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Original Article

Breast cancer-secreted miR-939 downregulates VE-cadherin and destroys the barrier function of endothelial monolayers

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ABSTRACT

Exosomes-secreted microRNAs play an important role in metastatic spread. During this process breast cancer cells acquire the ability to transmigrate through blood vessels by inducing changes in the endothelial barrier. We focused on miR-939 that is predicted to target VE-cadherin, a component of adherens junction involved in vessel permeability. By *in silico* analysis miR-939 was found highly expressed in the basal-like tumor subtypes and in our cohort of 63 triple-negative breast cancers (TNBCs) its expression significantly interacted with lymph node status in predicting disease-free survival probability. We demonstrated, *in vitro*, that miR-939 directly targets VE-cadherin leading to an increase in HUVECs monolayer permeability. MDA-MB-231 cells transfected with a miR-939 mimic, released miR-939 in exosomes that, once internalized in endothelial cells, favored trans-endothelial migration of MDA-MB-231-GFP cells by the disruption of the endothelial barrier. Notably, when up taken in endothelial cells exosomes caused VE-cadherin down-regulation specifically through miR-939 as we demonstrated by inhibiting miR-939 expression in exosomes-releasing TNBC cells.

Together, our data indentify an extracellular pro-tumorigenic role for tumor-derived, exosome-associated miR-939 that can explain its association with worse prognosis in TNBCs.

Introduction

Metastasis is the leading cause of mortality in cancer patients. In Europe, 10–20% of breast cancer (BC) patients relapse and develop distant metastatic disease after treatment [1,2]. The aggressiveness of metastatic BC underscores the need to elucidate the molecular mechanisms underlying the metastatic process in order to develop efficient treatment.

microRNAs (miRNAs) are small (19–22 nucleotides) non-coding RNAs able to suppress the translation of target mRNAs by binding to their 3′UTR and are critical regulators of cellular processes such as proliferation, differentiation, development and death, especially in cancer [3]. miRNAs can play a crucial role in modulating the tumor microenvironment by their internalization in exosomes and transfer to neighboring cells [4,5]. Exosomes are 30- to 100-nm membrane vesicles released by most cell types, and cancer patients are characterized by a higher number of blood circulating exosomes compared with healthy individuals [6,7]. The role of exosomes in mediating intercellular communication by interacting with recipient cells is well-established [8]: cancer cells release exosomes to transfer protein, soluble factors, RNAs and miRNAs to modulate the microenvironment [9–11] and alter vessel permeability [4]. Traversing the endothelial barrier is a crucial step in the metastatic process; indeed, this barrier regulates not only the passage of fluid and nutrients through the vascular wall, but also the transmigration of cells, such as leukocytes and tumor cells [12]. The loss of membrane-associated adhesion molecules such as cadherin 5 (CDH5, VE-cadherin) and zonula occludens 1 (ZO-1) on endothelial cells has been shown to favor the migration of invasive cancer cells [4,13,14].

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Due to increasing evidence of a role for miRNAs in mediating extracellular communication, we focused on miR-939, which is reported to be upmodulated in lung adenocarcinomas [15], hepatocellular carcinomas [16] and ovarian cancer tissues [17], and associated with prognosis in esophageal squamous cell carcinomas [18]. However, the precise function of miR-939 in breast cancer remains unclear. Here, we investigated the role of exosome-associated miR-939 in promoting blood vessel invasion by BC cells.

Materials and methods

Patient samples

RNA was extracted using the miRNeasy FFPE kit (Qiagen, Valencia, CA), from FFPE specimens of 63 TNBC patients diagnosed between 2002 and 2006 in our Institute (Fondazione IRCCS Istituto Nazionale dei Tumori) based on immunohistochemical criteria (<1% cell positivity for estrogen receptor, progesterone receptor and HER2 expression classified as 0 or 1+). All patients gave written consent to use of their FFPE specimens for future investigations and research purposes. All data were analyzed anonymously and all experiments were in compliance with the Helsinki Declaration. miR-939 was evaluated by qRT-PCR using the median value as cut-off to separate tumors into miR-939-positive and -negative groups.

Cell lines, transfection and actinomycin D treatment

All human BC cell lines and human embryonic kidney line HEK-293, purchased from ATCC (Rockville, MD, USA) were authenticated using the short tandem repeat profiling method in our Institute facility. For further information see Supplemental material and methods.

Primary human umbilical vascular endothelial cells (HUVECs) were harvested from the vein of human umbilical cords [19] and cultured for a maximum of 10 passages in MCDB131 medium supplemented with 20% FBS, 100 μg/ml ECGS (endothelial cell growth factor supplement, Sigma Aldrich, St. Louis, MO, USA), 100 μg/ml heparin (Sigma Aldrich), 1% penicillin/streptomycin and 1% L-glutamine on flasks pre-coated with 1% gelatin (Sigma Aldrich).

Cells were transfected with 100 or 50 nM of hsa-miR-939-5p mimic (miR-939) (Ambion®, Thermo Fisher Scientific) or miRCURY LNA™ microRNA inhibitor for hsa-miR-939-5p (LNA-939i) (Exiqon A/S, Vedbeak, Denmark), respectively, using RNAimax (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions.

To determine VE-cadherin mRNA stability, transfected HUVECs were treated with 1 μg/ml actinomycin D for 4, 8 and 24 h.

In silico analysis, plasmid construction and luciferase assay

To identify miR-939 targets, *in silico* analysis in the miRWalk 2.0 database was performed using DIANAmt, miRanda, miRDB, miRwalk and TargetScan 7.1 prediction tools.

A 986-bp region of the VE-cadherin (CDH5) 3′ untranslated region (3′UTR) including wild type or mutated hsa-miR-939-5p binding sites was cloned in the pmiR-GLO dual-luciferase miRNA target expression vector (Promega, Waltham, MA, USA) and a luciferase assay was performed.

A miR-939-5p Luciferase Reporter Vector (pmiR939-Reporter) (Signosis, Santa Clara, CA, USA) was used as reporter of miR-939 expression in cells. See Supplementary material and methods for detailed protocols.

Proliferation, migration and invasion assays

Detailed protocols can be found in Supplementary material and methods.

Isolation of exosomes and labeling

Exosomes were purified from 2.5−3 × 10^6 BC cells cultured for 48 and 72 h in medium supplemented with 10% exosome-depleted FBS prepared as previously described [20]. Exosomes were isolated using ExoQuick-TC exosome precipitation solution (System Biosciences, Mountain View, CA, USA) as described [5]. Exosomes obtained were dissolved in 60 μl of MCDB131, examined for protein concentration using the Bradford protein assay, and labeled with the green lipophilic fluorescent dye PKH67 (Sigma Aldrich) according to the manufacturer's instructions.

Tube formation, permeability and trans-endothelial migration assay

Tube formation assay was performed as described [21]. Permeability was assessed based on the passage of rhodamine B isothiocyanate-dextran (average MW 70,000; Sigma Aldrich) through HUVECs monolayer grown on 0.4-μm pore size filters (BD Bioscience) as reported [4]. Trans-endothelial invasion assay was performed as described [4]. See Supplementary material and methods.

Western blot and immunofluorescence analyses

The following antibodies were used for western blot analysis: polyclonal anti-human CD144 (VE-cadherin) (Cell Signaling, Danvers, MA, USA), 1:1000; anti-human CD63, clone H5C6 (Abcam, Cambridge, UK), 0.5 μg/mL, and anti-tubulin clone DM1A (Sigma Aldrich), 0.2 μg/mL. For the immunofluorescence analysis a polyclonal anti-human CD144 (VE-cadherin) (BMS158, eBioscience, San Diego, CA, USA), 6 μg/mL, was used. See Supplementary material and methods for detailed protocols.

RNA extraction and qRT-PCR analysis

RNA was extracted using QIAzol lysis reagent (Qiagen, Valencia, CA) according to manufacturer's instruction and quantified using the NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). For exosomes, a spike-in ath-miR-159a was added to QIAzol at a final concentration of 4 nM and used as internal control. miRNAs expression was analyzed using the Taqman microRNA reverse transcription kit and Taqman microRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Gene expression was analyzed using High Capacity RNA-to-cDNA kit (Applied Biosystems) and specific primers (Supplementary Table SI) for hsa-pri-miR-939 and CDH5. Detailed protocols in Supplementary material and methods.

Statistical analyses

Association between two categorical variables was tested by Fisher's exact test. Differences between means of independent groups were tested using unpaired Student's *t*-test.

An approach based on minimal-depth variable importance estimated by random survival forests [22] was used to select prognostic factors for disease-free survival (DFS). Adjusted hazard ratios (HRs) of prognostic factors together with 95% confidence intervals (CIs) were estimated by fitting multivariable Cox survival models. Synergy Index S was estimated as proposed in [23] equation (7). S > 1 indicated the presence of a synergistic interaction between two variables. Differences were considered significant at $P \leq 0.05$. Analyses were carried out using GraphPadPrism v5 (GraphPad software) and R 3.2.3 computing packages.

Results

miR-939 is expressed at higher levels in TNBC and associated with worse DFS

Analysis of the public available dataset *The Cancer Genome Atlas* (TCGA) showed a significantly higher expression of miR-939 in human BCs compared to normal mammary tissues (Fig. 1A). Furthermore, analysis of miR-939 expression levels in BC subtypes divided according to PAM50 classification [24] showed a significant up modulation of miR-939 in the highly aggressive basal-like tumor subgroup (Fig. 1B).

In our cohort of 63 TNBCs miR-939 expression revealed no association with clinicopathological variables (Supplementary Table SII). Permutation accuracy variable importance estimated by random survival forests [22] identified miR-939 expression as a prognostic factor for DFS, together with nodal involvement (N), multifocality and size (Supplementary Fig. S1A). Multivariate Cox survival analysis using the above prognostic covariates revealed a

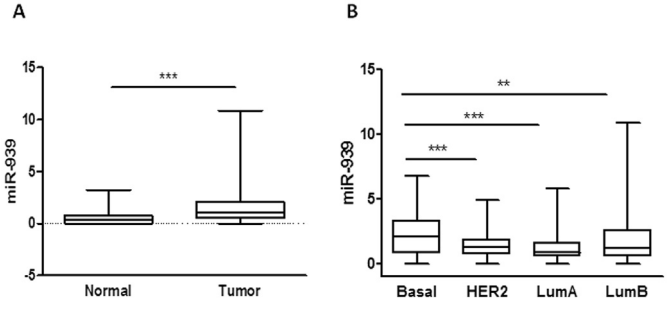

Fig. 1. miR-939 is expressed in human breast carcinomas. A, miR-939 expression in human breast carcinomas and normal mammary tissues in *The Cancer Genome Atlas* (TCGA) database. B, miR-939 expression in human breast carcinomas according to PAM50 classification. $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired Student's *t*-test.

significant interactions for DFS between miR-939 expression and N-status, with ~6-fold higher risk of relapse for patients with nodal involvement and tumors expressing high miR-939 levels compared to patients without nodal involvement and tumors with low miR-939 expression (Table 1 and Supplementary Fig. S1B). The Synergy index S evaluation [23] revealed that the interaction between miR-939 and N status is synergistic: S = 7.3 (95% CI = 1.77–30.09).

miR-939 directly targets VE-cadherin

MDA-MB-231 BC cells induced to overexpress miR-939 did not differ appreciably from non-induced cells with respect to proliferation, migration, or invasion ability (Supplementary Fig. S2). miRWalk analysis showed that miR-939 is predicted to target VE-cadherin, a key regulator of endothelial adherens junctions involved in the maintenance of contact between endothelial cells and in vessel permeability control. In addition, miR-939 showed the strongest Context++ score [25] among the eight miRNAs predicted to bind VE-cadherin 3′UTR by all prediction tools in miRWalk (Table 2).

To test whether miR-939 might play an extracellular role in disrupting endothelial junctions, HUVECs were transfected with miR-939 or a scramble (scr) oligonucleotide for 48 and 72 h and VE-cadherin modulation was assessed by Western blot and qRT-PCR. miR-939 overexpression, verified by qRT-PCR (Fig. 2A), induced a 40–60% reduction of VE-cadherin protein expression levels (Fig. 2B and C) but no significant reduction in VE-cadherin mRNA expression (Fig. 2D). Decay of VE-cadherin mRNA was similar in HUVECs transfected with miR-939, or scr, and treated with actinomycin D to inhibit mRNA new transcription (Supplementary Fig. S3A-B). These data indicate that miR-939 induces inhibition of mRNA translation but not its degradation.

To verify the direct regulation of VE-cadherin by miR-939, the 3′UTR of human *CDH5*, containing the three predicted miR-939 binding sites (Fig. 2E), was cloned into the reporter plasmid pmiR-GLO vector and a luciferase assay was performed. Luciferase activity in HEK-293 cells was reduced by 25% after miR-939 overexpression ($p = 0.019$) compared to scr (Fig. 2F). A similar reduction in luciferase activity was observed also in TNBCs cell lines (Supplementary Fig. S3C). The mutation of individual or all miR-939 binding sites in the CDH5-3′UTR prevented the down regulation of luciferase activity (Fig. 2F), consistent with the contribution of each miR-939 binding site to the downregulation of VE-cadherin expression.

Down-modulation of VE-cadherin by miR-939 decreases monolayer integrity of HUVECs

To further test the functional effect of miR-939 on endothelial cells, HUVECs transfected with miR-939 mimic or scr were analyzed by confocal microscopy for their ability to form a stable endothelial monolayer. Consistent with the adhesive role of VE-cadherin, the reduction of its expression in transfected HUVECs occurred at the junctional level with a loss of cell-cell contact (Fig. 3A). An *in vitro* assay to test whether the vascular damage induced by miR-939 impairs the vessel permeability showed that overexpression of miR-939 in these cells significantly increased the passage of rhodamine-labeled dextran probes (relative molecular mass 70 KDa) through HUVEC monolayers growing on 0.4-μm filters of a Boyden chamber compared to the negative control (Fig. 3B), indicating that miR-939 regulation of VE-cadherin expression impairs endothelial cell function.

Because VE-cadherin is known to be involved in the vasculogenesis of endothelial cells [26], the effect of miR-939 on vascular damage was evaluated in a tube formation assay. miR-939 overexpression for 72 h induced a clear inhibition of HUVECs vascular structure formation (Fig. 3C), down-modulating VE-cadherin protein expression as shown by Western blotting (Fig. 3D).

miR-939 is transferred to endothelial cells via exosomes

HUVECs cultured for 16 h with exosomes purified from the supernatant of MDA-MB-231 cells and labeled with PKH67 showed uptake and internalization of the exosomes, visualized as green vesicles in the cytoplasm (Supplementary Fig. S4A). Western blot analysis revealed the exosomal marker CD63 only in exosome lysates, while the cytoplasmic marker α-tubulin was present only in MDA-MB-231 cell lysates (Supplementary Fig. S4B), confirming these vesicles as exosomes [27]. In exosomes purified from MDA-MB-231 cells transfected for 48 h with mimic miRNA, levels of miR-939 were 150–200 times higher than in those derived from cells transfected with control (Fig. 4A). Treatment of HUVECs with exosomes containing miR-939 for 48 h induced an increase (4- to 5-fold, $p = 0.093$) of the cellular levels of mature miR-939 compared to levels in cells treated with control exosomes (Fig. 4B). By contrast, pri-miR-939 expression levels were the same in cells treated with miR-939 or control exosomes (Fig. 4C), indicating that the increased miR-939 found in HUVECs reflects exosome-mediated miRNA transfer and not induction of its transcription.

Exosome-associated miR-939 increases tumor cell trans-endothelial migration

Western blot analysis of VE-cadherin expression to assess consequences of miR-939 transfer from tumor cells to the endothelial monolayer revealed a 40% reduction of this protein in HUVECs at 48 h after treatment with miR-939-containing exosomes (Fig. 4D). In addition, confocal microscopy to assess endothelial junction integrity revealed a marked reduction of VE-cadherin and cell-cell contacts in HUVECs treated with miR-939-containing exosomes compared to controls (Fig. 4E). To further investigate the role of miR-939 in tumor progression, we simulated the step of metastatic

Table 1
miR-939 interacts with lymph node status in disease-free survival of TNBC primary tumors.

	HR[a]	95% C.I.	P-value
miR-939−/N−	1		
miR-939+/N−	1.59	0.37–6.77	0.533
miR-939−/N+	3.63	0.77–17.08	0.103
miR-939+/N+	5.81	1.41–23.39	0.015
Multifocality, yes	4.25	1.28–14.18	0.018
Size, >2 cm	2.84	0.93–8.64	0.066

[a] Adjusted hazard ratios from a Cox multivariate model.

Table 2
List of miRNAs targeting CDH5-3′UTR.

Gene	miRNA	Number of sites	Site type	Context ++ score[a]
CDH5	hsa-miR-939-5p	3	8mer	−0,25
CDH5	hsa-miR-762	3	8mer	−0,24
CDH5	hsa-miR-1343-5p	3	8mer	−0,23
CDH5	hsa-miR-1913	4	7mer-m8	−0,2
CDH5	hsa-miR-1254	3	7mer-a1	−0,2
CDH5	hsa-miR-324-3p	4	7mer-m8	−0,19
CDH5	hsa-miR-4254	3	7mer-m8	−0,17
CDH5	hsa-miR-1914-3p	3	7mer-m8	−0,1
CDH5	hsa-miR-3919	3	7mer-m8	−0,05

[a] Evaluated as in [25].

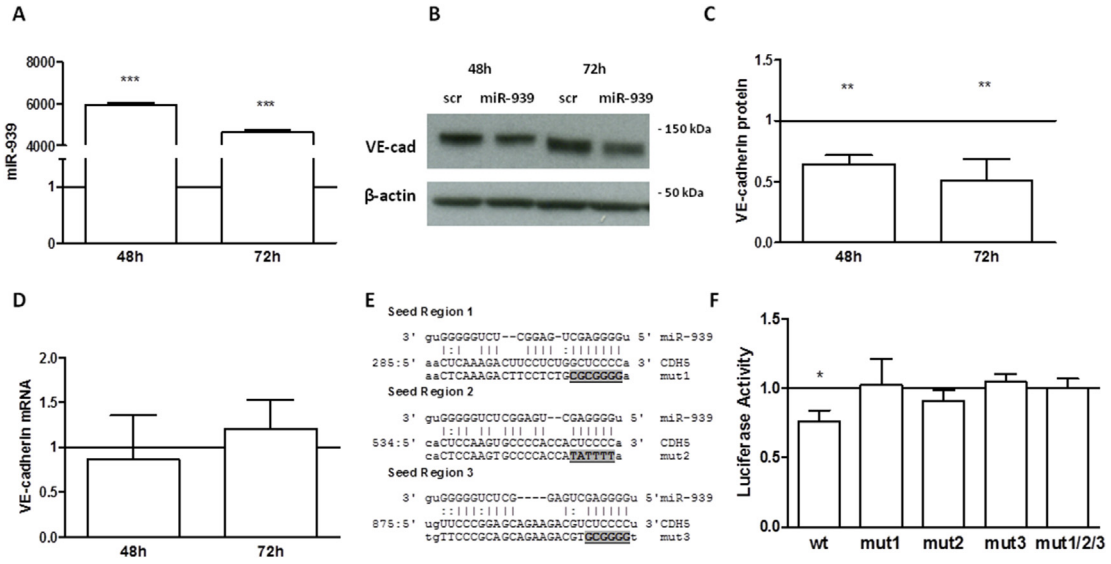

Fig. 2. miR-939 directly targets VE-cadherin. A, miR-939 expression in HUVECs transfected with 100 nM miR-939 mimic (miR-939) or scramble (scr) oligonucleotide as evaluated by qRT-PCR and normalized to RNU44. Data are shown as mean ± SD relative to the respective scr-transfected cells (set to 1), ($n = 2$). ***$P < 0.001$ by unpaired Student's t-test. B, representative Western blot of VE-cadherin in HUVECs transfected with miR-939 for 48 and 72 h. β-actin was used as protein loading control. C, Western blot quantification relative to VE-cadherin expression in scr-transfected cells. Data are mean ± SD ($n = 3$). **$P < 0.01$ by paired Student's t-test. D, qRT-PCR quantitation of VE-cadherin in HUVECs transfected with miR-939 for 48 and 72 h. Data are mean ± SD relative to the respective scr-transfected cells (48 h $n = 5$, 72 h $n = 3$). E, schematic representation of the interaction between miR-939 and the binding sites in wild-type CDH5-3′UTR. The 7-6 bp modified in the mutated forms (mut1-mut2-mut3) are in boldface. F, relative luciferase activity in HEK-293 cells transfected with miR-939 and wild-type (wt) or mutated CDH5-3′UTR. Data [mean ± SD ($n = 3$)] are shown as luciferase activity relative to the value obtained in the respective scr-transfected cells. *$P < 0.05$ by paired Student's t-test.

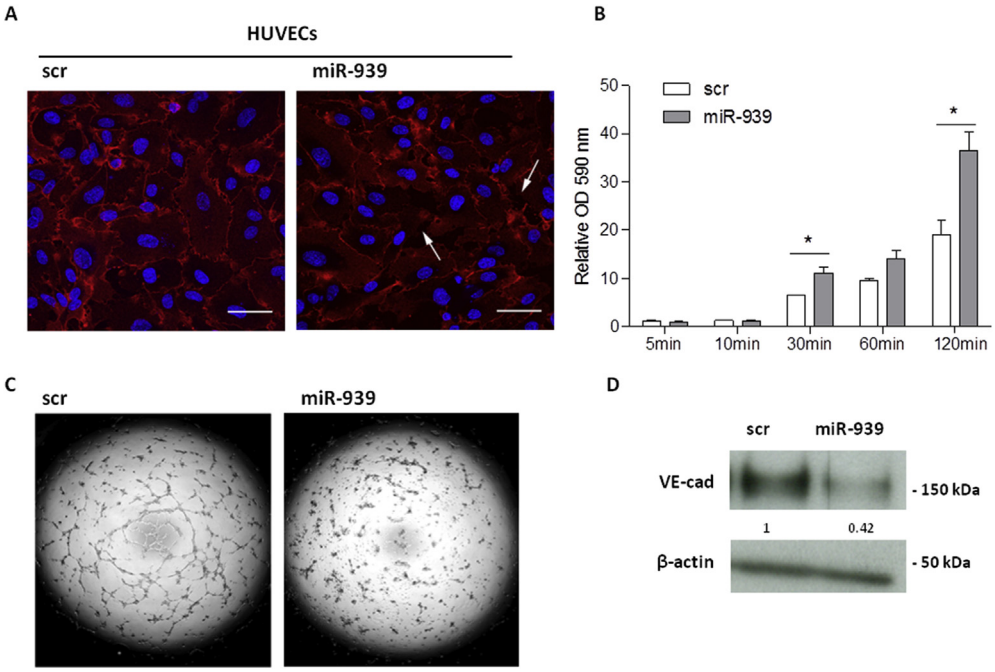

Fig. 3. miR-939 perturbs VE-cadherin activity in HUVECs. A, Confocal microscopy analysis of VE-cadherin (red) expression in HUVECs transfected with miR-939 or scr for 72 h. Nuclei were counterstained with DAPI (blue). Scale bars: 50 μm. Arrows indicate loss of cell—cell contact. Images are reported as extended depth-of-field and are representative of 3 independent experiments. B, Monolayer permeability of HUVECs transfected with miR-939 or scr evaluated based on absorbance (590 nm) in the lower chamber of a Boyden chamber with a HUVEC monolayer grown on a 0.4-μm filter. Data are mean ± SD (representative results of 2 experiments). *$P < 0.05$ by unpaired Student's t-test. C, Tube formation ability of HUVECs transfected with miR-939 or scr as evaluated by phase-contrast light microscopy (4X) (NIKON TE Eclipse-S). Results are representative of 2 independent experiments. D, Western blot of VE-cadherin in HUVECs at the time of tube formation assay. β-actin was used as protein loading control.

traversal of the endothelial barrier by plating HUVECs on a Transwell filter to form a monolayer and treating them with exosomes containing miR-939; after 48 h, MDA-MB-231-GFP (231-GFP) cells were seeded on the endothelial cells and left to migrate for 6 h. Analysis of trans-endothelial migration of cancer cells revealed a significant increase in the number of 231-GFP cells transmigrating through the endothelial monolayer treated with miR-939-containing exosomes compared to controls (Fig. 4F).

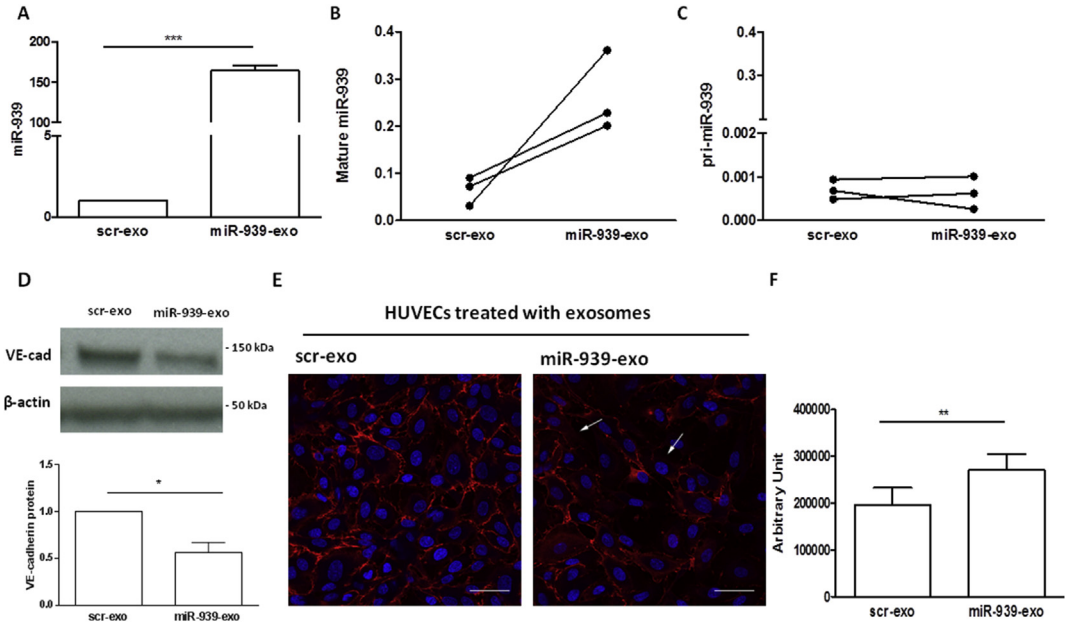

Fig. 4. miR-939 transfer increases trans-endothelial migration of TNBC cells by interfering with endothelial barrier integrity. A, Quantification of miR-939 expression by qRT-PCR in exosomes derived from MDA-MB-231 cells transfected with miR-939 or scr. miR-939 was normalized to the spike-in ath-miR-159a as reference. Data are mean ± SD (representative results of 2 experiments). $^{***}P < 0.001$ by unpaired Student's *t*-test. B and C, miR-939 and pri-miR-939 expression, respectively, in HUVECs as evaluated by qRT-PCR 48 h after treatment with exosomes. miR-939 and pri-miR-939 expression was normalized to RNU44 and GAPDH, respectively. Data are from 3 independent experiments. D, Representative Western blot of VE-cadherin in HUVECs treated for 48 h with exosomes derived from transfected MDA-MB-231 cells. β-actin was used as protein loading control. Data were quantified and expressed as relative amount ($n = 3$). E, Confocal microscopy analysis of VE-cadherin (red) expression in HUVECs treated for 48 h with miR-939-containing exosomes. Nuclei were counterstained with DAPI (blue). Images, representative of 3 independent experiments, were acquired in a single plane. Scale bars: 50 μm. Arrows indicate loss of cell-cell contact. F, Trans-endothelial migration of MDA-MB-231-GFP cells through HUVEC monolayers treated with miR-939-containing exosomes. MDA-MB-231-GFP was quantified using Image-Pro Plus 7.0.1 software by measuring the green fluorescence area on the bottom of the Transwell in each field. At least 3 fields were randomly selected. Data are mean ± SD ($n = 3$). $^{**}P < 0.01$ by paired Student's *t*-test.

TNBC cells regulate VE-cadherin in endothelial cells through exosome-associated miR-939

To test whether TNBC cell exosomes contain miR-939, we purified exosomes from supernatants of the TNBC cell lines MDA-MB-231, MDA-MB-468 and SUM149. As shown in Fig. 5, miR-939 is expressed both intracellularly (A) and in exosomes (B). To assess the ability of TNBC cell exosomes to regulate VE-cadherin in endothelial cells, HUVECs were treated for 24 h with 20 μg of purified exosomes and, consistent with previous results, VE-cadherin was down-regulated compared to untreated cells (Fig. 5C and Supplementary Fig. S5A). To confirm the direct involvement of miR-939 in down-modulating VE-cadherin, a specific mercury LNA™-miR-939-inhibitor (LNA939i) or a negative control (LNAneg) were transfected in MDA-MB-231, MDA-MB-468 and SUM149 cells (Supplementary Fig. S5B) and after 72 h, supernatants were collected to purify exosomes. Western blot analysis revealed the expected VE-cadherin reduction in HUVECs treated with exosomes from cells transfected with LNAneg as compared to untreated HUVECs (NT). Differently, HUVECs receiving LNA-939i-exosomes from MDA-MB-231 and SUM149 showed an increased VE-cadherin expression when compared to exo-LNAneg treated cells but not when compared to NT (Fig. 5D and Supplementary Fig. S5C). These data indicate that miR-939 is the main responsible for VE-cadherin protein regulation in HUVECs.

Discussion

In this study, we find that miR-939 is upregulated in human BCs, particularly in the most aggressive basal-like subtypes. Analysis of miR-939 expression in a cohort of 63 TNBC patients identified miR-939 as a negative prognostic factor for DFS supporting its pro-tumorigenic properties in this highly aggressive subtype. Indeed, lymph node-positive tumors overexpressing miR-939 have an increased risk of relapse as compared with those with lower levels of miR-939 and lacking lymph node involvement. These data suggest that while miR-939 alone is not sufficient to induce metastases, its activity contributes to the metastatic process in tumor cells able to disseminate to lymph nodes.

Unlike ovarian cancer cells [17], overexpression of miR-939 in MDA-MB-231 cells did not increase their proliferation and migration ability. Instead, proliferation was slightly decreased, consistent with the reported association of this miRNA to apoptosis [28]. Even though we cannot exclude a prometastatic effect of intracellular miR-939 in TNBC, our *in silico* analysis identifying VE-cadherin as a miR-939 target and our *in vitro* data support its role in cancer progression through the modulation of the surrounding microenvironment. Despite the association of miR-939 prognostic impact with its ability to increase intra-/extravasation awaits quantitation of circulating miR-939 in BC patients, an extracellular role for miR-939 is consistent with its presence in the blood of patients with lung [15] and hepatocarcinomas [16] and in patients with chronic neuropathic disorders [29].

Further, our finding that miR-939 overexpression in endothelial cells led to decreased VE-cadherin protein but not mRNA expression indicates that the mechanism exploited by miR-939 for target regulation is the inhibition of mRNA translation instead of mRNA degradation.

In addition to the validation of VE-cadherin as a miR-939 target by luciferase assay, our confocal microscopy analysis showed that disruption of VE-cadherin induces loss of contact between cells, consistent with the decreased ability of HUVECs to form tubules upon loss of VE-cadherin [30]. Tumor cells disseminating in the organism may exploit the impairment of the adherens junctions to

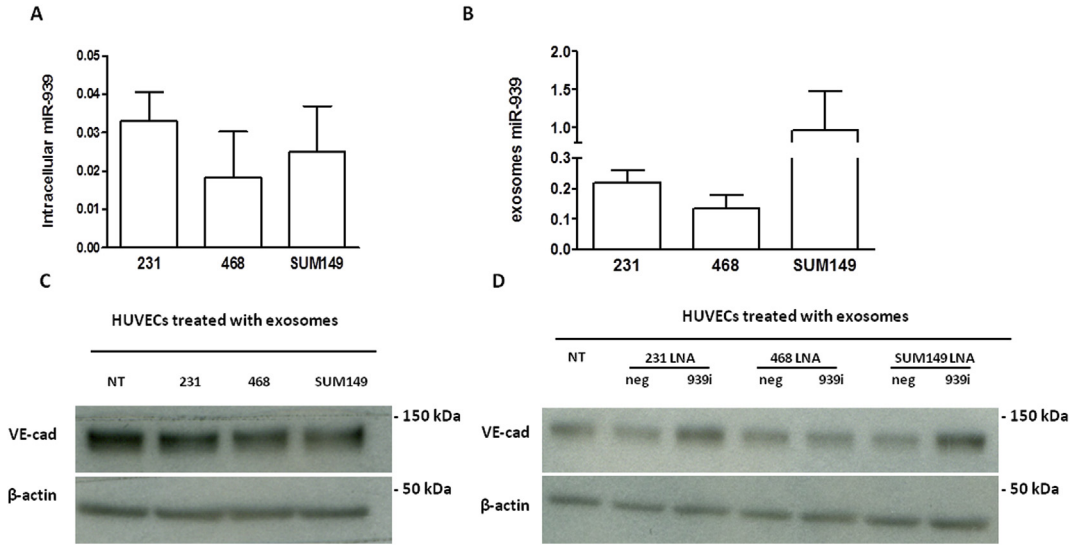

Fig. 5. miR-939 is expressed and released in exosomes of TNBC cell lines and its uptake in HUVECs downmodulates VE-cadherin. A and B, Intracellular and exosomal miR-939 expression levels, in MDA-MB-231, MDA-MB-468 and SUM149 cells as evaluated by qRT-PCR. U44 and miR-21 were respectively used for miR-939 normalization. Data are mean ± SD ($n = 2$). C and D, Western blot of VE-cadherin in HUVECs treated or not (NT) for 24 h with exosomes derived from BC cell wild-type (C) and from cells transfected with LNA-939 inhibitor (D). β-actin was used as protein loading control. Results are representative of 3 experiments.

enter into the vessels and spread to secondary sites. Our results suggest that miR-939 secretion is sufficient to open "gates" in tumor blood vessels for the traversal of cancer cells favoring hematogenous tumor spread. In early-stage metastatic BC, tumor cells disseminate through both lymphatic and hematogenous systems, although the factors determining the route remain unknown [31]. Since TNBC patients are characterized by capillary invasion more than lymphatic invasion [32], miR-939 expression could be of particular importance for tumor cells to acquire the capacity to metastasize to distant sites. However, the evidence that VE-cadherin is also present in lymphatic vessels, particularly in terminal lymphatic endothelial cells [33], raises the possibility that tumor cells exploit this mechanism to enter also lymphatic vessels.

Consistently, cancer-secreted miRNAs are increasingly recognized as mediators of cancer-endothelial cell crosstalk: tumor-derived exosome-mediated transfer of miR-105 to endothelial monolayers efficiently destroys tight junctions and the integrity of this natural barrier against metastasis [4]. Although it remains unclear whether miRNAs exist in the systemic circulation predominantly free, associated to proteins [34], or are transported via tumor-secreted microvesicles such as exosomes [35], there is evidence that exosome-associated miRNAs are more stable and thus more functional in modulating microenvironmental cell functions [36]. Indeed Zhou et al. [4] showed that only miRNAs internalized in exosomes can be up taken by recipient cells to exert their function. For this reason we only focused on miR-939-associated exosomes, even though miR-939 is reported equally distributed in exosomes and in serum [16]. The miR-939 sequence contains the loading motif recognized by the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNAB1) [37], indicating that it is actively loaded into exosomes. Thus, it is possible that tumor cells purposely release miR-939 into exosomes to modulate VE-cadherin expression in order to locally and transiently disrupt the barrier function of endothelial cells that normally express low miR-939 levels. Indeed, tumor-derived exosome-mediated transfer of miR-939 caused a reduction in VE-cadherin expression in the endothelial cell creating gap in the endothelial monolayer, as observed by transfecting miR-939 in HUVECs. Furthermore, miR-939 transfer into endothelial cells led to an increase in TNBC cells trans-endothelial migration due to the weakening of the endothelial barrier, as demonstrated in our trans-endothelial migration assay. Since monolayer integrity is maintained by adherens junctions through VE-cadherin but also by tight junctions that contain ZO-1, which is reportedly down-regulated via BC exosomes [4], we performed these experiments with exosomes from MDA-MB-231 cells transfected with miR-939 mimic in order to focus specifically on the effects caused by this miRNA.

Analysis of TNBC cell lines showed that these cells endogenously express and release sufficient miR-939 in exosomes to down-modulate VE-cadherin when taken up in HUVECs. Furthermore, the blockade of miR-939 with specific miRNA-LNA inhibitor in exosome-releasing MDA-MB-231 and SUM149 cells did not down-modulate VE-cadherin in HUVECs compared to controls. Thus, the regulation of VE-cadherin can be ascribed to miR-939 in TNBC cells that release high amount of this miRNA.

Together, our data demonstrate an association between miR-939 expression and worse prognosis in TNBC, likely due to its role in the extracellular milieu, where it increases cell trans-migration through the endothelial barrier by disrupting contact between endothelial cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.09.013.

Conflicts of interest

The authors declare no conflicts of interest.

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