Cell Tissue Res (2016) 365:621–641
DOI 10.1007/s00441-016-2430-x

REVIEW

Function of extracellular vesicle-associated miRNAs in metastasis

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Received: 15 March 2016 / Accepted: 7 May 2016 / Published online: 11 June 2016

Abstract Extracellular RNA (exRNA) is functionally transferrable from donor to recipient cells and is protected from RNAses by electrostatic interactions with proteins or by membrane encapsulation. In addition to bioactive RNA, extracellular vesicles (EVs) contain intraluminal and membrane-associated proteins. The cellular context and fitness affect the composition of EVs and thus the outcome of the communication between the EV-producer and recipient cells. Adaptive communication through EVs is particularly important between cancer cells and their local and distant environment and drives life-threatening metastatic progression. Small noncoding RNAs (miRNAs) have been reported in EV isolations and play a role in local invasion, angiogenesis, immune modulation, metastatic niche preparation, colonization and dormancy. The metastasis-related functions attributed to EV-associated miRNAs are currently increasing exponentially in the scientific literature. We must be aware that the correct and efficient separation of non-vesicular entities (soluble proteins, RNA-protein complexes and RNA-lipoprotein complexes) from EVs is necessary to determine the true contribution of EVs in any experiment that describes the molecular content or the functional consequences of the isolated material.

Keywords Extracellular vesicles · Exosomes · miRNA · Pre-metastatic niche · Cell-to-cell communication

Bert Dhondt and Quentin Rousseau contributed equally to this work.

This work was supported by the Fund for Scientific Spearheads of Ghent University Hospital, Concerted Research Actions from Ghent University, Stichting tegen Kanker, Vlaamse Liga tegen Kanker, a postdoctoral grant (to A.H.) from the Fund for Scientific Research-Flanders and funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement no. [316610] (to Q.R.).

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Introduction

Recently, we witnessed tremendous hype with regard to the potential of extracellular RNA (exRNA) as a novel communication type and biomarker in disease. Various NIH-funded exRNA communication projects have further boosted this hype. However, the discovery of exRNA is not new. Almost 50 years ago, Slavkin et al. (1969) identified RNA in the extracellular matrix (ECM) of embryonic rabbit tooth primordia. In two subsequent papers, Kolodny (1971, 1972) described the secretion of RNA by 3 T3 mouse fibroblasts and its transfer between cell cultures. Intracellular RNA, when purified from eukaryotic and bacterial sources (including chick embryo, rabbit liver and *Escherichia coli*) and added to cell culture medium, stimulated protein synthesis in chick embryo fibroblasts (Amos and Kearns 1962). The effects were RNA-specific, since ribonuclease treatment abolished the stimulation. Interestingly, antibody precipitation experiments and complement activation experiments suggested the protein synthesis of bacterial epitopes in chick embryo fibroblasts. These investigations showed that purified intracellular RNAs added into an extracellular environment are stable, can be taken up by cells and can be translated into proteins. Since the magnitude of the response was not correlated with the concentration of the exRNA, a follow-up study showed a

marked enhancement of RNA uptake by the addition of polybasic nuclear proteins such as protamines and histones (Amos and Kearns 1963). Interestingly, single-stranded RNA mixed with protamine forms particles and activates innate immune cells through Toll-like receptors independent of the RNA message. The size of the protamine-RNA particles is controllable and experiments suggest that 200-nm particles induce interferon-alpha production, whereas 1-μm particles induce tumor necrosis factor-alpha production showing that merely the particle size qualitatively affects the innate response (Rettig et al. 2010). Recent proteomic studies have demonstrated the presence of multiple histones in secretomes of eukaryotic cells, in lymph fluid and in human plasma (Goldfinch et al. 2008; Ji et al. 2013; Omenn 2005). Extracellular histones interact with multiple plasma proteins including coagulation factors, protease inhibitors and apolipoproteins. This histone interactome notably alters its extracellular function from cytotoxic to cytoprotective (Pemberton et al. 2010). ExRNA is most likely associated with positively charged proteins/protein complexes for protection against the abundant presence of extracellular RNAses. However, exRNA can also be membrane-protected. Extracellular vesicles (EVs) are bi-layered nano-sized membrane vesicles released by multiple cell types. In addition to bioactive RNA, EVs contain proteins and lipids and can enter the circulation to travel to distant tissues to influence various physiological and pathological functions. The RNA and protein content is transferable from donor cell to recipient cell and is functionally active in the donor cell (Demory Beckler et al. 2013; Higginbotham et al. 2011; Valadi et al. 2007). Protein variants, messenger RNA mutant/variants, and small noncoding RNAs (miRNAs) characteristic of disease have been detected in liquid biopsies of cancer patients (Skog et al. 2008). EVs contain specific repertoires of proteins and RNAs, indicating the existence of mechanisms that control the sorting of molecules into them. The cellular context affects the composition of EVs and, thus, the outcome of the communication between the EV-producer and recipient cells; this adaptivity is particularly important in the communication between cancer cells and their local and distant environments (Villarroya-Beltri et al. 2014). Much reported contents of EVs are the small noncoding RNAs of 18–24 nucleotides (nt) in length that control gene expression post-transcriptionally. These miRNAs are synthesized via the sequential actions of the Drosha and Dicer endonucleases and one of the strands of mature miRNA (guide strand) becomes incorporated into the RNA-induced silencing complex (RISC) involving DICER and argonaute proteins to target mRNAs (Bartel 2009; Maniataki and Mourelatos 2005; Rupaimoole et al. 2016). RISC-loaded miRNAs bind in a sequence-specific manner to mRNAs, initiating their repression through a combination of translational inhibition, RNA destabilization or direct RISC-mediated mRNA cleavage (Ambros 2004; Bartel 2009; Filipowicz 2005). Although miRNA biogenesis is a tightly regulated process, the deregulation of miRNAs, including their downregulation and upregulation, significantly affects the behavior of cancer cells and their interaction with local and distant environments (Rupaimoole et al. 2016). Better knowledge of EV-mediated miRNA communication will allow us to monitor the cells that are "speaking" and to "hear" the message by deciphering the molecular content of EVs. In the following sections, we will review the reported role of EV-associated proteins and miRNAs in the metastatic cascade (Table 1). Since extracellular miRNAs are not solely present in EVs, we add a critical technical note about the isolation of EVs and the relevant quality control procedures (or lack thereof; Table 2).

Local effects within primary tumor

The first step of the metastatic cascade is the invasion of cancer cells through the basal membrane followed by local infiltration of the stroma-rich ECM. The "epithelial to mesenchymal transformation" (EMT) is a much reported mechanism that has been implicated in invasion and ectopic survival (Hanahan and Weinberg 2011). Cancer cells might stimulate fibroblasts within the ECM and reciprocally profit from products produced by the fibroblasts; these fibroblasts are generally defined as cancer-associated fibroblasts (CAFs). Intravasation allows cancer cells to enter the circulation potentially leading to metastasis. Cancer cells stimulate the sprouting of new vessels within the tumor environment to allow the delivery of nutrients and oxygen and the evacuation of metabolic waste. The presence of leaky vessels promotes the extravasation of cancer cells. Escape from immune destruction and taking advantage of a pro-inflammatory environment are critical immune-editing steps for the primary tumor to proceed with metastasis. We need to understand that tumors are more than masses of proliferating cells carrying genetic mutations. Tumors are complex organs composed of diverse interdependent cell types such as CAFs, immune cells, and vascular endothelial cells that will communicate with cancer cells to participate in metastasis (Hanahan and Weinberg 2011). We will discuss the various EV-related communications between cancer cells and between cancer cells and their environment (Fig. 1). Since EVs are long-distance communicators, effects described in primary tumors can also have systemic effects and contribute to processes within the circulation and metastasis (De Wever et al. 2014).

Message from cancer cell to cancer cell

Cancer cells implement EVs to transmit aggressive traits toward neighboring cancer cells. Isogenic cell culture models with an identical genetic background but with different phenotypic

Table 1 Extracellular vesicle (*EV*)-derived small noncoding RNAs (*miRNAs*) in the metastatic process (*CCs* cancer cells, *CSCs* cancer stem cells, *CAFs* cancer-associated fibroblasts, *PTC* papillary thyroid cancer, *HCC* hepatocellular carcinoma, *TNBC* triple negative breast cancer, *GC* gastric cancer, *BM* bone marrow, *MCCs* metastatic cancer cells, *BrMC* brain metastatic cancer, *MM* multiple myeloma, *PCa* prostate cancer, *TCs* thyroid cells, *T-ICs* tumor-initiating cells, *BM* bone marrow, *MSCs* mesenchymal stem cells, *PMN* pre-metastatic niche, *LN* lymph node, *NA* not applicable, *NS* not specified, ↑ upregulated, ↓ downregulated, → leads to)

Tumor site	Origin of EVs	miRNA(s) studied	Recipient cells	Target(s) described	References
Primary tumor	Thyroid CCs	mir-145 ↓	Thyroid CCs	AKT3 ↑	Boufraqech et al. (2014)
	Pancreatic CCs	miR-212-3p ↑	Dendritic cells	RFXAP ↓	Ding et al. (2015)
	Lung CCs	miR-21, miR-29 ↑	Macrophages	TLR7 (mouse), TLR8 (human) [2]	Fabbri et al. (2012)
	CAFs (PCa)	miR-409 ↑	PCa cells	RSU1 ↓ and STAG2 ↓	Josson et al. (2014)
	PTC cells	miR-146b, miR-222 ↑	PTC cells, TCs	NS	Lee et al. (2015)
	HCC cells	miR-429 ↑	liver T-ICs	RBBP4 ↓ → E2F1/ OCT4 axis ↑	J. Li et al. (2015)
	TNBC cells	miR-134 ↓	TNBC cells	STAT5B ↑ → Hsp90 ↑	O'Brien et al. (2015)
	BM-MSCs	miR-15a ↓	MM cells	NS	Roccaro et al. (2013)
	Endothelial cells	miR-126 ↑	Leukemia cells	CXCL12 ↓ and VCAM1 ↓	Taverna et al. (2014)
	Leukemia cells	miR-92a ↑	Endothelial cells	ITGA5 ↓	Umezu et al. (2012)
	Hypoxic MM cells	miR-135b ↑	Endothelial cells	FIH-1 ↓ → HIF-1 ↑	Umezu et al. (2014)
	Endothelial cells	miR-214 ↑	Endothelial cells	ATM ↓	Van Balkom et al. (2013)
	GC-MSCs	miR-221↑	GC cells	p27, p57, BMF, BBC3, PTEN, PTPμ, TIMP [1]	Wang et al. (2014)
	Colorectal CCs	miR-92a ↑	Endothelial cells	*Dkk-3* ↓	Yamada et al. (2013)
	Colorectal CCs	miR-1246 ↑	Endothelial cells	PML ↓ → Smad1/5/8 ↑	Yamada et al. (2014)
	Macrophages	miR-223 ↑	Breast CCs	Mef2c ↓ → β-catenin ↑ (nucleus)	Yang et al. (2011)
	CCs	miR-9 ↑	Endothelial cells	SOCS5 ↓ → Jak-Stat pathway ↑	Zhuang et al. (2012)
Primary tumor	Ovarian CCs	Let-7 ↑, MiR-200 ↑	NA	NS	Kobayashi et al. (2014)
Metastases	Pancreatic CCs	miR-203 ↑	Dendritic cells	TLR4 ↓	M. Zhou et al. (2014)
Primary tumor	Breast MCCs	miR-105 ↑	Endothelial cells	ZO-1 ↓	W. Zhou et al. (2014)
Pre-metastatic niche					
Metastases	BrMC cells	miR-210↑,miR-19a↓, miR-29c↓	Non-Brain MCCs	NS	Camacho et al. (2013)
	HCC cells	11 miRNAs ↑ (miR-584, miR-517c, miR-378, miR-520f miR-142-5p, miR-451, miR-518d, miR-215, miR-376a[1], miR-133b, miR-367)	HCC cells	TAK1 ↓	Kogure et al. (2011)
	MCCs	mir-210 ↑	Endothelial cells	EFNA3 ↓	Kosaka et al. (2013)
	TNBC cells	miR-200 Cluster ↑	Breast -Poorly MCCs	Zeb2↓, Sec23a↓, Cdh1↑	Le et al. (2014)
	Gastric MCCs	Let-7 cluster ↑	NA	RAS and HMGA2[1]	Ohshima et al. (2010)
	BM-MSC	miR-23b ↑	Breast MCCs	*MARCKS* ↓	Ono et al. (2014)
	Bladder MCCs	miR-23b ↑	NA	PNRC2↑, KIAA1467↑, WBP2↑, ZEB1↑	Ostenfeld et al. (2014)
	Brain metastatic CCs	miR-181c ↑	Endothelial cells	PDPK1 ↓ → P-cofilin ↓	Tominaga et al. (2015)
	Lung MCCs	miR-192 ↑	Endothelial cells	IL-8↓, ICAM↓, CXCL1↓	Valencia et al. (2014)
	Brain Astrocytes	miR-19a ↑	BrMC cells	PTEN ↓	Zhang et al. (2015)
Pre-metastatic niche	Breast CCs	miR-122 ↑	PMN non-CCs	PKM ↓	Fong et al. (2015)
	Pancreatic CCs (Rat)	miR-494↑, miR-542-3p↑	LN stromal cells, lung fibroblasts	Cdh17↓, MAL↓, TRAF4↓ → MMP2↑, MMP3↑ and MMP14↑	Rana et al. (2013)
	Prostate C(S)Cs	miR-100-5p↑, miR-21-5p↑, miR-139-5p↑	Fibroblasts	MMP-2↑, −9↑, −13↑, RANKL↑	Sanchez et al. (2015)

[1] Not demonstrated/confirmed in the study

[2] Direct binding of the miRNA to the target

properties such as proliferation, motility, invasion and colony-forming capacity are ideal for studying this autocrine cross-talk. Indeed, EVs obtained from Hs578Ts(i)8 triple negative breast cancer cells confer increased invasion ability to the parental Hs578T cells. Furthermore, EVs from Hs578Ts(i)8 cells significantly stimulate proliferation, migration and invasion of other recipient breast cancer cells compared with EVs from Hs578T cells (O'Brien et al. 2013). In a follow-up study, this research group demonstrated that miR-134 (a predictive target of STAT5B, which controls the expression of the chaperone HSP90) is the most substantially downregulated miRNA in Hs578Ts(i)8 cells and EVs compared with the parental cells.

Table 2 Overview of technical information of reviewed studies. A semi-quantitative score from *A* to *C* (in *bold*) is given for the experiments (*A* sufficient technical information to repeat the experiment, *B* partial technical information, *C* no technical information to repeat experiment, # only used in some experiments, *(U)C* ultracentrifugation, *SDG* sucrose density gradient, *ODG* OptiPrep density gradient, *SC* sucrose cushion, *WB* Western blot, *CM* confocal microscopy, *TEM* transmission electron microscopy, *FM* fluorescent microscopy, *NTA* nanoparticle tracking analysis, *FAVS* fluorescence-activated vesicle sorting, *DLS* dynamic light scattering, *EM* electron microscopy, *IP* immuno-precipitation, *FC* flow cytometry, *AF4* asymmetric flow-field-flow-fractionation, *SEM* scanning electron microscopy, *ELISA* enzyme-linked immunosorbent assay, *NA* not applicable)

Structure	Isolation method	Characterization	Function in vitro	Function in vivo	Validation in patient material?	References
Exosomes	(U)C	WB (+: HSP70, TSG101, FLOT-1; −: VDAC), TEM	Uptake: **B**	NA	No	Demory Beckler et al. (2013)
	Kit	WB (CD63)	NA	NA	Yes	Boufraqech et al. (2014)
	Kit	WB (+: CD9, CD63, CD81; -: CNX, GM103), TEM	Uptake: **B** Education: **B**	NA	No	Camacho et al. (2013)
	(U)C	Proteomics, NTA, TEM	Education: **B**	Tracking: **A** Education: **A**	Yes	Costa-Silva et al. (2015)
	(U)C	WB (CD63, HSP70, TSG101), NTA, TEM	Education: **B**	NA	Yes	Ding et al. (2015)
	Kit	WB (CD63, CD9)	Education: **B**	Rescue: **B**	yes	Fabbri et al. (2012)
	(U)C + SDG	WB (+: CD9, Hsp70, TSG101, Flot-1; −: PARP, Lamin A/C, CRT, VDAC), FAVS, TEM	Uptake: **B** Education: **B**	NA	No	Higginbotham et al. (2011)
	(U)C	DLS	NA	Tracking: **A** Education: **A**	NA	Hood et al. (2011)
	(U)C	WB (Alix), NTA, TEM	Uptake: **B** Education: **B**	Tracking experiment: **A**	Yes	Hoshino et al. (2015)
	(U)C + ODG	WB (Alix, TSG101, Flot-1, Hsp70, CD9), TEM, CryoEM, Proteomics	NA	NA	No	Ji et al. (2013)
	(U)C	Proteomics	Education: **B**	Education: **B**	NA	Jung et al. (2009)
	(U)C	WB (CD63, CD9), NTA, TME, ExoELISA (CD63)	NA	NA	No	Kobayashi et al. (2014)
	(U)C	WB (Alix, HSP70, CD63), NTA, TEM	Uptake: **C** Education: **B**	Education: **B**	No	Kosaka et al. (2013)
	(U)C	WB (CD63, CD9, CD81, and Hsp70)	Education: **B**	NA	No	Lee et al. (2015)
	(U)C	WB (CD63), IP (CD63)	Education: **B**	NA	No	Lim et al. (2011)
	(U)C	WB (Hsp70, CD9), TEM	Education: **B**	Education: **B**	No	Liu et al. (2015)
	(U)C	WB (Alix, TSG101, CD63), CM, TEM	Uptake: **A** Education: **A**	NA	Yes	O'Brien et al. (2013)
	(U)C	WB (+: CD29/ITGB1, Alix, Tsg101; −: Grp78), TEM (CD63)	NA	NA	No	Ohshima et al. (2010)
	(U)C	WB (CD9, CD81), NTA, TEM	Uptake: **B** Education: **B**	Education: **A**	Yes	Ono et al. (2014)
	(U)C	WB (Alix, CD63, Hsp90, CD81, Tsg101) ; NTA	NA	NA	Yes	Ostenfeld et al. (2014)
	(U)C + SC	WB (Hsp70, Hsp 90, TSG101), NTA, TEM, Proteomics	Uptake: **B**	Tracking: **A**	Yes	Peinado et al. (2012)
	(U)C + SC	None	Uptake: **B** Education: **B**	Tracking: **A**	NA	Rana et al. (2013)
	Kit	WB (CD63, CD81), TEM (CD63, CD81)	Uptake: **B** Education: **B**	Education: **A**	No	Roccaro et al. (2013)
	(U)C + Kit	None	NA	NA	No	Sanchez et al. (2015)
	(U)C + SC	WB (HSC70, CD63), CM	Uptake: **B** Education: **B**	NA	No	Taverna et al. (2014)
	Kit	TEM, FM (CD63)	Uptake: **C** Education: **B**	NA	No	Umezu et al. (2012)
	Kit	WB(CD63, CD81), TEM, NTA	Education: **A**	Education: **C**	No	Umezu et al. (2014)
	(U)C + SDG #	WB(FLOT1), TEM	Uptake: **B** Education: **B**	Education: **B**	No	Van Balkom et al. (2013)
	Kit	WB (CD63), TEM	Uptake: **B** Education: **B**	NA	Yes	Wang et al. (2014)
	(U)C	WB (CD63, CD81, TSG101), FC (CD63), TEM	Uptake: **B** Education: **B**	Education: **C**	Yes	Zhang et al. (2015)
	(U)C	WB (CD63, HSP70, tsg101)		NA	No	M. Zhou et al. (2014)

Table 2 (continued)

Structure	Isolation method	Characterization	Function in vitro	Function in vivo	Validation in patient material?	References
			Uptake: **C** Education: **B**			
	(U)C	TEM	Uptake: **A** Education: **A**	Education: **A**	No	W. Zhou et al. (2014)
Extracellular vesicles	(U)C + SDG #	AF4, TEM	Uptake: **A** Education: **A**	Tracking: **A** Education: **A**	Yes	Fong et al. (2015)
	Kit	None	Uptake: **B**	NA	Yes	Josson et al. (2014)
	(U)C	WB (TSG101, ALIX; −: AGo2), NTA	Uptake: **B** Education: **A**	Education: **A**	No	Le et al. (2014)
	(U)C	WB (PDC6I/Alix, CD63, HSP90), TEM	Education: **C**	NA	Yes	O'Brien et al. (2015)
	(U)C	WB (+: CD63, CD9; -: Cytochrome C)	Uptake: **C** Education: **B**	Tracking: **B**	Yes	Tominaga et al. (2015)
	(U)C	WB (CD63 and Hsp70)	Uptake: **B**	Uptake: **B**	No	Zomer et al. (2015)
Microvesicles	(U)C	DLS, TEM	Uptake: **B** Education: **B**	Education: **A**	No	Grange et al. (2011)
	Kit	None	NA	NA	Yes	Yamada et al. (2013)
	(U)C	WB (CD63, CD81, CD9, TSG101), NTA	Uptake: **C** Education: **B**	NA	Yes	Yamada et al. (2014)
	(U)C	SEM	Uptake: **B** Education: **C**	NA	No	Yang et al. (2011)
	(U)C or Kit	None	Uptake: **B** Education: **C**	NA	No	Zhuang et al. (2012)
Microvesicles or exosomes	(U)C	TEM	Uptake: **A** Education: **A**	NA	Yes	Skog et al. (2008)
	Kit	WB (CD63)	Uptake: **B**	NA	Yes	J. Li et al. (2015)
Nanovesicles	(U)C	TEM	Uptake: **B**	NA	No	Kogure et al. (2011)
Exosome-like vesicles	(U)C	FC (CD63), WB (+: TSG101, ALIX; −: CNX), TEM, DLS, NTA	Uptake: **B** Education: **B**	Education: **A**	No	Valencia et al. (2014)

Overexpression of miR-134 in Hs578Ts(i)8 cells coincided with increased abundance of this miRNA in EVs (Turchinovich et al. 2011). Interestingly, treatment of Hs578Ts(i)8 cells with EVs obtained from Hs578Ts(i)8 cells overexpressing miR-134 significantly reduced STAT5B and HSP90 protein expression, motility and invasion and increased sensitivity to HSP90 drugs (O'Brien et al. 2015). Cancer cell lines with a different invasion potential can also be used to study the transfer of functional traits. The miR-200 family are well-known negative regulators of EMT and are located on chromosome 1 (miR-200a, miR-200b, miR-429) and 12 (miR-200c, miR-141). The miR-200a-c are expressed in OVCAR-3 ovarian cancer cells and are absent in the more invasive SKOV-3 cells, suggesting a gain of invasiveness by the downregulation of miR-200 family members (Kobayashi et al. (2014). This is consistent with the observation that primary cancer cells downregulate miR-200 expression at the invasive front, a hotspot of EMT (Hur et al. 2013; Paterson et al. 2013). The expression of miR-429, a member of the miR-200 cluster, is increased in EPCAM-positive liver tumor initiating cells. This miRNA directly targets RBBP4, a modulator of the transcriptional activity of E2F1 resulting in the upregulation of the stemness-related OCT4 gene. Importantly, treatment of EPCAM-negative hepatocellular carcinoma cells (HCCLM3) with EVs obtained from EPCAM-positive cells results in an increase of EPCAM-positive cells indicating that stemness traits are transferred between neighboring cells. Interestingly, the expression of miR-429 in the serum of patients with early stage hepatocellular carcinoma positively correlates with its expression levels in tissues; however, the presence of miR-429 in EVs in serum samples was not investigated (L. Li et al. 2015). Alternatively, EVs might discard anti-tumorigenic miRNAs to stimulate cancer progression. Boufraqech et al. (2014) detected elevated levels of miR-145 in EVs obtained from the serum of patients with thyroid cancer. MiR-145 inhibits the PI3K/AKT pathway by the direct inhibition of Akt3, resulting in reduced proliferation, an induction of cell cycle arrest, a caspase-dependent apoptosis and the inhibition of migration and invasion and is downregulated in thyroid cancer cells (TCP-1) compared with normal thyroid cells (Boufraqech et al. 2014).

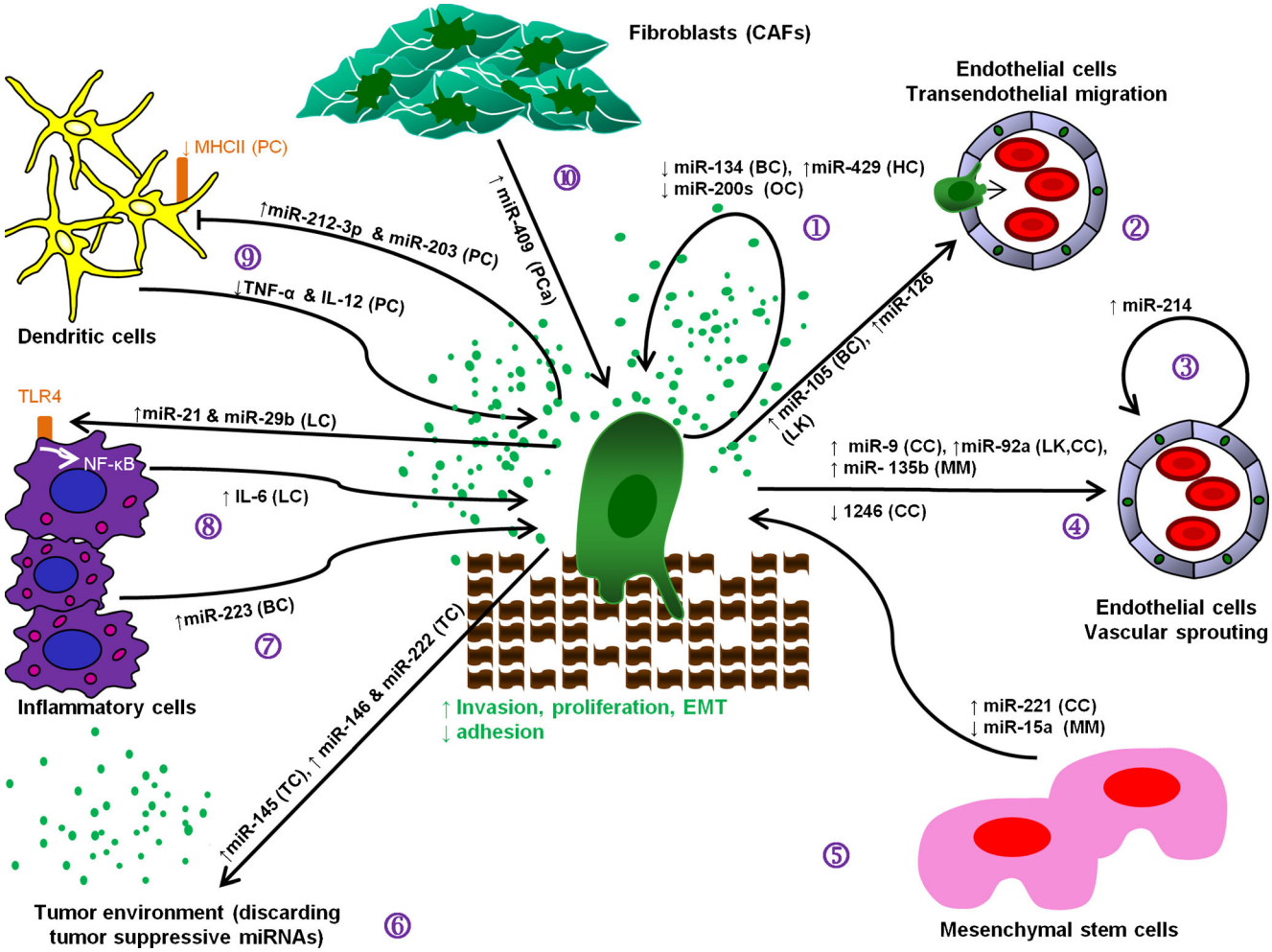

Fig. 1 Role of extracellular vesicle (EV)-derived small noncoding RNAs (miRNAs) at the primary tumor site. Cancer cells (CCs) make use of EV-mediated autocrine signaling to promote proliferation and migration (*1*). CCs release EVs enriched in miRNAs to target endothelial cells, promoting intravasation of CCs(*2*). Paracrine transfer of EV-derived miR-214 between endothelial cells promotes angiogenesis (*3*). CCs release EVs enriched in miRNAs, promoting angiogenesis after uptake by endothelial cells (*4*). Mesenchymal stem cells (MSCs) and bone-marrow-derived-MSCs (BM-MSCs) transfer miRNAs to CCs via EVs, stimulating proliferation and migration (*5*). CCs discard EVs containing tumor-suppressing miRNAs to increase their aggressiveness (*6*). Monocyte-derived EVs enriched in miR-223 drive breast cancer invasion (*7*). CCs transfer EV-derived miRNA to immune cells; miR-21 and miR-29b released from a primary lung tumor are able to bind to the Toll-like receptor-4 (*TLR4*) of macrophages to drive an interleukin-6 (*IL-6*)-mediated inflammatory response through nuclear factor kappa B (*NF-κB*) signaling (*8*). EV-derived miRNAs released from PC cells (*TNF-α* tumor necrosis factor-alpha, *MHCII* major histocompatability complex II) induce an immune tolerance (*9*). EV-mediated miR-409 transfer from cancer-associated fibroblasts (*CAFs*) to CCs promotes proliferation and epithelial to mesenchymal transformation (*EMT*) in prostate cancer (*10*). Cancer types are given as follows: *BC* breast cancer, *CC* colorectal cancer, *HC* hepatocellular carcinoma, *LC* lung cancer, *LK* leukemia, *MM* multiple myeloma, *OC* ovarian cancer, *PC* pancreatic cancer, *PCa* prostate cancer, *TC* thyroid cancer

Message from cancer cells to tumor environment

Angiogenesis, the formation of tumor-associated vessels, is the result of an interplay between cancer cells and endothelial cells and results in the sprouting of locally pre-existing vessels or the recruitment of bone marrow (BM)-derived endothelial progenitor cells. Indeed, tumor-endothelial co-cultures, in which HM7 colorectal cancer cells and primary endothelial cells are separated by a transwell membrane, reveal a general endothelial miRNA upregulation. This effect is not induced by treatment with angiogenic factors or by co-culture of human umbilical vascular endothelial cells (HUVECS) with cancer cells silenced for Drosha, the RNase III enzyme required for miRNA biogenesis. EVs isolated from SK23 melanoma induce miRNAs in HUVECs, whereas the knock-down of miR-9 in SK23 cells results in decreased miR-9 levels in isolated EVs coinciding with reduced miR-9 levels in EV-treated HUVECs. MiR-9 regulates the migration of HUVECs via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, since overexpression of miR-9 in HUVECs increases the phosphorylation of cAMP response element binding (CREB), STAT1 and 3 and JAK1.

Suppressor of cytokine signaling (SOCS)5, a negative regulator of the JAK-STAT pathway with two putative miR-9 binding sites, is indeed substantially decreased in response to miR-9. Subcutaneous implantation of HM7 cells results in elevated miR-9 levels in plasma and intratumoral injection of miR-9 antagomirs delays primary tumor growth and decreases angiogenesis as assessed by CD31 immunohistochemistry and the quantification of functional tumor vasculature by fluorescein-isothiocyanate-lectin perfusion (Zhuang et al. 2012). The miR-17-92 cluster (encoding miR-17, −18a, −19a/b, −20a and miR-92a) has also been implicated in angiogenesis. This cluster is highly present in the cell culture supernatant of leukemia K562 monocultures, but decreases in a transwell co-culture with HUVECs. Analysis of miR-17-92 cluster expression has confirmed their enrichment in the recipient HUVECs. EVs were isolated from leukemia cells transfected with Cy3-labeled miR-92a and treated or not with the EV-release inhibitor GW4869 (a specific neutral sphingomyelinase [nSMase] inhibitor). Incubation of HUVECs with EVs isolated from GW4869-treated leukemia cells diminished the incorporation of Cy3-miR-92a in HUVECs. Stimulation of HUVECs with Cy3-miR-92a did not influence growth, but stimulated migration through fibronectin-coated transwells and tube formation on Matrigel (Umezu et al. 2012). In accordance, another study demonstrated that miR-92a was transferred from DLD-1 colorectal cancer cells to HUVECs by EVs. MiR-92a was increased in EVs in the blood circulation of mice with colon cancer xenografts. In human primary colorectal tumors, miR-92a expression correlated with advanced clinical stages, tumor depth and size and was increased compared with adenomas. Transfection of HUVECs with miR-92a promoted cell growth and stimulated migration and tube formation (Yamada et al. 2013). EVs isolated from the supernatant of DLD-1 colorectal cancer cell cultures are enriched in miR-1246. In agreement, miR-1246 is frequently detected in EVs isolated from the plasma of mice bearing subcutaneous DLD-1 colorectal xenografts. Stimulation of HUVECs with DLD-1-derived EVs increases the intracellular level of miR-1246, down-regulates promyelocytic leukemia (PML) protein, which is an essential modulator of Smad 2/3 signaling and predicted target gene of miR-1246 and results in the decreased expression of the downstream Smad 2/3 signaling gene *PAI-1*. Smad 1/5/8 signaling is antagonized by Smad 2/3 signaling resulting in the increased expression of *Id1* and increased growth, migration and tube formation in EV-treated HUVECs. These effects are however attenuated after the treatment of HUVECs with EVs derived from antagomiR-1246 transfected-DLD-1 cells (Yamada et al. 2014). MiR-1246 is present in the EVs from the serum of patients with pancreatic cancer and can be used as a prognostic marker of this cancer (Madhavan et al. 2015).

Metastasis of multiple myeloma (MM) occurs in a manner similar to solid tumor metastasis through entry into the circulation and the re-entry of MM cells into new sites of BM. Azab et al. (2012) demonstrated that the dissemination of MM cells is promoted by hypoxia through the activation of proteins involved in the process of EMT. In addition, hypoxia-resistant multiple myeloma (HR-MM) cells produce two-fold more EVs than hypoxia-sensitive cells. These EVs enhance endothelial tube formation of HUVECs, dependent on miR-135b (Umezu et al. 2014).

Intravasation of cancer cells at the primary tumor site is a critical step toward metastasis and is at least in part orchestrated by miRNA-based communication between cancer cells and endothelial cells resulting in a disruption of the endothelial barriers. Indeed, miR-105, a regulator of the tight junction protein, zonula occludens-1 (ZO-1), is secreted in EVs from metastatic MDA-MB-231 breast cancer cells and transferred to primary human microvascular endothelial cells (HMVECs) to stimulate migration. Ectopic expression of miR-105 or treatment with EVs-derived from the MDA-MB-231 but not non-metastatic MCF-10A cells results in a significant decrease of ZO-1 expression at both the mRNA and protein level in HMVECs. Implementation of complementary in vitro permeability assays and three-dimensional vascular sprouting assays have confirmed that miR-105 destroys endothelial barriers and vasculature structure, respectively (W. Zhou et al. 2014). Chronic myelogenous leukemia cells (LAMA84) transfer miR-126-enriched EVs to HUVECs and negatively modulate the mRNA and protein expression of two predictive targets: CXCL12, a chemokine that binds specifically to the G-protein-coupled receptor CXCR4 and VCAM1, a cell-cell adhesion molecule. Decreased CXCL12 release from HUVECs concomitantly reduces the motility of LAMA84 cells toward conditioned medium of HUVECs in a transwell. Long-term treatment of endothelial cells with LAMA84 EVs downregulates VCAM1 mRNA and protein expression, resulting in the decreased adhesion of LAMA84 cells on HUVEC monolayers. In contrast, short-term treatments induce VCAM1 expression in HUVECs and increase the adhesion of LAMA84 cells. These results suggest a time-dependent mechanism whereby, during early contact, HUVECS induce CXCL12 and VCAM1 expression to attract cancer cells and stimulate their adhesion to endothelial cells, whereas after long-term exposure, CXCL12 and VCAM1 expression are diminished resulting in reduced chemokine concentrations and the loss of adhesion, stimulating intravasation (Taverna et al. 2014).

Several studies have demonstrated the involvement of the cross-talk between cancer cells and immune-cells, notably via EV-mediated miRNA transfer, to induce a metastatic inflammatory response. Fabri et al. (2012) suggested that the transfer of miR-21- and miR-29a-containing EVs from lung cancer cells to macrophages triggers a pro-metastatic inflammatory response by the binding of miR-21 and miR-29a as ligands to receptors of the Toll-like receptor (TLR) family. Their co-

localization and immunoprecipitation experiments revealed the binding of Dotap liposome-formulation-delivered miRNAs to TLR8-transfected HEK-293 cells. The functional activity of miRNA–TLR interaction and, accordingly, nuclear factor kappa B (NF-κB) pathway activation were confirmed by enzyme-linked immunosorbent assay measurements of the NF-κB–mediated secretion of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Incubation of macrophages with cell culture supernatant depleted of EVs, resulted in significantly reduced TNF-α and IL-6 secretion. In human primary lung tumors, only miR-29a–positive macrophages showed co-expression of IL-6 at the tumor interface. Finally, tail vein injection of Lewis lung carcinoma cells in wild-type or $TLR^{-/-}$ B6 mice demonstrated a paracrine loop of miR-21 and miR-29a to immune cells, resulting in TLR activation and increased lung metastasis (Fabbri et al. 2012). In addition, the education of HCT116 colorectal cancer cells with cell culture supernatant, conditioned from lipopolysaccharide-activated human monocytic cells (THP1), stimulated miR-21 and miR-29b release and invasion by cancer cells. Since miR-29b possesses a similar TLR-8-binding region as miR29a, the study further highlights the importance of this paracrine loop in the pro-metastatic inflammatory response, although the authors have not confirmed the encapsulation of these miRs in EVs (Patel and Gooderham 2015).

As a result of the cross-talk between cancer cells and innate and adaptive immune cells, tumors can evolve mechanisms to escape immune control by a process called immune editing, which provides a selective pressure in the tumor environment that can foster invasion and metastasis (Kim et al. 2007). EVs containing miR-212-3p isolated from pancreatic cancer cells (PANC-1) inhibit the mRNA and protein expression of the transcription factor regulatory factor X-associated protein (RFXAP) in immature dendritic cells (iDCs), resulting in a decrease of MHCII protein expression. This suggests that miR-212-3p regulates immune tolerance by reducing the presentation of pro-inflammatory signals to activate the immune system in response to the tumor (Ding et al. 2015). Furthermore, PANC-1 cells are able to decrease TNF-α and IL-12 secretion from dendritic cells (DCs) by releasing EVs containing miR-203 that, after uptake, decrease TLR-4 protein expression in DCs (M. Zhou et al. 2014).

Message from tumor environment to cancer cells

The tumor environment includes an assortment of cell types such as cancer-associated fibroblasts (CAFs), endothelial cells, normal epithelial cells, pericytes, mesenchymal stem cells (MSCs) and immune-related cells. The interactions between cancer cells and the tumor environment are known to facilitate metastatic progression (Greening et al. 2015).

Tumor-associated macrophages (TAMs; isolated and activated by IL-4 from human monocytes) transfer miR-223 to SKBR3 or MDA-MB-231 breast cancer cells via EVs. Transferred miR-223 increases Matrigel invasion by targeting the Mef2c-β-catenin pathway, leading to a nuclear accumulation of β-catenin (Yang et al. 2011).

Prostate CAFs highly express miR-409 and its expression is correlated with a higher Gleason score. Moreover, CAFs over-expressing miR-409-3p and miR-409-5p can transfer these miRNAs to cancer cells ($ARCaP_E$ and C4-2B) via EVs. The receiving prostate cancer cells show increased proliferation and EMT, characterized by decreased E-cadherin and increased vimentin mRNA expression. Subcutaneous co-injection of C4-2 cells with CAFs induces miR-409 expression in cancer cells and enhances primary tumor growth (Josson et al. 2014).

MSCs contribute to the complexity of the tumor environment. The comparison of miRNA expression in gastric cancer MSCs (GC-MSCs) with the non-cancerous tissue-derived MSCs (GCN-MSCs) shows three upregulated miRNAs (miR-214, miR-221 and miR-222). Moreover, GC-MSCs are able to promote gastric cancer cell (HGC-27) proliferation and migration in vitro and subcutaneous tumor growth. An increase of miR-221 and miR-222 expression is observed in recipient cancer cells in vivo and in vitro. The inhibition of miR-221 in GC-MSCs is able to block their tumor-supporting role. Therefore, the effect of GC-MSCs on cancer cells appears to be mostly driven by miR-221. Finally, GC-MSC-derived EVs have been shown to be able to promote the proliferation and migration of gastric cancer cells via the delivery of miR-221 to gastric cancer cells (Wang et al. 2014). Moreover, miR-221 downregulates some identified tumor suppressor genes: the cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57, the pro-apoptotic factors BMF and BBC3/PUMA, the PI3K/AKT pathway inhibitor PTEN, the cell adhesion regulator PTPm and the metallopeptidase inhibitor TIMP (Garofalo et al. 2012) and is known to promote EMT (J. Li et al. 2015; Shah and Calin 2011; Stinson et al. 2011).

EVs from BM-MSCs of MM patients contain lower amounts of miR-15a compared with those derived from healthy donor BM-MSCs. BM-MSCs release EVs containing miRNAs that can be transferred to multiple myeloma (MM) cells (MM.1S). EVs derived from BM-MSCs from healthy patients significantly reduce MM.1S cell proliferation and conversely, EVs derived from BM-MSCs from MM patients stimulate proliferation (Roccaro et al. 2013). Subcutaneous tumor load is stimulated if MM.1S cells are pre-educated with EVs from MM BM-MSC. Interestingly, an increase in MM invasion has been observed. The expression of the tumor-suppressive miR-15a is downregulated in MM BM-MSC cells and their EVs (Roccaro et al. 2009, 2013). Interestingly, IL-6 levels have been found to be increased in the conditioned

medium of MM cells exposed to BM-MSC–derived EVs from MM patients and IL-6 has been demonstrated to be functionally important in MM cell growth (Roccaro et al. 2013).

Tumor environment self-communication

Endothelial cells are also able of communicating with other endothelial cells via EVs to promote angiogenesis. MiR-214, a miRNA that controls endothelial cell function and angiogenesis and is derived from EVs of the endothelial cell line HMEC-1, is able to stimulate the migration and angiogenesis of neighboring HMEC-1. MiR-214 prevents senescence and allows blood vessel formation via the silencing of ataxia telangiectasia mutated (ATM) in recipient cells (Van Balkom et al. 2013).

Metastatic process

Circulating tumor cells (CTCs) need to survive in blood and lymph vessels before arresting at distant sites in the capillary beds (Hanahan and Weinberg 2011; Shibue and Weinberg 2011). Extravasated disseminated cancer cells can have three different fates: cell death, dormancy, or metastatic colonization. Interactions of the extravasated cancer cells with stimulatory signals from the environment will be fate-decisive (Shibue and Weinberg 2011).

Colonization is characterized by the survival and proliferation in a new environment to form metastasis (Hanahan and Weinberg 2011; Shibue and Weinberg 2011).

Dormancy allows the survival of the disseminated cancer cells without an apparent increase in cell number. In a dormant state, cancer cells stay in G_0-G_1 phase until appropriate environmental conditions occur to start cell cycle progression. Dormant cells are, in general, resistant to classic cancer management protocols and will eventually be responsible for future relapse (Sosa et al. 2014).

EMT is reversible and cells can undergo the reciprocal mesenchymal to epithelial transition (MET) process (Hanahan and Weinberg Robert 2011; Tsai and Yang 2013). This is an important step, because MET has been implicated in the establishment and stabilization of cancer cells at their metastatic location by allowing cancerous cells to regain epithelial properties and integrate into distant organs (Tsai and Yang 2013). In this section, we will discuss the impact of miRNA-containing EVs at the metastatic site (Fig. 2).

Cross communication between cancer cells

An intravital imaging study has shown the ability of malignant cells to phenocopy their metastatic behavior to less malignant cells, thereby increasing their migratory and metastatic capacity. Metastasized cells may influence the metastatic capacity of less malignant cancer cells at the primary tumor by long range EV transfer (Zomer et al. 2015).

Orthotopic 4T1E breast tumors form macro-metastases in the lung and are enriched in all miR-200 family members at the cellular and EV level. MiR-200 can be detected in the circulation of mice with 4 T1 tumors. EVs from miR-200–expressing cells convert poorly metastatic, non-miR-200-expressing 4TO7 cells to highly metastatic cells. Antagonizing the miR-200 family, in donor or recipient cancer cells, decreases the ability of 4TO7 cells to form metastases. Zeb2 and Sec23a, the direct target genes of miR-200s, are downregulated in the presence of EVs enriched in miR-200, whereas CDH1 (the E-cadherin gene, transcriptionally repressed by Zeb2) is up-regulated. In a xenograft model of human breast cancer, miR-200s are highly expressed and secreted in EVs derived from metastatic epithelial CA1a and BPLER cells but not from poorly metastatic mesenchymal MB-231 cells (Le et al. 2014). MB-231 cells are able to take up miR-200s from EVs derived from CA1a and BPLER cells and become more metastatic in a xenograft model (Korpal et al. 2011; Le et al. 2014). MiR-200 co-localizes with donor cell CD63, a marker for EVs but also with Ago2, a protein carrier of miRNAs. This work shows the importance of miR-200 family members in the suppression of EMT and stimulation of MET mainly by inhibiting the expression of Zeb1 and Zeb2 giving disseminated cancer cells the ability to grow at a metastatic site (Le et al. 2014). By microRNA polymerase chain reaction (PCR) array, a higher presence of miR-210 and lower presence of miR-19a and miR-29c are observed in EVs from brain tropic melanoma (70 W) and breast cancer cells (MDA-MB-231BR) compared with EVs from their non-brain metastatic parental cells. EVs from brain metastatic cell lines can increase the potential of poorly metastatic cells to adhere to endothelial cells and invade through Matrigel, suggesting that cancer cells from brain metastases can transfer EV-derived miR-221 to poorly metastatic cancer cells of the primary tumor (Camacho et al. 2013).

The Let-7 miRNA family, known as tumor suppressor miRNAs, targets oncogenes such as RAS and HMGA2 and is generally downregulated in solid primary tumors. Despite this, the metastatic gastric cancer cell line (AZ-P7a, which forms peritoneal metastases) expresses Let-7 family members abundantly, both at the intracellular and EV level, compared with its parental cell line having low metastatic potential (AZ-521). Thus, the EV-mediated release of let-7 miRNAs into the extracellular environment might be a mechanism allowing cancer cells to control intracellular tumor-suppressive Let-7s, thereby maintaining their oncogenic and invasive potential (Ohshima et al. 2010). Supportive evidence has come from another study. Isogenic bladder carcinoma cell lines with various metastatic properties secrete EVs enriched in tumor-suppressing miRNAs, such as miR-23b, miR-224 and miR-921. Moreover, miR-23b is poorly expressed in lymph node

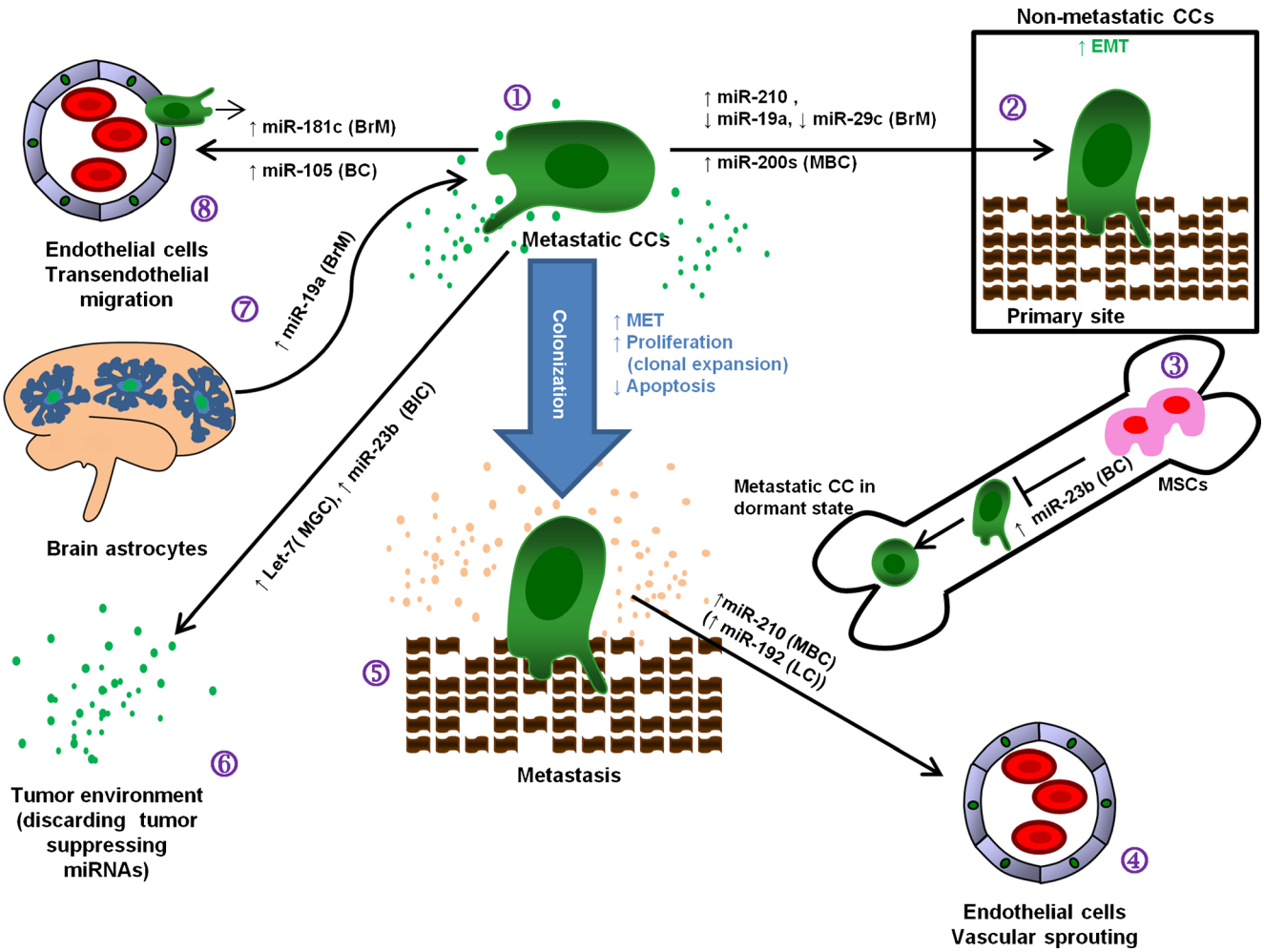

Fig. 2 Metastatic process. Metastatic cancer cells release extracellular vesicles (EVs) into the tumor environment and circulation (*1*). EV-derived miRNAs from metastatic cancer cells (CCs) can be transferred to poorly metastatic CCs and might be involved in EMT at the primary tumor site (*2*). EVs derived from the bone marrow environment induce dormancy in metastasized BC cells (*3*). Transfer of EV-derived miRNAs from CCs to endothelial cells promotes angiogenesis at the metastatic site (*4*). Disseminated cancer cells can colonize the metastatic site (*MET* mesenchymal to epithelial transition), after the uptake of EVs from the metastatic environment (*5*). Metastatic CCs discard tumor-suppressive miRNAs via EVs to increase their colonization (*6*). In the brain, astrocytes transfer EV-derived miR-19a to CCs, enhancing their proliferation and reducing apoptosis (*7*). EV-derived miR-181c and miR-105 are involved in extravasation (*8*). Cancer types are given as follows: *BrM* brain metastases, *BC* breast cancer, *MBC* metastatic breast cancer, *LC* lung cancer, *MGC* metastatic gastric cancer, *BIC* isogenic bladder carcinoma

metastases compared with the primary bladder tumor and is strongly released from metastatic FL3 cells compared with non-metastatic T24 cells. Ectopic miR-23b expression reduces the cellular invasion of FL3 cells but increases it for T24 cells. In addition, it promotes FL3 resistance to anoikis. Ectopic expression of miR-23b in FL3 cells reduces angiogenesis in a chorioallantois membrane assay and decreases pulmonary metastasis after tail vein injection in mouse models. These results suggest that miR-23b acts as a tumor suppressor at various steps of the metastatic process: invasion, anoikis resistance, angiogenesis and lung colonization. This suggests that the release of EVs is a mechanism allowing cancers cells to discard miR-23b, thereby increasing their ability to form metastases. Moreover, the small GTPases RAB27A and RAB27B have been identified as regulators of this EV-mediated miRNA secretion, indicating EV production and release through multi-vesicular body formation and fusion with the plasma membrane (Ostenfeld et al. 2014). Interestingly, high RAB27B expression has been correlated to poor prognosis of bladder cancer, as was previously shown for breast cancer (Hendrix et al. 2010). On a critical note, the role of miR-23b in cancer biology appears to be more complex. Indeed, many studies demonstrate that the downregulation of miR-23b promotes cancer progression in a variety of tumors through increased proliferation, migration and/or invasion, suggesting its role as a tumor-suppressive miRNA (Loftus et al. 2012; Majid et al. 2012, 2013; Pellegrino et al. 2013a, 2013b; Wang et al. 2013). Despite its described role as a tumor-suppressive miRNA in many tumors, other studies have

demonstrated that miR-23b can act as an oncomiR, stimulating tumor progression and might be a marker of bad prognosis (Chen et al. 2012; Jin et al. 2013; Li et al. 2013; Ma et al. 2014; Tian et al. 2013; Zaman et al. 2012). A recent review by Haier et al. (2016) highlighted the dual role of miR-23 ~ 24 ~ 27 clusters, including miR-23b, in cancer development, as they induce contradictory regulatory responses, acting both as oncogenic and tumor-suppressive miRNAs depending on the cellular context.

Nonetheless, from the results of both studies, we can hypothesize that the discarding of tumor-suppressing miRNAs via EVs is a mechanism allowing cancer cells to become more aggressive.

Message from cancer cell to tumor environment

Angiogenesis is required to sustain the primary tumor but is also important during metastatic colonization. We can assume that some miRNAs regulating angiogenesis in the primary tumor might also promote angiogenesis at metastatic sites. Nevertheless, some research groups have uniquely reported the role of EV-derived miRNAs in angiogenesis at the metastatic site.

EV-mediated miRNA transfer has been implicated in bone metastatic colonization by lung cancer cells. Indeed, miR-192 is strongly downregulated in A549-derived bone metastatic subpopulations. An anti-metastatic effect of miR-192 is demonstrated in invasion assays in vitro and in artificial metastasis assays in vivo with highly metastatic lung cancer cell lines over-expressing miR-192. Moreover, miR-192-mediated impairment of tumor-induced angiogenesis is observed in vivo by intratibial injection and in vitro by co-culture of cancer cells with HUVECs leading to a decrease in proliferation, migration and vessel connectivity. MiR-192 can be transferred by EVs to HUVECs and can impair tubulogenic activity through the downregulation of ICAM-1, CXCL-1, and IL-8. These effects are endothelial-cell-specific, since no effects have been observed on bone integrity. The subcutaneous injection of A549 lung cancer cells, overexpressing miR-192, decreases murine ICAM1 expression in tumor blood vessels. Systemically injected A549-derived EVs accumulate in BM and inhibit bone colonization by reducing angiogenesis (Valencia et al. 2014). The mouse mammary metastatic cancer cell line (4 T1) and human metastatic breast cancer cell line (MDA-MB-231) produce EVs containing more miR-210 than poorly metastatic cell lines. Moreover, miR-210 over-expressing EVs increase HUVEC migration and capillary formation, in vitro but is partially blocked by the introduction of a miR-210 inhibitor. These results suggest that miR-210 in EVs can be transferred to endothelial cells, suppressing its target genes and resulting in the activation of endothelial cells (Kosaka et al. 2013).

Extravasation of CTCs can be promoted via crosstalk between cancer cells and endothelial cells. The blood brain barrier (BBB) is mainly composed of endothelial cells with strong intercellular junctions, giving them low permeability for cancer cells. This implies that metastatic breast cancer cells need to communicate with endothelial cells to promote extravasation through the BBB to be able to form brain metastases. EV-related content might target the BBB as shown in an in vitro BBB model system consisting in primary cultures of brain capillary endothelial cells, brain pericytes and astrocytes. The effect of EVs on extravasation and increased brain metastasis has also been demonstrated in vivo by the tail vein injection of highly metastatic cell-line-derived EVs followed by intracardial injection of poorly metastatic cancer cells. EVs destroy the BBB through delocalization of actin fibers via the downregulation of 3-phosphoinositide-dependent protein kinase-1 (PDPK1) by miR-181c. PDPK1 degradation by EV-derived miR-181c results in the downregulation of phosphorylated cofilin and its associated modulation of actin dynamics (Tominaga et al. 2015). MiR-181c also promotes cancer cell invasion, proliferation and invasion in primary brain cancer (Li et al. 2014; Ruan et al. 2015) and has been implicated in various other types of cancer (Chen et al. 2015; Mori et al. 2015; Mosakhani et al. 2013; Zhang and Zhang 2015). EV-derived miR-105 has previously been shown to promote intravasation by creating gaps in the endothelial barrier but it can also promote the extravasation of cancer cells in secondary organs. Indeed, the tail vein injection of EVs with high expression of miR-105 enhances vascular permeability and stimulates lung and brain metastasis of intracardially injected MDA-MB231 breast cancer cells (W. Zhou et al. 2014).

In HCC, cancer cells have been shown to produce EVs that potentially contribute to local spread, intrahepatic metastasis and multifocal growth. These EVs contain eleven miRNAs (miR-584, miR-517c, miR-378, miR-520f, miR-142-5p, miR-451, miR-518d, miR-215, miR-376a*, miR-133b, miR-367), which are expressed in EVs derived from HCC cells. These EVs are able to modulate the expression of transforming growth factor activated kinase-1 (TAK1) to enhance the growth of recipient cells (Kogure et al. 2011).

Message from tumor environment to cancer cells

EV-mediated communication from the metastatic niche to the disseminated cancer cells can stimulate dormancy. Breast cancer patients develop BM metastatic disease decades after surgical and systemic treatment of the primary tumor, reflecting the survival of disseminated cancer cells in a dormant state (Lim et al. 2011; Ono et al. 2014). Lim et al. (2011) demonstrated that breast cancer cells, when co-cultured with a BM metastatic environment, arrest in the G_0 phase of the cell cycle because of the gap-junction-mediated import of BM-derived miR-127, −197, −222 and −223, targeting CXCL12 (C-X-C motif chemokine 12). Additionally, the quiescence of breast cancer cells is induced by BM metastatic-environment-

derived EVs, although their miRNA content was not determined (Lim et al. 2011). More convincing evidence regarding the role of BM-derived EVs in cancer cell dormancy was provided by Ono et al. (2014). The co-culturing of the bone metastatic breast cancer cell line BM2 with naive human BM-MSCs induced a change toward a dormant state and a decrease in proliferation, invasive capacity and sensitivity to the chemotherapeutic agent docetaxel. The incubation of BM2 cells with EVs isolated from BM-MSC cell culture supernatant resulted in EV uptake and the acquisition of a dormant phenotype. Analysis of the miRNA content of BM-MSC-derived EVs demonstrated the overexpression of miR-23b compared with EVs from adult fibroblasts. To investigate the role of this miRNA in metastatic breast cancer cell quiescence, BM2 cells were transfected with miR-23b and a dormant phenotype was induced through the suppression of a cell cycling and motility target gene, Myristoylated Alanine-Rich protein Kinase C Substrate (MARCKS). Furthermore, miR-23b expression was increased in BM2 cells treated with BM-MSC-derived EVs, whereas MARCKS expression was significantly decreased. Knockdown of MARCKS in BM2 cells also promoted the acquisition of dormancy. BM2 cells treated with BM-MSC-derived EVs exhibited substantially decreased orthotopic tumor growth, whereas xenografts derived from BM2 cells transfected with miR-23b did not exhibit the same degree of reduced proliferation, suggesting that factors additional to miR-23b contribute to this effect. Finally, metastatic breast cancer cells in patient BM were observed among BM-MSCs and had increased miR-23b and decreased MARCKS expression (Ono et al. 2014).

MiRNA containing EVs from the metastatic environment assist disseminated cancer cells to adapt to their new location. For example, PTEN, a tumor suppressor, shows reduced expression in disseminated cancer cells in the brain but not in other organs. Interestingly, PTEN-low primary cancer cells are not at the origin of PTEN loss in brain metastases. Cultured cells from PTEN-low brain metastases can regain PTEN expression, indicating a reversible epigenetic PTEN loss regulated by the brain environment. Zhang et al. (2015) showed that astrocytes induce a significant decrease of PTEN at the mRNA and protein level in cancer cells without affecting promoter methylation or activity, suggesting miRNA-dependent activity. Interestingly, astrocyte-specific depletion of the miR-17 ~ 92 cluster blocks PTEN downregulation in the disseminated cancer cells and suppresses brain metastatic growth. Moreover, the EV-mediated transfer of miR-19a is at the origin of PTEN downregulation in brain metastasis. PTEN loss in disseminated cancer cells enhances proliferation and reduces apoptosis via the recruitment of IBA1-expressing myeloid cells, leading to increased secretion of the chemokine CCL2 (Zhang et al. 2015).

Pre-metastatic niche

The "seed and soil" hypothesis of metastatic spread, proposed by Stephen Paget in 1889, states that for engraftment and outgrowth of cancer cells (the "seeds") in distant tissues to occur, the presence of a receptive organ-specific environment (the "soil") is required (Paget 1989). Before the introduction of this concept, it was thought that the distribution of cancer throughout the body was a matter of chance and that the dissemination of malignant cells to distant organs was caused by cancer cell emboli in the blood or lymphatic vessels (Virchow 1858). By analyzing 735 autopsy records of fatal breast cancer, Paget documented that metastasis to visceral organs and bones did not occur in a random pattern. The discrepancy between the receptiveness of certain organs to metastases and their relative blood supply meant that metastatic spread could not be explained by cancer cell emboli alone. Therefore, he concluded that disseminating cancer cells were carried in all directions throughout the circulation but could only survive and grow in the compatible milieu of certain organs (see Paget 1989).

Building on Paget's hypothesis, the idea was introduced that, before metastatic dissemination, primary tumor-secreted factors prime secondary sites for metastatic cancer cell engraftment and proliferation through to the development of a suitable microenvironment (the pre-metastatic niche; Psaila and Lyden 2009). The first evidence for this concept was provided by Kaplan et al. (2005) who demonstrated that BM-derived hematopoietic progenitor cells (HPCs), expressing vascular endothelial growth factor receptor 1 (VEGFR1), home to tumor-specific pre-metastatic sites before the arrival of cancer cells. $VEGFR1^+$ HPCs express VLA-4, whereas tumor-secreted factors up-regulate fibronectin, a VLA-4 ligand, in fibroblasts at distant pre-metastatic sites, creating a permissive niche for incoming tumor cells. The site of pre-metastatic $VEGFR1^+$ HPC distribution is tumor-type-specific. Furthermore, cell culture supernatants obtained from distinct tumor types with unique patterns of metastatic spread redirect fibronectin expression and $VEGFR1^+$ HPC colonization, thereby transforming the metastatic profile. Antibodies blocking VEGFR1 function or the removal of $VEGFR1^+$ cells from the BM of wild-type mice inhibit the formation of these pre-metastatic cell clusters and prevent tumor metastasis (Kaplan et al. 2005).

Subsequent research has provided additional insights into the pathophysiology of the pre-metastatic niche. Several tumor-secreted factors have been found to influence the recruitment of specific BM-derived cells (BMDCs) involved in pre-metastatic niche formation, such as HPCs and myeloid cells. These render the secondary environment receptive to tumor growth by the secretion of matrix proteins, angiogenic factors and cytokines (Peinado et al. 2011; Psaila and Lyden 2009; Quail and Joyce 2013).

The release of soluble cytokines and chemokines by the primary tumor is currently considered as the main mechanism

underlying the generation of suitable niches in distant organs (Peinado et al. 2011; Psaila and Lyden 2009; Quail and Joyce 2013). However, recent research increasingly supports a role for EVs in the intercellular transfer of information between the primary tumor and its pre-metastatic microenvironment, uncovering a second distinct mechanism of pre-metastatic niche formation by EVs, in addition to the secretion of soluble factors (Fig. 3). On a critical note, the precise biological role and clinical significance of the pre-metastatic niche in patients with cancer is not yet fully understood and future research should address these issues (Massague and Obenauf 2016). In addition, in vivo observations based on the injection of large amounts of EVs from cultured cancer cells might not completely reflect what happens in a living organism.

Lymphatic metastasis

Several studies have demonstrated that tumor-derived EVs home to regional lymph nodes and facilitate lymphatic metastasis through an EV-mediated process of pre-metastatic niche preparation. Hood et al. (2011) described the selective homing of melanoma-derived EVs to sentinel lymph nodes compared

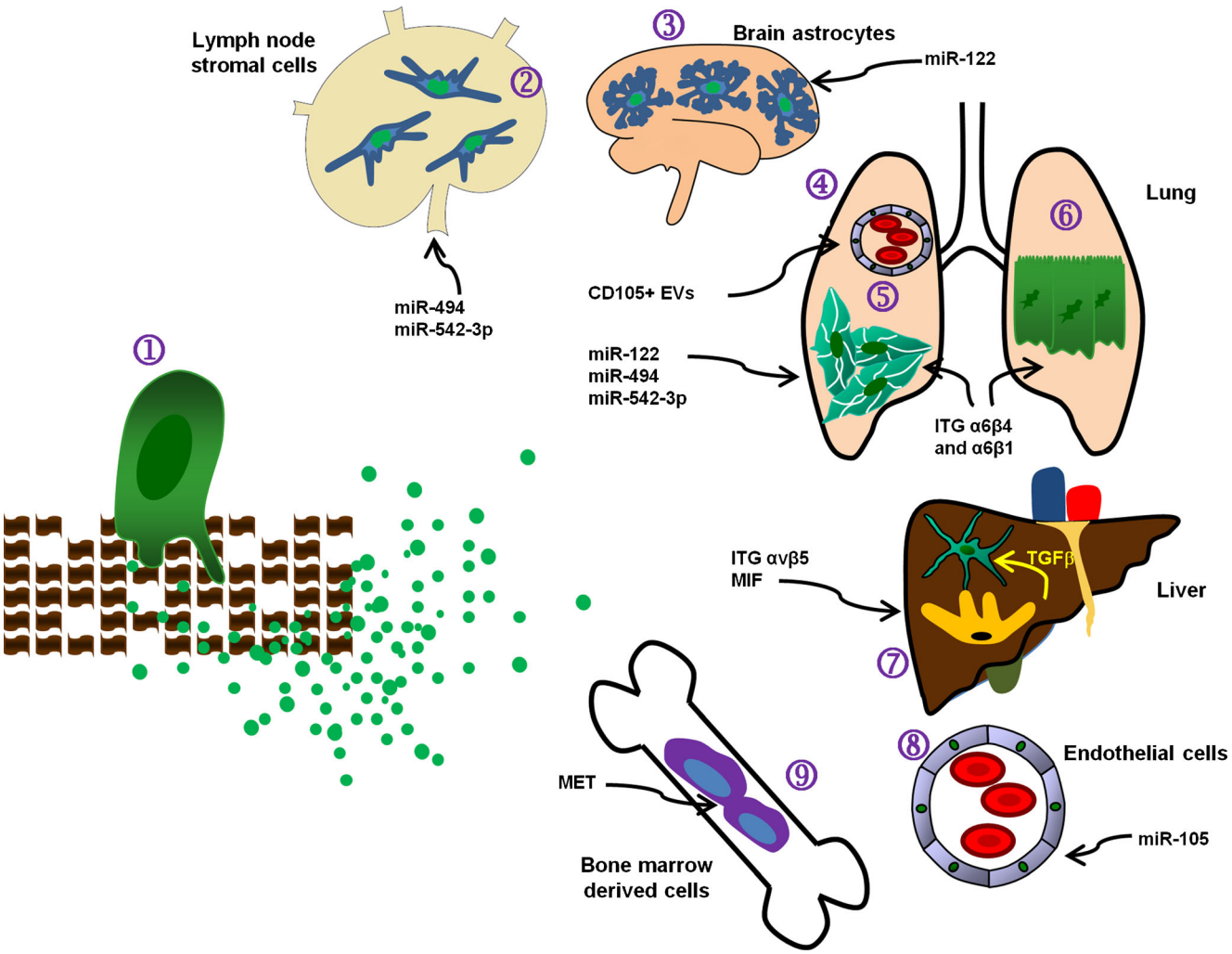

Fig. 3 Role of EVs in preparation of the pre-metastatic niche. Cancer cells in the primary tumor release extracellular vesicles (*EVs*) into the circulation, driving pre-metastatic niche formation (*1*). EVs enriched in miR-494 and miR-542-3p target lymph node stromal cells (*2*). EV-derived miR-122 targets brain astrocytes, reprogramming glucose metabolism (*3*). $CD105^{+}$ renal cancer-stem-cell-derived EVs target lung endothelial cells and induce angiogenesis (*4*). EVs enriched in the abovementioned miRNAs also target lung fibroblasts (*5*). EVs expressing ITGα6β4 and ITGα6β1 are lung tropic and show uptake by lung fibroblasts (*5*) and epithelial cells (*6*) in laminin-rich lung niches and stimulate the formation of a metastasis-enhancing inflammatory environment. Cancer-cell-derived EVs target Kupffer cells to initiate pre-metastatic niche formation in the liver (*7*). EVs expressing tumor EV integrin (*ITGαvβ5*) are liver tropic and fuse with Kupffer cells in fibronectin-rich liver environments. EV-derived macrophage migration inhibitory factor (*MIF*) triggers the release of fibrotic cytokines (TGFβ) by Kuppfer cells. This stimulates the deposition of fibronectin by hepatic stellate cells, creating a supportive environment for disseminated cancer cells (*7*). EV-derived miR-105 targets blood vessel endothelial cells, breaking down vascular integrity and enhancing extravasation of disseminating cancer cells (*8*). EV-derived MET promotes bone-marrow-derived cell mobilization to pre-metastatic sites (*9*)

with control liposomes of a similar size after intra-footpad injection in mice. Melanoma-derived EVs mediated the induction of genes associated with angiogenesis, extracellular matrix remodeling and cell recruitment and metastatic melanoma cells were recruited to EV-rich sites within these preconditioned lymph nodes (Hood et al. 2011). In a pancreatic cancer model, intra-footpad injection of a cell culture supernatant isolated from the highly metastatic rat adenocarcinoma cell line ASML^{wt} modulated the draining lymph node and lung tissue to support the settlement of subsequently injected poorly metastatic ASML-CD44v^{kd} cells lacking the expression of the metastasis-promoting CD44v6 molecule. Fractionation of the cell culture supernatant revealed an important contribution by EVs, independent of CD44v6, complemented by a CD44v6-dependent soluble matrix (Jung et al. 2009). Subsequent research attributed the pre-metastatic priming to the transfer of EV-encapsulated miRNAs targeting the stromal compartment at these remote sites. Tumor-secreted EVs, enriched in miR-494 and miR-542-3p, were taken up by lymph node stromal cells and lung fibroblasts, regulating the expression of cadherin-17, MAL (Myelin And Lymphocyte protein) and TRAF4 (TNF receptor-associated factor 4) genes and leading to the up-regulation of matrix metalloproteinases MMP2, MMP3 and MMP14 (Rana et al. 2013). More recently, gastric-cancer-derived EVs have been reported to contribute to lymphatic metastasis and pre-metastatic niche formation, their participation in this process being dependent on CD97iso expression on gastric cancer cells. Intra-footpad injections of EVs derived from highly metastatic SGC-L cells but not SGC-L/CD97^{kd} cells, strongly promote both SGC-L and SGC-L/CD97^{kd} cell accumulation in the draining lymph nodes. Although immunohistochemistry has demonstrated increased CD55, CD44v6, a5b1, CD31, EpCam and CD151 expression in non-metastatic lymph nodes after EV and tumor cell injection, no mechanism of pre-metastatic niche formation has been elucidated (Liu et al. 2015).

Angiogenesis and endothelial permeability at pre-metastatic niche

CD105^{+} renal cancer stem cell (CSC)-derived EVs have been shown to confer an angiogenic phenotype on human endothelial cells in vitro and to promote angiogenesis in vivo. In addition, treatment of severe combined immunodeficient (SCID) mice with CD105^{+} CSC-derived EVs induced the formation of a pre-metastatic niche, greatly enhancing lung metastasis after the injection of renal carcinoma cells. Characterization of the RNAs, shuttled by CD105^{+} CSC-derived EVs, defined a set of proangiogenic mRNAs and miRNAs. RNase pretreatment of CD105^{+} EVs significantly inhibited their biologic effects (Grange et al. 2011). The authors interpreted this as an indication that RNA transfer by CSC-derived EVs plays a role in establishing the pre-metastatic niche. However, since RNA contained within EVs is protected from the effects of external RNases, these findings indicate instead that co-isolated soluble RNA is, at least in part, responsible for the observed effects. In addition, the specific RNAs involved in this pathophysiological process remain unidentified (Grange et al. 2011). Peinado et al. (2012) reported that EVs from highly metastatic B16-F10 melanoma cells increased the metastatic behavior of primary tumors. This process was found to be mediated through the horizontal transfer of the receptor tyrosine kinase MET to BMDCs and downstream signaling, promoting BMDC mobilization to pre-metastatic sites. Melanoma-derived EVs also induced endothelial permeability at these sites and reprogrammed BMPCs toward a pro-vasculogenic phenotype, positive for c-Kit, Tie2 and Met. Reducing Met expression in EVs and decreasing EV production through Rab27A RNA interference each prevented pro-metastatic BMDC education and metastasis. In addition, a plasma EV-specific melanoma signature comprised of TYRP2 (Tyrosinase-related Protein-2), VLA-4 (Very Late Antigen-4), HSP70 (Heat Shock Protein 70), an HSP90 (Heat Shock Protein 90) isoform and the MET oncoprotein, predicting tumor stage and metastatic progression was identified (Peinado et al. 2012). An increase in endothelial permeability and thus vascular leakiness, might also be induced by the effect of miRNAs contained in tumor-secreted EVs. W. Zhou et al. (2014) demonstrated the enrichment of miR-105 in EVs secreted by metastatic MDA-MB-231 breast cancer cells. EV-derived miR-105 targeted the tight junction protein ZO-1, down-regulating tight junctions on endothelial cells and thus destroying the vascular endothelial barrier function at the local tumor site and at the pre-metastatic site. Therefore, EV-derived miR-105 exerts a dual metastasis-promoting effect by promoting the intravasation of metastatic cells at the primary site and the formation of a pre-metastatic niche, enhancing extravasation of disseminated cancer cells. Anti-miR-105 treatment restored vascular integrity and suppressed metastasis in vivo, whereas the overexpression of miR-105 in a poorly metastatic breast cancer cell line induced vascular permeability and promoted metastasis. Increased levels of circulating miR-105, predominantly enriched in EVs, detected in serum samples of pre-metastatic breast cancer patients correlated with metastatic progression, suggesting clinical application as a predictive or early diagnostic marker (W. Zhou et al. 2014).

Reprogrammed energy metabolism

Reprogrammed energy metabolism as a means to fuel malignant cell growth and division is an emerging hallmark of cancer (Hanahan and Weinberg 2011). Normal cells rely on mitochondrial oxidative phosphorylation to account for their energy needs. Instead, even under aerobic conditions, cancer

cells limit their energy metabolism largely to glycolysis, leading to a state of "aerobic glycolysis", a phenomenon called the "Warburg effect". This seemingly counterintuitive switch in energy metabolism, in which the poor efficiency of ATP production relative to oxidative phosphorylation has to be countered by mechanisms that increase glucose uptake and utilization, allows the diversion of glycolytic intermediates into various biosynthetic pathways required for the increased assembly of new cancer cells (Vander Heiden et al. 2009). Recently, Fong et al. also demonstrated that cancer cells have the ability to alter glucose metabolism at distant organ microenvironments to promote metastasis. In a metastatic breast cancer cell line, they showed that cancer cells from the primary tumor suppress glucose uptake by non-cancer cells in the pre-metastatic niche by secreting miR-122 enriched EVs, thereby increasing nutrient availability for disseminated cancer cells. Mechanistically, vesicle-derived miR-122 targets the glycolytic enzyme pyruvate kinase (PKM), resulting in the down-regulation of GLUT1 glucose transporters. To verify these concepts in vivo, EVs containing low and high levels of miR-122 were intravenously injected in mice, followed by cancer cell injection. High miR-122 levels resulted in increased metastasis to the brain and lungs, while concurrently decreasing glucose uptake attributable to modulations in PKM and GLUT1 expression. The same observations were made in a model using orthotopic xenograft tumors of a poorly metastatic breast cancer cell line over-expressing miR-122. Anti-miR-122 intervention in a xenograft model derived from highly metastatic breast cancer cells, naturally secreting large amounts of miR-122 EVs, alleviated cancer-induced glucose re-allocation and reduced metastasis to the brain and lungs (Fong et al. 2015). Interestingly, elevated levels of circulating miR-122 in the serum of early stage breast cancer patients have previously been associated with metastatic progression (Wu et al. 2012), making it a potential target for therapeutic intervention.

Inflammatory environment

Research by Costa-Silva et al. (2015) has elucidated the sequential steps through which pancreatic cancer EVs promote liver metastasis by initiating the formation of a pre-metastatic niche. Whereas the injection of pancreatic ductal adenocarcinoma (PDAC) EVs in mice, followed by the intravenous injection of PDAC cells, increased the metastatic burden in the liver, education by the injection of normal pancreatic tissue-derived EVs did not. PDAC-secreted EVs were preferentially taken up by Kupffer cells in the liver. EV-derived MIF (macrophage migration inhibitory factor) subsequently triggered the release of several fibrotic cytokines, TGFβ (transforming growth factor-beta) in particular, activating hepatic stellate cells to produce and deposit fibronectin (FN). The fibrotic microenvironment created through this process promoted the recruitment of BMDCs, including macrophages and neutrophils, establishing the pre-metastatic niche that allows the arrest and proliferation of disseminated PDAC cells. Treatment with a TGFβ type 1 receptor inhibitor during the process of EV education reduced FN deposition and macrophage migration to the liver. The same observation was made in an FN knockdown murine model. Macrophage depletion reverted the effects of PDAC-derived EV education on liver metastasis. Additionally, EVs derived from MIF-knockdown PDAC cells lost the ability to elicit the sequential steps of pre-metastatic niche formation, reducing their competence to promote liver metastasis. Clinically, plasma EV MIF levels from stage I PDAC patients who later developed liver metastasis were markedly higher, compared with patients whose pancreatic tumors did not progress, suggesting that EV-derived MIF might be a prognostic marker for the development of PDAC liver metastasis (Costa-Silva et al. 2015).

Organotropism

Although several of the abovementioned studies provided important insights into the mechanisms through which EVs contribute to metastasis formation, none of them elucidated the factor(s) that directs the specificity of these EVs for certain pre-metastatic sites. Hoshino et al. (2015) recently demonstrated that organotropic metastasis is determined by tumor EV integrins (ITGs) fusing with tissue-specific target cells, thereby initiating the process of pre-metastatic niche formation. They observed that EVs from mouse and human lung-, liver- and brain-tropic breast and pancreatic cancer cells were taken up preferentially by resident cells at their predicted destination. Additionally, the injection of lung-tropic EVs in mice enhanced the lung metastatic capacity of subsequently injected lung-tropic cancer cells and, interestingly, redirected the metastasis of bone-tropic cancer cells. This observation suggests that organotropic tumor EVs stimulate the formation of a pre-metastatic niche facilitating the metastatic colonization of these sites by cancer cells poorly capable of doing so. Further analysis by quantitative mass spectrometry and Western blot revealed that ITGs α6β4 and α6β1 were abundantly present in lung-tropic EVs, whereas ITGαvβ5 was primarily found in liver-tropic EVs. Subsequently, lung-tropic EVs expressing ITGα6β4 and ITGα6β1 were shown to be co-localized with S100A4-positive fibroblasts and surfactant protein-C-positive epithelial cells in laminin-rich lung niches, whereas ITGαvβ5-expressing EVs co-localized with $F4/80^{+}$ macrophages and fused with Kupffer cells in FN-rich liver microenvironments, suggesting the selective adherence of these specific ITGs to ECM-enriched areas in the lung and liver. Knockdown of ITGβ4 expression and the blocking of EV ITGβ4 binding to laminin by HYD-1 peptide markedly reduced EV uptake and metastasis in the lung. The same

observations were made for EV uptake and metastasis in the liver through ITGβ5 knockdown or the inhibition of fibronectin binding by RGD peptide. Gene expression analysis by RNA sequencing demonstrated that EV integrin uptake by resident cells in the distant tumor environments activated pro-inflammatory S100 gene expression. Additionally, Src phosphorylation was activated in an ITGβ4-dependent manner (Hoshino et al. 2015). The authors concluded that, in addition to their adhesive properties, EV-derived integrins initiate pre-metastatic niche formation through metastasis promoting S100 proteins (Lukanidin and Sleeman 2012) and Src signaling (Kim et al. 2009). Finally, clinical data showed that plasma EVs isolated from breast cancer patients who later developed lung metastasis, expressed high levels of ITGβ4. Similarly, EV ITGαv levels at diagnosis were higher in patients who had pancreatic cancer and who developed liver metastasis compared with those that did not. These data indicate that EV-derived integrins can be used as biomarkers predicting likely sites of metastasis (Hoshino et al. 2015).

Osteoclast differentiation?

Gene expression analysis from prostate cancer bulk and CSC-derived EVs demonstrated their enrichment in miR-100-5p and miR-21-5p. Additionally, miR-21-5p was differentially overexpressed in EVs derived from bulk cells compared with CSCs, whereas the opposite was true for miR-139-5p. Transfection of normal prostate fibroblasts (WPMY-1) with miR-100-5p, miR-21-5p and miR-139-5p increased fibroblast migration and the expression of MMP2, MMP9, and MMP13, related to ECM restructuring and of RANKL (receptor activator of NF-κB ligand), related to osteoclast recruitment and cell migration. Based on these results, the authors of this study (Sanchez et al. 2015) hypothesized that prostate cancer EVs act at the distant bone microenvironment, preparing the pre-metastatic niche through the RANKL-mediated initiation of the bone vicious cycle that activates osteoclastogenesis. However, fusion of these vesicles with target cells at the bone microenvironment and the proposed mechanism of pre-metastatic niche preparation through miRNA-mediated RANKL-signaling were not experimentally validated (Sanchez et al. 2015).

Role of EVs in transit (interaction with CTCs)

Metastatic colonization is a highly inefficient process. Blood-borne cancer cells are exposed to the innate immune system, hemodynamic shear forces and oxidative stress. Therefore, the vast majority of cancer cells entering the circulation fail to form metastases. Important factors supporting the survival of CTCs are platelets. In transit, CTCs are coated with platelets and components of the coagulation system, which support their survival by protecting them from immune recognition and by escorting them to the site of extravasation (Massague and Obenauf 2016; Quail and Joyce 2013).

Early work by Poste and Nicolson (1980) demonstrated that the phytohaemagglutinin/polyethylene glycol-induced fusion of plasma membrane vesicles from highly metastatic F10 melanoma cells with poorly metastatic F1 cells significantly enhanced the ability of blood-borne F1 cells to arrest in the lung microcirculation and to establish metastases. This research primarily suggested that the differences in the abilities of melanoma cell sublines to localize in the lung are determined by differences in cell surface properties. Nonetheless, with the current advances in EV research, we can postulate a role for EVs in the survival of CTCs by facilitating their arrest in the microcirculation of target organs. Once these metastatic cells have infiltrated into the more protective environment of the vessel wall, they may be less susceptible to hemodynamic shear stress and immune mechanisms (Poste and Nicolson 1980).

Technical note

We have reviewed the role of membrane-encapsulated miRNAs in cancer progression published in 47 original research manuscripts. These studies involved various terminologies to describe the assessed EVs (exosomes: $n=32$; extracellular vesicles: $n=6$). Although the origin of exosomes and microvesicles has been defined precisely, current methodology does not allow the experimental separation or discrimination of the different EV types of similar sizes. Therefore, we used the term EVs (extracellular vesicles) throughout this review. In addition, when analyzing the functional role of EV-specific miRNAs, the isolation method to purify EVs from biofluids is of uppermost importance. Indeed, differential centrifugation and commercial precipitation kits are known to co-isolate protein aggregates and lipoproteins, non-membrane-containing particles rich in RNA, a finding that obscures the identification of EV-specific miRNAs (Van Deun et al. 2014). In addition, miRNAs are thought to be more enriched in protein complexes than in EVs (Arroyo et al. 2011). Future studies should identify the contribution of free circulating miRNA captured in protein complexes and EV encapsulated miRNAs to cancer cell biology and reveal their (differential or equal) importance for cancer diagnosis, prognosis and therapy monitoring. Since the 29 and 11 studies reviewed here have, respectively, used differential centrifugation only (not with other purification steps) and commercial precipitation kits to identify EV-specific miRNA profiles and their functional effects, the reader should be aware of the possibility that the described role of EVs in miRNA transfer in cancer progression can be only partially or not even attributed to EVs. Although ISEV2014 minimal experimental requirements recommend

the validation of observed functional effects with pure EV populations obtained by density gradient centrifugation, only four studies actually implement this isolation method (Lotvall et al. 2014). Only 13 studies characterized the isolated EVs by different complementary methods such as protein analysis particle analysis, and electron microscopy, whereas five studies performed no characterization at all. Strikingly, not one study analyzed the presence of Argonaute-2 protein to assess the level of contamination with miRNA-bound protein complexes. RNase treatment is often performed to exclude the contribution of other extracellular RNAs to functional effects. However, the efficiency of this treatment is mostly not evaluated and Argonaute-2 complexes are resistant to this treatment (Turchinovich et al. 2011). To demonstrate the functional role of EV encapsulated miRNAs, in vitro uptake is usually analyzed by flow cytometry, confocal microscopy, or quantitative reverse transcription plus PCR (Q-RT-PCR) analysis of the miRNA(s) of interest. However, in the EV uptake assays, 26 out of 30 studies did not indicate the treatment dose or the number of recipient cells. In this review, in vitro and in vivo studies were assessed by using a semi-quantitative method with A: containing sufficient information (EV dose, number of recipient cells, timing of experiments, EV treatment methods) to repeat the experiment, B: containing partial information given with the omission of the number of recipient cells or EV dose and C: no or almost no information being given concerning the experimental protocol (cf. Table 2). Only three studies involve a time-dependent evaluation and not one study includes an EV dose–response curve. In addition, in order to assess the functional effects of EV education, complementary methods such as Q-RT-PCR of target genes, western blot analysis and migration, invasion, proliferation and tube formation assays have been implemented but again, the doses of EVs provided to a certain amount of recipient cells for a certain period of time are often not specified, further hindering the interpretation of the research data. In conclusion, a combination of the correct isolation methods and the detailed experimental description of the treatment dose, time and number of recipient cells is a prerequisite to foster our knowledge about the role of EV-encapsulated miRNAs in various steps of cancer progression.

Concluding remarks

MiRNAs have been reported in EV isolations and play a role in local invasion, angiogenesis, immune modulation, metastatic niche preparation, colonization and dormancy. To improve our knowledge of EV-enclosed miRNAs further, future research should attempt to include a validation of functional results with pure EV populations in a dose-dependent and time-dependent manner and to provide all experimental details in order to increase reproducibility. In addition, by using density gradients, Argonaute-2 miRNA/protein complexes can be separated from EVs allowing the identification of miRNAs uniquely enriched in EVs. Finally, cancer cells load EVs, specifically or unspecifically, with pro-tumorigenic miRNAs to transmit aggressive traits to neighboring cancer cells and on the other hand, cancer cells implement identical mechanisms to discard anti-tumorigenic miRNAs. The mechanisms by which cancer cells are able to distinguish these two types of EVs have not been elucidated yet. Indeed, some experiments have revealed unexpected results, such as the inhibition of proliferation in normal cells after treatment with EVs derived from cancer cells (Lee et al. 2015). However, in these experiments, the contribution of the tumor environment has not been considered. One possibility is that these EVs are preferentially sequestered in stromal cells in which miRNAs can exert different functions from those in cancer cells.

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