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Correlation Between Endoglin and Malignant Phenotype in Human Melanoma Cells: Analysis of hsa-mir-214 and hsa-mir-370 in Cells and Their Extracellular Vesicles

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Abstract

Endoglin (CD105) is an auxiliary receptor of transforming growth factor (TGF)-β family members that is expressed in human melanomas. It is heterogeneously expressed by primary and metastatic melanoma cells, and endoglin targeting as a therapeutic strategy for melanoma tumors is currently been explored. However, its involvement in tumor development and malignancy is not fully understood. Here, we find that endoglin expression correlates with malignancy of primary melanomas and cultured melanoma cell lines. Next, we have analyzed the effect of ectopic endoglin expression on two miRNAs (hsa-mir-214 and hsa-mir-370), both involved in melanoma tumor progression and endoglin regulation. We show that compared with control cells, overexpression of endoglin in the WM-164 melanoma cell line induces; (i) a significant increase of hsa-mir-214 levels in small extracellular vesicles (EVs) as well as an increased trend in cells; and (ii) significantly lower levels of hsa-mir-370 in the EVs fractions, whereas no significant differences were found in cells. As hsa-mir-214 and hsa-mir-370 are not just involved in melanoma tumor progression, but they can also target endoglin-expressing endothelial cells in the tumor vasculature, these results suggest a complex and differential regulatory mechanism involving the intracellular and extracellular signaling of

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hsa-mir-214 and hsa-mir-370 in melanoma development and progression.

Keywords

Cancer · Melanoma · miRNAs · Extracellular vesicles · Exosomes · Endoglin · TGF-β · BMP

Abbreviations

AKT	Serine/threonine-specific protein kinase B (PKB)
ALIX	ALG-2-interacting protein X
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAFs	Cancer-associated fibroblasts
circRNA	Circular RNA
CMV	Citomegalovirus
CYLD	Cylindromatosis
DMEM	Dulbecco's modified Eagle medium
EMT	Epithelial-mesenchymal transition
EVs	Small extracellular vesicles
FBS	Fetal bovine serum
HA	Hemagglutinin
HEPES	4-[2-Hydroxyethyl]-1-piperazineethanesulfonic acid
HHT	Hereditary hemorrhagic telangiectasia
hPSCs	Human pancreatic stellate cells
HRP	Horseradish peroxidase
hsa-mir	Homo sapiens microRNA
MAFs	Melanoma-associated fibroblasts
MEK	Mitogen-activated ERK kinase
mir	MicroRNA
miRNA	MicroRNA
NP-40	Nonidet P-40
PAGE	Poly-acrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PMEL	Premelanosome protein
qRT-PCR	Real-time quantitative reverse transcription PCR
SDS	Sodium dodecyl sulfate
TAMs	Tumor-associated macrophages
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
TYR2	Tyrosinase 2

14.1 Introduction

The mechanism involved in tumor development and dissemination of cancer cells is still poorly understood and numerous proteins, miRNAs and signaling pathways have been reported to regulate this process [1, 2]. Among these, endoglin, an auxiliary receptor of the transforming growth factor β (TGF-β) family, has emerged as a promising therapeutic target [3, 4]. Endoglin (Eng; CD105) is a 180-kDa disulfide-linked homodimeric transmembrane glycoprotein [5, 6] highly expressed by proliferating endothelial cells in tumor associated neoangiogenesis [7], as well as in a large number of cancers with poor prognosis [8–13]. The role of endoglin in tumor progression and metastasis has been studied in several cancer cell types using in vitro and in vivo models [14–21]. In this regard, an active role of endothelial endoglin in extracellular extravasation of healthy and metastatic tumor cells has been postulated [22, 23]. Furthermore, endoglin-targeted therapy for malignant melanoma is currently been investigated with promising results [24–26]. While endoglin is heterogeneously expressed by primary and metastatic melanoma cells, its involvement in the malignant and metastasis processes is not fully understood [8, 27–30]. Given the high mortality rate of this type of skin cancer and the unresponsiveness of some patients to current immunological treatments, a better knowledge of the mechanisms and active players involved in melanoma growth and development, including endoglin, is a subject of scientific and clinical interest [31, 32].

While endoglin is a type 1 transmembrane glycoprotein with cytoplasmic, transmembrane and extracellular regions, almost 90% of the protein is encompassed within its extracellular region [5]. For this reason, the extracellular region of endoglin has focused many structural and functional studies [6, 33]. Structurally, the extracellular region of endoglin contains two distinct domains: (i) a conserved Zona Pellucida (ZP) juxtamembrane domain at the C-terminus consisting of ∼ 260 amino acids (Lys362-Asp561) with eight conserved cysteine residues and divided in two well-defined subdomains (ZP-C and ZP-N); and (ii) a domain at the N-terminus named orphan (orphan domain; OD) due to its lack of significant homology with other protein families [34, 35]. The orphan domain is involved in recognition of TGF-β family ligands [35, 36], whereas the ZP domain is involved in the interaction with members of the integrin family via its arginine-glycine-aspartic acid (RGD) motif located within the ZP-N subdomain [37]. The cellular and pathophysiological function of endoglin has been widely studied in endothelial cells, which are the target in hereditary hemorrhagic telangiectasia type 1 (HHT1), a vascular disease caused by heterozygous mutations in the endoglin gene. HHT1 is associated with telangiectases in skin and mucosa, as well as with arteriovenous malformations in lung, liver, and brain [38, 39]. As an auxiliary receptor of the TGF-β system, endoglin can bind with high affinity to bone morphogenetic protein (BMP)-9 and BMP-10 ligands [36] and interact with the type I and II serine/threonine kinase TGF-β receptors, including ALK1 and ALK5 (type I receptors) and the type II TβRII [40, 41] to modulate cellular responses to different TGF-β family members. Several lines of experimental evidence suggest that binding of BMP9 to endoglin potentiates ALK1 signaling, including the fact that mutations in the gene coding for ALK1 (ACVRL1) are responsible for a second form of HHT (HHT2), whereas heterozygous and homozygous mutations in GDF2, the gene encoding BMP-9, lead to an HHT-like variant [38, 42]. Signaling triggered by BMP-9 through the endoglin/ALK1 route mediates, via the Smad1/5/8 pathway, the expression of a wide range of genes, including the gene for the helix-loop-helix transcription factor inhibitor of differentiation 1 (ID1), a negative transcriptional regulator which is involved in the development of malignant melanoma [43–45]. Beyond the TGF-β/BMP-related functions, endoglin is also involved in integrin-mediated cell adhesion via its RGD motif in its extracellular ZP-N subdomain. Thus, endoglin has shown functional binding activity to integrins, such as α5β1 or αIIbβ3 from leukocytes, smooth muscle cells and platelets [22, 37, 46]. Of note, integrins, the major family of cell adhesion receptors in humans, play a key role in tumor growth and metastasis and several studies have investigated the contribution of integrins to the phenotypic aggressiveness of melanoma [47, 48]. In this line, differential expression of integrins in primary cutaneous melanoma has been used to distinguish indolent from aggressive, prometastatic melanoma. Also, some integrins preferentially direct circulating melanoma cells to specific organs, promoting the development of metastases. For example, melanoma cells expressing β1 or β3 integrins, both endoglin interactors, tend to metastasize to the lungs or generate lymph node metastases, respectively. In addition to their relevant role in mediating invasion and metastasis, integrins are not only promising biomarkers, but also attractive therapeutic targets in melanoma [47, 48]. Given the role of integrins in tumor angiogenesis, tumor cell migration and proliferation, and organ-specific metastasis in malignant melanoma, it can be postulated that endoglin, as integrin counter-receptor, will have a relevant impact in melanoma development.

In addition to the membrane-bound form of endoglin, a circulating form of endoglin packed into small extracellular vesicles (EVs) has been described in several pathological conditions, such as preeclampsia, liver disease or thromboembolic pulmonary hypertension [49–52]. Heterogeneous EVs, including exosomes, can be secreted by all cell types carrying various bioactive cargos such as proteins, RNAs, lipids or metabolites [53].

They are emerging as key regulators of intercellular communication in health and disease with potential relevance as biomarkers and therapeutic strategies in different pathological conditions [54, 55]. EVs can transfer their bioactive cargo from donor to recipient cells and influence the biological function of the target cell. In this regard, a functional role for circulating endoglin in EVs has been postulated in several studies, including a protective mechanism supporting endothelial cell survival and angiogenesis [49]. In addition, endoglin^{+} EVs have been proposed as a biomarker for preeclampsia and metastatic breast cancer [10, 50]. Among the different bioactive cargos of EVs are microRNAs (miRNAs, miRs), which are small endogenous non-coding RNAs that regulate gene expression. During the last decade, compelling evidences support the involvement of cellular and EVs miRNAs in cancer. Among others, miRNAs may act as either tumor suppressors or oncogenes, activating invasion and metastasis, or inducing angiogenesis; as therapeutic targets; and as potential biomarkers for cancer diagnosis, and prognosis [56–59]. Aberrant expression of miRNAs occurs in several human cancers, including melanoma. Thus, dysregulation of miRNAs has been linked to suppression, progression, differentiation, development, and prognosis of melanoma [60–62]. Some miRNAs are specific for one or more skin cancer type, such as hsa-mir-21 and hsa-mir-221, which are observed in cutaneous melanoma and squamous carcinoma; while hsa-mir-155 has been detected in melanoma and cutaneous lymphoma. In this work, we have focused our studies on the pleiotropic hsa-mir-214 and hsa-mir-370, as they are predicted and have been shown to target endoglin [63, 64]. Both, hsa-mir-214 and hsa-mir-370 are dysregulated in several other tumors, besides skin cancers, displaying contrasting behavior. Regardless of whether hsa-mir-214 levels are upregulated or downregulated in skin cancer and melanoma, its dysregulation always correlates with metastasis or poor progression [65, 66]. In the case of hsa-mir-370, controversial findings have also been reported since its upregulation correlates with progression and poor prognosis in breast and prostate cancer [67, 68], as well as promotion of cell apoptosis and inhibition of proliferation in human gastric cancer [69]. By contrast, (i) downregulation of hsa-mir-370 in esophageal squamous-cell carcinoma is associated with cell proliferation and cancer progression [70], and (ii) hsa-mir-370 acts as a tumor suppressor in hepatocellular carcinoma [71]. Interestingly, enforced expression of hsa-mir-370 in melanoma cell lines promotes proliferation, inhibits apoptosis and enhances invasion [72]. Overall, these contradictory results suggest that the function of these miRNAs is highly dependent on the cancer cell context, likely due to their differential cell source, cell target, expression level and/or specific mRNA targeting in each case.

Here we have delved into role of endoglin in human melanoma. We find a correlation between expression levels of endoglin with malignancy in primary melanomas and cultured melanoma cell lines. In addition, overexpression of endoglin in a melanoma cell line leads to dysregulated levels of hsa-mir-214 and hsa-mir-370, mRNAs involved in melanoma tumor progression and endoglin regulation. These results suggest that endoglin is actively involved in development and dissemination of malignant melanoma, and identify endoglin as a potential therapeutic target to block tumor progression.

14.2 Methods

Immunohistochemistry of melanoma tissues: A total of 73 human specimens (3 benign nevi, 73 malignant melanomas) were analyzed with the corresponding informed consent and ethical protocols approved by the Clinical Investigation Ethical Committee. Immunohistochemistry was performed on 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue samples using an anti-endoglin monoclonal antibody (SN6h, Dako). The staining results were independently analyzed by two expert pathologists who were blinded to the staging and clinical features of the subjects.

Cell culture: WM-164, SK-Mel-28, SK-Mel-103, and SK-Mel-147 cell lines were kindly provided by Dr. María S. Soengas (Spanish National Cancer Research Centre (CNIO), Madrid, Spain). Cells were cultured in DMEM (Lonza BE12-604F) supplemented with 10% heat-inactivated filtered fetal bovine serum (FBS) (Gibco) and 20 µg/mL gentamycin (Lonza 17-519Z). This melanoma cell line was routinely tested for mycoplasma contamination.

Lentiviral production and generation of human ENG stably overexpressing WM-164 cells: Lentiviral plasmids expressing human endoglin containing a hemagglutinin (HA) tag (pLV-CMV-IRES-Puro/hEng) and the corresponding empty vector (pLV-CMV-IRES-Puro/Ø) were kindly provided by Professor Peter ten Dijke (LUMC, Leiden, The Netherlands). These vectors were used in conjunction with the packaging plasmids p8.91 and pSVCG. HEK 293T cells were seeded in a 10-cm plate and transfected with 5 µg p8.91, 2.5 µg pSVCG and 5 µg pLV-CMV-IRES-Puro/Ø or pLV-CMV-IRES-Puro/hENG, using Lipofectamine® 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions. After 10–12 h, medium was changed by fresh culture medium (DMEM) and cells were incubated for additional 48 h. Culture supernatants containing lentiviral particles were harvested, clarified by centrifugation at 1500 rpm for 5 min, and filtered through a 0.45 µm filter. Lentiviral particles at 1:3 dilution were used to infect WM-164 cells in suspension in the presence of 4 µg/mL polybrene (Sigma). After incubation for 24 h, medium was replaced by fresh culture medium. Twenty four hours later, infected cells were selected in the presence of 0.4 µg/mL puromycin (Sigma), and the resulting endoglin-overexpressing WM-164 cells (WM-164 ENG) were validated by immunoblot and flow cytometry analyses.

Immunoblot assays: Cells were washed twice with PBS and lysed in cold lysis solution containing 50 mM HEPES pH 7.5, 0.4 M KCl, 10% glycerol, 1% NP-40 and protease inhibitors (PhosSTOP™, Sigma Aldrich). Lysates were sonicated for 1 min and centrifuged at 13,000 rpm for 10 min at 4 °C. Supernatant fractions were used for Western blot analyses. Protein extracts or purified EVs were quantified for protein content using the bicinchoninic acid assay (Pierce™ BCA Protein Assay kit, Thermo Scientific). Equal amounts of extracted protein or purified EVs from each sample were resuspended in Laemmli buffer and, subsequently, incubated at 95 °C for 10 min. Samples were separated by SDS-PAGE and then transferred onto a PVDF membrane (Invitrogen). Protein-bound membranes were blocked with 0.1% Tween-20 (Sigma-Aldrich) in Tris-buffered saline (TBS) containing 5% BSA or 2.5–3% milk (TBS-T), and phosphatase inhibitor cocktail (0.2 mM sodium orthovanadate, 5 mM sodium beta-glycerophosphate and 10 mM sodium fluoride) for 2 h at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies specific for: human endoglin (1:1000 in TBS-T/BSA, Abcam #169545); ALIX (1:1000 in TBS-T/milk, Cell Signal #2171); MEK 1/2 (1:1000 in milk; Cell Signaling #8727S); AKT (1:1000 in milk, Cell Signaling #9272); β-actin (1 µg/mL in TBS-T/BSA, Sigma #A1978); and GAPDH (1:500 in TBS-T/BSA, Abcam #9484). Then, membranes were washed with TBT-T and incubated for 1 h at room temperature with the corresponding secondary HRP-linked antibodies. After rinsing with TBS-T, protein bands were revealed using SuperSignal™ West Pico PLUS Chemiluminescent substrate (Thermo Scientific) to enhance HRP luminescence, followed by analysis using the Molecular Imager® Gel Doc™ XR+ System with Image Lab™ software (Bio-Rad).

Immunofluorescence flow cytometry: Cell surface expression of endoglin in WM164 cells was analyzed by flow cytometry. After collecting and washing transfected cells in PBS by soft centrifugation at 1000 rpm 8 °C for 5 min, non-specific binding was blocked for 20 min at 4 °C with sterile-filtered 1% BSA in PBS (PBS-BSA). Cells were then incubated for 1 h at 4 °C with a mouse monoclonal antibody against human endoglin (P4A4, anti-CD105, 1/100; Developmental Studies Hybridoma Bank-DSHB-

University of Iowa, USA) or against the hemagglutinin (HA) tag (1/100; MilliporeSigma). As a negative control, cells were stained with isotype control antibodies (Immunostep, Salamanca, Spain) at the same concentration as the corresponding primary antibody. Following incubation with primary antibodies, cells were washed with PBS, and incubated with Alexa-Fluor-488-conjugated anti-mouse antibody (1/200, Molecular Probes) for an additional period of 45 min. Samples were then washed, resuspended in cold PBS, and analyzed with a FC500 Beckman Coulter flow cytometer using the FlowLogic software. Endoglin protein levels were measured using the fluorescence intensity mean and expressed as fold induction relative to empty-transfected cells.

EVs isolation by sequential ultracentrifugation, characterization and analyses: Cells were cultured in media supplemented with 10% EVs-depleted FBS. Serum was depleted of bovine EVs by ultracentrifugation at 100,000 g for 70 min at 10 °C and then filtered. Supernatant fractions collected from 48 to 72 h exponentially growing cell cultures were pelleted by centrifugation at 500 g for 10 min at 4 °C to remove any cell contamination. In addition, possible apoptotic bodies and large cell debris were removed from supernatants by centrifugation at 12,000 g for 20 min at 10 °C. EVs, including exosomes were then collected by spinning at 100,000 g for 70 min at 10 °C. The pellet with EVs was then washed in 20 mL of PBS and collected again by ultracentrifugation at 100,000 for 70 min at 10 °C (Beckman, L100 X-P). The final pellet of EVs was resuspended in PBS. EVs size and particle number were analyzed using Nanosight (Nanoparticle Tracking Analysis-NTA) and its protein content was measured by BCA.

RNA isolation, cDNA synthesis and quantitative RT-PCR: microRNA (miRNA) and total RNA were isolated from cells using the miRNeasy Micro kit (Qiagen), according to the manufacturer´s instructions. To quantify specific microRNAs, first they were reversed transcribed using TaqMan™ MicroRNA Reverse Transcription kit and then, PCR was performed using Taqman Universal PCR Master mix (Applied Biosystems) and specific and pre-designed Taqman® MicroRNA assays (hsa-miR-214: ID 002306 and hsa-miR-370: ID 002275). For quantification of gene expression, RT-PCR was performed with SuperScript™ II (Invitrogen) and FastStart Essential DNA Green Master (Roche) using the primers shown in Table 14.1. qRT-PCR was performed on Light Cycler 96 (Roche), according to the following PCR settings: initial denaturation for 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C for miRNA assays and 30 s at 60 °C in the case of gene expression assays. Both miRNA and total RNA quantifications were performed in triplicates. Gene and miRNA expressions were analyzed using the delta-deltaCT method for relative quantification and all samples were normalized to the corresponding housekeeping gene, hsa-miR-16 ID 000391 and human mRNA β-actin.

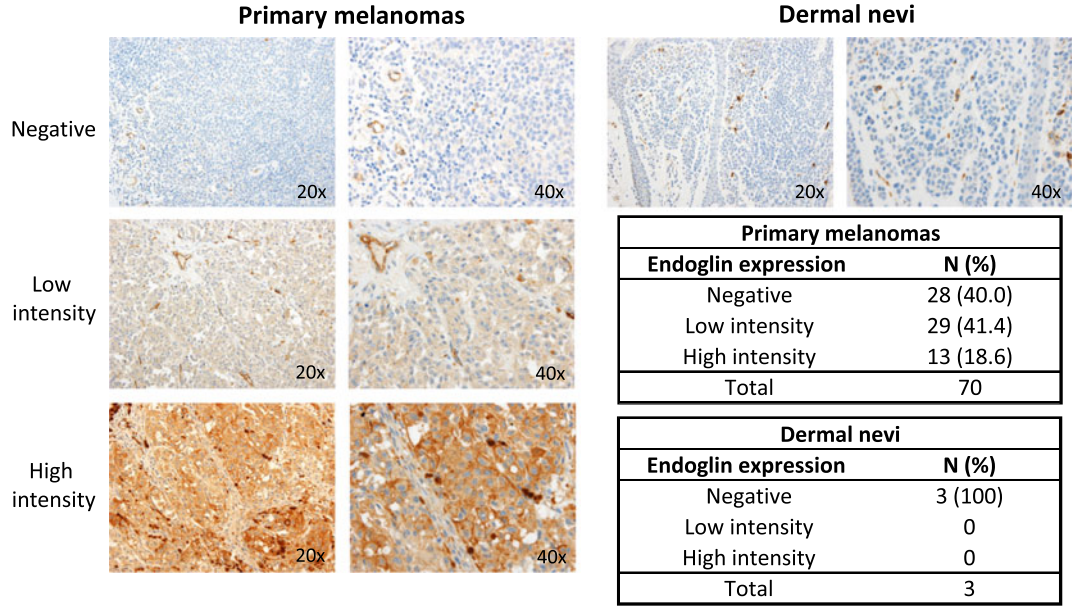
14.3 Results

Endoglin expression in primary melanomas and cultured cells

Endoglin expression was assessed in a cohort of primary melanomas and dermal nevi by immunohistochemistry. As expected, staining of endoglin was observed in endothelial cells from primary melanomas and dermal nevi. While endoglin staining was not detected in dermal nevi, 41.4 and 18.6% of primary melanomas showed low or high endoglin expression, respectively, in tumor cells (Fig. 14.1). These data suggest that, compared to normal nevi, melanoma tumors show markedly increased levels of endoglin. This prompted us to study whether endoglin expression correlates with melanoma malignancy or metastatic potential. Endoglin expression levels were also analyzed by immunoblotting in a panel of different melanoma cell lines (Fig. 14.2). While the non-metastatic or low metastatic melanoma cell lines (WM-164 and SK-Mel-28, respectively) showed low levels of endoglin, the more metastatic cell lines (SK-Mel-147 and SK-Mel-103)

Table 14.1 Sequences of primers used for qRT-PCR

Human gene	Sequence (5′-3′)
ENG (forward)	CTGCTGCTGAGCTGAATGAC
ENG (reverse)	AGTTCCACCTTCACCGTCAC
PMEL (forward)	CTCATTCCAGCTCAGCCTTC
PMEL (reverse)	CAGATAGCCACTGGGGTCAT
TYR2 (forward)	TACGGCGTAATCCTGGAAAC
TYR2 (reverse)	ATTGTGCATGCTGCTTTGAG
MLANA (forward)	GCTCATCGGCTGTTGGTATT
MLANA (reverse)	ATAAGCAGGTGGAGCATTGG
MITF (forward)	AACTCATGCGTGAGCAGATG
MITF (reverse)	TACTTGGTGGGGTTTTCGAG
VEZF-1 (forward)	AGAGGAAGGACCGGATGACT
VEZF-1 (reverse)	ACTCAGGAGCTTCCCACAGA
ACTB (forward)	GGACTTCGAGCAAGAGATGG
ACTB (reverse)	AGCACTGTGTTGGCGTACAG



Primary melanomas	
Endoglin expression	**N (%)**
Negative	28 (40.0)
Low intensity	29 (41.4)
High intensity	13 (18.6)
Total	70

Dermal nevi	
Endoglin expression	**N (%)**
Negative	3 (100)
Low intensity	0
High intensity	0
Total	3

Fig. 14.1 Endoglin expression in primary melanomas. The presence of endoglin in human dermal nevi (n = 3) and primary melanoma (n = 70) tissues was analyzed by immunohistochemistry and endoglin staining was quantified and classified as negative, low intensity or high intensity by the pathologist

had higher levels of endoglin expression (Fig. 14.2). These results suggest that endoglin expression correlates with malignancy in primary melanomas as well as in cultured melanoma cell lines.

Characterization of ectopically overexpressed endoglin in the WM-164 cell line

To investigate the impact of endoglin in the malignant phenotype of melanoma cells, the low metastatic melanoma cell line WM-164 was

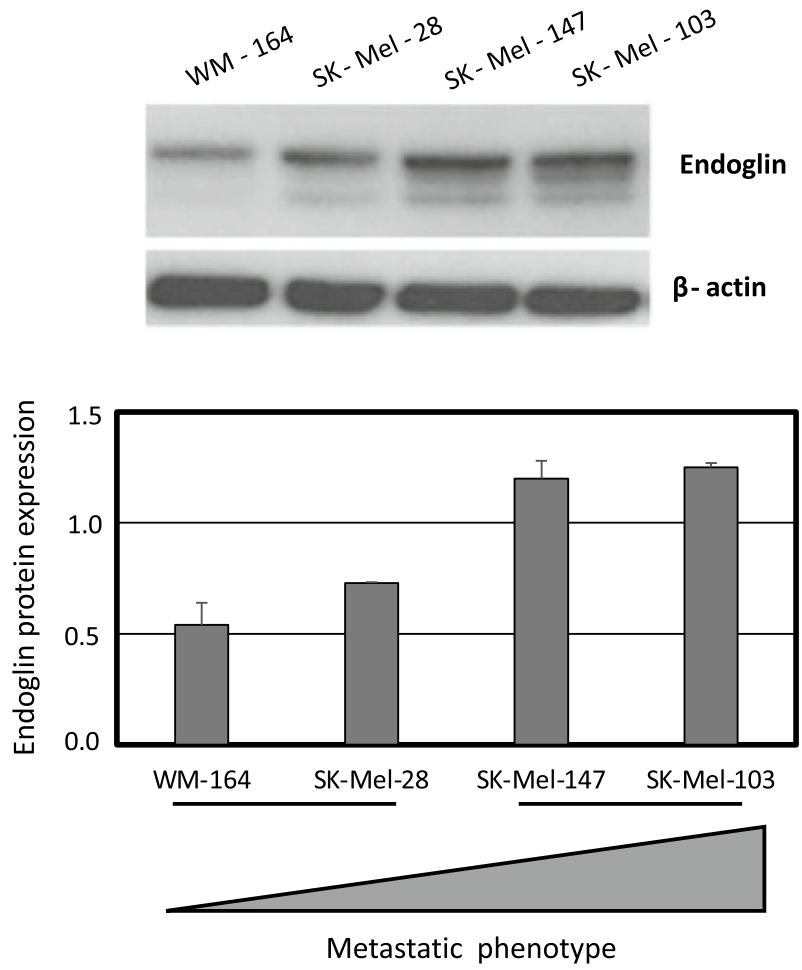


Fig. 14.2 Endoglin expression in primary melanomas and melanoma cell lines. Western blot analysis (upper panel) of endoglin expression in four melanoma cell lines with different metastatic phenotype. Quantification of endoglin staining relative to β-actin is shown in the lower panel

transduced with a lentivirus encoding HA-tagged human endoglin. Following cell infection and puromycin selection, we verified the ectopic expression of endoglin by immunoblot analysis in cellular extracts and EVs fractions (Fig. 14.3 a). As expected, endoglin-transduced cells and derived EVs showed a clear signal of ectopic endoglin relative to mock-transduced cells. To confirm the correct isolation of the EVs, the expression of ALIX, a broad biomarker of EVs, was tested. ALIX was not detected in cellular extracts, whereas a weak band was observed in EVs from mock- and endoglin-transduced cells (Fig. 14.3a), confirming the proper quality of purified EVs. To assess whether endoglin overexpression could be affecting other relevant signaling pathways, we analyzed total protein levels of MEK and AKT by immunoblotting. No significant differences were found in MEK and AKT protein levels between mock- and endoglin-transduced cells (Fig. 14.3b). The expression of ectopic endoglin in transduced WM-164 cells was also analyzed by flow cytometry using anti-HA or anti-endoglin (P4A4) monoclonal antibodies (Fig. 14.4). The strong expression of cell surface endoglin was demonstrated in endoglin-transduced WM-164 cells compared to the weak labelling of mock-transduced cells, as evidenced by the histograms obtained with anti-HA (Fig. 14.4a, left panel) and P4A4 anti-endoglin (Fig. 14.4a, right panel and Fig. 14.4b) monoclonal antibodies. Taken together, immunoblot (Fig. 14.3) and flow cytometry (Fig. 14.4) analyses demonstrate that

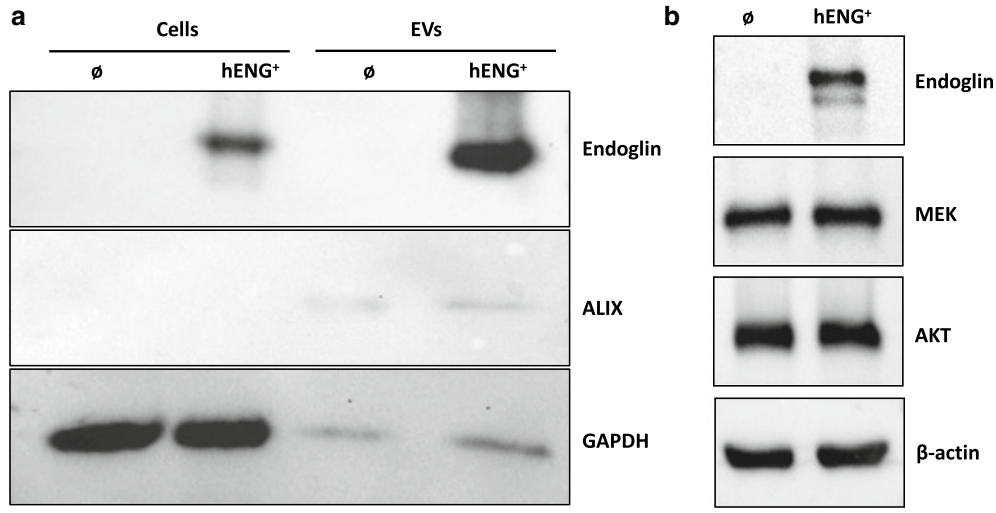


Fig. 14.3 Western blot analysis of endoglin-expressing WM-164 cells. **a** Analysis of cellular lysates and EVs from mock-transduced (ø) or endoglin-transduced (hENG$^+$) WM-164 cells using antibodies to endoglin (anti-HA), the EVs marker ALIX or GAPDH, as a loading control. **b** Analysis of cellular lysates from mock-transduced (ø) or endoglin-transduced (hENG+) WM-164 cells using antibodies to endoglin (anti-HA), total MEK, total AKT or β-actin, as a loading control. Representative Western blots are shown

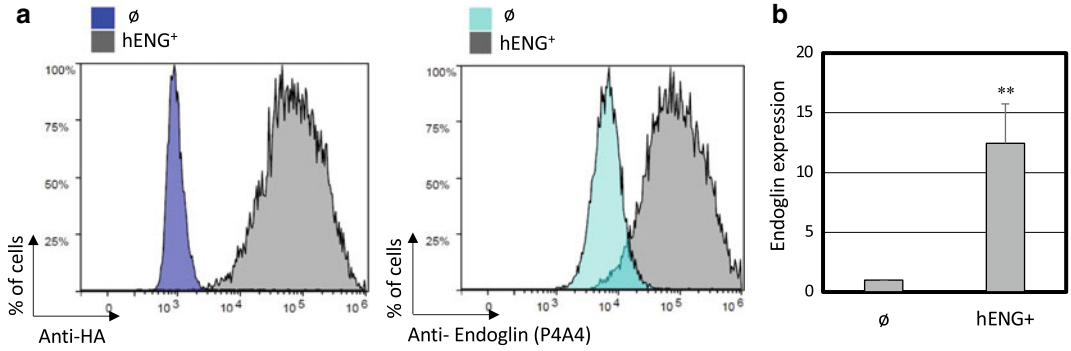


Fig. 14.4 Flow cytometry analysis of endoglin-expressing WM-164 cells. **a** Cell surface expression of endoglin was analyzed in mock-transduced (ø) or endoglin-transduced (hENG$^+$) WM-164 cells using anti-HA or P4A4 antibodies that recognize the recombinant endoglin. Representative flow cytometry histograms are shown. **b** Endoglin protein levels were measured in cells stained with P4A4 anti-endoglin antibody (n = 4 per condition) as in panel (**a**). $^{**}p < 0.01$; by two-tailed student's t-test

lentiviral transduction of WM-164 cells efficiently yields endoglin overexpression at their cell surface. The results from Figs. 14.1 and 14.2 suggesting that endoglin expression correlates with malignancy of melanomas prompted us to analyze the malignant phenotype of the endoglin-expressing WM-164 cells by measuring the levels of PMEL and TYR2, two well-known melanoma markers. PMEL (Premelanosome protein) is expressed by melanocytes and melanoma cells, and is widely used as a melanoma marker in serum samples. Compared with normal melanocytes, PMEL is over-expressed at all stages of melanoma progression [73, 74]. Tyrosinase TYR2 is involved in melanogenesis and mediates anti-apoptotic effects in human melanoma cells [75]. Analysis by qRT-PCR of endoglin-transduced WM-164 cells and their

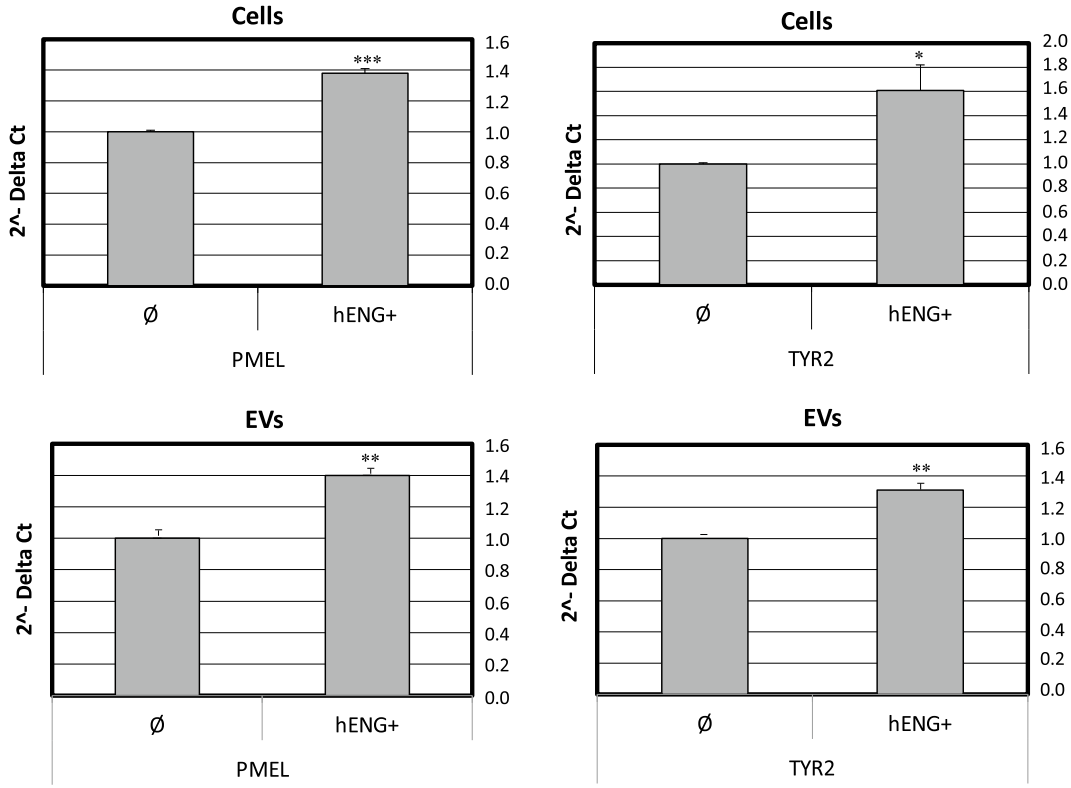


Fig. 14.5 Analysis of the melanoma marker genes *PMEL* and *TYR2* in endoglin-expressing WM-164 cells. Cells transduced with endoglin (hENG+) or an empty vector (ø) were analyzed by qRT-PCR using primers specific for *PMEL* and *TYR2*. Representative qRT-PCR from cells (upper panels) or their derived EVs (lower panels) are shown (n = 3 per condition). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; by two-tailed student's t-test

derived EVs showed that *PMEL* and *TYR2* mRNA levels were significantly higher than those of mock-transduced WM-164 controls (Fig. 14.5). These results further support the involvement of endoglin in melanoma progression.

Effect of endoglin expression on additional markers of melanoma cancer cells

To further assess whether endoglin upregulation exerted a functional effect during melanoma development and progression, three additional markers were analyzed: (i) MLANA (also known as MART-1, Melanoma antigen recognized by T-cells 1); (ii) MITF (Microphthalmia-associated transcription factor); and (iii) VEZF-1 (Vascular endothelial zinc finger 1). Of note, MLANA and MITF are relevant proteins involved in melanocyte and melanoma biology. MLANA is a cytoplasmic protein expressed by normal melanocytes and benign nevi and it is used in the clinic to detect and confirm melanocytic tumors [76, 77]. In addition, MITF has been described as the main transcription factor regulating key processes in melanoma cell development, growth, survival, proliferation, differentiation and invasion [78, 79]. Also, VEZF-1 is a Krüppel-like zinc finger protein that contributes to cancer pathogenesis [80, 81]. qRT-PCR analysis showed that *MLANA*, *MITF* and *VEZF-1* mRNA levels were significantly increased in endoglin-expressing WM-164 cells, but not in EVs, both compared to mock-transduced cells (Fig. 14.6). These findings further support the active role of endoglin in melanoma development and progression.

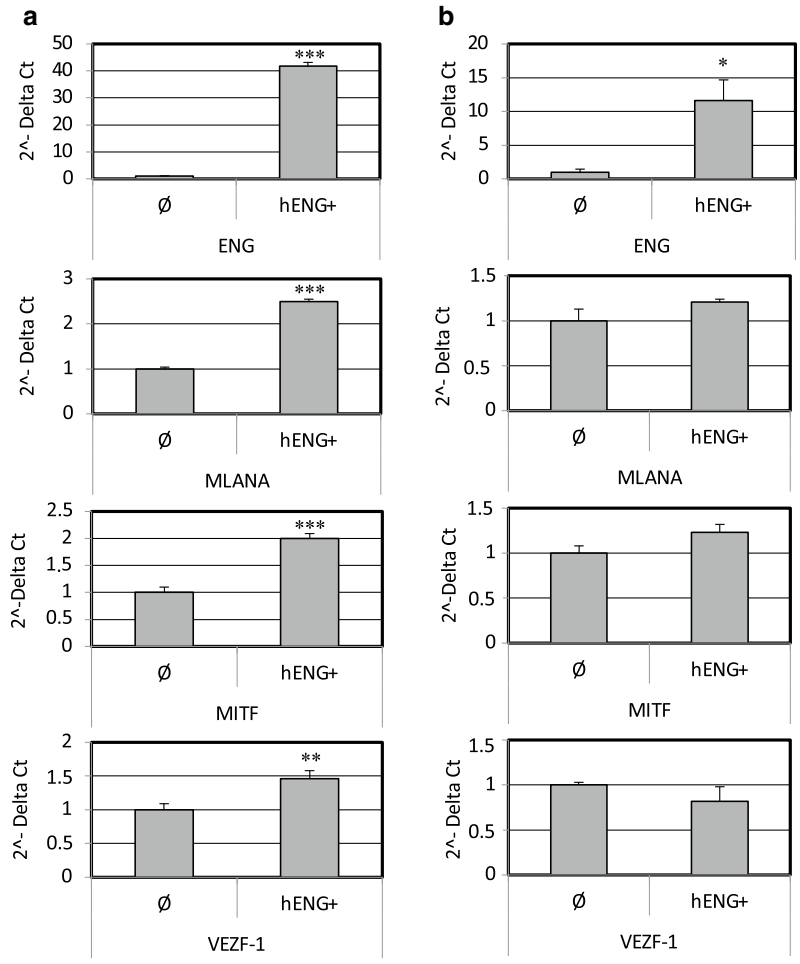


Fig. 14.6 Analysis of *MLANA*, *MITF* and *VEZF-1* genes in WM-164 cells transduced with empty (ø) or endoglin-expressing (hENG+) vectors. qRT-PCR of *ENG*, *MLANA*, *MITF* and *VEZF-1* genes was carried out using specific primers in cells (**a**) or their derived EVs (**b**) (n = 3 per condition). *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$; by two-tailed student's t-test

Effect of ectopic endoglin expression in the levels of hsa-miR-214 and hsa-miR-370

Emerging evidence support the involvement of cellular and EVs miRNAs in cancer progression, diagnosis, and prognosis [56–59], including melanoma [60–62]. Therefore, we investigated the effect of endoglin in the levels of miR-214 and miR-370 as they (i) have been found dysregulated in several cancer types, including skin cancers, (ii) are predicted to target endoglin [64], and (iii) differential expression of circulating miR-370 has been reported in plasma from patients with hereditary hemorrhagic telangiectasia type 1 (HHT1), an autosomal dominant disorder due to mutations in the endoglin gene [64]. We then measured by qRT-PCR hsa-miR-214 and hsa-miR-370 levels in cells and EVs from endoglin-expressing WM-164 cells (Fig. 14.7). Levels of hsa-miR-370 were similar in control and endoglin-expressing WM-164 cells, whereas the expression of hsa-miR-214 showed a non-significant increased trend in endoglin-positive WM-164 cells compared to controls (Fig. 14.7, left panels). In EVs from endoglin-transduced WM-164 cells, the levels of hsa-miR-370 displayed a significant reduction, while those of hsa-miR-214 showed a significant increase compared to mock-transduced WM-164 cells (Fig. 14.7, right panels). These results suggest that endoglin expression in melanoma involves the dysregulation of hsa-miR-214 and hsa-miR-370, which in turn could modulate melanoma progression.

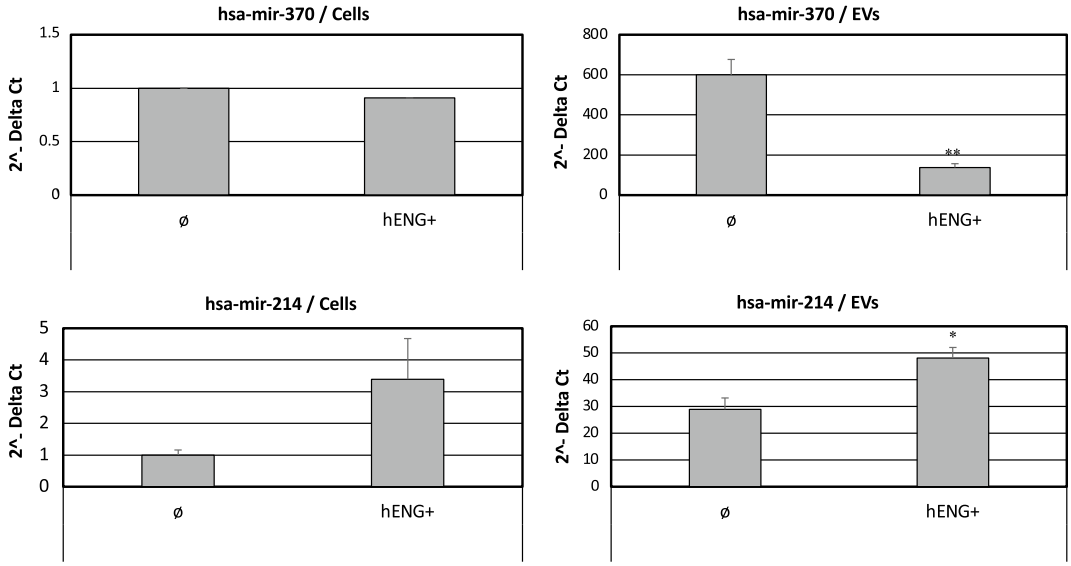


Fig. 14.7 Analysis of miRNAs hsa-mir-370 and hsa-mir-214 in endoglin-expressing WM-164 cells. Cells transduced with endoglin (hENG+) or an empty vector (ø) were analyzed by qRT-PCR using primers specific for hsa-mir-370 and hsa-mir-214. Representative qRT-PCR from cells (left panels) or their derived EVs (right panels) are shown (n = 4 per condition). The miRNA expression levels are displayed relative to WM-164 cells transduced with the empty vector. $*p < 0.05$; $**p < 0.01$ by two-tailed student's t-test

14.4 Discussion

In this work, we demonstrate a correlation between endoglin expression and tumor malignancy in primary melanoma and cultured melanoma cell lines. We have also deepened into the underlying endoglin-dependent molecular mechanisms, mainly focusing on the role of microRNAs in this process. Besides its physiological role in angiogenesis, endoglin has also emerged as a promising therapeutic target in recent years since endoglin expression has been reported either in tumor vessels or neoplasm in tumor cells, including melanoma, renal cell carcinoma (RCC), leukemias, certain subtypes of sarcomas, and breast, ovarian, endometrial, and prostate cancer. The role of endoglin in tumor cells depends on the cellular context. In this regard, and in line with our results obtained in melanoma, endoglin would be promoting tumor development and progression, playing an important role in oncogenic signalling (Fig. 14.8); whereas in other cases it has been associated with tumor suppression [4, 15, 82, 83]. In melanoma, endoglin has been pointed out to be essential for tumor plasticity, playing a key role in the interplay between TGF-β and BMP signalling pathways. Accordingly, endoglin downregulation hinders anchorage-independent growth and invasiveness and abrogates tumor growth in preclinical models of melanoma [8]. Moreover, experiments with shRNA against endoglin have shown to significantly reduce proliferation, survival and migration of melanoma cells [26, 30]. Recently, the therapeutic efficacy of a fusion protein containing endoglin single-chain variable fragment and IP10 (Endoglin-scFv/IP10) has been demonstrated. Indeed, this fusion protein inhibited proliferation and angiogenesis, while stimulating apoptosis within melanoma tissue [25]. In this context, our results further support the hypothesis that endoglin mediates malignant melanocyte transformation in WM-164, as the levels of the well-known melanoma markers PMEL and TYR2 increase upon endoglin overexpression. Furthermore, an increased trend of hsa-mir-214 levels is observed

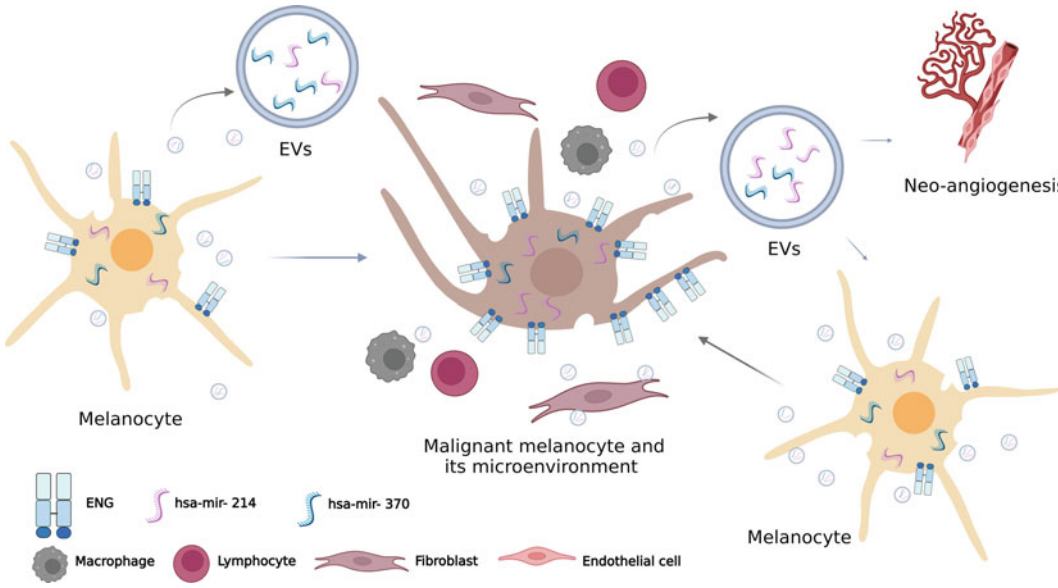


Fig. 14.8 Hypothetical model of the role of endoglin in melanoma progression. Endoglin expression is upregulated in malignant melanoma compared to primary melanocytes, contributing to the malignant phenotype, at least by dysregulating hsa-mir-214 and hsa-mir-370. EVs released by malignant melanocytes and loaded with abnormal levels of hsa-mir-214 and hsa-mir-370 can target cells from the tumor microenvironment, including primary melanocytes, endothelial cells from the tumor vasculature, melanoma-associated fibroblasts (MAFs), lymphocytes or tumor-associated macrophages (TAMs), leading to enhanced melanoma tumor growth and development. Created with BioRender.com

in endoglin-transduced melanoma cells. Interestingly, hsa-mir-214 dysregulation has been widely described in several tumors, including melanoma.

Cancer-derived extracellular vesicles, including EVs, can target different cell types in the tumor microenvironment modulating tumor growth and metastasis [84–86]. Of note, cellular endoglin expression significantly regulates both hsa-mir-214 and hsa-mir-370 in EVs, of which endoglin is also a component. Thus, compared to EVs from control cells, endoglin overexpressing cells show reduced levels of hsa-mir-370 while increased content of hsa-mir-214 in EVs. We hypothesize that these dysregulated microRNAs in EVs may play a relevant role in tumor development and metastasis (Fig. 14.8). For example, the reduction of hsa-mir-370 levels in EVs from endoglin-expressing melanoma cells could favour the process of neo-angiogenesis, which is necessary for tumor growth. This can be achieved because endoglin is negatively regulated by hsa-mir-370 [63], and endoglin is highly expressed by actively proliferating endothelial cells of the tumor vasculature [7]. Consequently, EVs from the primary melanoma tumors carrying lower levels of hsa-mir-370 would favour migration, proliferation, differentiation and adhesion of endothelial cells. Given the reported role of hsa-mir-214 in tumor progression [65, 66], increased levels of hsa-mir-214 in EVs from melanoma cells may act in a paracrine manner once taken up by neighbour melanocyte cells, thereby transforming them and contributing to tumor growth and development (Fig. 14.8). The EVs-mediated targeting of hsa-mir-370 and hsa-mir-214 may not be limited to neoangiogenic vessels or melanocytes, as an effect on additional non-cancer cells from the tumor environment is expected as well [87]. Apart from malignant cells, non-cancerous cells, including adipocytes, endothelial cells of tumor vessels, lymphocytes, tumor-associated macrophages (TAMs), and cancer-associated fibroblasts (CAFs), as well as

molecules produced and released by them, constitute the tumor microenvironment [88, 89]. Active and mutual interactions, through a paracrine signalling or circulatory and lymphatic systems, between tumor cells and the tumor microenvironment have been described to play decisive roles in tumor initiation, development and progression, metastasis, and response to therapies [90, 91]. Consequently, the tumor environment has received increased attention in the recent cancer literature [92, 93]. For instance, melanoma-associated fibroblasts (MAFs) have been described to have a role in melanoma progression, therapy resistance and immunosurveillance [94–96]. Moreover, a variety of immune cells, i.e., T and B lymphocytes, macrophages, neutrophils, dendritic and natural killer cells support the growth and invasiveness of melanoma cells, using multiple mechanisms. Among them, it is remarkable the downregulation in T lymphocytes of anti-apoptotic proteins, including Bcl-2, caused by melanoma-derived EVs containing miRNAs, such as hsa-mir-690 [97, 98]. A recent study has shown that hsa-mir-125b-5p transferred by cutaneous melanoma-derived EVs induces a tumor-promoting TAM phenotype in macrophages [99]. A role for EVs carrying hsa-mir-370 or hsa-mir-214 on malignant progression has been outlined. Breast cancer cells-secreted EVs with hsa-mir-370-3p cargo can aggravate breast cancer through downregulation of the cylindromatosis (CYLD) tumor suppressor in fibroblasts concomitantly with activation of the NF-κB signaling pathway, thereby promoting the tumor cell functions [100]. Interestingly, expression of endoglin, a target of hsa-mir-370, in CAFs regulates invasion and stimulates colorectal cancer metastasis [101]. Also, by sponging hsa-mir-370-3p, the circular RNA (circRNA) circ_0020710 can promote melanoma cell proliferation, migration and invasion in vitro, as well as tumor growth in vivo through the upregulated expression of the CXCL12 [102], a chemokine known to regulate melanoma metastasis to distant sites [103]. In the case of hsa-mir-214, its downregulation in CAFs contributes to migration and invasion of gastric cancer cells through induction of epithelial-mesenchymal transition (EMT) [104]. Accordingly, hsa-mir-214-3p has been proposed as a novel therapeutic target in pancreatic CAFs and human pancreatic stellate cells (hPSCs), as its inhibition led to inhibition of TGF-β-induced differentiation of pancreatic CAFs and reduced expression of myofibroblast markers during the differentiation of hPSCs to myofibroblasts [105]. Furthermore, a role of tumor-secreted miR-214 in the conversion of $CD4^+$ T cells into immune-suppressive regulatory T cells, promoting tumor immune escape has been described [106]. Future independent studies remain to be performed to better understand the functional impact of the endoglin-induced dysregulated microRNAs in melanoma cells and their microenvironment, as well as the possible mechanisms involved.

Along with the hsa-mir-214 and hsa-mir-370 cargos, EVs derived from endoglin-enriched melanoma cells, also contain the protein endoglin, in agreement with previous reports in EVs from endoglin-expressing endothelial cells or primary hepatic stellate cells [49–51]. Although endoglin$^+$ EVs have been proposed as biomarkers for metastatic breast cancer [10], the putative functional role of this endoglin cargo in cancer remains to be elucidated. It is well established that endoglin specifically binds integrins [22, 37, 46] and tumor cell-derived EVs contain integrins involved the generation of pre-metastatic niches in specific tissues promoting organ-specific metastases of several types of cancer including melanoma [47, 84, 107]. Accordingly, it is tempting to speculate that by interacting with integrins, endoglin could be involved in these malignant processes. Further investigations are needed to better understand the role of endoglin in melanoma development.

Statements and Declarations

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Disclosure of Interests All authors declare they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent for participation and publication was obtained from all individual participants included in the study.

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