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# UNIVERSITÀ DEGLI STUDI DI TORINO

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**Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung pre-metastatic niche.**

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**Running title:** Renal Cancer Stem Cell Microvesicles

**Key Words:** tumor micro vesicles; exosomes; tumor initiating cells; metastases.

## **Abstract**

Recent studies suggest that tumor-derived microvesicles (MVs) act as a vehicle for exchange of genetic information among tumor and stromal cells, engendering a favorable microenvironment for cancer development. Within the tumor mass, all cell types may contribute to MV shedding, but specific contributions to tumor progression have yet to be established. Here we report that a subset of tumor initiating cells expressing the mesenchymal stem cell marker CD105 in human renal cell carcinoma release MVs that trigger angiogenesis and promote the formation of a pre-metastatic niche. MVs derived only from CD105-positive cancer stem cells conferred an activated angiogenic phenotype to normal human endothelial cells, stimulating their growth and vessel formation after *in vivo* implantation in immunocompromised SCID mice. Further, treating SCID mice with MVs shed from CD105-positive cells greatly enhanced lung metastases induced by i.v. injection of renal carcinoma cells. Molecular characterization of CD105-positive MVs defines a set of pro-angiogenic mRNAs and microRNAs implicated in tumor progression and metastases. Our results define a specific source of cancer stem cell-derived MVs that contribute to triggering the angiogenic switch and coordinating metastatic diffusion during tumor progression.

## Introduction

Recent studies demonstrated that exosomes/microvesicles (MVs) released by cells act as mediator of intercellular communications (1-3). Tumor cells produce large amount of MVs that may enter in the circulation and in other biological fluids (4,5). It has been suggested that MVs, due to their pleiotropic effect, could be involved in cancer development, progression and formation of the pre metastatic niche (6). MVs contain mRNAs, microRNAs (miRNAs) and proteins that could be transferred to target cells inducing epigenetic changes (7-10). Moreover, tumor derived MVs may transport to neighbouring cells the products of oncogenes (11). Emerging evidence suggests that, in cancer patients, circulating miRNAs are stable in blood, probably due to their incorporation in exosomes-microvesicles allowing their use as novel diagnostic markers (12).

It is generally recognized that tumors contain a heterogeneous population of cells with different proliferation and differentiation potential. The majority of cells that form tumors are fated to differentiate and ultimately to stop dividing. At variance, a minor population of cells, defined as cancer stem cells or tumor-initiating cells, possess self-renewal capability and can induce tumors in immune-compromised animals (13). Recently, we identified in human renal cell carcinoma a subset of tumor initiating cells expressing the mesenchymal stem cell marker CD105 that display stem cell properties, such as clonogenic ability, expression of Nestin, Nanog, Oct3-4 stem cell markers and lack of differentiative epithelial markers (14). This CD105<sup>+</sup> population has the capacity to generate epithelial and endothelial cells and serially transplantable tumors *in vivo* (14).

Previous studies demonstrated that normal stem cells are an abundant source of MVs which may act as paracrine mediators by a horizontal transfer of genetic information (7, 8, 15).

The aim of the present study was to evaluate whether MVs released by CD105<sup>+</sup> cancer stem cells of renal carcinomas may modify tumor microenvironment by triggering angiogenesis and may favour the formation of a pre metastatic niche.

## **Material and methods**

### **Cell culture**

Human umbilical vein endothelial cells (HUVEC) were obtained and characterized as previously described (8). CD105<sup>+</sup> cancer stem cells, three deriving CD105<sup>+</sup> clones, CD105<sup>-</sup> tumor cells and unsorted tumor cells were previously isolated and characterized (14). Briefly, cell suspension obtained from five specimens of renal carcinomas of patients undergoing radical nephrectomy with informed consent were either used to generate unsorted tumor cells or sorted by anti-CD105 magnetic beads (MACS system, Miltenyi Biotec) (14). To avoid the presence of non neoplastic contaminating cells, CD105<sup>+</sup> cancer stem cells were grown in expansion medium without serum (14) or were cloned. Three clones originating from 3 different renal cell carcinomas were used. The CD105<sup>-</sup> population was unable to generate clones. The CD105<sup>+</sup> clones as well as the total CD105<sup>+</sup> cell population were negative for the endothelial or haematopoietic markers CD31, VEGF receptor 2 and CD45. In addition, they showed cancer stem cells properties as expression of stem cell markers and lack of differentiative markers, ability to growth in spheres and the ability to initiate tumors and generate serially transplantable tumors with a number of cells as low as 100 cells/mice (Table S1). All cell types were thawed, used within 2 months and the phenotype characterized by FACS analysis and immunofluorescence immediately before the generation of MVs. The previously described (16) K1 renal tumor line was thawed and characterized by FACS immediately before their use for metastases generation.

### **Isolation and characterization of MVs**

MVs were obtained from cell supernatants by ultracentrifugation as previously described (8). The protein content of MV preparations was quantified by Bradford method (Bio-Rad). In selected experiments, MVs were labelled with the red PKH26 dye (Sigma). The mean diameter of MVs and

zeta potential were determined using a Malvern dynamic light-scattering spectrophotometer (Malvern Zetasizer 3000HS) and by transmission electron microscopy (17). Cytofluorimetric analysis was performed as described (17) using the following FITC- or PE- conjugated antibodies: CD44 (Dakocytomation), CD73 and CD29 (BD Biosciences), CD105,  $\alpha$ 5-integrin,  $\alpha$ 6-integrin and HLA class I (BioLegend). FITC or PE mouse isotypic IgG (Dakocytomation) were used as control. Beads of different sizes (1, 2 and 4  $\mu$ m, Invitrogen) were used as size markers. In selected experiments, CD105<sup>+</sup> MVs derived from cloned CD105<sup>+</sup> cancer stem cells were treated with 1U/ml RNase (Ambion) for 3 h at 37 °C (RNase CD105<sup>+</sup> MV) (9, 10). After RNase treatment the reaction was stopped by addition of 10 U/ml RNase inhibitor (Ambion) and MVs were washed by ultracentrifugation. The efficacy of RNase treatment was evaluated by MV-RNA analyses by Agilent 2100 bioanalyzer (Agilent Technologies) and by 0.6% Agarose gel electrophoresis.

### **mRNA analysis**

RNA from MVs was isolated using the RNAqueous®-Micro kit (Ambion). RNA was quantified spectrophotometrically (Nanodrop ND-1000) and the RNA quality was assessed by Agilent 2100 Bioanalyzer. mRNA expression levels were analysed using the RT<sup>2</sup> Profiler™ PCR array system (SABiosciences-Qiagen) to profile 84 genes involved in angiogenesis by real time-PCR. A pool of RNA from 4 MV preparations (400 ng CD105<sup>+</sup> or CD105<sup>-</sup> MVs) was retrotranscribed and run on 7900HT RT-PCR instrument (Applied Biosystems). Raw Ct values were calculated using SDS software version 2.3 using automatic baseline and threshold. Quantitative RT-PCR (qRT-PCR) validation of gene array data was performed using SYBR green technique (Supplementary).

### **miRNA analysis**

RNA was isolated using *mirVana* miRNA Isolation Kit (Ambion). TaqMan MicroRNA Assay Human Panel Early Access kit (Applied Biosystems) was employed to profile 365 mature miRNAs

by qRT-PCR. Sixty ng of RNA from CD105<sup>+</sup> or CD105<sup>-</sup> MVs were analysed. Raw Ct values were calculated using the SDS software. miRNAs with raw Ct values greater than 35 in both preparations were not included in the analysis, as considered non-specific (18, 19). Using filtering criteria, 82 and 87 miRNAs present in CD105<sup>+</sup> and CD105<sup>-</sup> MVs, respectively, were included in the analysis. As the snoRNAs (internal controls) were undetectable in MV preparations, endogenous control was calculated using the mean value of 4 of the most stable miRNAs between CD105<sup>+</sup> MVs and CD105<sup>-</sup> MVs (hsa-miR 181b, 27a, 484, 324-3p) (20, 21). Relative quantification (RQ) was obtained using the equation  $2^{-\Delta\Delta CT}$  ( $\Delta\Delta CT$  is the difference between  $\Delta CT$  CD105<sup>+</sup> MVs and  $\Delta CT$  CD105<sup>-</sup> MVs;  $\Delta CT = \text{mean Ct miRNA} - \text{mean Ct of endogenous control}$ ). In order to confirm some miRNAs identified by microarray analysis, qRT-PCR, using SYBR green technique was performed (Supplementary).

### **Gene targets analysis**

The software TargetScan (<http://www.targetscan.org>) was employed to predict genes target for upregulated miRNAs in CD105<sup>+</sup> MVs. To define a core list, genes that were target of at least by 5 miRNAs were selected. This group of genes was searched for GO (Gene Ontology) term enrichment, using the Gene Ontology annotations (<http://www.geneontology.org>). We used Fisher's exact test to evaluate GO keywords overrepresentation. A p-value  $<10^{-4}$  was considered as statistically significant for GO terms overrepresentation.

### **Internalization of MVs**

HUVEC labelled with CFSE (Vybrant CFDA SE Cell Tracer Kit, Molecular Probe) were incubated for 1 hour at 37°C with PKH26 labelled CD105<sup>+</sup> and CD105<sup>-</sup> MVs and, after washing were analysed by confocal microscopy (LSM 5 Pascal, Carl Zeiss Int.) (17). Hoechst 33258 dye (Sigma) was added for nuclear staining.



### ***In vitro* angiogenesis assay**

*In vitro* formation of capillary-like structures was performed on growth factor reduced Matrigel (BD Biosciences) (8). HUVEC ( $3 \times 10^4$  cells/well) were seeded onto Matrigel-coated wells in RPMI+5% FCS with or without 30  $\mu\text{g/ml}$  MVs. Cell organization onto Matrigel was microscopically recorded after 16 hours. Data were expressed as the mean  $\pm$ SD of tubule length in arbitrary units per field.

### **Invasion, apoptosis and adhesion assays**

The effect of CD105<sup>+</sup> MVs, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and unsorted tumor MVs on Matrigel invasion and apoptosis resistance of HUVEC and on adhesion of K1 tumor cells to HUVEC were evaluated. Invasion was evaluated in 24 wells cell culture inserts (BD Biosciences) with a porous membrane (8.0  $\mu\text{m}$  pore size) precoated with 100  $\mu\text{g/well}$  Matrigel as described (21). Total area of invaded Matrigel (magnification  $\times 100$ ) was evaluated by MicroImage analysis system (Cast Imaging srl). Apoptosis was performed using TUNEL assay (ApopTag® Fluorescein Direct In Situ Apoptosis, Millipore). Adhesion assay was performed on HUVEC monolayer pre-treated for 24 hours at 37°C in RPMI+5% FCS with or without MVs. Renal K1 tumor cells ( $5 \times 10^5$ /well), labelled with CFSE, were added to the endothelial monolayer. The adhesion assay in static conditions was evaluated after 6 hours. After washings, cells adherent to HUVEC were counted by fluorescence microscopy (magnification  $\times 200$ ) in 10 fields and expressed as mean  $\pm$ SD of cells per field.

### ***In vivo* angiogenesis**

Animal experiments were performed according to the guidelines for the care and use of research animals and were approved by the local Ethic Committee. HUVEC, pre-stimulated with or without 70  $\mu\text{g}$  MVs, were implanted subcutaneously into SCID mice (Charles River) within Matrigel (22).

At day 10 mice were sacrificed and the Matrigel plug recovered. Angiogenesis was calculated as the mean  $\pm$ SD of the number of vessels with red cells inside per total area of haematoxylin and eosin stained sections. Immunohistochemistry was performed using anti HLA class I (Santa Cruz Biotechnology) and anti von Willebrand factor (vWF) (Dakocytomation) antibody.

### ***In vivo* metastasis**

SCID mice were injected intravenously daily for 5 days with 70  $\mu$ g of MVs in 100  $\mu$ l PBS. On day 5, mice received an intravenous injection into the tail vein of  $6 \times 10^5$  renal K1 tumor cells. Mice were sacrificed after 5 weeks and organs (lung, spleen, liver, kidney) were collected for histology. Lung metastasis were counted in 5 non sequential serial sections; results were expressed as mean  $\pm$ SD of metastasis per lung (23). On day 5, 8 mice treated with CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and PBS (vehicle) were sacrificed and lung processed for histology, RNA extraction and murine endothelial cells sorting using magnetic beads anti-CD146 (MACS system) (Fig. S1 and Supplementary material). Immunohistochemistry was performed using anti MMP2, MMP9 metalloproteinase (Santa Cruz Biotechnology) antibodies. Cytofluorimetric analysis on lung endothelial cells was performed using anti-CD31, anti-CD146 (BD Biosciences), anti-CD45 (Miltenyi), anti-VEGF Receptor 1 (R&D) and anti- $\alpha$ 6-integrin (Biolegend) mAbs. qRT-PCR for murine MMP9, MMP2, and VEGF was performed using SYBR green technique on total lung tissues and endothelial cell fractions (Supplementary material).

### **Statistical methods**

Differences were determined by Student t test or by ANOVA followed by the Newmann-Keuls' multicomparison test when appropriate. A p value of  $<0.05$  was considered significant.

## Results

### Characterization of MVs shed by CD105<sup>+</sup> renal cancer stem cells

MVs released from CD105<sup>+</sup> cancer stem cells (n=5) and deriving clones (n=3) were compared with MVs released from CD105<sup>-</sup> tumor cells (n=5). MVs generated by CD105<sup>+</sup> cancer stem cells and derived cloned and by the CD105<sup>-</sup> tumor cells had the same morphology and size, ranging from 10 to 100 nm as determined by Zeta-size and electron microscopy (Fig. 1A, B). Moreover, they showed the same zeta potential of  $-22.4 \pm 3.5$  mV. By cytofluorimetric analysis, MVs were detected below the forward scatter signal corresponding to 1 $\mu$ m beads. The main difference between MVs derived from CD105<sup>+</sup> cancer stem cells and CD105<sup>-</sup> tumor cells was the expression of CD105 only present on MVs derived from CD105<sup>+</sup> cells (CD105<sup>+</sup> MVs), but not on those derived from CD105<sup>-</sup> cells (CD105<sup>-</sup> MVs). Both CD105<sup>+</sup> and CD105<sup>-</sup> MVs expressed CD44 and adhesion molecules such as  $\alpha 5$  and  $\alpha 6$  integrins (Fig. 1C, D) as the cells of origins, whereas CD29 was barely detectable in CD105<sup>+</sup> MVs and negative in CD105<sup>-</sup> MVs (not shown). Both MV types did not expressed HLA class I (Fig. 1) and CD73 (not shown).

### Characterization of RNAs shuttled by MVs

We performed a bioanalyzer profile of total RNA present in CD105<sup>+</sup> MVs from cloned cancer stem cell preparations and CD105<sup>-</sup> MVs. Both MVs contained RNA of different size suggesting the presence of mRNAs and of small RNAs compatible with the presence of miRNAs, whereas the ribosomal subunit 28S and 18S were barely detectable (Fig. 2A). In the CD105<sup>+</sup> MVs, we observed an enrichment of small RNAs of the size of miRNAs ( $42.3 \pm 2.5\%$ ) in comparison with CD105<sup>-</sup> MVs ( $20.2 \pm 1.7\%$ ) (Fig. 2B). miRNA expression by MVs shed from CD105<sup>+</sup> and CD105<sup>-</sup> cells was then screened by qRT-PCR profiling 365 human mature miRNAs. CD105<sup>+</sup> and CD105<sup>-</sup> MVs revealed the presence of 82 and 87 miRNAs, respectively. Twenty four were significantly up regulated in CD105<sup>+</sup> MVs in respect to CD105<sup>-</sup> MVs whereas 33 miRNAs were significantly down regulated

(Table 1). In order to confirm data obtained from miRNA screening, single miRNAs were selected and analyzed in 3 different preparations of CD105<sup>+</sup> and CD105<sup>-</sup> MVs by qRT-PCR (Supplementary Table S2). To characterize the biological processes modulated by the up regulated miRNAs present in CD105<sup>+</sup> MVs, we analyzed their target genes predicted by TargetScan algorithm, selecting those genes targeted by almost 5 miRNAs. This list counted 157 genes (Supplementary Table S3). We performed the functional characterization of the genes target list searching for Gene Ontology keywords enrichment and we found a strong overrepresentation of terms belonging to crucial biological processes like transcription, metabolic process, nucleic acid binding, cell adhesion molecules and regulation of cell proliferation (Fig. 2C and Supplementary Table S4).

Moreover, we investigated whether CD105<sup>+</sup> MVs contained mRNAs involved in the stimulation of angiogenesis in comparison with CD105<sup>-</sup> MVs. mRNAs of genes involved in angiogenesis were detected only in CD105<sup>+</sup> MVs. In particular, they contained mRNAs for growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors 2 (FGF2), angiopoietin1 and ephrin A3 and for MMP2 and MMP9. Each mRNA detected was confirmed on 3 different CD105<sup>+</sup> MV preparations using qRT-PCR.

### ***In vitro* activation of HUVEC by CD105<sup>+</sup> MVs**

To evaluate whether MVs derived from CD105<sup>+</sup> renal cancer stem cells could be responsible of stimulating tumor angiogenesis and invasion, we tested their effect in comparison with MVs from CD105<sup>-</sup> tumor cells. We first evaluated the uptake of CD105<sup>+</sup> and CD105<sup>-</sup> MVs labelled with PKH26 dye by HUVEC, after 1 hour incubation at 37°C. HUVEC incorporated in equal manner both CD105<sup>+</sup> and CD105<sup>-</sup> MVs (Fig. 3A).

CD105<sup>+</sup> MVs from cancer stem cells and deriving clones stimulated HUVEC to organize *in vitro* into capillary-like structures on Matrigel. In contrast CD105<sup>-</sup> MVs did not induce formation of capillary-like structures. MVs derived from unsorted tumor cells also induced formation of capillary like structures, but the pro-angiogenic effect of MVs from CD105<sup>+</sup> sorted cells was significantly

greater (Fig. 3B and C). Moreover, CD105<sup>+</sup> MVs, but not CD105<sup>-</sup> MVs, significantly enhanced the invasion of HUVEC through transwells coated with Matrigel, in respect to CD105<sup>-</sup> MVs as well as to MVs from unsorted tumor cells (Fig. 4A and B). CD105<sup>+</sup> MVs also induced a greater apoptosis resistance in HUVEC treated with 100 ng/ml of doxorubicin (Fig. 4C). In order to investigate whether MV treatment could modify the adhesive property of endothelial cells, HUVEC were pre-treated with different MVs and after 6 hours the adhesion of renal tumor cells was evaluated. CD105<sup>+</sup> MVs significantly enhanced the adhesion of tumor cells in respect to CD105<sup>-</sup> MVs and unsorted tumor MVs (Fig. 4D). MVs from unsorted tumor cells induced invasion, apoptosis resistance and tumor cells adhesion in HUVEC that were greater in respect to CD105<sup>-</sup> MVs or vehicle, suggesting that the effects observed by tumor cell derived MVs should be ascribed to MVs released from cancer stem cells.

RNase pre treatment of CD105<sup>+</sup> MVs significantly reduced *in vitro* capillary-like formation (Fig. 3B), as well as the enhanced invasion, apoptosis resistance and adhesion properties (Fig. 4), suggesting a role of RNA molecular species carried by MVs.

### ***In vivo* effects of CD105<sup>+</sup> MVs**

In order to evaluate whether CD105<sup>+</sup> MVs were able to stimulate angiogenesis *in vivo*, we injected MV-stimulated HUVEC within Matrigel subcutaneously in SCID mice. CD105<sup>+</sup> MVs from cloned cancer stem cell preparations stimulated the growth of HUVEC that formed dense clusters containing small vessels organized into patent capillaries connected with the murine vasculature and into large aneurismatic structures (Fig. 5A). The cells grew into Matrigel and the vessels expressed the endothelial marker vWF and their human nature was showed by staining for HLA class I (Fig. 5B). HUVEC challenged with vehicle or with CD105<sup>-</sup> MVs or RNase CD105<sup>+</sup> MVs did not organize or proliferate into the Matrigel. MVs from unsorted tumor cells induced HUVEC proliferation and organization into small vessels, but the extent of angiogenesis was significantly

lower than that induced by CD105<sup>+</sup> MVs (Fig. 5C).

To evaluate whether CD105<sup>+</sup> MVs contribute to establish a pre metastatic niche we injected i.v SCID mice for 5 days with 70 µg of MVs, followed by i.v. injection of 6x10<sup>5</sup> renal tumor cells. After 5 weeks, organs were recovered (liver, spleen, kidney and lung) and the incidence of metastasis was evaluated. Metastasis clearly detectable were found only in lungs (Fig. 6A). Whereas in mice injected with vehicle alone or with CD105<sup>-</sup> MVs or RNase CD105<sup>+</sup> MVs the number of metastasis induced by renal tumor cells was very low, a significant increase in the number of metastasis was observed in mice pre-treated with CD105<sup>+</sup> MVs or MVs from unsorted tumor cells. However, CD105<sup>+</sup> MVs were significantly more efficient in inducing metastasis than unsorted tumor MVs (Fig. 6A). To evaluate whether the administration of MVs modify lung microenvironment, the expression of VEGFR1, VEGF, MMP9 and MMP2 was studied. By cytofluorimetric analysis VEGFR1 expression in CD146<sup>+</sup> sorted lung endothelial cells was enhanced by CD105<sup>+</sup> MVs but not by CD105<sup>-</sup> MVs (Fig. 6B). By qRT-PCR, CD105<sup>+</sup> MVs, but not CD105<sup>-</sup> MVs, significantly enhanced MMP9 expression in total lung tissue and VEGF and MMP2 in sorted lung endothelial cells (Fig. 6C). The enhanced expression of MMP9 and MMP2 in lung after treatment with CD105<sup>+</sup> MVs was confirmed by immunohistochemistry (Fig. 6D). Whereas MMP2 staining was mainly confined to lung vessels, the staining of MMP9 was more diffuse and the alveolar epithelial cells were positive.

## Discussion

Previous studies demonstrated an angiogenic potential of MVs derived from tumors but did not characterize the cells of origin (11, 24-26). Herein, we demonstrated that in renal cancer the MVs that retain the angiogenic properties were those derived from cancer stem cells. Indeed, MVs released from cancer stem cells induced *in vitro* and *in vivo* angiogenesis and favoured lung metastasis. These properties were ascribed only to the MVs released from the CD105<sup>+</sup> cell fraction as those derived from the CD105<sup>-</sup> tumor cells were ineffective. Indeed, CD105<sup>+</sup> MVs contained pro angiogenic mRNAs and miRNAs that may be involved in tumor progression and metastases.

Recently, circulating MVs were described in patient with various tumors (27-32) suggesting that they may serve as diagnostic and prognostic tool (33-35). In the contest of cancer, several studies pointed on the potential role of tumor derived MVs in the interaction with stromal cells and in the formation of pre metastatic niche (36-39). The potential of MVs to reprogram recipient cells was first established by Ratajczak J et al (7). Several subsequent studies indicate that mRNA delivered by MVs can be translated into the corresponding proteins by target cells (8, 9, 40).

In the present study we investigated whether MVs derived from cancer stem cells posses biological activities, that may account for the induction of a favourable environment for tumor growth and invasion. We found that MVs derived from CD105<sup>+</sup> renal cancer stem cells differ for their content of mRNAs and miRNAs in respect to the CD105<sup>-</sup> renal cancer cell population. In particular, CD105<sup>+</sup> MVs contained several pro-angiogenic mRNAs such as VEGF, FGF, angiopoietin1, ephrin A3 and MMP2, MMP9 that were absent in CD105<sup>-</sup> tumor MVs. The presence of the pro-angiogenic mRNAs correlated with an *in vitro* and *in vivo* angiogenic effect of CD105<sup>+</sup> MVs. The pro-angiogenic effect of CD105<sup>+</sup> MVs can be ascribed to their ability to induce endothelial cell growth, organization, invasion of matrix and resistance to apoptosis. An angiogenic effect of MVs was previously described for MVs derived from unfractionated tumor cells of lung cancer, ovarian cancer and glioblastoma, as well as from some tumor cell lines (11, 25, 26, 33). Beside mRNAs,

MVs were shown to contain and to deliver functional miRNAs to target cells (9, 20). CD105<sup>+</sup> MVs were enriched in miRNAs in respect to the CD105<sup>-</sup> MVs. The Gene Ontology analysis of predicted target genes indicated that CD105<sup>+</sup> MVs shuttled a selected pattern of miRNAs that may modulate several biological functions relevant for cell growth, regulation of transcription, cell matrix adhesion and synthesis of macromolecules. Among the miRNAs shuttled by CD105<sup>+</sup> MVs we detected miR-200c, miR-92 and miR-141 that were described significantly up regulated in patients with ovarian (28, 41), colorectal (42) and prostate cancer (43) respectively. These miRNAs were suggested as marker of unfavourable prognosis (44). In addition we detected several miRNAs such as miR-29a, miR-650, miR-151 that were associated with tumor invasion and metastases (45-47). Moreover miR-19b, miR-29c, miR-151 were observed up regulated in renal carcinomas in comparison with normal renal tissue (48) and they were significantly enriched within miRNAs present in CD105<sup>+</sup> MVs.

It has been recently suggested that tumor derived MVs may contribute to the formation of a pre metastatic niche (37, 38). Herein we demonstrated that MVs derived from CD105<sup>+</sup> renal cancer stem cells, but not from CD105<sup>-</sup> tumor cells, were able to significantly enhance lung metastases formation when injected prior of a renal tumor cell line. Indeed, CD105<sup>+</sup> MVs but not CD105<sup>-</sup> MVs, significantly enhanced the expression of VEGFR1, VEGF and MMP2 in CD146-sorted lung cells containing endothelial cells and a small population of leucocytes and of MMP9 in the whole lung. Previous studies demonstrated that these factors are involved in the generation of lung premetastatic niche (49,50). Our results confirm that MVs create a receptive microenvironment to coordinate metastatic diffusion (37) and identify the specific contribution of MVs derived from cancer stem cells.

A recent study indicated that tumor stem cells not only initiate tumors but may promote metastases in virtue of their peculiar content of tumorigenic miRNAs (46). MVs may transfer products of oncogenes to bystander cells inducing changes in their phenotype (11). The result of the present



study suggests that the RNA content of MVs plays a critical role as the RNase treatment of MVs significantly inhibited the *in vitro* and in particular the *in vivo* biological effects of CD105<sup>+</sup> MVs. This suggest that the effects of CD105<sup>+</sup> MVs could be at least in part accounted to epigenetic changes induced by transfer of mRNAs and/or miRNAs.

In conclusion the results of the present study suggest that in renal cancer the MVs that favour tumor growth and invasion were those derived from the cancer stem cells rather than from the whole tumor cell population. This MVs by enhancing tumor vascularization and by contributing to the establishment of a pre metastatic niche may sustain an unfavourable outcome of the tumor.

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## Legend for figures

### Figure 1. Characterization of MVs

A and B. Representative micrographs of transmission electron microscope analysis of CD105<sup>+</sup> MVs (A) and CD105<sup>-</sup> MVs (B) showing a spheroid shape (Original magnification: x10,000; bar = 100 nm). CD105<sup>-</sup> MVs display the same morphology and size (not shown). C. Representative FACS analyses of CD105<sup>+</sup> MVs showing the size (with 1, 2 and 4  $\mu\text{m}$  -beads used as internal size standards) and the expression of CD105,  $\alpha 6$  integrin, CD44,  $\alpha 5$  integrin and HLA class I (thick lines) surface molecules. In the CD105,  $\alpha 6$  integrin, CD44,  $\alpha 5$  integrin experiments the Kolmogrov-Smirnov statistical analyses between relevant antibodies and the isotypic control was significant ( $p < 0.001$ ). No significant expression of HLA class I was observed. D. Representative Cytofluorimetric analyses of CD105<sup>-</sup> MVs showing the expression of  $\alpha 6$  integrin, CD44,  $\alpha 5$  integrin (Kolmogrov-Smirnov statistical analyses between relevant antibodies and the isotypic control was significant:  $p < 0.001$ ). CD105 was negative. Dot lines indicate the isotypic controls. MV preparations derived from 3 CD105<sup>+</sup> clones, 5 CD105<sup>+</sup> uncloned cancer stem cells and 5 CD105<sup>-</sup> tumor cells were analyzed with similar results.

### Figure 2. Characterization of MV RNAs

A. Representative bioanalyzer profile of the RNAs contained in CD105<sup>+</sup> MVs derived from CD105<sup>+</sup> clones and in CD105<sup>-</sup> MVs showing that the ribosomal subunit 28S and 18S were absent or barely detectable. B. Representative bioanalyzer profile of small RNAs performed RNA subtypes present in CD105<sup>+</sup> MVs and CD105<sup>-</sup>. Three different samples tested in triplicate were analyzed with similar results. C. Gene Ontology enrichment analysis of target genes of at least 5 up regulated miRNAs in CD105<sup>+</sup> MVs. Fisher's exact test to evaluate GO keywords overrepresentation was used. A p-value  $< 10^{-4}$  was considered statistically significant for GO terms. Overrepresented biological processes



are grouped according to their common ancestor.

**Figure 3. Internalization of MVs in HUVEC and *in vitro* angiogenic effect.**

A. Representative confocal microscopy analysis of red-labeled MVs in HUVEC stained with CFSE (green). Seven experiments were performed with similar results (Original magnification x630). B. Quantitative evaluation and C representative micrographs showing the formation of capillary like structure formed by HUVEC seeded on Matrigel-coated plates in serum starvation condition (RPMI) and stimulated with 30  $\mu\text{g/ml}$  of CD105<sup>+</sup> MVs from uncloned and cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs derived from cloned cancer stem cells, CD105<sup>-</sup> MVs and MVs from unsorted tumor cell (TMV). Data are expressed as the mean  $\pm$ SD of the length of capillary-like structure after 16 hours, evaluated by the computer analysis system in arbitrary units (AU) in at least 10 different fields at x200 magnification. Four different experiments per group were performed in duplicate. ANOVA with Newman Keuls' multicomparison test was performed: \*  $p < 0.05$ , CD105<sup>+</sup> MV versus RPMI, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV; §  $p < 0.05$  TMV versus RPMI and CD105<sup>-</sup> MV.

**Figure 4. Effect of MVs on endothelial cell invasion, apoptosis resistance and on tumor cell adhesion to endothelium.**

A. Quantitative evaluation and B representative micrographs showing the invasion of Matrigel-coated transwells by HUVEC stimulated with 30  $\mu\text{g/ml}$  of MVs from uncloned and cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and TMVs. Invasion was evaluated after 24 hours. Data are expressed as the mean  $\pm$ SD of the area occupied by cells on total well-surface area evaluated by the computer analysis system in arbitrary units (AU) at x100 magnification. ANOVA with Newman Keuls multicomparison test was performed, \*  $p < 0.05$ : CD105<sup>+</sup> MV versus RPMI, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV; §  $p < 0.05$ : TMV versus RPMI and CD105<sup>-</sup> MV. C. Quantitative evaluation of apoptosis of HUVEC cultured in presence of 100 ng/ml of

doxorubicin plus vehicle or 30 µg/ml of MVs from uncloned and cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and TMVs. Apoptosis was evaluated by TUNEL assay after 24 hours as percentage (mean ±SD of cells per field) of apoptotic cells per field. As control, cells were cultured in EBM in absence of doxorubicin. ANOVA with Newman Keuls' multicomparison test was performed, \* p< 0.05: doxorubicin treatment in presence of vehicle alone, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV induced significant apoptosis versus doxorubicin untreated (EBM); § p<0.05: CD105<sup>+</sup> MV significantly inhibited apoptosis versus all other doxorubicin treatment (vehicle, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV). D. Quantitative evaluation (mean ±SD of cells per field) of adhesion of 5x10<sup>5</sup> K1 tumor cells labelled with CSFE to a monolayer of HUVEC unstimulated (RPMI) or stimulated with 30 µg/ml of MVs from cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and TMVs. ANOVA with Newman Keuls' multicomparison test was performed: \* p< 0.05, CD105<sup>+</sup> MV versus RPMI, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV; § p<0.05 TMV versus RPMI and CD105<sup>-</sup> MV. For all the experimental condition 5 different experiments were performed in duplicate.

**Figure 5. *In vivo* angiogenesis of HUVEC stimulated with CD105<sup>+</sup> MVs.**

HUVEC (1x10<sup>6</sup>) treated with vehicle or 70 µg of CD105<sup>+</sup> MVs from cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs and TMVs were injected subcutaneously within Matrigel in SCID mice and mice were sacrificed 10 days after. A. Representative micrographs of haematoxylin and eosin staining of section of Matrigel showing dense cluster of cells infiltrated by small vessels and microaneurismatic structures containing erythrocytes in HUVEC stimulated with CD105<sup>+</sup> MVs. TMVs induced only formation of small vessels. B. Representative micrograph of immunostaining for the endothelial antigen (vWF) and for HLA class I antigen (Original magnification x200). C. Quantitative evaluation of neo-formed vessels was expressed as number of vessels per total area of Matrigel. Data are expressed as mean ±SD of 8 individual experiments for

each condition. ANOVA with Newman Keuls' multicomparison test was performed, \*  $p < 0.05$ : CD105<sup>+</sup> MV versus vehicle, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV; §  $p < 0.05$ : TMV versus vehicle, RNase CD105<sup>+</sup> MV and CD105<sup>-</sup> MV.

**Figure 6. Effect of MVs on lung metastasis formation.**

SCID mice (5/group) were treated for 5 days with i.v. injections of vehicle or 70 µg of CD105<sup>+</sup> MVs from cloned cancer stem cell preparations, RNase CD105<sup>+</sup> MVs, CD105<sup>-</sup> MVs or TMVs. K1 renal tumor cells ( $6 \times 10^5$ ) were injected i.v. on day 5 and mice sacrificed 5 weeks later. A. quantitative evaluation of metastases performed in 5 non consecutive sections of whole lungs and expressed as mean  $\pm$ SD per lung and representative haematoxylin and eosin stained lung sections (Original magnification x200). ANOVA with Newman Keuls' multicomparison test was performed, \*  $p < 0.05$ : CD105<sup>+</sup> MV versus vehicle, RNase CD105<sup>+</sup> MV, CD105<sup>-</sup> MV and TMV; §  $p < 0.05$ : TMV versus vehicle, RNase CD105<sup>+</sup> MV and CD105<sup>-</sup> MV. B. Representative cytofluorimetric analysis of VEGFR1 expression by CD146<sup>+</sup> sorted lung endothelial cells obtained from mice treated for 5 days with 70 µg of CD105<sup>+</sup> MVs (red line) or CD105<sup>-</sup> MVs (dark line) or with vehicle alone (dotted line). The percentage of positive cells was: CD105<sup>+</sup> MVs= $63 \pm 3.1\%$ ; CD105<sup>-</sup> MVs= $36 \pm 2.7\%$ ; vehicle= $40 \pm 2.9\%$ . Eight mice per group were studied with similar results. C. Quantitative RT-PCR analysis of VEGF, MMP2 and MMP9 mRNA expression in total lung and in CD146<sup>+</sup> endothelial cells of mice treated for 5 days with 70 µg of CD105<sup>+</sup> MVs or CD105<sup>-</sup> MVs or with vehicle alone. Data were normalized to actin mRNA and to 1 for vehicle. Eight mice per group were studied with similar results. ANOVA with Newman Keuls' multicomparison test was performed, \*  $p < 0.05$ : CD105<sup>+</sup> MV versus CD105<sup>-</sup> MV. D. Representative immunohistochemistry for MMP9 and MMP2 on lung sections obtained from mice treated for 5 days with 70 µg of CD105<sup>+</sup> MVs or CD105<sup>-</sup> MVs or with vehicle alone showing MMP9 staining of vessels and alveolar epithelial cells (arrows and inset) and MMP2 staining of vessels (inset) in CD105<sup>+</sup> MV

treated mice. (Original magnification: MMP9 x200; MMP2 x400; insets x620).