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CD90+ liver cancer cells modulate endothelial cell phenotype through the release of exosomes containing H19 lncRNA



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Abstract

Background: CD90+ liver cancer cells have been described as cancer stem-cell-like (CSC), displaying aggressive and metastatic phenotype. Using two different *in vitro* models, already described as CD90+ liver cancer stem cells, our aim was to study their interaction with endothelial cells mediated by the release of exosomes.

Methods: Exosomes were isolated and characterized from both liver CD90+ cells and hepatoma cell lines. Endothelial cells were treated with exosomes, as well as transfected with a plasmid containing the full length sequence of the long non-coding RNA (lncRNA) H19. Molecular and functional analyses were done to characterize the endothelial phenotype after treatments.

Results: Exosomes released by CD90+ cancer cells, but not by parental hepatoma cells, modulated endothelial cells, promoting angiogenic phenotype and cell-to-cell adhesion. lncRNA profiling revealed that CD90+ cells were enriched in lncRNA H19, and released this through exosomes. Experiments of gain and loss of function of H19 showed that this lncRNA plays an important role in the exosome-mediated phenotype of endothelial cells.

Conclusions: Our data indicate a new exosome-mediated mechanism by which CSC-like CD90+ cells could influence their tumor microenvironment by promoting angiogenesis. Moreover, we suggest the lncRNA H19 as a putative therapeutic target in hepatocellular carcinoma.

Keywords: Exosomes, Long-non-coding RNA H19, CD90+ liver cancer cells, Angiogenesis

Background

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide [1]. Primary HCC lesions can be removed completely when detected at an early stage, but intrahepatic recurrence of HCC and extrahepatic metastasis are very frequent, giving rise to a poor prognosis for patients [2, 3]. It is widely accepted that both differentiated hepatocytes and cells with progenitor characteristics, known as cancer stem cells (CSCs), can cause

HCC [4–7]. Forty percent of HCCs are clonal, and potentially derived from progenitor/stem cells. Moreover, these cells have a critical role in the development and progression of HCC [8]. Liver CSCs have been isolated from primary HCC specimens and patients' sera as circulating cells, and from HCC cell lines by use of surface antigens [9–11]. CD90, the epithelial cell adhesion molecule (EPCAM) and CD133 have been found to recognize three distinct cell populations that differ from one another in features and behavior in determining cancer phenotypes [12].

CD90 (Thy-1) is a 25–37 kDa glycosphosphatidylinositol (GPI)-anchored protein expressed by several cells such as T-cells, neurons, endothelial cells and fibroblasts. It is involved in cell-to-cell and cell-matrix interaction, apoptosis, adhesion, migration, fibrosis, and

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cancer development [13]. Concerning the liver, the expression of CD90 has been linked to hepatic stem/progenitor cells [14] and, during tumor growth, it has been correlated with an aggressive phenotype [15], and associated with low differentiated HCC and poor prognosis [16–18]. CD90+ CSCs obtained from HCC cell lines, from tumor tissues and peripheral blood as circulating cancer cells displayed, in contrast to the other CSC populations, a mesenchymal phenotype and, most importantly, a greater capacity to metastasize when injected into immunodeficient mice [11, 12, 19]. Moreover, recent data associate CD90 expression with early HCC recurrence [20]. Gene expression and miRNA analysis in CD90+ HepG2 cells have revealed an imbalance in the expression of apoptotic and anti-apoptotic genes compared with CD90 negative cells [21]. However, we are still far from understanding the molecular mechanisms underlying the more aggressive and metastatic phenotype of these cells compared with the other liver cancer cells.

Tumor development is dependent on the reciprocal interactions between cancer cells and the surrounding microenvironment. It is well known that in addition to pathways involving cell-to-cell contact and the release of soluble factors, cancer cells are able to communicate with the tumor microenvironment (e.g., myeloid cells, fibroblasts, endothelial cells) through the intercellular exchange of proteins and genetic materials via exosomes [22].

Exosomes are spherical membrane vesicles of endocytic origin, with an average size of 40 to 150 nm [23], released by both normal and diseased cells after the fusion of multivesicular bodies with the plasma membrane. First considered as collectors of cellular waste materials, exosomes have assumed a leading role in the regulation of the tumor microenvironment. Depending on their content, exosomes can affect tumor cells and surrounding stroma by influencing major cellular pathways, such as apoptosis, cell differentiation, angiogenesis, and metastasis [24]. These vesicles act as cargos that release bioactive molecules e.g., lipids, proteins, and nucleic acids in target cells. Interestingly, recent observations have identified a vesicle-mediated transfer of lncRNAs as an important mechanism in the development of HCC [25]. In this paper we demonstrate that CD90+ cells, derived from HCC cell lines, release exosomes that, in turn, are able to influence endothelial cells by promoting angiogenesis and stimulating their adhesive properties. Furthermore, our results suggest the lncRNA H19 as a possible mediator of angiogenic effects.

Results

CD90+ cells show a mesenchymal phenotype and actively release exosomes

As reported by Yang and colleagues [11], highly positive CSC-like CD90+ cells were isolated by cell sorting,

starting from Huh7 cell line presenting a mean of 4 % CD90+ cells and 2 % CD90 high-expressing cells. After sorting, the purity of the selected CD90+ population was monitored during cell passages by FACS analysis, and the cells were kept in culture until they maintained a positivity for CD90 of over 90 % (at approximately the 40th passage). Isolated CD90+ cells, in contrast to the parental Huh7 and as already described by others [12], showed a mesenchymal phenotype, revealing a delocalized E-Cadherin and a lack of expression of HNF4 α , a master regulator of hepatocytic differentiation (Fig. 1a). On the contrary, most of the cells were positive for vimentin, a component of intermediate filaments in mesenchymal cells (Fig. 1a). In order to evaluate the ability of Huh7 and its CD90+ subpopulation to release nanovesicles, the conditioned medium was collected, and the vesicles isolated as described by members of our group [26, 27]. Measures obtained by DLS revealed, in the ultracentrifuged cell culture medium, vesicles with an average size in diameter of 50 nm and 100 nm from CD90 + or Huh7 cell medium, respectively (Fig. 1b). This in line with the exosomes dimensions between 30 and 150 nm [28]. Moreover, Western blot analyses showed that Alix and Tsg101 markers are expressed but not enriched in exosomes released by CD90 + Huh7 (Fig. 1c).

Exosomes released by CD90+Huh7 cells affect HUVECs by promoting tube formation and cell-cell adhesion

CD90 + CSCs have been associated with metastasis and early recurrence in HCC [12, 20]. In order to evaluate whether the CD90+Huh7 cells were able to influence the tumor microenvironment, we treated HUVECs with exosomes released by CD90+ Huh7 cells or Huh7 parental cells (CD90 + exo and Huh7exo). Endothelial cells rapidly internalized exosomes from both cell types; uptake was evident after one-hour of incubation at 37 °C, and increased over the course of six hours (Fig. 1d). Eighteen hours after exosome treatment, real-time PCR analysis revealed that the addition of CD90 + exo, but not of Huh7exo, highly increased the mRNA levels of the pro-angiogenic factor VEGF and its receptor VEGF-R1 in endothelial cells (Fig. 2a). ELISA assay showed that HUVECs treated with CD90 + exo released three-fold more VEGF (Fig. 2b, left panel). Moreover, a significant increase in the number and the length of tubular-like structures was observed when HUVECs were treated with CD90 + exo compared with Huh7exo (Fig. 2b, middle and right panels).

Liver CD90+ CSCs were found circulating in HCC patients and in metastatic colonies [19]. For this reason, we tested the ability of exosomes released by the hepatoma cell line or by sorted CD90+ cells to modulate the adhesion to an endothelial cell monolayer, a crucial event for intra- or extra-vasation. As revealed by real-

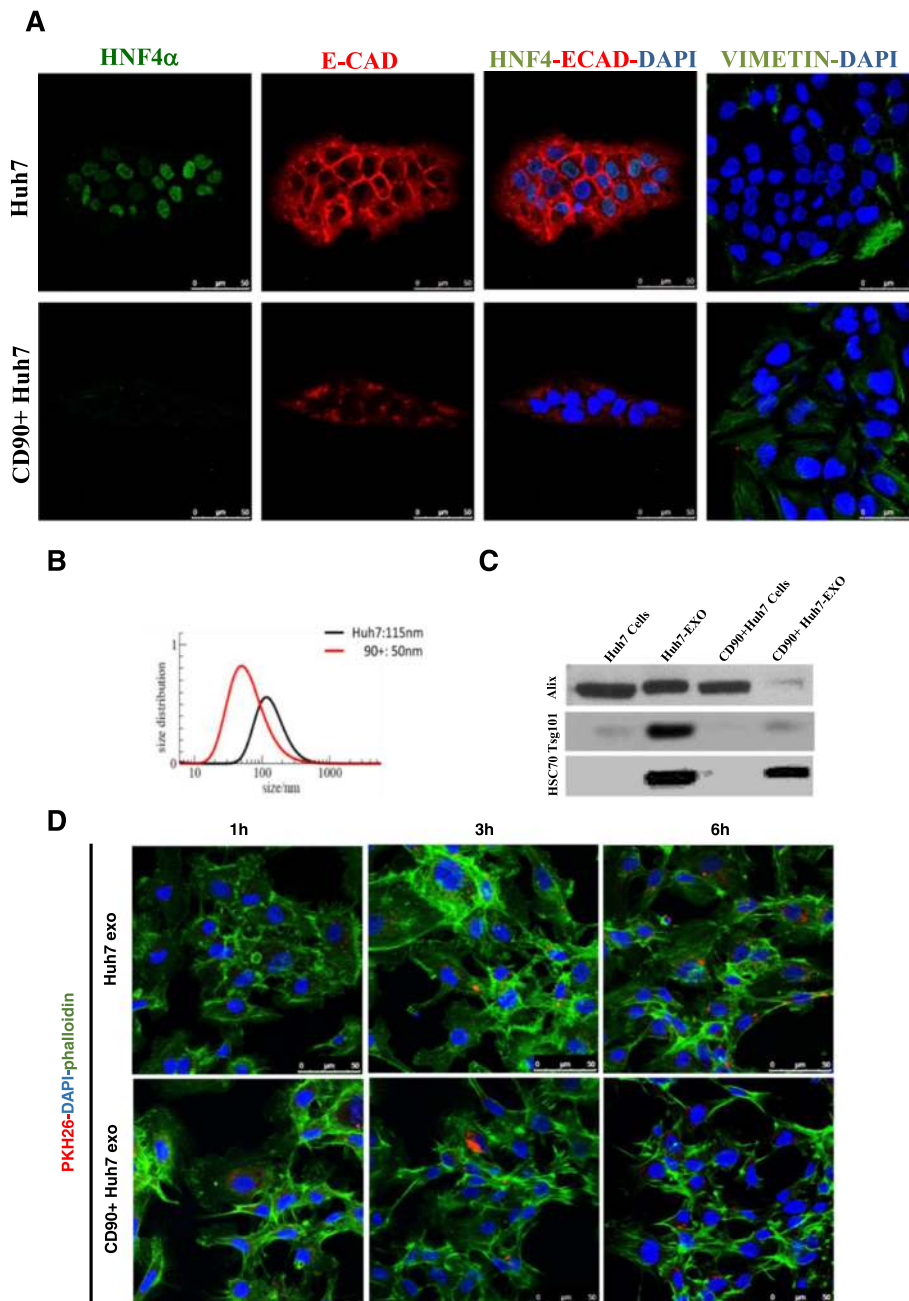
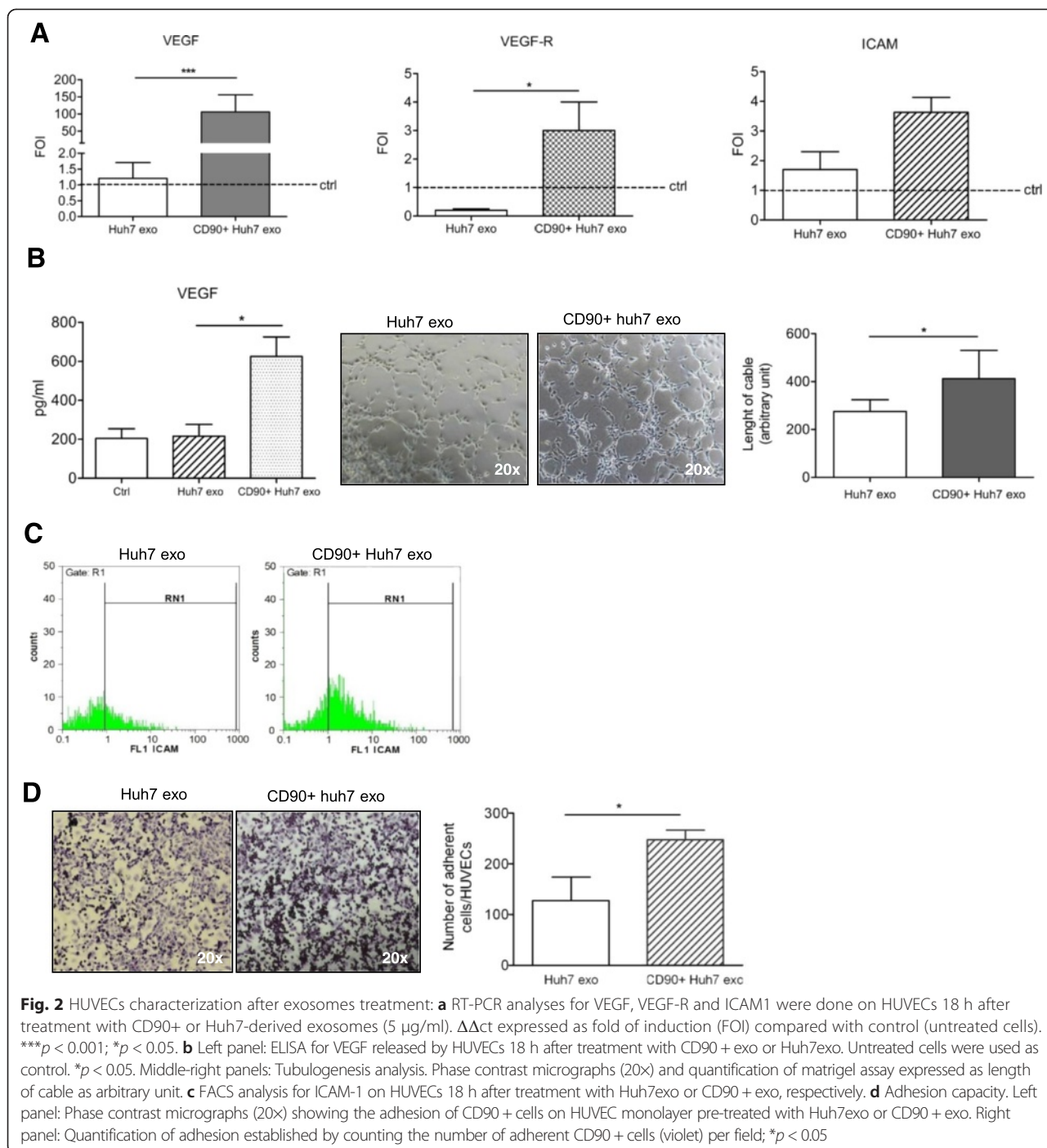


Fig. 1 CD90+ population. **a** Huh7 and sorted CD90+ Huh7 were stained for hepatocytic (HNF4 α), epithelial (E-Cadherin) and mesenchymal (Vimentin) markers, in blue the nuclear staining with DAPI. Characterization of isolated exosomes. **b** Dynamic light scattering of vesicles isolated from Huh7 (in black) and from CD90+ Huh7 cells (in red). **c** Western blot for endosomal markers Alix, Tsg101 and HSC70 in Huh7 and CD90+Huh7 population with their relative exosomes. **d** Confocal microscopy analysis on HUVECs treated for 1, 3, and 6 h with 5 μ g/ml of exosomes from CD90+ or Huh7 cells. HUVECs were stained with phalloidin Alexa Fluor488 (green), nuclear counterstaining was done using DAPI (blue), exosomes were labelled with PKH26 (red)

time PCR (Fig. 2a), and confirmed by FACS analysis (Fig. 2c), treatment of HUVECs with CD90+ -derived exosomes modulated intercellular adhesion molecules, inducing an increase in the expression of ICAM-1. No significant differences were found in VCAM and VE-Cadherin gene expression (data not shown). To

validate our data, we did an adhesion assay, pre-treating endothelial cells with exosomes. As shown in Fig. 2d, CD90+ exo caused a two-fold increase in adhering cells compared with pre-treatment Huh7 exo.

To further confirm our observation, the same experiments were performed with SkHep, a hepatoma cell line



already characterized as 100 % CD90+, and displaying mesenchymal stem cell characteristics [29]. Additional file 1 illustrates the characterization of exosomes released by SkHep and their uptake by HUVECs, that present different features compared to CD90+ Huh7 derived exosomes. In addition, measures obtained by DLS revealed, in the ultracentrifuged SKHep culture medium, exosomes with an average size in diameter of 70 nm expressing high level of the exosomal markers

TSG101 and HSC70. As observed for CD90 + exo, the SkHep-derived exosomes induced a pro-angiogenic stimulus in endothelial cells, modifying their transcriptional profile and enhancing tube formation in matrigel, as well as increasing the adhesive properties of HUVECs.

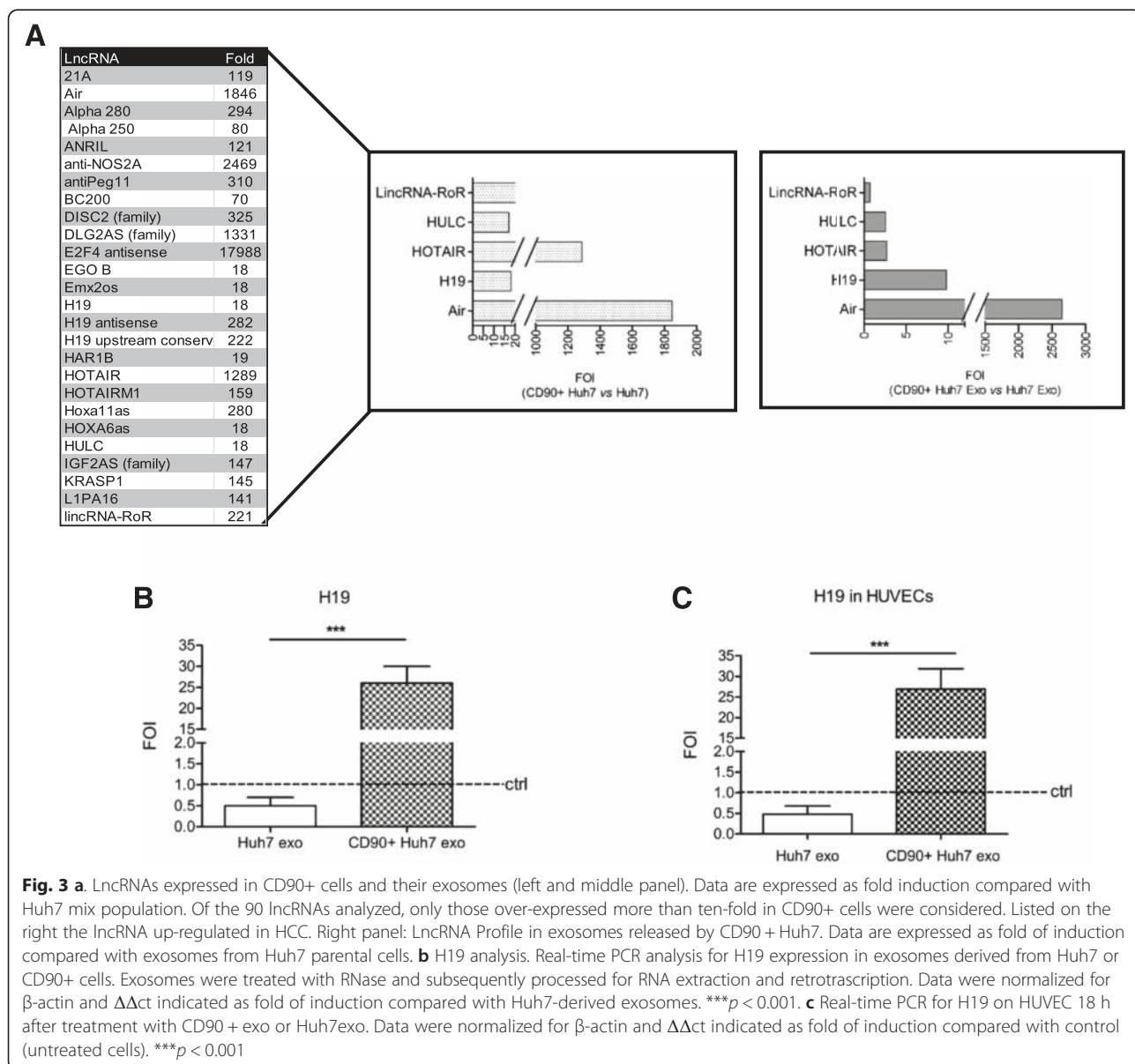
In summary, our results showed that exosomes released by CSC-like CD90+ liver cells, but not from hepatoma cells, induce pro-angiogenic stimuli in HUVECs,

and influence the adhesion between CD90+ cells and endothelial cells.

CD90+ cells express the lncRNA H19 and release it via exosomes

It has been confirmed that dysregulation of lncRNAs is associated with several human tumors and, recently, a contribution of lncRNAs to hepatocarcinogenesis was found [30–32]. In order to clarify the molecular mechanism driving the modifications induced in HUVECs by CD90+ -derived exosomes we did an lncRNA profile study in CD90+ cells and parental Huh7 by analyzing the expression of 90 different lncRNAs. In Fig. 3a (left and middle panel), the RNAs over-expressed in CD90+Huh7 cells compared with Huh7 parental cells with at least a ten-fold

increase are listed. Among these, Air, Hotair, LincRNA-ROR, Hulc, and H19 have already been identified as positively correlated with hepatocellular carcinoma [31, 33, 34]. We focused our interest on H19, expression of which has been previously associated with metastasis [35, 36]. In line with recent articles, which have demonstrated that hepatocellular carcinoma cells release exosomes containing lncRNA [25, 37], we investigated the expression in exosomes, of those lncRNAs that we found overexpressed in cells. As shown in Fig. 3a right panel the LncProfiler performed on CD90+ Huh7 and Huh7-derived exosomes evidences that H19 was 10-fold up-regulated in exosomes derived from CD90+ Huh7, compared to parental cell line. The Real-time PCR confirmed that vesicles released by CD90+ cells (both sorted or SkHep cells) are highly



enriched in H19 transcript compared with vesicles from Huh7 parental cells (Fig. 3b, S1e). Moreover, treatment with CD90+ -derived exosomes induced in HUVECs an increase in H19 transcript (Fig. 3c, S1f). These data suggest a transport of H19 lncRNA from CD90+ cells to HUVECs, even if we cannot exclude a stimulation of endogenous lncRNA.

lncRNA H19 stimulates angiogenesis and promotes the adhesion of CD90+Huh7 cells to endothelial cell monolayer

To investigate a possible role of H19 as mediator of pro-angiogenic and adhesive stimuli in HUVECs, we transfected endothelial cells with the entire sequence of the lncRNA H19 (pH19). As shown in Fig. 4a, H19 overexpression in HUVECs induced a transcriptional modulation similar to that obtained after CD90+ exo treatment. Real-time PCR indicated that the over expression of H19 induced a significant increase in the VEGF and ICAM1 transcripts, while, no modulation compared to controls was observed for the transcription of VEGF-R1, VCAM and VE-cadherin (Fig. 4a right panel). The ELISA assay (Fig. 4a left panel) found, for the first time to our knowledge, a substantial increase in VEGF release induced by lncH19. Moreover, a rise in the number and length of tubes was found in HUVECs transfected with pH19 (Fig. 4b), while FACS analysis (Fig. 4c) indicated an increase in the number of ICAM-1-expressing cells induced by H19 overexpression, thus explaining the more adhesive phenotype of HUVECs. The adhesion assay, in fact, revealed a two-fold increase in adhering CD90+ cells when HUVECs were transfected with pH19 (Fig. 4d).

Overall, these data demonstrate, for the first time to our knowledge, the ability of the lncRNA H19 to stimulate angiogenesis, and to favor cell-cell interaction, allowing us to postulate H19 as a possible mediator of pro-metastatic properties of exosomes released by CD90+ cells. To confirm our hypothesis, lncH19 was silenced in HUVECs concomitantly with CD90+ exo treatment. As shown in Fig. 5a, the silencing of H19 abrogated the exosome-mediated induction of VEGFR1, while no modulation was revealed in the expression of ICAM1. Concerning the VEGF, even if the reduction of transcript did not appear significant (5a), the release of VEGF protein induced by exosome treatment was totally inhibited by H19 silencing (5b).

Discussion

CD90+ liver CSCs have been found in primary tumors, and circulating in the blood of HCC patients, and are associated with early recurrence, metastasis, and poor prognosis [19, 20]. Our study highlights the ability of CSC-like CD90+ cells, but not hepatoma

cells, to influence endothelial cell phenotype through the release of exosomes.

In a solid tumor, the CSC's niche is composed of an extracellular matrix (ECM), mesenchymal stem cells, tumoral cells, immune cells, and endothelial cells, all of which converge in determining the fate of CSCs through extracellular signals [38]. Little is known about the modulation of the tumor microenvironment by CSCs. Several studies have described exosomes as signaling extracellular organelles that modulate the tumor microenvironment, promoting angiogenesis and tumor progression [27, 39]. Our data indicate that exosomes released by CSC-like CD90+ liver cells are able to promote an angiogenic phenotype in cultured endothelial cells. CD90+ -derived exosomes induced in HUVECs an increase in the production and secretion of VEGF, the most powerful pro-angiogenic cytokine, as well as of its receptor VEGF-R1. This increase was accompanied by an amplification in the number and length of tube-like structures formed by HUVECs in culture.

It is abundantly documented that metastatic processes induce changes in the endothelial surface antigens, with an increase in adhesion molecules, which, in turn, favor the adhesion and the consequent intra- or extra-vascularization of metastatic cells. We found that exosomes released by CD90+Huh7 cells, and not by hepatoma cells, increased the number of HUVECs expressing ICAM-1 and, more extensively, increased the adhesion between endothelial cells and the CSC-like CD90+ cells. Our data also indicate that the CD90+ released exosomes may be able to promote metastasis.

Recently, Patel et al. demonstrated that lncRNA could be selectively packaged in extracellular vesicles released by hepatoma cell lines and transported to other cells, with subsequent modulation of cellular function [25, 40]. lncRNA are emerging as molecular players in several biological processes acting at epigenetic, transcriptional and post-transcriptional levels or processing small non-coding RNAs [41]. H19 was among the first lncRNAs to be identified and studied principally for its monoallelic expression, and as regulator of IGF2 abundance [42, 43]. As already described for other lncRNAs, H19 can work as a microRNA sponge, miRNAs precursor, or epigenetic modulator [44, 45], and has been found overexpressed in several tumors, and able to promote tumor growth [46, 47] and progression [47, 35, 36]. Concerning the liver, H19 has been clearly involved in hepatocarcinogenesis [48] and hepatic metastases [49]. Several indications correlate H19 with angiogenesis [50, 51]. Northern analysis has indicated a high expression of H19 during development of rat aorta that decreases in differentiated tissue and, interestingly, re-appears following vascular injury *in vivo* and *in*

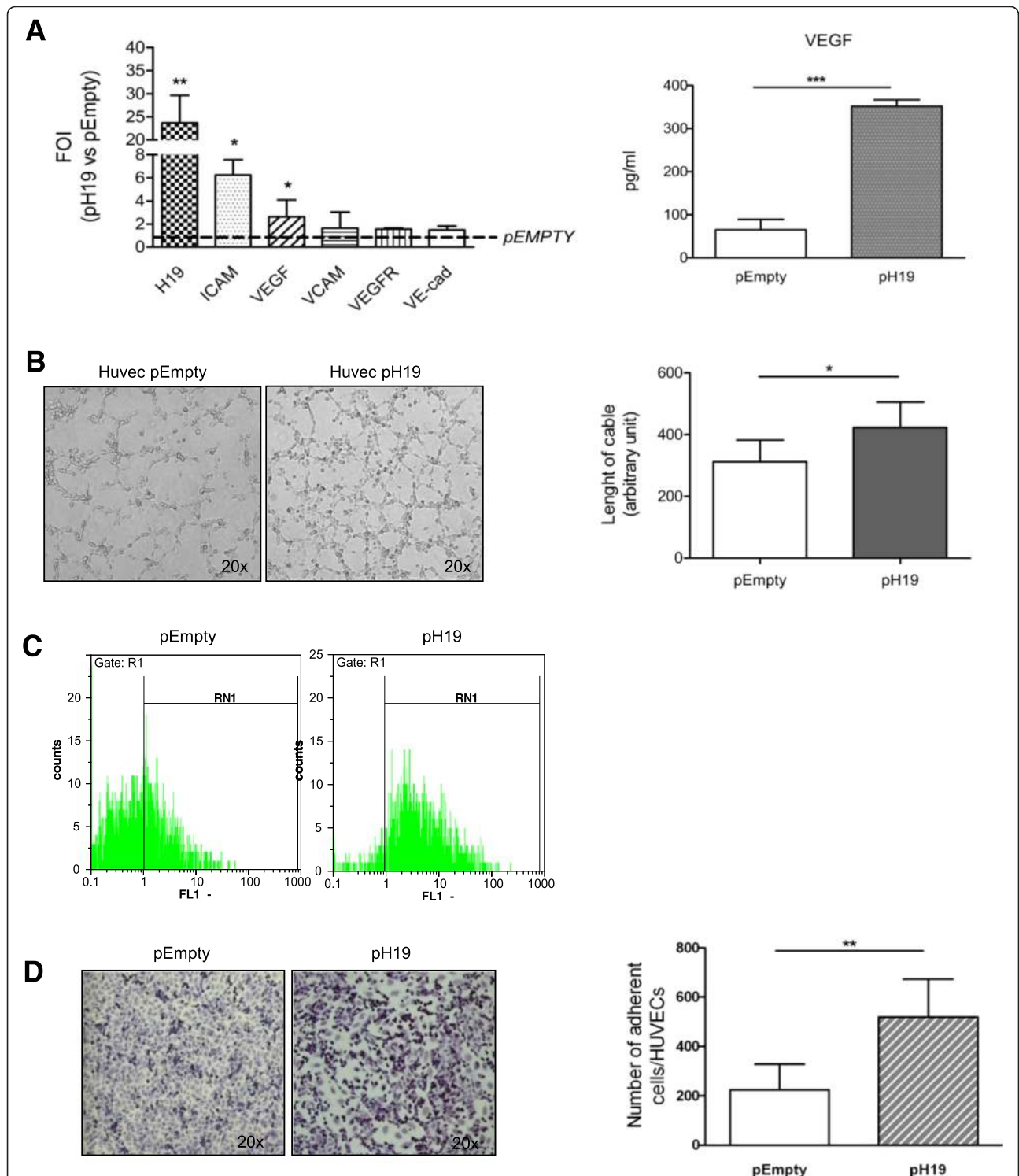


Fig. 4 H19 overexpression. **a** Left panel: Real-time PCR performed on HUVECs 18 h post-transfection. Data were normalized for β -actin and $\Delta\Delta Ct$ expressed as fold of induction pH19 vs. pEmpty $**p < 0.01$; $*p < 0.05$. Right panel: ELISA assay for VEGF level in supernatant from HUVECs 18 h after transfection. $***p < 0.001$. **b** Left Panel: Phase contrast (20x) of tubulogenesis assay performed 18 h after transfection. Right panel: quantification of matrigel assay expressed as length of cable as arbitrary unit $*p < 0.05$. **c** FACS analysis for ICAM expression in HUVEC transfected cells. **d** Left Panel: Phase contrast micrographs (20x) showing the adhesion of CD90+ cells on HUVEC monolayer transfected with pEmpty or pH19. Right Panel Quantification was calculated by counting the number of adherent CD90+ cells (violet) per field. $**p < 0.01$

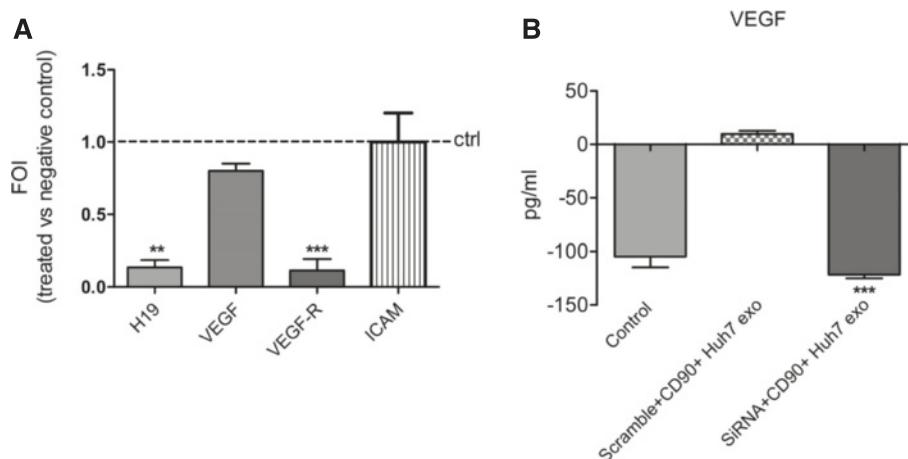


Fig. 5 a Real-time PCR for H19, VEGF, VEGFR1 and ICAM1 from HUVECs transfected with H19 siRNA or negative scramble and treated with CD90 + exo. Data were normalized for β -actin and $\Delta\Delta$ ct expressed as fold of induction siRNA H19 versus negative control. ** $p < 0.01$, *** $p < 0.001$ **b** ELISA assay for VEGF detection on the supernatant from HUVECs treated as indicated above. *** $p < 0.001$

vitro [50], though no observations of the overexpression of H19 in endothelial cells have been published.

In this study, we demonstrate, for the first time to our knowledge, that H19 is highly expressed in a subpopulation of hepatoma cells that expose the surface antigen CD90 and are characterized, by others, as CSC-like cells [11, 12, 15, 29]. We found that CD90+Huh7 cells package lncRNA H19 inside exosomes, thus delivering it to possible target cells. Exosomes released by CD90+ liver cancer cells could be internalized by endothelial cells, influencing these in a pro-metastatic way. Moreover, we identified in H19 an important player of this process. H19 overexpression in endothelial cells is able to up-regulate the VEGF production and release, increase the ability of HUVEC cells to arrange *in vitro* tubular-like structures, and promote heterotypic adhesion between endothelial cells and CSC-like liver cells. Silencing experiments revealed lncRNA H19 as the principal player of the exosome-mediated VEGF increase, while suggested the presence of other molecular actors that, transported or induced by CD90+ -derived exosomes, and together with H19, affect endothelial cells in a pro-metastatic way. However, the mechanisms of action through which this lncRNA controls an endothelial phenotype remain to be elucidated.

Conclusion

Our *in vitro* experiments demonstrated that CD90+ liver cancer cells release exosomes that, in turn, are able to affect endothelial cells in a pro-metastatic way. Exosomes derived by CD90+Huh7 cells and H19 may represent two new therapeutic targets for reducing recurrence and metastasis of HCC.

Material and methods

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Verviers, Belgium) and grown in endothelial growth medium (EGM, bullet kit, Lonza) according to supplier's instructions. Huh7 cells and Sk-Hep cells were cultured in DMEM medium (Euroclone, UK), and supplemented with 10 % fetal bovine serum (Euroclone, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Euroclone, UK).

Sorting CD90+Huh7 cells

Huh-7 human hepatocellular carcinoma cells were stained with anti-CD90 PE (BD Pharmingen™ 555596), and surface marker was determined by flow cytometry. CD90+ and CD90- cells were sorted through a FACSAria I (BD Biosciences). A purity check was done after the sorting by re-running a small fraction of the sorted populations. All cells showed over 85 % purity.

Immunocytochemistry

Immunocytochemistry was done on PFA 4 % fixed cells, and stained with the following antibodies: the primary antibodies were anti-E-Cadherin (BD Biosciences 610181), anti-HNF4a (Abcam ab41898), and anti-Vimentin (Epitomics, 2707-1); the secondary antibodies were Alexa-Fluor 488 and Alexa-Fluor 594, from Molecular Probes. The nuclei were stained with NucRed® Live 647 (Catalog number: R37106, Life Technologies), and preparations were analyzed by confocal microscopy (Leica TSC SP8).

Exosome preparation and characterization

Huh7, CD90+ Huh7 and Sk-Hep cells were grown with 10 % ultracentrifugated FBS, and conditioned medium was collected 48 h after culture; exosomes were subsequently isolated by serial centrifugation [26]. Briefly, culture medium was centrifuged subsequently for 5 min at 300 × g, 15 min at 3,000 × g, 30 min at 10,000 × g and ultracentrifuged 90 min at 100,000 × g in a Type 70 Ti, fixed angle rotor. Pelleted exosomes were washed and then resuspended in PBS. Exosome protein content was determined with the Bradford assay (Pierce, Rockford, IL, USA). On average we recovered 10 micrograms of vesicles from 25 ml of conditioned medium from 3×10^6 cells. The intensity autocorrelation functions of diluted vesicle samples were measured by dynamic light scattering (DLS) using a Brookhaven Instruments BI-9000 correlator and a BI200-SM goniometer, equipped with a solid-state laser tuned at 532 nm. The size distribution was determined from the vesicle diffusion coefficients by standard analysis [52]. Thirty µg of protein for each sample, exosomes, and cells, were analyzed by western blot for Alix (3A9-Cell Signaling Technology #2171S), Tsg101 (Santa Cruz Biotechnology sc-7964) and HSC70 (Santa Cruz Biotechnology sc-7298).

Uptake of exosomes by HUVECs

Exosomes from Huh7, CD90+ Huh7 and SkHep cells were labeled with PKH26 according to supplier's instructions, suspended in low serum medium (5 µg/ml), and incubated with HUVECs for 1, 3, and 6 h at 4° or 37 °C. After incubation, cells were processed as previously described [26].

HUVECs treatment

HUVECs were grown at a density of 100.000cells/well in a 12 wells plate, and treated for 18 h with 5 µg/ml of exosomes in low serum medium; untreated cells were considered control. Plasmid for psiCHECK2-H19 and the Empty vector psiCHECK2 (kindly provided by Dr Y. Huang [45]), H19 siRNA (SR319206B Origene Technologies) and scramble negative control (SR30004 Origene Technologies) were transfected in HUVECs with Attractene Transfection Reagent (cat.number.1051531, Quiagen) following manufacturer's indications.

RNA extraction and real-time PCR

RNA was extracted using the commercially available illustra RNAspin Mini Isolation Kit (GE Healthcare), according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RT-QPCR was done in 48-well plates using the Step-One Real-Time PCR system (Applied Biosystem). Real-time PCR was performed in duplicates for each data

point. For sybr-green method the oligonucleotide used were *β-actin* for 5'-ATCAAGATCATTGCTCCTCCTGA-3' rev 5'CTGCTTGCTGATCCACATCTG-3'; H19 for 5'-GCACCTTGACATCTGGAGT-3' rev 5'-TTCTTTCCAG CCCTAGCTCA-3'; *VEGF* for 5'-CGAGGGCCTGGAGTG TGT-3' rev 5'-CGCATAATCTGCATGGTGATG-3'; *VEGF-R1* for 5'-CGGTCAACAAAGTCGGGAGA-3' rev 5'-CAG TGCACCACAAAGACACG-3'; *VE-CADHERIN* for 5'-GATCAAGTCAAGCGTGAGTCG-3' rev 5'-AGCCTCT CAATGGCGAACAC-3'. *VCAM1*, *ICAM*, *H19* and *β-actin* transcript levels were measured by TaqMan Real-Time PCR using the TaqMan gene expression assay: Hs00174239_m1, HS 00277001_m1, Hs00262142_g1 and Hs99999903_m1, respectively (Life Technologies). Changes in the target mRNA content relative to house-keeping were determined with the $\Delta\Delta\text{ct}$ Method.

Endothelial tube formation assay

HUVECs were seeded at 50,000 cells/well in growth factor-reduced Matrigel-coated 24 well plate and incubated up to 2 h at 37 °C. Tube formation was examined under an inverted microscope and photographed at 20× magnification. The length of the cables was measured manually with IMAGE-J software (<http://rsbweb.nih.gov/ij/>).

FACS analysis

Two hundred thousand (200,000) cells were washed in PBS and incubated with 0.5 µg ICAM-1-FITC (sc-107, Santa Cruz). Viable cells were gated by forward and side scatter, and analyzed on 100,000 acquired events for each sample. Samples were analyzed on a Partec CyFlow Space using the Partec FloMax® software.

Adhesion assay

In order to evaluate the ability of CD90+ Huh7 cells and SkHep cells to adhere to HUVECs, an adhesion assay was performed, as previously described [26].

ELISA

HUVEC conditioned medium was collected 18 h after exosome treatment or transfection with pH19 or pEmpty. VEGF concentrations were quantified using the ELISA kit (KHG0111, LifeTechnologies), according to manufacturer's protocol.

Array for long non-coding RNA

In order to study lncRNA expressed in the sorted population, a LncProfiler lncRNA qPCR array was performed (System Bioscience) on Huh7, CD90 + Huh7 cells and their exosomes following manufacturer's indications. After amplification, $\Delta\Delta\text{ct}$ of CD90 + Huh7 was normalized on $\Delta\Delta\text{ct}$ of Huh7, and data were expressed as fold induction of the sorted population compared with the parental cells.

Statistical analysis

In vitro experiments were repeated three times, giving reproducible results. Data are presented as mean values \pm standard deviation (SD) of three independent experiments. Statistical analysis was done using Prism 4 (GraphPad Software Inc., San Diego, CA, USA); one-way ANOVA (non-parametric) was performed, followed by Dunnett's multiple comparison test.

Additional file

Additional file 1: (a) Characterization of isolated exosomes.

Left panel: DSL for exosomes released by SKHep Middle panel: Western blot for Tsg101 and HSC70 in SKHep cells and their relative exosomes. Right panel: Confocal microscopy analysis on HUVECs treated for 1, 3 and 6 hours with 5 mg/ml of SKHep-derived exosomes. HUVECs were stained with phalloidin Alexa Fluor (green), nuclear counterstaining was performed using DAPI (blue), exosomes were labelled with PKH26 (red). **(b) Target analysis.** Real time-PCR analysis on HUVECs treated for 18 h with 5 mg/ml of SKHep-derived exosomes. Normalized for b-actin the DDct were indicated as fold of induction respect to control (untreated cells). ** $p < 0.05$. **(c) Tubulogenesis of HUVECs after exosomes treatment.** Matrigel assay performed on HUVECs cells after 18 hour of 5 mg/ml SKHep-derived exosomes. Left panel: phase contrast, magnification 20X. Right panel: quantification of matrigel assay expresses as length of cable as arbitrary unit ** $p < 0.01$. **(d) Adhesion assay of SKHep cells on HUVECs.** Left panel: phase contrast, magnification 10X. Right Panel: quantification of Huh7 or SKHep cells adherent on HUVECs, performed by counting the number of CD90+adherent cells (violet) per field *** $p < 0.001$. **(e) Analysis on H19 expression in exosomes.** Real time-PCR analysis for H19 expression performed on SKHep-derived exosomes respect to Huh7 derived exosomes. Normalized for b-actin the DDct were indicated as fold of induction. ** $p < 0.01$ **(f) Comparison of H19 expression in HUVECs after exosomes treatment.** Real time-PCR analysis for H19 expression on HUVECs treated for 18 h with 5 mg/ml of SKHep or Huh7 exosomes. Normalized for b-actin the DDct were indicated as fold of induction respect to control (untreated cells). (DOCX 855 kb)

Abbreviations

lncRNA: Long-non-coding RNA; HCC: Hepatocellular carcinoma; HUVEC: Human umbilical vein endothelial cell; CSC: Cancer stem cell; VEGF: Vascular endothelial growth factor; ICAM-1: Intercellular adhesion molecule-1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AC, RA, VC, ALD contributed to the conception and design of the study. AC, RA, VC, ALD, LS, SB, SR, MM, RA contributed to the generation, collection, assembly, analysis and/or interpretation of data. AC, RA, VC, ALD, MT, CM, FD, GDL, contributed to drafting or revision of the manuscript. All authors approved the final version of the manuscript.

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