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Review

Extracellular vesicle-mediated modulation of angiogenesis

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Summary. Angiogenesis is a tightly regulated process where a number of different players are involved. Recently, a role for membrane vesicles actively released from cells has been proposed. Virtually all cell types may release non-apoptotic membrane vesicles in the nano-size range containing critical components of the cell of origin. The two main categories of these vesicles include exosomes and microvesicles that differ for biogenesis but, sharing several features and mechanisms of action, have been collectively named extracellular vesicles (EV). EV are able to transfer from one cell to another bioactive lipids, proteins and nucleic acids that may induce changes in the phenotype and functions of the recipient cells. This new mechanism of cell to cell communication has been involved in modulation of the angiogenic process. Tumor cells, inflammatory cells and stem/progenitor cells were shown to release EV with angiogenic properties, suggesting that they may act on vascular remodeling in different physiological and pathological conditions. In this review we discuss the evidence for the role and the mechanisms of action of EV in vascular homeostasis and in the angiogenic processes occurring in tumors, inflammation and tissue regeneration.

Key words: Exosomes, Microvesicles, Angiogenesis

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Introduction

Angiogenesis is a physiological process of new vessel formation consequent to destabilization of pre-formed vessels. It consists of endothelial cell sprouting followed by canalization and final stabilization of the vascular wall. This process not only occurs in the embryo during fetal life, but also in the adult organism, where it is involved in homeostasis, tissue repair and tumor progression (Carmeliet and Jain, 2011). The players of angiogenesis, namely endothelial cells, pericytes and extracellular matrix (ECM), interact with each other through multiple signals that provide a coordinated regulation of different phases of angiogenesis. An impaired regulation is involved in multiple pathological processes that may be characterized either by reduced, abnormal or enhanced angiogenesis (Carmeliet and Jain, 2011; Katoh, 2013). Apoptotic remodeling, matrix deposition or degradation, proliferation and migration of endothelial cells and pericytes, are driven by soluble mediators and cell-to-cell and cell-to-matrix interactions. Among soluble mediators we can find matrix metalloproteinases (MMP), which are implicated in ECM remodeling, in pathways involved in regulation of activation/quiescent state of endothelial cells, and in their migration and proliferation. Other regulating factors are vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), angiopoietins (ANGPT1 and ANGPT2), Notch ligands [jagged 1 (JAG1) and Delta like ligand 4 (Dll4)], and fibroblast growth factor (FGF). These mediators have been shown to contribute in modulation of the angiogenic phenotype of endothelial

cells by differentially activating eNOS, SRC, RAS-ERK, PI3K-AKT, Notch, WNT and Hedgehog signaling pathways (Coultas et al., 2005). Whereas pericyte-derived ANGPT1 maintains quiescent endothelial cells by activating TEK/TIE2 receptor, ANGPT2 stimulates endothelial sprouting by inhibiting TEK (Fagiani and Christofori, 2013). Similarly, sprouting is inhibited and vessels are stabilized by Dll4-Notch signaling, whereas JAG1-Notch signaling has an opposite effect resulting in angiogenic sprouting (Bridges et al., 2011). TGF-β also has differential effects depending on receptor/pathway activation. TGF-β prevents the endothelial cell sprouting when it activates Smad2/3 cascade through TGFβR1/ALK5. Conversely, TGF-β stimulates endothelial cell proliferation and migration when it activates Smad1/5 cascade through ACVRL1/ALK1 receptor (Gaengel et al., 2009). By activating its receptor VEGFR2, VEGF triggers eNOS, RAS-ERK, SRC and PI3K-AKT signaling pathways in endothelial cells, inducing enhanced permeability, migration and proliferation (Olsson et al., 2006). FGF2 may stimulate angiogenesis either indirectly through stimulation of endothelial cell VEGF secretion or directly through its receptor FGFR1 (Presta et al., 2005). To this complex array of soluble mediator/receptor interaction and dependent pathway activation the potential contribution of cell-secreted vesicles has recently been added. These vesicles may induce epigenetic changes in endothelial cells and pericytes by delivering biologically active lipids and proteins/receptors. Moreover, vesicles may vehiculate extracellular RNAs able to modify the phenotype and function of recipient cells. RNA encapsulation in vesicles allows protection from nuclease degradation and sharing between neighboring and distant cells (Camussi et al., 2014). In the present review we will discuss the evidence for the involvement of cell-secreted vesicles in modulation of angiogenesis.

Extracellular vesicle-mediated intercellular communication

Extracellular vesicles (EV) are defined as a set of secreted membrane vesicles. This group includes exosomes, which are vesicles released from the endosomal compartment, and vesicles, which are shed from the cell surface and are indicated in the literature with different names, such as ectosomes, microvesicles or shedding vesicles. These two major classes of EV display similar functions despite their differences in origin and size. In particular, they transfer, from one cell to another, cellular constituents that may account for an extension of the functional properties of originating cells. In fact, these vesicles contain cytoplasmic proteins, nucleic acids, surface receptors, and lipid raft-interacting proteins (Lötvall et al., 2014). Moreover, EV encapsulation of nucleic acids, including mRNA, long non-coding RNA (lncRNA) and microRNA (miRNA), and in some instances also DNA, were found to protect from enzymatic degradation and to allow exchange of genetic information among cells (Ratajczak et al., 2006b; Deregibus et al., 2007; Valadi et al., 2007; Balaj et al., 2011). A common pattern of these molecules is shared by all EV, whilst other proteins and nucleic acids are characteristic of the cell of origin. Moreover, EV content may vary depending on the metabolic and the activation state of the cells. For example, it has been shown that EV derived from tumor and neutrophil cells carry MMP and proteolytic enzymes involved in inflammation and tumor progression and that platelet derived-EV express integrins and P-selectin involved in the coagulation cascade (Cocucci et al., 2009).

Once released from the cell of origin, by membrane shedding or exocitosis of multi vesicular bodies, EV may remain in the extracellular space interacting with recipient cells in a paracrine fashion or undergo degradation. Otherwise, vesicles may migrate far from the site of origin by entering in biological fluids, like blood, urine and cerebrospinal fluid (Cocucci et al., 2009).

Much evidence supports the role of EV as important effectors in cell-to-cell communication as they may exchange multiple signals between cells (Ratajczak et al., 2006a). As shown by Raposo et al. (1996), EV released from B lymphocyte may serve as a vehicle for presentation of the peptide-MHC–II complex to T cells, thus acting as a signaling complex. Moreover, EV may deliver surface receptors or biologically active proteins. For instance, EV released from tumor cells carry Fas ligand that may induce apoptosis of T cells favoring an immune escape (Kim et al., 2005), and EV released from platelets carry adhesion molecules that may induce pro-adhesive properties in target endothelial cells (Barry et al., 1998).

EV were also shown to transport growth factors that may promote angiogenesis. For instance, cancer stem cells may release EV containing MMP2/9, angiopoietin 1, ephrin A3, FGF, and VEGF (Grange et al., 2011). EV can transfer several other biologically active cytoplasmic proteins. For example caspase-1 which, once released from activated monocytes, may induce apoptosis of vascular smooth muscle cells (Sarkar et al., 2009), and oncogenic products, like EGFRvIII (Al-Nedawi et al., 2008), which can be conveyed by tumor derived EV to recipient cells inducing a phenotypic switch. In addition, EV released from cancer cells were shown to carry bioactive lipids, such as sphingomyelin, able to activate migration and invasion of endothelial cells (Kim et al., 2002).

EV interaction with target cells

EV are able to interact with target cells through various mechanisms. Since vesicles are membrane-bound structures, it has been postulated that they may fuse and release their content in recipient cells. The mechanism is largely unknown. However it has been proposed that the uptake may occur by receptor-mediated interactions or by direct fusion of EV with cell

plasma membrane. Recently, syncytin-1, which belongs to a family of mammalian fusogens, has been implicated in the cell uptake of placental EV (Vargas et al., 2014). After internalization EV can deliver mRNA and miRNA (Deregibus et al., 2007; Valadi et al., 2007; Skog et al., 2008), which may undergo translation into protein or regulate gene expression (Ratajczak et al., 2006a; Bruno et al., 2009; Aliotta et al., 2010). This hypothesis is supported by studies based on lipid fusion assays, which observed that the transfer of miRNA shuttled by EV to dendritic cells (DCs) depends both on actin-mediated phagocytosis and on cholesterol rich plasma membrane microdomain fusion (Parolini et al., 2009; Montecalvo et al., 2012). Furthermore, an acidic pH, which is typical of hypoxic microenvironment of solid tumors, facilitates EV membrane fusion and release of their content into the cytoplasm (Taraboletti et al., 2006).

Another way of EV internalization is receptor-mediated endocytosis. Many surface molecules may mediate exosome uptake. For example, P-selectin glycoprotein ligand-1 expressed by macrophage-derived EV mediates binding to platelets (Cocucci et al., 2009), phosphatidylserine receptors favor entry into macrophages (Miyanishi et al., 2007), and heparan sulfate proteoglycans induce vesicle capture in many cell types (Christianson et al., 2013).

Through fusion with target cells, EV may convey receptors and adhesion molecules which exert an active role in recipient cells. For instance, platelet-derived-EV can transfer the CD41 ligand to endothelial cells (Barry et al., 1998) or to tumor cells (Janowska-Wieczorek et al., 2001) with consequent enhancement of their pro-adhesive properties.

Endocytosis may also occur by a receptor independent mechanism, as a consequence of small EV interactions with clathrin coated vesicles or, for larger EV, by mechanisms of phagocytosis and micro-pinocytosis (Tian et al., 2014). Once incorporated by cells, EV may release their content in the cytoplasm or undergo digestion after fusion with lysosomes.

However, internalization is not always necessary for transmission of signals. In fact, another mechanism of EV-to-cell communication is based on direct cell stimulation. For instance, platelet-derived-EV may directly activate intracellular signaling pathways in endothelial (Barry et al., 1997), inflammatory (Miyamoto et al., 1998), and human hematopoietic cells (Ratajczak et al., 2006b), thus favoring tumor invasion and diffusion.

EV induced phenotypic and functional changes in recipient cells

The delivery of transcriptional regulators via EV may lead to the epigenetic and functional re-programming of target cells, underlying that EV carry a high plasticity potential, which may influence cell fate. Studies on the paracrine action of stem cells have shown that EV derived from stem cells may mimic several biological effects of the cell of origin. As mentioned above, Ratajczak and coworkers (Ratajczak et al., 2006b) demonstrated that vesicle-delivered proteins and mRNA are critical for maintaining stemness and pluripotency of bone marrow stem cells. Embryonic stem cells-derived EV express stem cell-specific ligands that stimulate expansion and self-renewal of adult stem cells. Furthermore, EV carry a specific pattern of mRNA derived from the cell of origin, which could be horizontally transferred to recipient cells increasing their stem potential (Ratajczak et al., 2006b).

Studies by the group of Quesenberry on the ability of bone marrow cells to acquire a non-hematopoietic phenotype showed that EV derived from injured lung may induce the expression of lung specific mRNA and proteins in marrow cells (Aliotta et al., 2007). The mechanism was related to the transfer of non-coding regulatory RNAs, such as miRNA and lncRNAs from injured lung cells to bone marrow cells. Therefore, EV can either directly shuttle RNAs or transcription factors, which are able to modify the expression of tissue-specific mRNA and to induce phenotypic changes. This seems to be a general phenomenon since it was observed in various tissues, such as bone marrow, liver, brain and heart (Aliotta et al., 2010). Especially the delivery of transcription factors seems to be the basis of the stability of EV-induced cellular reprogramming along time. This was shown by co-culture experiments with either rat or mouse bone marrow cells incubated with lung derived EV from the opposite species. For up to 12 weeks bone marrow cells maintained expression of pulmonary epithelial cell genes of the opposite species, which decreased after this period. However, a *de novo* mRNA transcription pattern remained, indicating EV induction of stable transcriptional changes (Aliotta et al., 2012). Moreover, these authors proposed that the stem cell plasticity is dependent not only on EV and other environmental stimuli but also on cell cycle transit (Quesenberry et al., 2010).

Deregibus et al. (2007) observed that EV derived from endothelial progenitor cells contain a particular subset of mRNA, some of which are involved in angiogenesis. These vesicles undergo integrin-mediated internalization in endothelial cells and their content activates signaling pathways like PI3K/AKT and eNOS, leading to the activation of an angiogenic program. Furthermore, a GFP-mRNA was internalized in vesicles and transferred in endothelial cells, where GFP protein was synthesized. EV effect was abrogated by treatment with a high non physiological dose of RNase. These data suggest that the RNA delivered by EV participate in vesicle effects.

EV derived from endothelial cells may exert an autocrine/paracrine action critical for maintenance of vascular homeostasis. Sheldon et al. (2010) demonstrated the exchange of Dll4 between neighboring endothelial cells *via* EV with consequent inhibition of Notch signal ensuing in stimulation of angiogenesis.

Taken together these studies suggest that EV may

exert an important role in physiological conditions and in the exchange of information between healthy and diseased tissues in several pathological states, including inflammation and cancer. EV may act either locally or at distant sites (Castellana et al., 2009) reached by a random diffusion through tissue and body fluids or by a more specific mechanism of homing guided by surface receptors (Hood et al., 2011).

EV-induced modulation of tumor microenvionment

It is well known that tumor cells release a large amount of EV enriched in proteins and nucleic acids with an oncogenic potential. EV released by cancer cells may modify the tumor microenvironment to favor tumor growth and metastasis (Janowska-Wieczorek et al., 2005; Jubb et al., 2009). For instance, tumor EV carry MMP, which mediate ECM disruption and release of growth factors favoring tumor cell migration (Hendrix et al., 2010). Moreover, tumor EV contain cytokines, which stimulate immune and inflammatory responses (Thery et al., 2009), and VEGF, which exerts pro-angiogenic and pro-inflammatory functions (Taraboletti et al., 2006). Vesicles derived from leukemia cells also favor tumor development by promoting inflammation and reprogramming mesenchymal and endothelial stem cells towards cancer-associated fibroblast phenotype (Paggetti et al., 2015). Moreover, the microenvironment hypoxic conditions amplify the pro-angiogenic and pro-metastatic potential of EV favoring reduction of cell-to-cell and cell-to-ECM adhesion and increasing invasiveness. In fact, proteomic analysis of tumor EV cargo revealed a significant enrichment in proteins involved in ECM remodeling, focal adhesion, angiogenesis, and immune cell recruitment (Park et al., 2010). Another study performed on glioblastoma showed that in a hypoxic status, which reflects the low oxygen conditions of patient tumors, EV are enriched in hypoxia-regulated mRNA and proteins, such as PDGF, MMP, IL-8, caveolin 1, and lysyl oxidase. These EV were shown to be responsible for the exchange of information between malignant and vascular cells, for endothelial release of growth factors and cytokines, for activation of PI3K/AKT signaling pathway, and for migration of pericytes. Therefore, tumor EV may exert an autocrine stimulation of tumor cell proliferation and migration (Kucharzewska et al., 2013).

It is well known that angiogenesis is essential for tumor cell survival and that the uncontrolled growth of the tumor mass leads to a reduction of the oxygen level in the surrounding microenvironment. Given that tumor hypoxia plays a major role in stimulation of new-vessel formation process, but also in vesicle secretion and content, it is not surprising that cancer EV sustain tumor vascularization (Wysoczynski and Ratajczak, 2009; Park et al., 2010). This hypothesis is supported by the observation that Rab22a, a GTPase which controls vesicle budding from the plasma membrane, is regulated by hypoxia inducible factors (HIF). Furthermore, the overexpression of this protein improves metastasis formation, whereas its silencing counteracts tumor spread (Wang et al., 2014).

Tumor derived EV have also been shown to modulate the immune response to tumor (Robbins and Morelli, 2014). EV may present tumor antigens. However, several studies indicated that tumor EV may favor the immune-escape by inhibiting NK and T cell-mediated immune response (Kim et al., 2007).

Table 1. Angiogenic properties of tumor-derived EV on endothelial cells.

EV origin	Effect	Factors	References
Ovarian carcinoma	Tubule formation	VEGF	Taraboletti et al., 2006
Glioblastoma	Migration of pericytes, migration of tumor cells	PDGF, MMP, IL-8, caveolin 1, lysys oxidase	Kucharzewska et al., 2013
	Endothelial Tubule Formation	EGF-R, VEGF, FGF, IL-8, IL-6, angiogenin, TIMP-1, TIMP-2	Skog et al., 2008
Pancreatic cancer	Vessel branching	Tspan8	Gesierich et al., 2006
Glioma	Increased expression of VEGF and VEGF-R2 in endothelial cells	EGFRvIII (EGF-R oncogenic form)	Al-Nedawi et al., 2008
Colorectal cancer and glioma	Switch to tip-cell phenotype, new vessel formation	Dll4	Jubb et al., 2009; Sheldon et al., 2010
Renal cancer stem cells	Induction of angiogenesis *in vitro* and *in vivo*, promotion of lung metastasis	VEGF, FGF, angiopoietin1, ephrin A3, MMP2, MMP9	Grange et al., 2011
Breast cancer	Induction of angiogenesis	miR210	King et al., 2012; Kosaka et al., 2013
Ovarian and lung cancer	Induction of angiogenesis	miR214	Van Balkom et al., 2013
Leukemia	Induction of angiogenesis	miR17-92 cluster	Umezu et al., 2013
Renal cancer stem cells	Tumor invasion and metastasis	miR200c, miR92, miR141, miR29a, miR650, miR151	Grange et al., 2011
Carcinoma cells (under hypoxic conditions)	Induction of angiogenesis and metastasis	MMP, angiogenin, VEGF, PDGF, IL-1, IL-3	Park et al., 2010
MSC (under hypoxic conditions)	Induction of angiogenesis	VEGF, angiogenin, UPAR, MCP-1, IGF, Tie-2/TEK, IL-6	Chen et al., 2014

Furthermore, it has been shown that T lymphocyte cytotoxicity may be restored by interleukin-12 anchored to EV by reversing the JAK/STAT pathway (Zhang et al., 2015). This opens the possibility of EV manipulation for therapeutic purposes.

Role of EV in tumor angiogenesis

Evidence of tumor-derived EV implication in angiogenesis was provided by Kim et al. (2002) who reported that these vesicles stimulate migration of endothelial cells and formation of new vessels through a sphingomyelin mediated interaction. Further studies focused on pro-angiogenic proteins shuttled by EV. Taraboletti et al. (2002) observed that EV deliver active MMP (i. e. MMP-2, -9, and MT1-MMP) able to degrade ECM, favoring proliferation and migration of endothelial cells and consequent formation of new vessels. Gesierich et al. (2006) reported that tetraspanin proteins involved in motility, adhesion, and angiogenic modulation are abundantly expressed in exosomes compared to parent cells. In particular, pancreatic cancer derived-EV express Tspan8, which triggers vessel branching. The same protein, complexed with other adhesion molecules, such as CD49d, was identified as a mediator for the recognition and internalization of EV in endothelial cells (Nazarenko et al., 2010). Indeed, after internalization, EV upregulate expression of pro-angiogenic factors like VEGF, VEGF-R2 and von Willebrand factor (Nazarenko et al., 2010). EV derived from glioma carry the mutated oncogenic form of the epidermal growth factor receptor (EGFRvIII). This factor is responsible for the activation of MAPK and Akt pathways, which lead to the increased expression of VEGF and VEGF-R2 in endothelial cells (Al-Nedawi et al., 2008). EV interaction with the target cells can be prevented by Annexin-V, which masks phosphatidyl-serine residues on EV surface, impeding the internalization in the endothelial cells and the angiogenic signaling (Al-Nedawi et al., 2009). EGFRvIII mutated tumor form plus EGF-R and TGFβ were also found in brain tumor released exosomes (Graner et al., 2009). Millimaggi et al. (2007) reported that EV from ovarian cancer cells shuttle the extracellular MMP inducer CD147, which is involved in the activation of the angiogenic program in endothelial cells. This effect was lost using a small interfering RNA (siRNA) against CD147. Skog et al. (2008) studied the content of vesicles derived from glioblastoma and found many pro-angiogenic mRNA and proteins, such as EGF-R, VEGF, FGF, IL-8, IL-6, angiogenin, TIMP-1 and TIMP-2. These factors stimulate endothelial tubule formation. Moreover, EV released by colorectal cancer and glioma cells carry Dll4. Usually this ligand is highly expressed by activated endothelial cells and inhibits Notch signaling pathway thus favoring the acquisition of a tip-cell phenotype by endothelial cells. The transfer of Dll4 activates quiescent endothelial cells leading to formation of filopodia and new vessels (Jubb et al., 2009; Sheldon et al., 2010).

Park et al. (2010) analyzed the protein content of EV released by carcinoma cells under hypoxic conditions. They found proteins reported to facilitate angiogenesis and metastasis such as MMP, angiogenin, VEGF, PDGF, IL-1, IL-3. A recent study analyzed the content of vesicles from mesenchymal stromal cells (MSC) released under hypoxic conditions and observed again an enrichment in VEGF and angiogenin, plus receptor of urokinase-type plasminogen activator (UPAR), monocyte chemotactic protein-1 (MCP-1), IGF, Tie-2/TEK, and IL-6 compared to MSC and EV released in normal conditions (Chen et al., 2014). This suggests that under hypoxic conditions different cells react with a common mechanism leading to the release of vesicles containing several pro-angiogenic factors. Moreover, a recent study by Lindoso et al. (2015) showed that EV from cancer stem cells may reprogram MSC by inducing the expression of CXCR4 and CXCR7, which are associated with migration ability, of IL-8, Osteopontin, and Myeloperoxidase, which favor angiogenesis and

Table 2. Stem/progenitor cell-derived EV and angiogenic tissue regeneration.

EV origin	Effect	Factors	References
MSC	Promotion of cutaneous wound healing	Wnt4	Zhang et al., 2015
	Repair of chronic mucosal injury	Annexin 1	Leoni et al., 2015
	Promotion of angiogenesis and myogenesis	miR494	Nakamura et al., 2015
ASC	Stimulation of angiogenesis	TCF4, HES1, HGF	Eirin et al., 2014
	Stimulation of angiogenesis	c-kit, SCF, APRIL, artemin, MFG-E8, MMP-20, angiopoietin like factor	Lopatina et al., 2014a
	Stimulation of angiogenesis	miR148a, miR378, miR532-5p, let-7f	Eirin et al., 2014
	Stimulation of angiogenesis	miR17, miR21, miR126, miR130a, miR296, miR210	Lopatina et al., 2014b
EPC	Stimulation of angiogenesis, inhibition of apoptosis	α4 and β1 integrins	Deregibus et al., 2007; Cantaluppi et al., 2012b
	Promotion of angiogenesis, kidney protection from ischemia-reperfusion injury	miR126, miR296	Cantaluppi et al., 2012a
Myocardial progenitor cells	Endothelial cell migration and wound healing	MMP, EMPRIN	Vrijsen et al., 2010
Cardiomyocytes	Stimulation of angiogenesis	Hsp20	Zhang et al., 2012

tumor growth.

One more important class of mediators involved in EV angiogenic signaling is represented by nucleic acids, in particular mRNA and miRNA. Studies on glioma and colorectal cancer showed that EV carry several transcripts involved in angiogenesis, cell migration, and proliferation (Skog et al., 2008; Hong et al., 2009). They also confirmed that mRNA shuttled by EV is translated into proteins within recipient cells (Skog et al., 2008) and that EV enhance endothelial cell proliferation and tubule formation (Hong et al., 2009). Grange et al. (2011) studied EV derived from a subset of renal cancer cells. Vesicles from cancer stem cells, but not from other tumor cells, were shown to contain mRNA and miRNA that exert angiogenic effects. Administration of these EV *in vivo* were found to induce local vessel branching and to prepare lung microenvironment for tumor cell migration and metastatization. Evidence also suggests that EV derived from MSC can promote tumor engraftment and growth through the upregulation of VEGF and CXCR4 mRNA and protein and the consequent activation of ERK1/2 pathway leading to an increase in tumor vascularization (Zhu et al., 2012).

Hypoxia seems to increase the compartmentalization in vesicles of the pro-angiogenic miR210 (King et al., 2012), which is also contained in breast cancer derived EV (Kosaka et al., 2013). These vesicles were shown to induce HUVEC reprogramming and consequent capillary formation, and to promote lung metastatization. King et al. (2012) showed that the increased EV secretion, which occurs under hypoxic conditions, is reduced by siRNA-mediated silencing of HIF. On the contrary, EV production can be increased by HIF pharmacological stimulation.

Certain miRNA shuttled to endothelial cells promote angiogenesis by favoring cell migration. Among these, miR17–92 cluster represses the expression of adhesion molecules (Umezu et al., 2013). Indeed, endothelial cell-, ovarian- and lung cancer–derived exosomes are enriched in angiogenesis-stimulating miR214. Anti–miR214 treatment was shown to inhibit endothelial migration and sprouting (van Balkom et al., 2013).

Role of EV in inflammatory angiogenesis

Aberrant angiogenesis occurs in inflammation statuses associated with several disorders, among which are cardiovascular diseases (Dignat-George and Boulanger, 2011), atherosclerosis (Leroyer et al., 2008; Philippova et al., 2011) and proliferative diabetic retinopathy (Chahed et al., 2010). Also in these cases EV may be involved and cooperate in disease progression.

In normal subjects, circulating EV are mainly derived from platelets. However, in patients with vascular injury an increase of monocyte and endothelial cell derived EV has been observed. Moreover, the level of circulating EV is increased in inflammation and may promote activation of monocytes and endothelial cells leading to chronic vascular injury, such as atherosclerosis (Angelillo-Scherrer, 2012; Loyer et al., 2014).

Leroyer et al. (2008) postulated that vesicles derived from human atherosclerotic lesions may be responsible for intraplaque neovascularization through a CD40-dependent mechanism of stimulation of endothelial cells. Philippova et al. (2011) observed that an increased expression of T-cadherin on the surface of EV derived from endothelial cells is a marker of endothelial dysfunction, which occurs in early phases of atherosclerosis. Indeed, this molecule may activate an angiogenic pathway through Akt phosphorylation in endothelial cells boosting the atherosclerotic damage.

Recent evidence suggests that EV may also play a role in progression of diabetic retinopathy. Chahed et al. (2010) found increased levels of platelets and endothelial cell-derived EV in vitreous samples of diabetic patients, compared to controls. They identified EV derived from endothelial cells as shedding products prevalently of neo-formed retinal endothelial cells, while platelet-derived EV originate from plasma. Given that these vesicles appeared to promote endothelial proliferation *in vitro* and angiogenesis in a Matrigel plug model, the authors postulated that they may contribute to new vessel formation in the retina of patients. Furthermore, after two different treatments for diabetic retinopathy, these vesicles were shown to be abundantly reduced.

Another work by Beltramo et al. (2014) focused on the effect of MSC-derived EV on retinal pericytes.

These cells may be lost in early phases of the disease, favoring the proliferation of endothelial cells. The authors cultured MSC in physiological and diabetic-like conditions, then incubated EV with pericytes. They observed that EV are internalized into target cells and, in a time-dependent way, pericytes detach and start to migrate, with a major effect of EV obtained in diabetic-like conditions. They suggested that, in this way, EV may indirectly favor angiogenesis as a consequence of vascular destabilization. By adding EV to endothelial cells/pericytes co-cultures in Matrigel, they observed the formation of new vessels, confirming also a direct pro-angiogenic property of EV. Furthermore they hypothesized that this process is supported by MMP-2, whose active form is expressed by EV and EV-stimulated pericytes.

A different study evaluated the angiogenic properties of exosomes derived from different retinal cells and reported that vesicles released from retinal astroglial cells may play an anti-angiogenic role by suppressing retinal vascular leakage and reducing choroidal neovascularization. Data obtained by protein array showed that these vesicles are enriched in proteins with anti-angiogenic properties, like endostatin, pigment endothelium-derived factor and TIMP-1, but also of MMP and chemokines that, depending on the context, may either inhibit or promote angiogenesis. In fact,

angiostatin and endostatin, two anti-angiogenic factors, derive from MMP cleavage of plasminogen and collagen XVIII. Indeed, these vesicles appeared to suppress macrophage infiltration, contributing to the reduction of inflammation (Hajrasouliha et al., 2013).

Other studies analyzed EV role in the inflammatory status. Yamamoto et al. (2015) recently demonstrated that EV released by endothelial cells during inflammation are involved in the cell-to-cell communication with pericytes and vascular smooth muscle cells by transferring selected miRNA. These EV induce in the recipient cells an increased expression of VEGF-B mRNA and protein that modulate vascular remodeling (Yamamoto et al., 2015). The analysis of proteins, mRNA, and miRNA in BALF has also suggested a potential contribution of EV in progression of lung allergic reactions (Fujita et al., 2014).

Aliotta et al. (2009) have recently shown an EV mediated pulmonary vascular remodeling in an experimental model of pulmonary hypertension. Vascular damage consisted of fibroblast proliferation and increased collagen deposition in the ECM, vascular smooth muscle hypertrophy and hyperplasia, and vascular endothelial cell abnormal proliferation. Of interest, EV derived from diseased mice are able to reproduce these lesions in normal mice.

On the other hand, EV derived from normal endothelial cells containing several anti-inflammatory miRNA were found to inhibit monocyte activation and to reduce inflammation both *in vitro* and *in vivo*.

In particular, the EV-mediated transfer of miR10a, which targets components of the NF-κB pathway, such as IRAK4, has been implicated in repressing the inflammatory signaling (Njock et al., 2015).

It has been shown that EV released by Gram-negative bacteria induce inflammation by a IL-17A-dependent activation of neutrophils and by upregulation of elastase activity, promoting development of lung emphysema (Kim et al., 2015).

A recent work has assessed EV ability to transfer information from immune cells to neurons, especially during inflammation status. The authors used transgenic mice which expressed Cre recombinase enzyme specifically in the hematopoietic lineage. They observed the expression of an ubiquitary reporter gene in Purkinje neurons of the cerebellum, not due to cell fusion. Indeed, the recipient cell miRNA profile was shown to be different from that of non-recombined cells. Moreover, the reporter gene expression seemed to be markedly higher after peripheral inflammation and it spread via EV to other brain cells, like dopaminergic neurons of the substantia nigra (Ridder et al., 2014).

Another work focused on vesicles derived from immune cells. It reported that EV derived from T cells express thrombospondin-1 and CD47 as surface receptors. These proteins mediate EV internalization in T cells and endothelial cells. In the latter cells, CD47 modifies gene expression and response to VEGF favoring new vessel formation (Kaur et al., 2014).

Role of EV in angiogenesis-dependent tissue regeneration

Much evidence shows that EV released from stem/progenitor cells may stimulate angiogenesis in the regenerative process after tissue injury. EV-induced cell fate modulation has been shown to contribute to the paracrine action of MSC. Bone marrow MSC-derived EV contain selected patterns of mRNA and of miRNA involved in the control of transcription, cell proliferation and immune regulation (Bruno et al., 2009; Collino et al., 2010). We provided evidence for an EV-mediated transfer of RNAs showing that miRNA may downregulate *in vitro* their specific targets and that at least some mRNA are translated into protein both *in vitro* and *in vivo*. Preclinical studies demonstrated that MSC-derived EV mimic the protective and regenerative effects in several models of acute and chronic kidney and liver injury leading to functional and morphological recovery (Bruno et al., 2009; Herrera et al., 2010; Biancone et al., 2012). Bio-distribution studies provided evidence for selective accumulation of EV in the injured kidneys (Grange et al., 2014). The notion that the biological action of EV is related to the delivery of extracellular RNAs has also been supported by experiments showing the expression of human IGF-1R mRNA carried by MSC-derived vesicles in murine proximal tubular cells and their synthesis (Tomasoni et al., 2013). Moreover, EV depleted of miRNA produced by Drosha knock-down MSC were unable to stimulate kidney regeneration suggesting a crucial function of EV-carried miRNA (Collino et al., 2015).

Other factors are involved in the pro-angiogenic activity of MSC-derived EV. The transfer of Wnt4 protein has been shown to promote cutaneous wound healing by inducing activation of β-catenin (Zhang et al., 2015). Annexin A1 has also been implicated in the repair of chronic mucosal injury induced by EV (Leoni et al., 2015).

As mentioned above, endothelial progenitor cell (EPC)-derived EV were shown to activate an angiogenic program in the endothelium both *in vitro* and *in vivo*. This effect was accounted to the transfer of selected pro-angiogenic mRNA and miRNA (Deregibus et al., 2007; Cantaluppi et al., 2012a). In an experimental model of renal ischemia-reperfusion injury (IRI), characterized by endothelial injury, Cantaluppi et al. (2012b) showed that EPC-derived EV carrying miR126 and miR296 protect the kidney and promote reparative angiogenesis. When miR126 and miR296 were silenced, the healing effect of EV was lost, further supporting the role of miRNA in EV-mediated angiogenesis. Another work by Cantaluppi et al. (2012a) showed that the same vesicles may facilitate pancreatic islet engraftment by promoting islet vascularization in a mouse model of islet xenotransplantation. They observed both *in vitro* and *in vivo* that these EV improve β cell survival and insulin secretion, endothelial cell proliferation, migration, and tubule formation. Also in this case miR126 and miR296

turned out to be responsible for the pro-angiogenic EV effect.

Enrichment in miR126 was also observed in EV from $CD34^+$ peripheral blood mononuclear cells and progenitor cells. These EV, after endothelial cell uptake, mediate the new vessel formation. Furthermore, a reduced level of miR126 was observed in $CD34^+$ cells of patients with type 2 diabetes. This may be the basis of the impaired angiogenesis (Mocharla et al., 2013).

In a mouse model of hindlimb ischemia, Ranghino et al. (2012) found that revascularization is significantly improved by treatment with EPC-derived EV. EV treatment markedly reduces the sequel of ischemia, including foot and limb necrosis, and improves perfusion, as detected by Laser Doppler Blood Flow analysis. The number of capillaries is increased, as seen by immunohistochemistry, the damage of muscle is reduced, and the regeneration of small rounded myofibers is increased. RNA inactivation, as well as EV depletion of miRNA obtained by DICER knock-down-EPC, were shown to abrogate the proangiogenic effect. Moreover, when compared with EV derived from fibroblasts, that are ineffective, EPC-derived EV contain an enhanced amount of pro-angiogenic miRNA.

Nakamura et al. (2015) recently demonstrated that MSC-derived EV enhance myogenesis and angiogenesis by transferring miR494 thus favoring muscle regeneration.

In the heart, IRI has also been shown to be limited by MSC-derived EV. Experiments performed in a murine model of myocardial infarction demonstrated a significant reduction of infarct size after treatment with MSC-derived EV (Lai et al., 2011; Timmers et al., 2011) by a mechanism related to the delivery of miRNA (Zhu and Fan, 2012).

A potential cardio-protective action of EV derived from myocardial progenitor cells and cardiomyocytes has also been investigated. EV released from myocardial progenitor cells were shown to contain MMP and ECM metalloproteinase inducer (EMMPRIN) and stimulate endothelial cell migration and *in vitro* wound-healing, suggesting a potential angiogenic activity (Vrijsen et al., 2010). Zhang et al. (2012) showed that cardiomyocyte-derived EV carry Hsp20, which is able to stimulate angiogenesis via a VEGFR2 triggered pathway.

Another potential source of angiogenic EV is the adipose-derived mesenchymal stem cells (ASC).

ASC-derived EV contain several pro-angiogenic

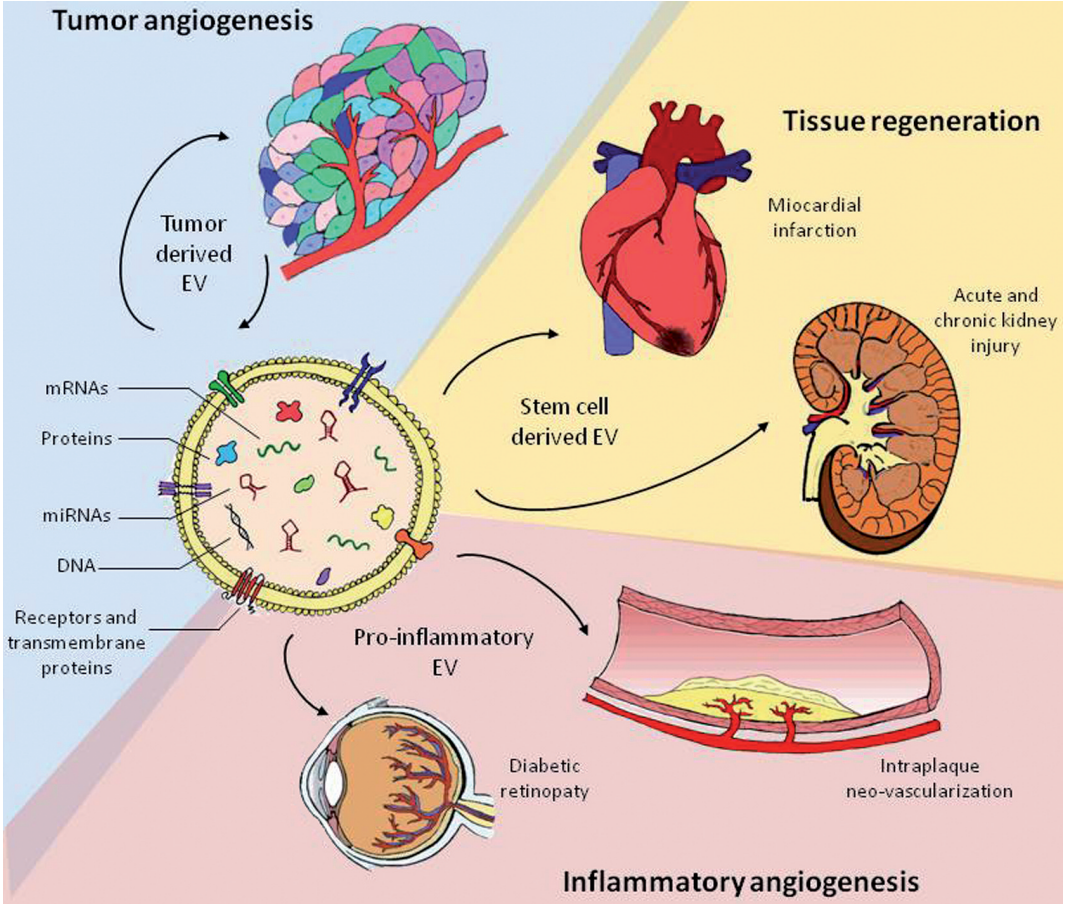

Fig. 1. Angiogenic potential of EV. EV by delivering their cargo to endothelial cells may be involved in aniogenic homeostasis, in the angiogenesis associated with tissue repair and in the altered angiogenesis occurring in tumors and inflammation.

factors, such as TCF4, HES1, HGF and several miRNA. In particular, these EV are enriched with miR148a, miR378, miR532-5p and let-7f, targeting transcription factors and genes involved in a variety of cellular pathways, including angiogenesis (Eirin et al., 2014). Moreover, we found several pro-angiogenic factors carried by these EV, including c-kit, SCF, APRIL, artemin, MFG-E8, MMP-20 and angiopoietin-like factor, as well as pro-angio-miRNA, such as miR17, miR21, miR126, miR130a, miR296 and miR210 (Lopatina et al., 2014a). However, the expression of these angiogenic factors in EV is modulated by the microenvironment. When ASC are exposed to PDGF, the expression of pro-angiogenic factors is markedly enhanced (Lopatina et al., 2014a). In contrast, when exposed to bFGF, a decreased level of pro-angiogenic factors and an upregulation of the anti-angiogenic factors were observed (Lopatina et al., 2014b). Vessels generated by EV derived from bFGF-stimulated ASC are less numerous but larger and more stabilized. bFGF is known to play a critical role in vascular remodeling with a complex action that balances the angiogenic activity with stabilization of neo-formed vessels. These findings suggest that the cargo of vesicle may vary depending on the context. Also the metabolic state of the originating cells is relevant for the EV biological activities. When compared with normal, EV from obese subjects showed a reduced angiogenic potential (Togliatto et al., 2016). EV from obese subjects contain reduced amount of VEGF, MMP-2, and of miR126. The reduced expression of miR126 in obese EV induces an up-regulation of Spred1 and an inhibition of Erk1/2 MAPK pathway in endothelial cells, leading to a reduced angiogenic response. These observations suggest that obesity influences the EV pro-angiogenic cargo and may raise concerns about the suitability of EV obtained from ASC derived from obese subjects for therapeutic purposes (Togliatto et al., 2016).

Moreover, a recent study on exosome from iPSC-derived MSC shows that, when administered to endothelial cells, these vesicles are able to upregulate the expression of several pro-angiogenic genes, to promote cell proliferation and migration, and to favor the formation of new vessels. Furthermore, the intramuscular injection of the same vesicles in a model of ischemic injury ameliorates hind limb ischemia by increasing vessel formation and muscle perfusion (Hu et al., 2015).

Conclusions

It is well established that EV participate in cell-to-cell communication and that they deliver information by surface receptor-mediated signalling and by transfer of their content of lipids, proteins, mRNA and non-coding RNA in recipient cells. As a consequence of these events, EV may influence cell fate and reprogram the phenotype and the functions of recipient cells (Quesenberry et al., 2015a). The ability of EV to modulate angiogenesis varies depending on their cargo and on cellular source (Fig. 1). Angiogenesis is a fundamental process for tumor survival and growth, and for development of inflammation. It is also the basis of the reparative process after injury. The angiogenic potential of EV has been related to their content in pro-angiogenic proteins, mRNA and miRNA. In the context of tumor biology, EV may promote sprouting of endothelial cells and reorganization in capillary-like structures (Kim et al., 2002; Skog et al., 2008). The hypoxic conditions of the tumor microenvironment promote EV release from tumor cells and the enrichment of EV in angiogenic factors (Park et al., 2010; King et al., 2012; Chen et al., 2014). These findings suggest the possibility to use tumor-derived EV as diagnostic and prognostic markers.

Endothelial progenitor cell-derived EV and endothelial EV may play a relevant role in vascular homeostasis and it has been suggested a paracrine and autocrine action of these vesicles in modulation of angiogenesis.

On the other hand, EV derived from stem cells, such as MSC or ASC, have a regenerative potential, especially in those pathological status that require angiogenesis for tissue repair. In these conditions, EV may represent a new therapeutic tool that can substitute stem cells (Quesenberry et al., 2015b). EV present a variety of advantages compared to stem cells, for example reduced safety risks, such as maldifferentiation, tumorigenesis or risks connected with cell transplantations (Fleury et al., 2014). Being a biological component of normal plasma, the acute infusion of EV in different experimental models avoided toxic effects.
Another powerful feature of EV is the possibility to be engineered with surface molecules targeting endothelium and to be enriched of selected therapeutic mRNA/miRNA or proteins. Nevertheless, it is necessary to perform further studies in order to understand the long-term effects of EV on target cells and potential side effects. Furthermore, protocols of isolation and characterization should be improved and studies of potency should be performed in order to standardize EV production.

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