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Angiogenesis is promoted by hypoxic cervical carcinoma-derived extracellular vesicles depending on the endothelial cell environment

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ABSTRACT

Introduction: Cancer needs perfusion for its growth and metastasis. Cancer cell-derived extracellular vesicles (CA-EVs) alter the tumor microenvironment (TME), potentially promoting angiogenesis. We hypothesize that conditions in the tumor, e.g., hypoxia, and in the target cells of the TME, e.g., nutrient deprivation or extracellular matrix, can affect the angiogenic potential of CA-EVs, which would contribute to explaining the regulation of tumor vascularization and its influence on cancer growth and metastasis.
Methods: CA-EVs were isolated and characterized from cervical carcinoma cell lines HeLa and SiHa cultured under normoxia and hypoxia, and their angiogenic potential was evaluated in vitro in three endothelial cells (ECs) lines and aortic rings, cultured in basal (growth factor-reduced) or complete medium.
Results: Hypoxia increased EV production 10–100 times and protein content 2–4 times compared to normoxic CA-EVs. HeLa-EVs contained six times more RNA than SiHa-EVs, and this concentration was not affected by hypoxia. Treatment with CA-EVs increased tube formation and sprouting in ECs and aortic rings cultured in basal medium and long-term stabilized the stablished vascular networks formed by ECs cultured in complete medium.
Conclusion: Hypoxia differentially affects CA-EVs in a cell line-dependent manner. The cellular environment (nutrient availability and extracellular matrix scaffold) influences the effect of CA-EV on the angiogenic potential of ECs.

1. Introduction

Cancer is a leading cause of disease-related death worldwide. Interactions between the tumor and the host promote tumor cells' growth, survival, angiogenesis, and metastasis [1]. During tumor growth, the hypoxic environment induces upregulation of molecules such as hypoxia-inducible transcription factor alpha (HIF-1α) and vascular endothelial growth factor (VEGF) [2,3]. Such factors activate the nearby vascular wall, particularly endothelial cells (ECs), which transiently specialize into tip, stalk, and phalanx cells to migrate, proliferate, or stabilize the new vessel [4]. A gradient of growth factors carefully regulates this ECs specialization during angiogenesis to ensure the appropriate proportion of ECs subpopulations to build a functional mature vessel [5]. The biochemical composition and mechanical properties (e.g., stiffness) of the extracellular matrix (ECM) influence this process [6]. Therefore, it makes sense to evaluate angiogenesis in ECM derived from the tumor basement membrane (Matrigel or Geltrex) and in natural-derived ECM such as from organs.

The pathological deregulation of the molecular gradients causes an imbalance in the proportion of these cellular subpopulations. Tumor vessels have impaired maturation and leak, which is convenient for a growing tumor because it provides “efficient” nutrients but little oxygen. This correlates with the metabolic profile of tumor cells, intratumoral ECs, and angiogenic tip cells, which are highly glycolytic and less

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efficient in oxidative phosphorylation to generate ATP [7,8]. This process is accompanied by loss of ECs markers, such as surface-expressed platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and von Willebrand factor (vWF) [9], with alteration of essential homeostatic functions for ECs, such as cell signaling, adhesion, leukocyte diapedesis and barrier function [10,11].

Extracellular vesicles (EVs) are membrane bags produced by all living cells; they carry a plethora of lipids, polysaccharides, proteins, and nucleic acids, which act as endocrine or paracrine signals, crucial in angiogenesis, tumor growth, and metastasis [12]. Tetraspanins such as CD9 and CD63 are involved in their biogenesis [13] and serve as EV markers [14]. A hypoxic tumor environment is likely to alter the secretion of EVs and enrich their cargo with proangiogenic proteins (i.e., IL8, IGFB, VEGF, FGF) and miRNAs to stimulate tumor perfusion, promoting growth and metastasis [15,16].

Generating knowledge about the regulatory mechanisms of vascularization can contribute to a better understanding of the pathophysiology involved and eventually propose new molecular treatment points. We hypothesize that cancer cell-derived EVs (CA-EVs) generate a proangiogenic environment enhanced by hypoxia, influenced by the growth factors availability and ECM type. To test it, we isolated CA-EVs from HeLa and SiHa cells cultured under normoxic and hypoxic conditions; after EVs characterization and uptake test by ECs, we evaluated their angiogenic potential in 2D (tube formation assay in HUVEC,

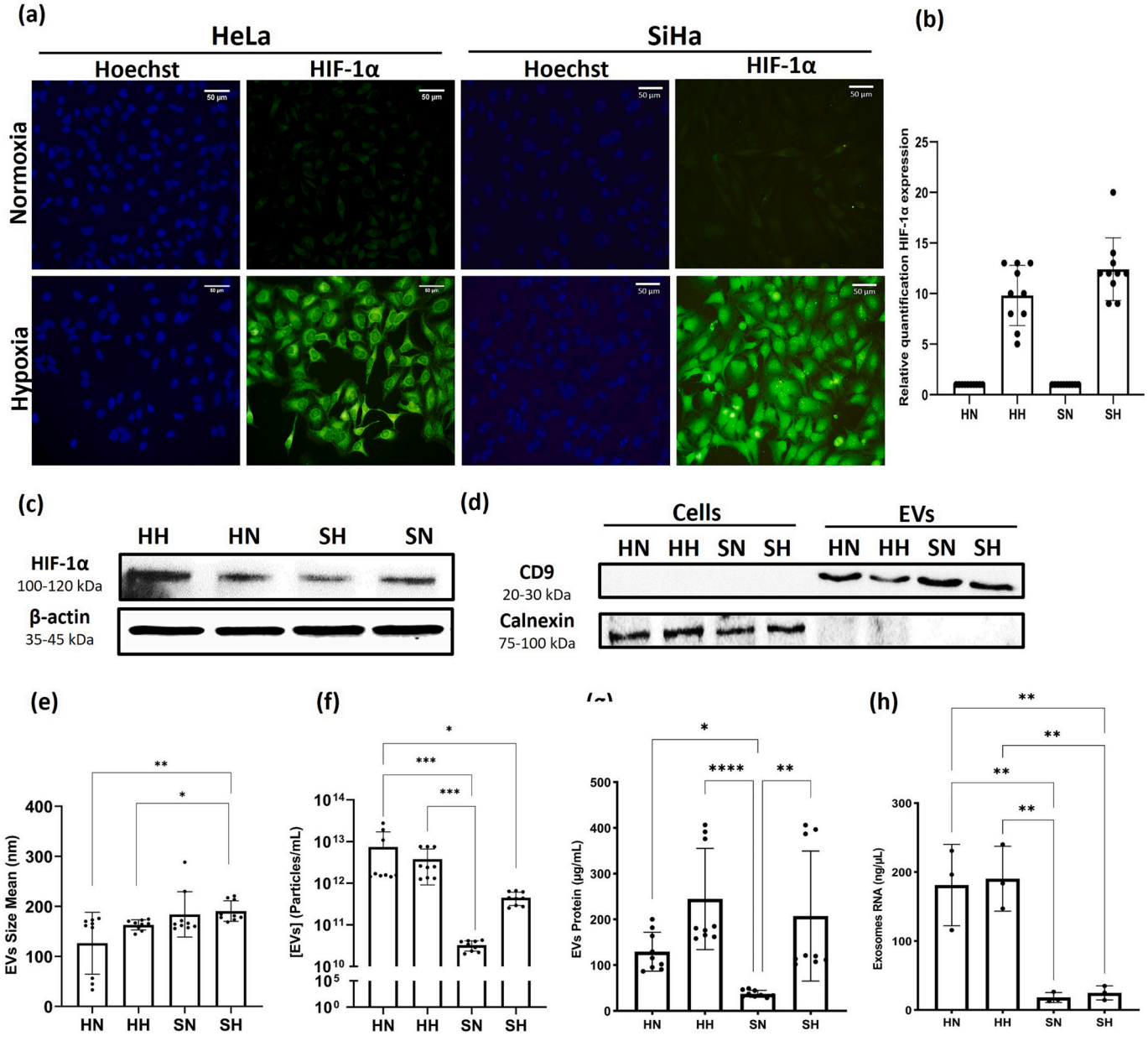

Fig. 1. Characterization of the CA-EVs. (a) Representative immunofluorescence of HIF-1α (green) in HeLa and SiHa cells cultured in normoxia and hypoxia for 24 h. Nuclei stained with Hoechst (blue). n = 5 in triplicate. Scale bar 50 µm (b) Relative fluorescence quantification of HIF-1α data shown in (a). Data represents mean integrated density values of corrected total cell fluorescence ± s.d. n = 5 in triplicate. (c) Immunoblotting confirmed the increase in HIF-1α expression in HeLa and SiHa after culture in hypoxia. (d) Immunoblotting of CD9 and calnexin in cells or isolated EVs. The EVs (e) mean size and (f) particle concentration was estimated using NTA. n = 3, five replicate captures of 60 s/sample. Kruskal Wallis tests Multiple comparisons. (g) EV protein concentration (microBCA). Kruskal Wallis tests Multiple comparisons. (h) EVs RNA concentration. One-way ANOVA analysis. All results are representative of at least n = 3 in triplicate, and each value is the mean ± s.d. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. HN, HeLa Normoxia; HH, HeLa Hypoxia; SN, SiHa Normoxia; SH, SiHa Hypoxia derived EVs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

HMEC-1, and HPMEC) and 3D (aorta sprouting assay) models. The growth factors availability and ECM influence on the vascular network formation were tested.

2. Materials and methods

The complete Material and Method can be found in Appendix 1. Supplementary Materials and Methods. In short, the exosomes were isolated from HeLa and SiHa cells, characterized by performing Flow Cytometry and EVs uptake assay. Rat aortas were used for ex vivo aorta sprouting assay.

3. Results

3.1. Hypoxia and serum deprivation do not affect carcinoma cell lines morphology and viability

Hypoxia and/or serum deprivation for 24 h caused no alteration in cell morphology (Appendix 2: Supplementary Figures, SF. 2a), size (FSC values), or granularity (SSC values) (SF. 2b) in cervical carcinoma cell lines HeLa and SiHa. The apoptosis ratio, i.e., the sum of early apoptosis ratio (Q3) and late apoptosis ratio (Q2), was lower than 3% for all experimental groups. After 24 h of culture, apoptosis and necrosis percentages did not differ between all experimental groups, with 96–98% of non-apoptotic/necrotic cells (Q4) for all treatments (SF. 2c). For both HeLa and SiHa, there was an about sixfold increase in the expression of HIF-1α in hypoxic compared to normoxic culture conditions (Fig. 1a-c).

3.2. Hypoxia differentially affects some EV characteristics according to the cell line

The expression of CD9 in EVs derived from both cell lines was not affected by hypoxia. In contrast, the cellular marker calnexin was only expressed in the cell lysates (Fig. 1d). HeLa and SiHa-EVs assessed by atomic force microscopy showed the typical morphological characteristics of small EVs, i.e., exosomes (SF. 3), appearing as nanovesicles with a condensed core [17]. Although both HeLa and SiHa-derived EVs fell within the 30–200 nm size range (Fig. 1e), the mean size of SiHa hypoxia-derived EVs (SH-EVs) was larger than HeLa normoxia-derived EVs (HN-EVs; $p < 0.01$) and HeLa hypoxia-derived EVs (HH-EVs; $p < 0.05$) (Fig. 1e). That SiHa-EVs are larger was further confirmed when analyzing the mode of the EVs size (SF. 3). No differences in size distribution within the same cell line in normoxia or hypoxia were observed (SF. 3, Fig. 1e).

The EVs normalization and quantification, i.e., particle number, protein, and RNA, are described in materials and methods. The EVs' concentration was not altered by hypoxia in HeLa-EVs (~10 [12]/mL) (Fig. 1f). In contrast, the concentration of SiHa-EVs in normoxia was about one order of magnitude lower than in hypoxia (~10 [10]/mL and ~10 [11]/mL, respectively) (Fig. 1f). SiHa cells produced 10–100-fold fewer EVs than HeLa irrespective of oxygen tension ($p < 0.05$) (Fig. 1f). Hypoxia almost doubled the protein content in HeLa-EVs compared to normoxia (~244 μg/mL vs ~129 μg/mL) (Fig. 1i). The EVs derived from normoxic SiHa (SN-EVs) showed the lowest protein concentrations (~37 μg/mL) compared to hypoxic SiHa (~207 μg/mL, $p < 0.01$), and all HeLa-EVs (HN-EVs, $p < 0.05$ and HH-EVs, $p < 0.01$) (Fig. 1g). Interestingly, the RNA concentration of EVs was not affected by the hypoxia treatment in both cell lines (Fig. 1h). For HN-EVs, the RNA concentration was 181 ng/μL, and for HH-EVs was 190 ng/μL (ns); for SN-EVs was 18 ng/μL and for SH-EVs was 25 ng/μL (ns) (Fig. 1j). The RNA concentration in SiHa-EVs was approximately tenfold lower compared to HeLa-EVs ($p < 0.01$) (Fig. 1h). These results show that external disturbances such as hypoxia can alter EVs' release and protein concentration in a cell line-dependent fashion. However, differences in some characteristics of EVs may be inherent to the cell type.

3.3. EVs are internalized by endothelial cells

The uptake by HUVECs of DiI-labelled CA-EVs was investigated by quantification of fluorescence intensity using fluorescence microscopy (SF. 4a) and flow cytometry (SF. 4b). To verify that the detected signal was from internalized and not from surface-bound fluorescent EVs, the surface-bound were stripped by treatment with trypsin after 1 h incubation at 4 °C. The fluorescent signal in HUVECs incubated with DiI-EVs at 4 °C (0 h) was close to the background. After incubation with DiI-labelled EVs, the total mean fluorescence intensity (MFI, geometric mean) of HUVEC increased from the normalized value 1 at 4 °C (0 h) to 490 ± 122 for HN-EVs, 1057 ± 145 for HH-EVs, 971 ± 178 for SN-EVs and 846 ± 111 for SH-EVs after 24 h as more stained EVs were internalized (SF. 4c). Judging by the intensity/cell, the HUVECs uptake of HH-EVs and SN-EVs was more efficient compared to HN-EVs uptake ($p < 0.05$) (SF. 4c). The cytoplasmic localization (not only membrane-bound) of EVs was corroborated by fluorescence microscopy (SF. 4). FACS results confirmed a similar EV uptake observed in both cell types regardless of oxygen tension. >98% of cells took up the EVs within 24 h with no significant differences ($p > 0.05$) (SF. 4 d). Both results showed efficient and time-dependent HeLa and SiHa-derived EVs internalization by HUVEC.

3.4. Carcinoma cell-derived EVs promote angiogenesis in vitro

The tube formation capacity of HUVEC was virtually absent when grown in basal medium compared to complete medium, considered a proangiogenic control (Fig. 2a). The EV treatment rescued the tube formation capacity of the ECs grown in basal medium (Fig. 2a). The total length and number of branches in the cells grown in the basal medium increased at least twofold upon treatment with HH-EVs ($p < 0.0001$) and HN-EVs ($p < 0.01$) (Fig. 2b, c). Although increased, the total branch length between cells treated with SiHa-EVs in normoxia or hypoxia did not reach significance (Fig. 2b). However, an increase in the number of branches of cells treated with SiHa-EVs in normoxia and hypoxia was found (Fig. 2c). These results can probably be ascribed to an increase in cell viability favored by the EVs, reflected by the EVs-associated increased mitochondrial activity as measured by MTT conversion in HUVEC compared to basal untreated cells (Fig. 2d). Compared to the complete medium, the mitochondrial activity in a basal medium decreased to 27% after 24 h of treatment. Cells grown in basal medium treated with CA-EVs showed an increased mitochondrial activity by >100% compared to non-EVs treated HUVECs ($p < 0.01$) (Fig. 2d). HeLa-EVs treatment increased the HUVEC mitochondrial activity irrespective of hypoxic preconditioning (ns). In contrast, HUVECs treated with SN-EVs increased by 15% the mitochondrial activity when compared to SH-EVs ($p < 0.05$) (Fig. 2d).

3.5. Carcinoma cell-derived EVs stabilize the sprouting over time

The CA-EVs treatment did not increase but stabilized, the sprouting in several EC types growing in complete medium (Fig. 3). Total branching length and total segment length of branches on HUVEC (Fig. 3a, b), HMEC-1 (Fig. 3c, d) and HPMEC (Fig. 3e, f) treated with the EVs did not differ from non-treated controls until 24 h. At 48 h, the tubes started to collapse in all non-EV treated cells and were almost non-detectable at 72 h (Fig. 3a-f). Although the treatments with EVs did not enhance the tube formation in cells grown in complete medium, they noticeably stabilized the tubes in all EC types, allowing them to remain intact until 48 h in HPMEC and until 72 h in HUVEC and HMEC-1 (Fig. 3a-f).

3.6. CA-EVs treatment decreases the expression of endothelial markers

We investigated whether the CA-EVs treatment might be linked to vWF or PECAM-1 expression. The endothelial markers vWF and PECAM-

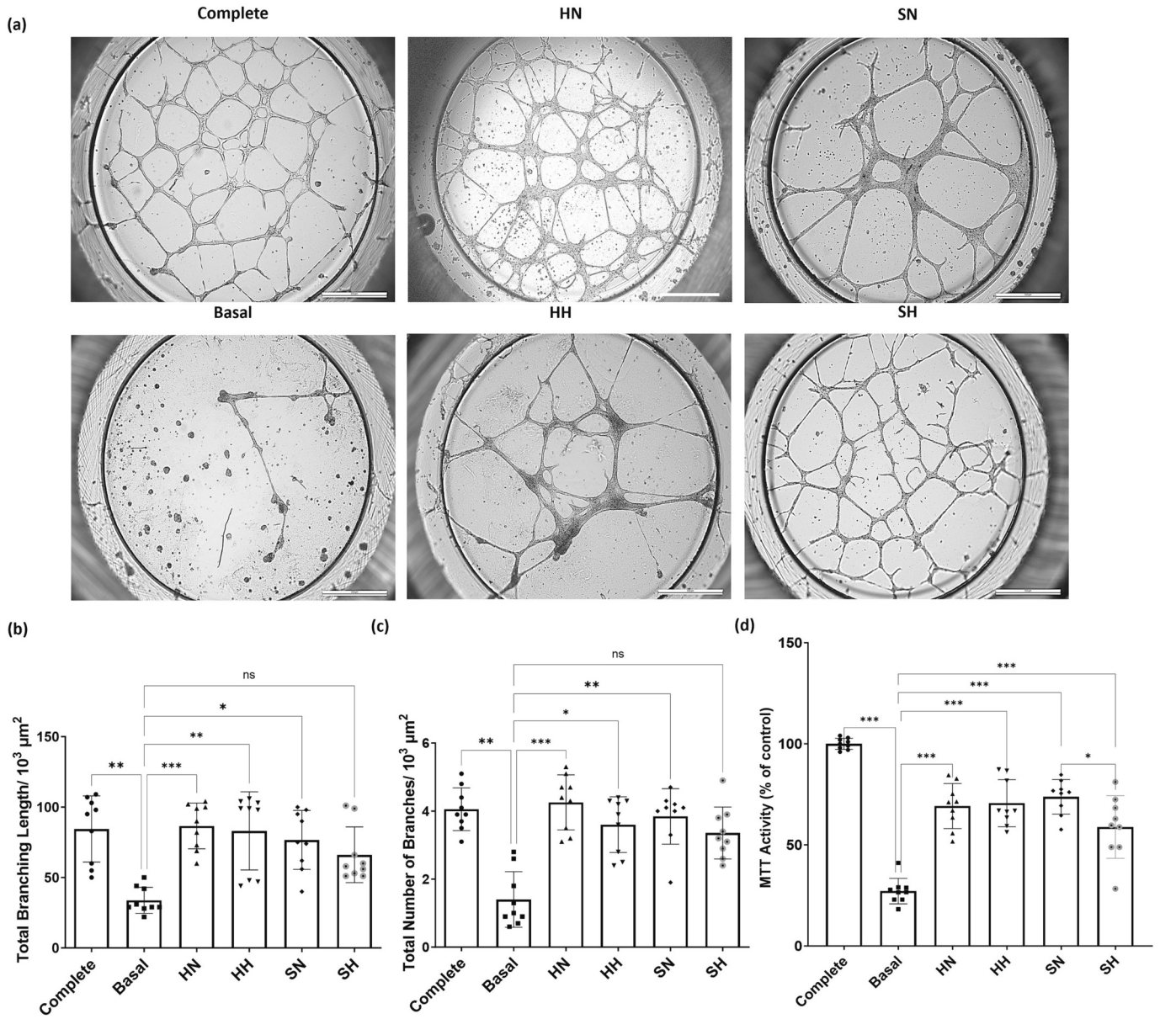

Fig. 2. CA-EVs increased the pro-angiogenic ability and mitochondrial activity of ECs cells in basal conditions. (a) HUVEC cells were treated for 24 h with CA-EVs (1000 particles/cell) and evaluated for tube formation after 24 h growing in complete (upper left panel) or basal (other panels) medium. Calibration bar 500 µm. (b) The total length and (c) the number of branches demonstrate the clear rescuing effect of EVs on the tube formation ability of the cells. (d) Increased mitochondrial activity, as assessed through the MTT assay. All results are representative of at least n = 3 in triplicate, and each value is the mean ± s.d. Two-way ANOVA test with Tuckey's post-test Multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

1 were downregulated in HUVECs incubated for 24 h with CA-EVs (Fig. 4a). HUVECs grown in the complete and basal medium had a comparable expression of these markers (Fig. 4b-c). vWF fluorescence decreased after 24 h of EV treatment, irrespective of cell source or hypoxic preconditioning ($p < 0.001$). Treatment with HH-EVs induced a larger reduction in vWF levels compared to HN-EVs ($p < 0.01$). Treatment with SiHa-EVs induced a similar reduction in vWF levels independent of oxygen tension (Fig. 4b). Conversely, PECAM-1 was differently affected by EV treatment. Levels of PECAM-1 were similarly decreased after 24 h of HeLa-EVs treatment, irrespective of the oxygen tension ($p < 0.001$). Interestingly, the treatment with SN-EVs slightly decreased the PECAM-1 expression ($p < 0.05$), and SH-EVs did not alter the PECAM-1 level compared to the controls (Fig. 4c).

3.7. Carcinoma cell-derived EVs promote angiogenesis ex vivo

Aortic rings embedded in Geltrex or dECM after 5 (data not shown) and 10 days of culture had sprouted and were viable, as demonstrated by the positive Calcein AM staining and absence of PI-staining (Fig. 5a). CA-EVs increased the number of sprouts compared to non-EV treated aortic rings grown in basal medium (Fig. 5a). This increase in the sprouting capacity was evidenced by a 3–4-fold increase in the vessel length, total number of junctions and number of endpoints in aorta rings treated with HH-EVs and SN-EVs ($p < 0.01$) (Fig. 5b-d). Lacunarity (measurement of gaps sizes in the networks) was increased in aorta rings in basal medium. The increase in all the branching parameters was correlated to a reduction in the lacunarity in aorta rings treated with all CA-EVs ($p < 0.05$) (Fig. 5e). After skeletonization, morphological analysis of aorta rings showed that the sprouting topology varied depending on the type of matrix, in which dECM hydrogels were stiffer and richer in collagen

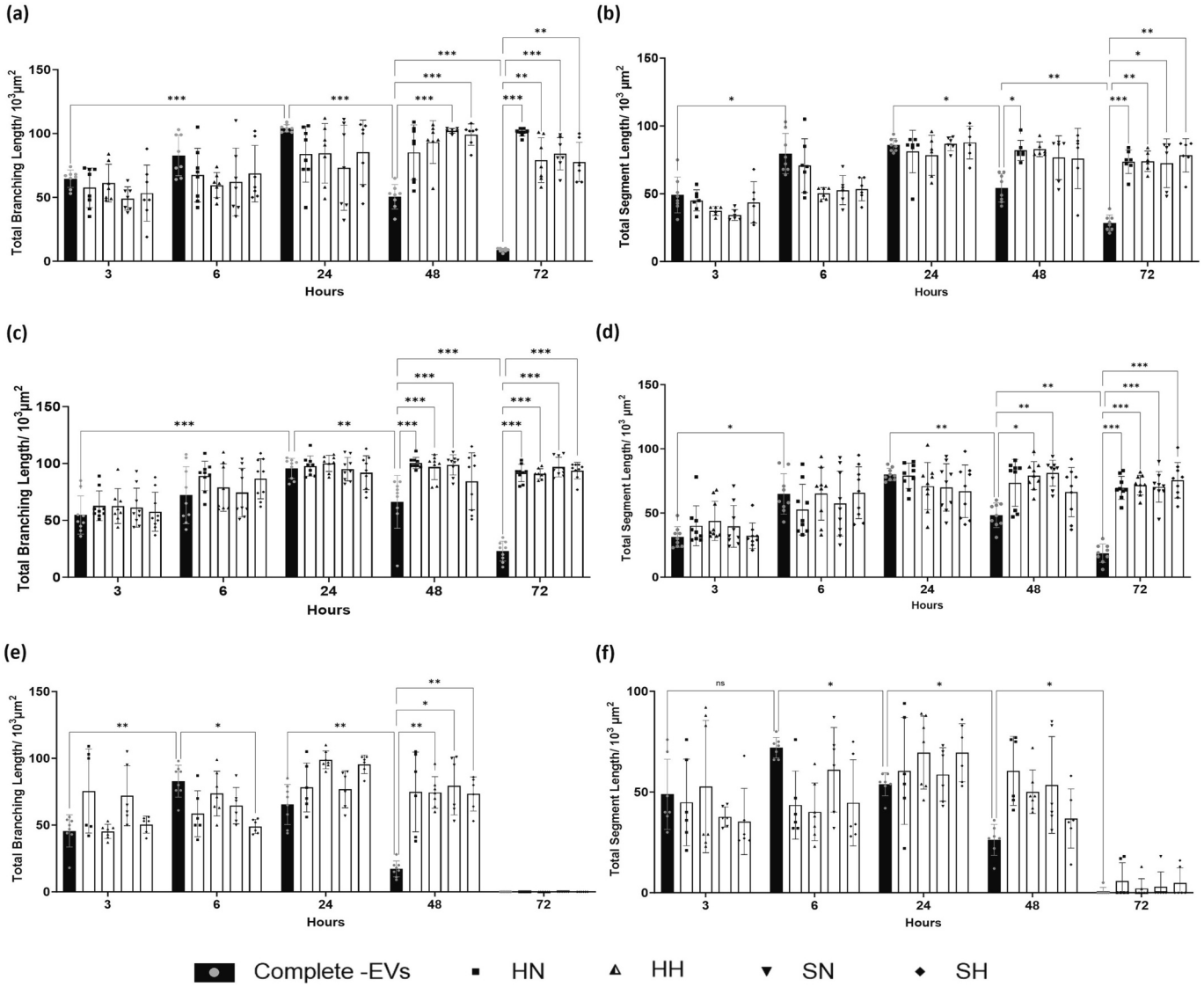

Fig. 3. CA-EVs do not increase but stabilize the network in different EC types over time. HUVEC (a-b), HMEC-1 (c-d), and HPMEC (e-f) grown in complete medium were treated for 24 h with CA-EVs (1000 particles/cell) and evaluated for tube formation. Complete -EV indicates the control group left untreated. The decrease of the black bar at 48 and 72 h in all panels demonstrates the normal destabilization of the network as a function of time. The network remained stable until 48–72 h when the cells were pre-treated with the EVs. All results are representative of at least n = 3 in triplicate, and each value is the mean ± s.d. Two-way ANOVA test with Tuckey's post-test Multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001. Representative images of this experiment are presented in Supplementary Fig. 5.

type I than Geltrex hydrogels.

4. Discussion

Our study presents five main findings: i) hypoxia differentially affects some characteristics of EVs in a cell line and culture condition-dependent fashion. EVs treatment ii) does not increase but does stabilize the vascular networks formed by ECs cultured in a complete medium; iii) restores the ability to form tubes and sprouting in transformed and primary ECs and tissues cultured in a nutrient-reduced medium; and iv) decreases the expression of the EC markers vWF and PECAM-1 in HUVEC. Also, v) the topology of the sprouting is affected by the rigidity of the extracellular matrix.

Our results coincide with previous studies showing that oxygen tension modulates EVs' release rate and molecular composition [15,18,19]. Cervical cancer cell transformation with oncogenic types of human papillomavirus (HPV, e.g., HeLa: HPV18 and SiHa: HPV16) influences the expression of the two primary HPV oncogenes, E6 and E7, which are sufficient to stabilize HIF-1α expression in normoxia [20], as we observed in our results. This basal expression of HIF-1α in normoxic HeLa and SiHa could help explain why our EVs' proangiogenic potential after 24 h of culture in hypoxia was not increased. However, it did increase the HIF-1α expression in cells, the EVs release, and its protein content without altering their RNA concentration. Those findings do not rule out the possibility that the molecular profile carried by these EVs changes, which justifies future studies to clarify possible changes in such information.

The lack of a standardized EV quantification method for cellular treatments hinders reliable and comparable analyzes of molecular and functional assays. As EVs contain a heterogeneous mixture of components [21], the protein or RNA concentration does not allow us to infer that different samples have the same number of EVs. Several investigations show an effect of EVs derived from hypoxia [22]. We found that in SH cells, the EV concentration increased approximately 10-fold compared to SN cells. However, hypoxia did not cause a significant change in EV concentration in HeLa cells. In our hands, hypoxic preconditioning did not increase the already strong vascularization potency of EVs derived from carcinoma cells when the cells were treated with the

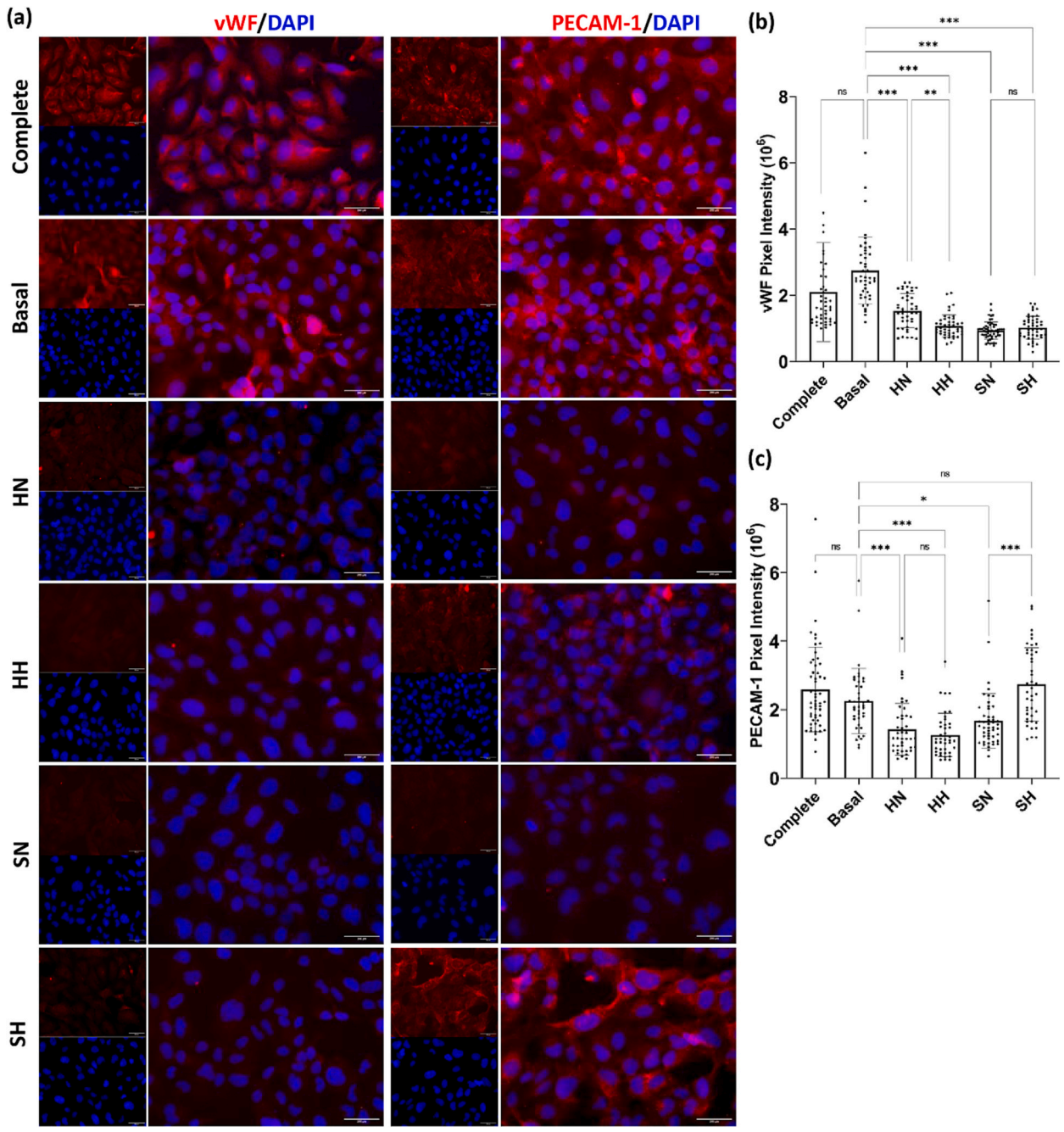

Fig. 4. CA-EVs treatment downregulates endothelial markers in HUVECs. The expression of von Willebrand factor (vWF, a-b) and Platelet endothelial cell adhesion molecule-1 (PECAM-1, a-c) were severely reduced in HUVECs after 24 h incubation with EVs. All results are representative of at least n = 3 in triplicate, and each value is the mean ± s.d. Two-way ANOVA test with Tuckey's post-test Multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.

same concentration of vesicles. These results indicate that CA-EVs effectively enhance the ability of ECs cultured in basal medium to induce the formation of capillary-like structures, resulting in a greater number and longer sprouts, which is essential in the angiogenic process.

In a complete medium, EVs did not stimulate tube formation but increased their stabilization. This is a novel finding since most studies reported the effect of EVs on angiogenesis induction [18,22,23] but do not report a stabilization of the tubes over time. The vascular stabilization has been explained by different factors, such as the regulation of cytoskeletal components [24]. Under normal conditions, EC directs their sprout towards higher levels of shear stress, leading to the pruning of vessels exposed to low blood flow while stabilizing the connections of vessels exposed to high blood flow [25]. Non-canonical Wnt signaling (Wnt5 and Wnt11) plays a role in this mechanism, interfering with the ability of ECs to sense shear stress levels, stabilizing vascular networks by reducing endothelial shear sensitivity, and preventing premature vessel regression [26]. Furthermore, it is important to consider that EVs carry metabolites that could partially and temporarily replace the tumor deficiencies in the metabolic supply, which arrive through a dysfunctional vasculature.

Our findings were in primary EC of umbilical cord macrovasculature (HUVEC) and two cell lines of dermal (HMEC-1) and pulmonary (HPMEC) microvasculature, suggesting that this EV-induced tube stabilization may occur in different calibers and vascular beds. Tumor vessels diverge from normal tissue vessels by having weak anatomy and hierarchy, being fragile and leaky. Their compromised function

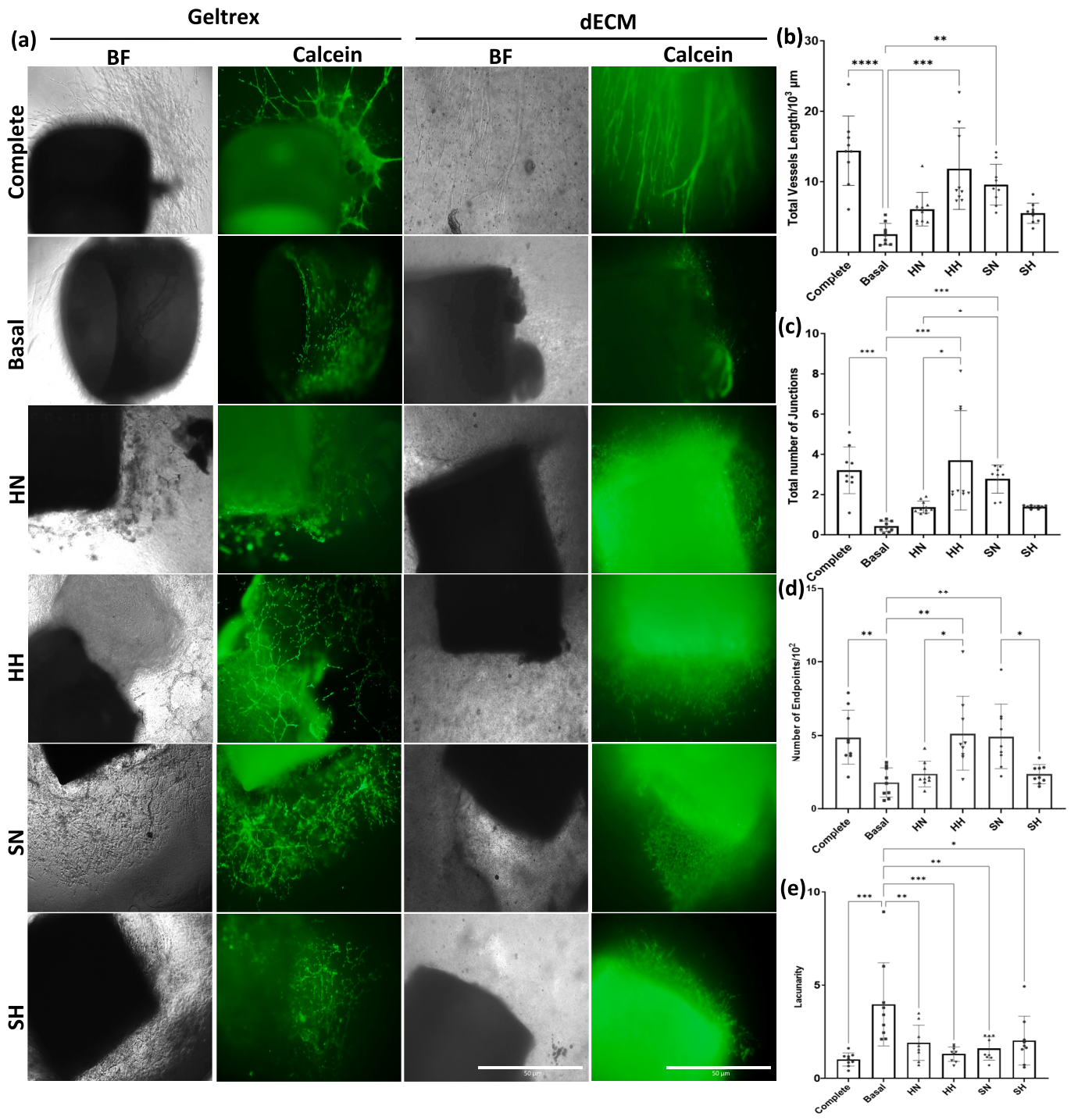

Fig. 5. CA-EVs potentiate microvessel sprouting after 10 days in culture. (a) Representative images (bright field -BF- and Calcein AM, green) of microvessel sprouting from rat aortic rings embedded either in Geltrex or dECM; grown in complete, basal, or basal medium plus CA-EVs (HN, HH, SN, SH). Quantification of sprouting parameters in Geltrex: (b) vessel length, (c) total number of junctions, (d) number of endpoints, and (e) lacunarity. All results represent at least n = 3 in triplicate, and each value is the mean ± s.d. Kruskal-Wallis test. *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

generates areas of inefficient blood perfusion, which gives them an irregular distribution of antiangiogenic therapy. According to our results, EVs do not increase but stabilize the vascular area in the well-nourished ECs. In contrast, they restore the angiogenic capacity in malnourished ECs. With this, EVs can contribute to changing the resistance to therapy, probably by maintaining tumor blood flow that the dysfunctional vessels cannot provide on their own or by providing at least momentary nutritional support per se.

Vascular damage and pathological vascularization are associated with a decrease in EC markers, i.e., vWF and PCAM-1, and an increase in the circulation [12]. In addition to tube stabilization, EV treatment decreased vWF and PECAM-1 in ECs. Loss of vWF in EC decreases αvβ3 expression, which may cause increased VEGFR-2 signaling. This increased and dysregulated VEGFR-2 signaling causes dysfunctional angiogenesis, leading to dysplastic blood vessels [24].

Treatment of aortic rings with CA-EVs, mainly with HH-EVs and SN-

EVs, increased microvessel sprouting compared to the untreated control (basal medium), confirming in a more robust model what we found in ECs. This response could be mediated by the transmission of angiogenic molecules such as proteins and miRNAs from cancer to ECs. Our results are consistent with studies showing that EVs, particularly derived from cells cultured in hypoxia, lead to an increase in migration, invasion, and angiogenesis [26]. Most of these studies were performed in in vitro or in vivo models with normal growth factor conditions [22,27–31]. Interestingly, in our assays, CA-EVs promoted significant angiogenesis features, including EC migration, sprouting, and formation of capillary-like structures, both in vitro and ex vivo, independently of growth factor signaling (for example, VEGF, FGF, and angiopoietins). This demonstrates a proangiogenic effect of EVs under minimal growth factor conditions, highlighting the ability of these vesicles to bring ECs out of quiescent mode and activate their angiogenic switch.

Finally, we observed a different branching pattern in aortic segments cultured in different ECMs but treated with the same EVs. Mechanical forces and flow patterns are important in modulating signaling pathways in ECs, and matrix stiffness regulates interactions with ECs and soluble growth factors [32]. VEGF binding, internalization, and signaling increase with substrate stiffness [33,34], enhancing angiogenesis and tip cell formation in vitro and in vivo [35]. Since different types of scaffolds provide mechanical support for angiogenesis, EVs can sensitize and enhance the effect of these scaffolds on ECs [36,37]. Further studies involving EVs and 3D structures for culture and physiological and pharmacological study are warranted.

Our results suggest that altered tissue mechanics, characteristic of many pathological states such as cancer progression, influence the vascular phenotype and subsequently generate an alternative or increased response to the same stimulus. The study of EVs and the mechanisms that affect vascular homeostasis – cellular patterns, shape changes, migration in centers of angiogenic activity, and coordination of these cellular behaviors with angiogenic genetic programs and external mechanical factors (such as the ECM) - is an exciting field of research, which is far from being fully understood. Much work must be done to elucidate the contribution of EVs in different extracellular contexts to specific ECs behaviors involved in vascular adaptation to cancer.

5. Conclusion

We have extended the observation that oxygen tension modulates EV characteristics to cervical carcinoma cells. We found that these EVs can directly increase the tube formation and stabilization in different ECs lines, helping them to overcome the lack of classical angiogenic growth factors. This may suggest that under most conditions (i.e., high or low oxygen, high or low presence of growth factors), certain tumors have an EV-dependent safety factor that allows them to regulate and potentiate the angiogenesis around their environment.

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CRediT authorship contribution statement

E. Orozco-García: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. **V. Getova:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **J.C. Calderón:** Writing – review & editing. **M.C. Harmsen:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review & editing. **R. Narvaez-Sanchez:** Funding acquisition, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the study's design, in the collection, analyses, interpretation of data, or the writing of the manuscript.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2024.107276.

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