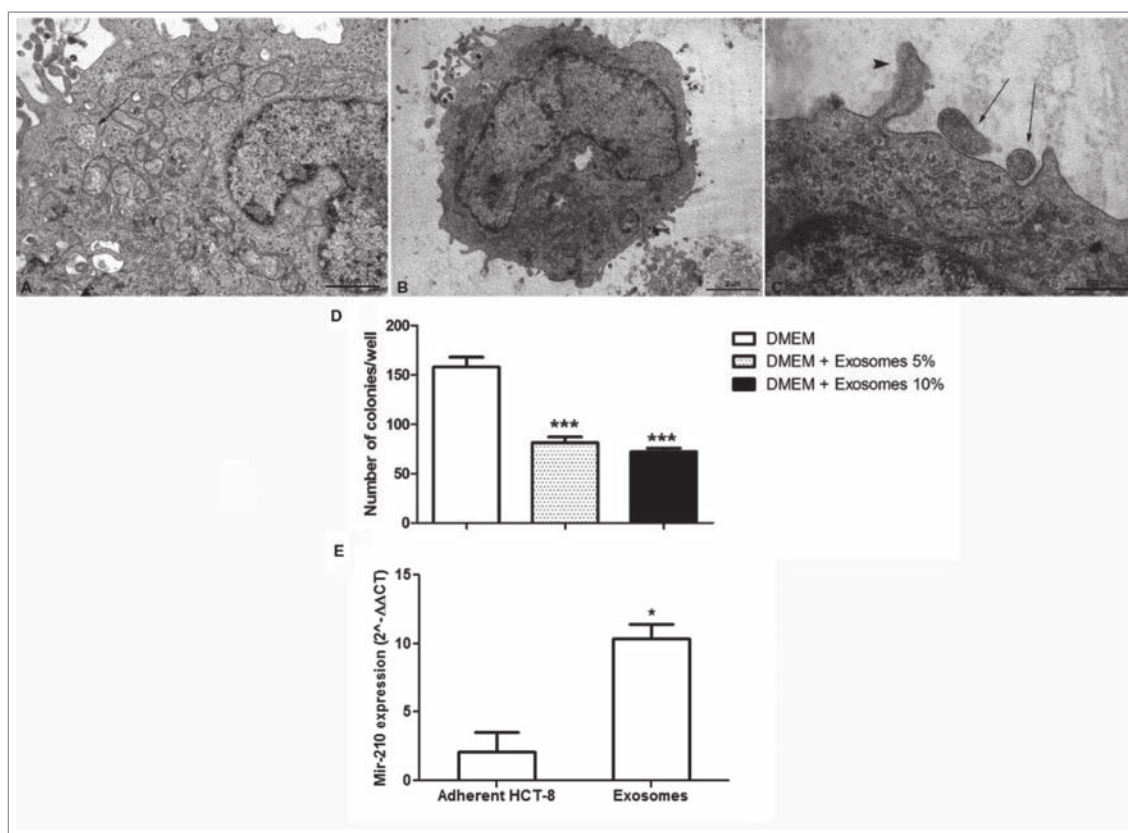
Figure 3. Morphological analysis of exosomes. (Panel A) Transmission electron microscopy of HCT-8 cells showing micro vesicles enclosing bilipidic layer-bound exosomes (black arrow) (20,000X, scale bar 1 μm). (Panel B) Metastatic cell cultured with 10% exosome suspension (10,000X, scale bar 2 μm). (Panel C) Higher magnification of panel B. Two microvescicles are located closed to cell membrane (black arrows), one appears fused with cell membrane (arrow head); (50,000X, scale bar 500 nm). (Panel D) Number of adherent new colonies formed by metastatic cells in the presence and absence of exosomes ***$p < 0.001$ vs DMEM. (Panel E) miR-210 expression in adherently growing HCT-8 and in exosomes, analyzed using real-time RT-PCR. *$p < 0.05$.

not only a key event for epithelial cells to acquire a motile and invasive phenotype, but also an essential process for anoikis resistance.[8,18] Among the hallmarks of EMT *in vitro* and *in vivo*, the up-regulation of mesenchymal markers (N-cadherin and vimentin) and the down-regulation of epithelial markers (E-cadherin and cytokeratins) were frequently reported.[19] In our results, the conversion from an epithelial into a mesenchymal phenotype, suggests that HCT-8 cells spontaneously undergo through an EMT process and acquire a metastatic potential. The loss of E-cadherin expression in fact, allows tumor cells to dissociate from the primary tumor, to overcome anoikis and disseminate.[20] Successful metastatic colonization seems to require also the reverse process known as

mesenchymal-epithelial transition (MET). Infact, to invade other sites, cancer cells, that have undergone EMT, must re-acquire epithelial characteristics to anchor in the surrounding tissue.[21] Interestingly, our metastatic cells, only if seeded in cell-free wells, formed new, adherent, E-cadherin positive and vimentin negative colonies suggesting that the occurrence of MET might be regulated by a paracrine signal sent by adherent HCT-8 cells to the metastatic ones.

In recent years, exosomes have received increasing attention for their role in regulating and transferring molecules responsible for tumor progression and metastasis.[22] We therefore investigated if exosomes might represent a mechanism trough which adherent HCT-8 cells communicate with neighboring disseminated cancer cells and modify their adhesion. The ability of HCT-8 cells to actually secrete exosomes was confirmed by transmission electron microscopy: cytoplasmic structures identical to those described in exosomes secreting cells were infact identified.[22] Electron microscopy also demonstrated the presence of extracellular microvesicles nearby metastatic cells incubated with exosomes secrete by adherent HCT-8 cells. This finding may be indicative of both exosome release and uptake but the lack of intracellular multi-vesicle bodies enclosing exosomes in metastatic cells, strongly suggests the interaction with exogenous exosomes secreted from adherently growing HCT-8. This is also supported by the discovery that this interaction has a functional consequence on metastatic cells: infact, when exosomes isolated from primary HCT-8 culture media were
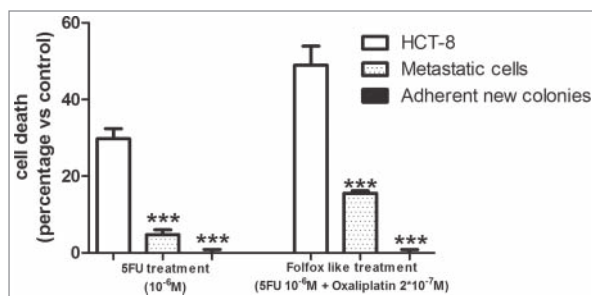

Figure 4. Chemotherapy sensitivity in the 3 different cell clones. Data are expressed as means $\pm$ SEM of 3 independent experiments. ***$p < 0.001$ vs adherent HCT-8 cells.
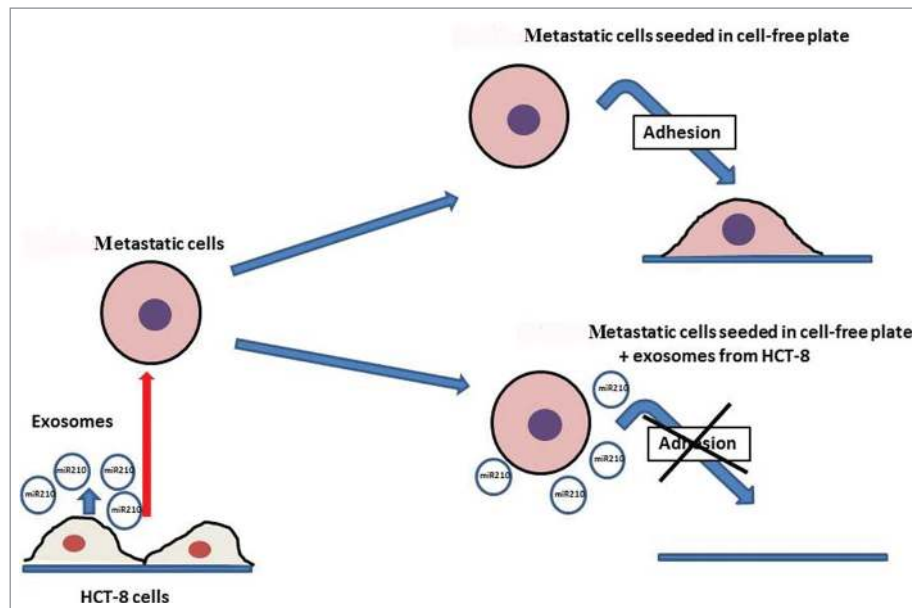
Figure 5. Schematic diagram depicting the major findings of this work.

administered to metastatic cells, the number of colonies formed by metastatic cells in cell-free wells, was significantly reduced. In the last few years, a number of studies have demonstrated that exosomes contribute to EMT, invasion and metastasis.[20] The novelty of our findings is the documentation that exosomes modify the adhesion dynamic of neighboring metastatic cells and are involved in the MET process. Moreover, E-cadherin re-expression is usually induced by factors secreted by a secondary organ microenvironment.[21] Here we demonstrated that the adhesion of metastatic cells and the re-expression of E-cadherin is also driven in a cell-free well where they were no longer in the presence of the originating HCT-8 cells. This finding suggests that in its own local microenvironment, the primary tumor itself, by secreting exosomes, hinders E-cadherin re-expression in nearby metastatic cells possibly to preserve its local permissive milieu.

To investigate the mechanism involved, we measured the expression of mir-210, known to be raised in colorectal cancer patients and to correlate to the occurrence of distant metastasis.[12] miR-210 was significantly up-regulated in exosomes compared to its intracellular levels in adherently growing HCT-8 cells and correlated to anoikis resistance and EMT markers. Mudduluru and coworkers, recently performed a systematic analysis of the metastasis-associated miRNome.[22] These authors demonstrated that colorectal cancer cell lines transfected with miR-210 displayed a significant decrease in E-cadherin expression and a parallel increase of N-cadherin expression, an increased migration and invasive capacity and an enhanced ability to metastasize *in vivo*. The expression of miR-210 was found to correlate with EMT markers also in human gastric and ovarian cancer cells.[23-24]

To better understand the characteristics of metastatic cells and of the new adherent colonies undergoing MET, a chemotherapy sensitivity assay was performed. The three different cell populations were exposed to 5FU or to a combination of 5FU plus oxaliplatin (5:1) to mimic FOLFOX treatment, commonly used strategies to treat colorectal cancer.[25] We demonstrated

that metastatic cells undergoing EMT were much more resistant to standard chemotherapy compared to the adherent cells. Studies using colorectal cell lines have shown that EMT inducers also increased resistance to chemotherapy.[26] Conversely, oxaliplatin-resistant colorectal cell lines showed phenotypic and gene expression changes consistent with EMT.[27] Recently, lung adenocarcinoma cell lines exhibiting TGF-β/FGF-2-induced EMT, were also chemoresistant to gefitinib and chemically induced EMT reversion only partly restored chemosensitivity.[28] Studies investigating the potential survival advantage of cancer cells undergoing MET toward conventional chemotherapy are scarce and it is unclear whether EMT-driven therapy resistance is retained after cells undergo MET. We are among the first to demonstrate that cells undergoing MET are insensitive to conventional chemotherapy highlighting the importance to characterize this phenomenon to comprehend the EMT/MET axis in cancer and resistance to therapy. Infact, whereas the inhibition of EMT may be effective in preventing the invasive dissemination of organ-confined cancers, in the latter stages, it could also accelerate MET-dependent colonization of new sites of dissemination and increase chemoresistance.

Cancer cells manipulate their microenvironment to optimize conditions for growth and metastasis in multiple ways. Our hypothesis is that in our model, through the delivery of mir-210 to nearby metastatic cells, primary tumor gains a double evolutionary advantage: on one hand, it preserves its local cancer-growth permissive milieu in terms of space and nutrients and on the other hand, it also redirects metastatic homing to distant sites. Infact we demonstrated that disseminated cancer cells which leave the "primary site" revert to the epithelial state in the absence of exosomes containing mir-210. Thus, in the absence of the EMT-inducing signals received from the "primary site," metastatic cancer cells may fall back into an epithelial state when entering into new, free sites of dissemination. This evolutionary vision of cancer agrees with the "Seed and soil" theory formulated by Paget[29] in which metastatic cells are

"seeds" and a particular microenvironment or niche, serves as the "soil." In this vision, exosomes represent an instrument of communication which allows the primary tumor not only to create favorable conditions of "soil" in remote niches but also to prevent metastatic cells adhesion in its own "soil" avoiding unfavorable local conditions. In the last few years, this ecological vision of cancer has been suggested to be useful to understand the biology of metastasis and stimulate the discovery of new therapeutic strategies.[30]

As summarized in Fig. 5, we have demonstrated that exosomes containing miR-210 mediate the cross-talk between primary cancer cells and nearby disseminated cells, and redirect metastatic cells homing to free sites of dissemination, leading to the discovery of a new molecular mechanisms involved in the primary cancer niche preservation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Author's contribution

E.B. and C.L performed the experiments. E.B. analyzed the data, performed mir-210 expression and wrote the manuscript. D.G. was responsible of embedding and cutting for electron microscopy. L.C. performed electron microscopy analysis, planned and conceived the experiments and coordinated the research group.

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