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## Extracellular Vesicles

*Chantal Boulanger, Guest Editor*

### Extracellular Vesicles in Angiogenesis

Dilyana Todorova, Stéphanie Simoncini, Romaric Lacroix, Florence Sabatier, Françoise Dignat-George

**Abstract:** During the past decade, extracellular vesicles (EVs), which include apoptotic bodies, microvesicles, and exosomes, have emerged as important players in cell-to-cell communication in normal physiology and pathological conditions. EVs encapsulate and convey various bioactive molecules that are further transmitted to neighboring or more distant cells, where they induce various signaling cascades. The message delivered to the target cells is dependent on EV composition, which, in turn, is determined by the cell of origin and the surrounding microenvironment during EV biogenesis. Among their multifaceted role in the modulation of biological responses, the involvement of EVs in vascular development, growth, and maturation has been widely documented and their potential therapeutic application in regenerative medicine or angiogenesis-related diseases is drawing increasing interest. EVs derived from various cell types have the potential to deliver complex information to endothelial cells and to induce either pro- or antiangiogenic signaling. As dynamic systems, in response to changes in the microenvironment, EVs adapt their cargo composition to fine-tune the process of blood vessel formation. This article reviews the current knowledge on the role of microvesicles and exosomes from various cellular origins in angiogenesis, with a particular emphasis on the underlying mechanisms, and discusses the main challenges and prerequisites for their therapeutic applications. (*Circ Res.* 2017;120:1658-1673. DOI: 10.1161/CIRCRESAHA.117.309681.)

**Key Words:** cell-derived microparticles ■ endothelial cells ■ exosomes ■ extracellular vesicles  
■ regenerative medicine

Angiogenesis, defined as the formation of new blood vessels from a pre-existing vascular network, naturally occurs in an organism during growth and development and also in response to injury to restore a tissue's blood supply and promote wound healing. The new vessels can be formed by either sprouting angiogenesis, where endothelial cells (ECs) form sprouts that grow toward an angiogenic stimulus, or intussusceptive angiogenesis, where interstitial tissues invade the existing vessels and form transvascular tissue pillars that expand and split the vessel.<sup>1</sup> Sprouting angiogenesis comprises several steps: enzymatic degradation of the vessel's basement membrane, EC proliferation, migration, sprouting, branching, and tube formation. The stabilization and maturation of the newly formed vascular structures require the recruitment of pericytes, the deposition of extracellular matrix, and mechanical stimulation by the shear stress. In healthy tissues, angiogenesis is

tightly regulated by a precise balance between stimulatory and inhibitory signals.<sup>2</sup> Abnormal blood vessel growth occurs when this balance is disturbed and is a major cause of numerous diseases, such as cancer, atherosclerosis, corneal neovascularization, rheumatoid arthritis, or ischemic diseases.

During the past decade, the vesicles released by different cell types have been shown to be important mediators during the process of blood vessel formation and, as such, they have attracted particular interest among researchers from various fields of biology and medicine, including angiogenesis.<sup>3,4</sup> Longtime considered as inert debris or a hallmark of cell injury, extracellular vesicles (EVs), which include apoptotic bodies, microvesicles, and exosomes, have emerged as an important tool for intercellular communications in normal physiology and in pathophysiological conditions.<sup>5,6</sup> Indeed, EVs function as the carriers of small bioactive molecules, such as

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### Nonstandard Abbreviations and Acronyms

<b>EC</b>	endothelial cells
<b>ECFC</b>	endothelial colony-forming cells
<b>EPC</b>	endothelial progenitor cells
<b>EVs</b>	extracellular vesicles
<b>MSC</b>	mesenchymal stem/stromal cells

peptides, proteins, lipids, and nucleic acids, that act as regulators in the recipient cells in a paracrine or endocrine manner.<sup>7–9</sup>

This article reviews the current knowledge on the role of microvesicles and exosomes from various cellular origins in angiogenesis, with a particular emphasis on the underlying mechanisms, and discusses the main challenges and prerequisites for their therapeutic applications.

### Biogenesis of EVs

EVs are defined as heterogeneous plasma membrane vesicles released from various types of cells into biological fluids under both normal and stressed conditions.<sup>5</sup> According to their size and biogenesis pathways, EVs can be divided into 3 main types: exosomes, microvesicles (also called microparticles), and apoptotic bodies. Table 1 shows the main characteristics of these different types of EVs.

Exosomes are assumed to represent a homogeneous population with a size between 30 and 120 nm in diameter, and they typically display a cup-like shape.<sup>10</sup> However, it was demonstrated that cells release distinct subpopulations of exosomes with heterogeneous sizes and compositions that elicit differential molecular and biological properties.<sup>11</sup> Exosomes are derived from the endosomal system and are generated by the intraluminal budding of endosomal compartments, forming the intraluminal vesicles (ILVs) in intracellular multivesicular bodies. ILV formation constitutes the starting point of the exosome biogenesis process. The main mechanism of ILV formation is mediated by the Endosomal Sorting Complexes Required for Transport machinery.<sup>12</sup> However, ILV invagination and exosome secretion are also regulated in an Endosomal Sorting Complexes Required for Transport-independent manner that includes tetraspanin microdomains and lipid rafts.<sup>8,13,14</sup> After multivesicular bodies fuse with the plasma membrane of the parent cell, ILVs are released as exosomes into the extracellular environment. Similar to the various mechanisms proposed for the biogenesis of exosomes, a set of mechanisms has also been proposed for their release, involving Rab GTPases (Rab11/35, Rab27), the aforementioned tetraspanin and the SNARE (soluble *N*-ethylmaleimide-sensitive attachment protein receptor) complex.<sup>15–17</sup>

In contrast to exosomes, microvesicles, with a size ranging from 100 to 1000 nm in diameter, represent a more heterogeneous population and are formed by the outward blebbing of the plasma membrane. The release is visible within a few seconds after stimulation.<sup>16</sup> In response to stimuli, outward blebbing may be dependent on various enzymes and mitochondrial or calcium signaling. During the blebbing process, a cytoskeleton reorganization and alterations of phospholipid symmetry occur. These processes may differ significantly between cell

types.<sup>18–20</sup> In this way, the modification of membrane asymmetry promotes the redistribution of aminophospholipids, principally phosphatidylserine, to the outer part of the plasma membrane. Microvesicle formation seems to occur selectively in the lipid-rich microdomains of the membrane, such as in lipid rafts or caveolae domains.<sup>21,22</sup>

Interestingly, several studies have reported that changes in the plasma membrane may be independent of asymmetry loss. These processes involve the Endosomal Sorting Complexes Required for Transport pathway or tetraspanin microdomains.<sup>14,17</sup> The few studies that have analyzed the molecular mechanisms of microvesicle production clearly show that multiple complex pathways are involved. Depending on the starting stimulus, these include myosin light chain and Rho-associated kinase I and II, nuclear factor- $\kappa$ B, tumor necrosis factor-related apoptosis-inducing ligand, or p38 mitogen-activated protein kinase.<sup>6</sup>

Apoptotic bodies are another type of EVs that are exclusively released during the last steps of apoptosis by caspase-mediated cleavage and the consequent activation of Rho-associated kinase I.<sup>17,23</sup> They are characterized by the presence of an externalized phosphatidylserine and a permeable membrane, and they are larger than exosomes and microvesicles. Several reports indicate that apoptotic bodies contain a variety of cellular materials, such as histones, DNA, cellular organelles, and membrane/cytosolic components.<sup>17,24</sup> However, little is known about their molecular composition, and only a few recent studies have provided proteomic characterization.

Importantly, whether the passive transfer of cargo by apoptotic bodies has a functional involvement in intercellular communication is not well known, and it requires more studies.

### Molecular Properties of EVs

In the past decades, numerous works have focused on providing an exhaustive and comprehensive characterization of EV content, leading to the creation of specialized online databases, such as EVpedia and vesiclepedia,<sup>25,26</sup> which record the bioactive molecules and markers observed within these vesicles. Vesicle content specifically reflects the vesicle's localization, cellular origin, and mechanism of secretion (Table 1).<sup>17,27,28</sup> However, a set of proteins is commonly found in EVs. These proteins are associated with vesicle trafficking and biogenesis, such as tetraspanins (CD81, CD9, and CD63), specific stress proteins (HSP90 [heat shock proteins]), members of the Endosomal Sorting Complexes Required for Transport complexes (Tsg101, Alix), proteins involved in membrane fusion (Rabs, ARF6), and signaling proteins.<sup>8,15,29</sup> It is now agreed on that these proteins, which were considered for years to be specific to exosomes, can also be detected in larger vesicles, such as microvesicles (Table 1). In addition, several recent reports point to a nonuniform distribution of these proteins in all EV subpopulations.<sup>12,30</sup> Importantly, EVs also carry membrane receptors on their surface that are characteristic of the cells that they have been generated from. In addition to their surface molecules, EVs also carry important soluble mediators, such as cytokines, growth factors, and transcription factors.<sup>8,26</sup> In addition, their lipid content displays a particular organization

**Table 1. Exosomes, Microvesicles, and Apoptotic Bodies: Main Characteristics**

Characteristic	Exosome	Microvesicle	Apoptotic Bodies
Size, nm	30–120	100–1000	800–5000*
Morphology	Cup-shaped	Heterogeneous	Heterogeneous
Origin	Multivesicular body	Plasma membrane	Plasma membrane
Formation mechanisms	Exocytosis of MVB	Budding from plasma membrane	Budding from plasma membrane
Pathways	ESCRT-dependent	Ca <sup>2+</sup> -dependent	Apoptosis-related pathways
	Tetraspanin-dependent	Stimuli- and cell-dependent (various pathways)	
	Ceramide-dependent		
Timing of release	Ten minutes or more	A few tenths of a second	
Enriched protein marker	CD81, CD63, Alix, Tsg101	Selectins, integrin, CD40	Caspase 3, histones
Composition	Protein, lipids, coding RNA, noncoding RNA, DNA*	Protein, lipids, cell organelles, coding RNA, noncoding RNA, DNA*	Cell organelles, proteins, nuclear fractions, coding RNA, noncoding RNA, DNA

ESCRT indicates Endosomal Sorting Complexes Required for Transport; MVB, multivesicular body.

\*Data that are controversial in the literature (as discussed in Biogenesis of EVs and Molecular Properties of EVs sections of this article).

and composition that can be distinct from the parent cell, even though they share common features.<sup>8,31,32</sup>

The lipids that are generally enriched in EVs are sphingomyelin, cholesterol, phosphatidylserine, ceramide, and glycosphingolipids, which confer an EV structure that is close to the raft domains. Phosphatidylserine represents one of the characteristic EV markers that is expressed on their outer surface. Interestingly, microvesicles lacking phosphatidylserine have been recently identified.<sup>33–35</sup> This finding suggests that phosphatidylserine externalization might not be the sole mechanism of EV biogenesis. A lack of phosphatidylserine-positive staining might also be because of very low expression of phosphatidylserine, which is undetectable by the current methods. However, a lack of phosphatidylserine-positive staining might also result from phosphatidylserine engagement with lactadherin, Del-1, or protein S, thereby preventing its recognition by labeled Annexin V. Furthermore, lipids are emerging as important factors in EV functions. Several lipids, such as eicosanoids, fatty acids, and cholesterol, have been described as key mediators that are able to activate signaling in the recipient cells. In addition to proteins and lipids, EVs can also incorporate genetic material, such as small and long, coding and noncoding RNA (mRNA, miRNA, and lncRNA), and other cytosolic components and molecules.<sup>36–39</sup> Some studies have also reported the presence of genomic and mitochondrial DNA in the EVs.<sup>40,41</sup> The mechanisms of DNA packaging in the EVs are still unclear, but it may involve cell apoptosis,<sup>42</sup> the existence of plasma membrane associated DNA,<sup>43</sup> or the release of genomic DNA in the cytosol.<sup>44–46</sup>

Interestingly, the lipid-bilayered EVs encapsulate their genetic cargo and protect it from enzymatic digestion.<sup>47</sup> EVs may therefore serve as nucleic acid delivery vehicles, representing a new mechanism of genetic exchange between cells.<sup>36,48,49</sup> For example, several studies have demonstrated that encapsulated mRNA can be exchanged between cells and can induce the reprogramming of hematopoietic progenitors and ECs.<sup>3,37</sup> EVs can directly shuttle RNAs and transcription factors, which are capable of inducing phenotypic changes in the target cells.<sup>50,51</sup>

In addition, EVs carry a broad range of miRNAs, which are known to modulate gene expression by translational inhibition or by promoting the degradation of target mRNAs.<sup>52</sup> Of note, EVs can be packaged with disease- and activation-specific bioactive molecules.<sup>53</sup> Also, the microvesicles released from leukemia cells have been demonstrated to increase the global DNA methylation levels in recipient cells.<sup>54</sup> Thus, the EV-mediated horizontal transfer of genetic material may directly modulate the phenotype and behavior of the recipient cells.<sup>3,55,56</sup>

### EV-Mediated Intercellular Communication/Interaction With Target Cells

Many studies have documented the biological role of EVs as the mediators of cell communication. EVs convey information to the recipient cells that are present in the surrounding extracellular environment through several mechanisms. Some EVs can deliver their content through different types of endocytosis, such as clathrin-mediated endocytosis that is dependent or independent of receptors, macropinocytosis, and raft domain-mediated endocytosis.<sup>57,58</sup> Once absorbed into the endosomal–lysosomal system, EVs can fuse with the organelle membrane and dump their contents into the cytoplasm. They can also fuse with the membrane of the recipient cell to release their cargo intracellularly, either directly or through specific receptors.<sup>59</sup> They may also release their contents into the extracellular space and activate a fast response in the neighboring cells.<sup>16</sup> Finally, the membrane surfaces of EVs can trigger signaling cascades through receptor/ligand interactions without internalization.<sup>5,16,60</sup> Thus, EVs have the potential to deliver complex information to multiple cells in their tissue environment. They function as dynamic systems and are capable of adapting their content, and consequently their function, depending on both the cellular source and the stimulus that engendered their biogenesis.

### EVs and Angiogenesis

EVs are generated from different cell types, and they can modulate angiogenesis by stimulating or inhibiting it. These effects are highly dependent on the EV content and surface

molecule expression, which can be highly modulated by the stimulus used to induce EV production.

Table 2 summarizes the main mechanisms involved in the modulation of angiogenesis by EVs. During the past 10 years, a large amount of studies has documented the role of EVs in angiogenesis and highlighted their therapeutic potential. However, some of the earlier studies need to be revisited as they attributed the biological effect of the EVs without confirming the purity of the vesicle preparation. Here, we focus on the EVs derived from ECs, endothelial colony-forming cells, mesenchymal stem/stromal cells, platelets, leukocytes, and erythrocytes and discuss the underlying mechanisms.

### EC-Derived EVs

Angiogenesis is a dynamic and tightly regulated process in which EC are in constant communication with their environment through multiple paracrine factors and cell-to-cell and cell-to-matrix interactions. Among the extracellular factors, the vesicles released by ECs have been described as important mediators in the formation of blood vessels. Figure 1 illustrates the main mechanisms involved in the modulation of angiogenesis by endothelial EVs. The angiogenic potential of the vesicles shed by EC was documented for the first time in 2002.<sup>82</sup> This study evidenced that the EVs released by EC contain  $\beta$ 1 integrin and the enzymatically active matrix metalloproteinases matrix metalloproteinase-2 and matrix metalloproteinase-9. The EVs were capable of promoting EC invasion and capillary-like structure formation in vitro.<sup>82</sup> In addition, stimulation with VEGF (vascular endothelial growth factor) and FGF-2 (fibroblast growth factor-2) resulted in an increased amount of both the active and proenzyme forms of vesicle-associated metalloproteinases.<sup>82</sup>

Recently, it was shown that the EVs generated in response to interleukin-3 stimulation promoted angiogenesis through the transfer of miR-126-3p and pSTAT5 into the recipient EC,

leading to a lower Spred-1 level and increased ERK1/2 activation and cyclin D1 transcription (Figure 1).<sup>61</sup> The involvement of miR-126 in angiogenesis was previously documented in another study, which showed that the presence of miR-126 in apoptotic bodies induced CXCL12 expression in the target cells and the recruitment of progenitor cells in mice with atherosclerosis and conferred an atheroprotective effect by increasing plaque stability.<sup>62</sup> In addition, other micro-RNAs present in the EC-derived exosomes, such as miR-214, was shown to promote angiogenesis both in vitro and in vivo by repressing ATM expression and preventing senescence.<sup>64</sup> During blood vessel formation, the selection of tip- and stalk cells depends on  $\delta$ -like 4/Notch signaling.<sup>97</sup> Interestingly, it was demonstrated that this signaling does not necessarily require direct cell contact, as  $\delta$ -like 4 can be incorporated into endothelial exosomes and transferred to the target EC.<sup>98</sup> These  $\delta$ -like 4 containing exosomes stimulated the formation of capillary-like structures in vitro and in vivo through the internalization of the Notch receptor, followed by its degradation.<sup>98</sup> Microparticles released from the microvascular EC were shown to contain a tissue factor and to trigger angiogenesis ex vivo and postischemic collateral vessel formation in vivo.<sup>91</sup> The proangiogenic effect of the microparticles was mediated by  $\beta$ 1 integrin interaction with the target EC, leading to Rac1-ERK1/2-ETS (Ras-related C3 botulinum toxin substrate 1-extracellular signal-related kinase 1 and 2-avian erythroblastosis virus E26 homolog-1) signaling and CCL2 production (Figure 1).<sup>91</sup> EC-derived EVs also generate monomeric CRP (C-reactive protein), which was shown to be highly angiogenic.<sup>79-81</sup> In addition, it was demonstrated that endothelial microparticles play an important role in plasmin generation, which can affect the in vitro tube formation of endothelial progenitor cells.<sup>83</sup> This effect was dose dependent, as low amounts of endothelial microparticles increased tube formation, whereas a high concentration had an inhibitory effect.<sup>83</sup> In line with this study, an increased concentration of EC-derived microparticles inhibited angiogenesis in the cultured sections of hearts by inducing eNOS (endothelial nitric oxide synthase) dysfunction.<sup>99</sup> Recently, it was shown that EC-derived EVs inhibit tube-like structure formation by microvascular EC in vitro and in vivo. This antiangiogenic effect of EVs was triggered by reactive oxygen species in an NADPH (nicotinamide adenine dinucleotide phosphate) oxidase and Src family kinase-dependent manner and required the expression of CD36 on the target EC (Figure 1).<sup>68</sup> The involvement of oxidative stress in the antiangiogenic effect of EV was also documented by other studies, showing that endothelial microparticles impaired acetylcholine-induced endothelial vasorelaxation and nitric oxide (NO) production in rat aortic rings and inhibited in vitro angiogenesis by increasing the oxidative stress.<sup>70,71</sup>

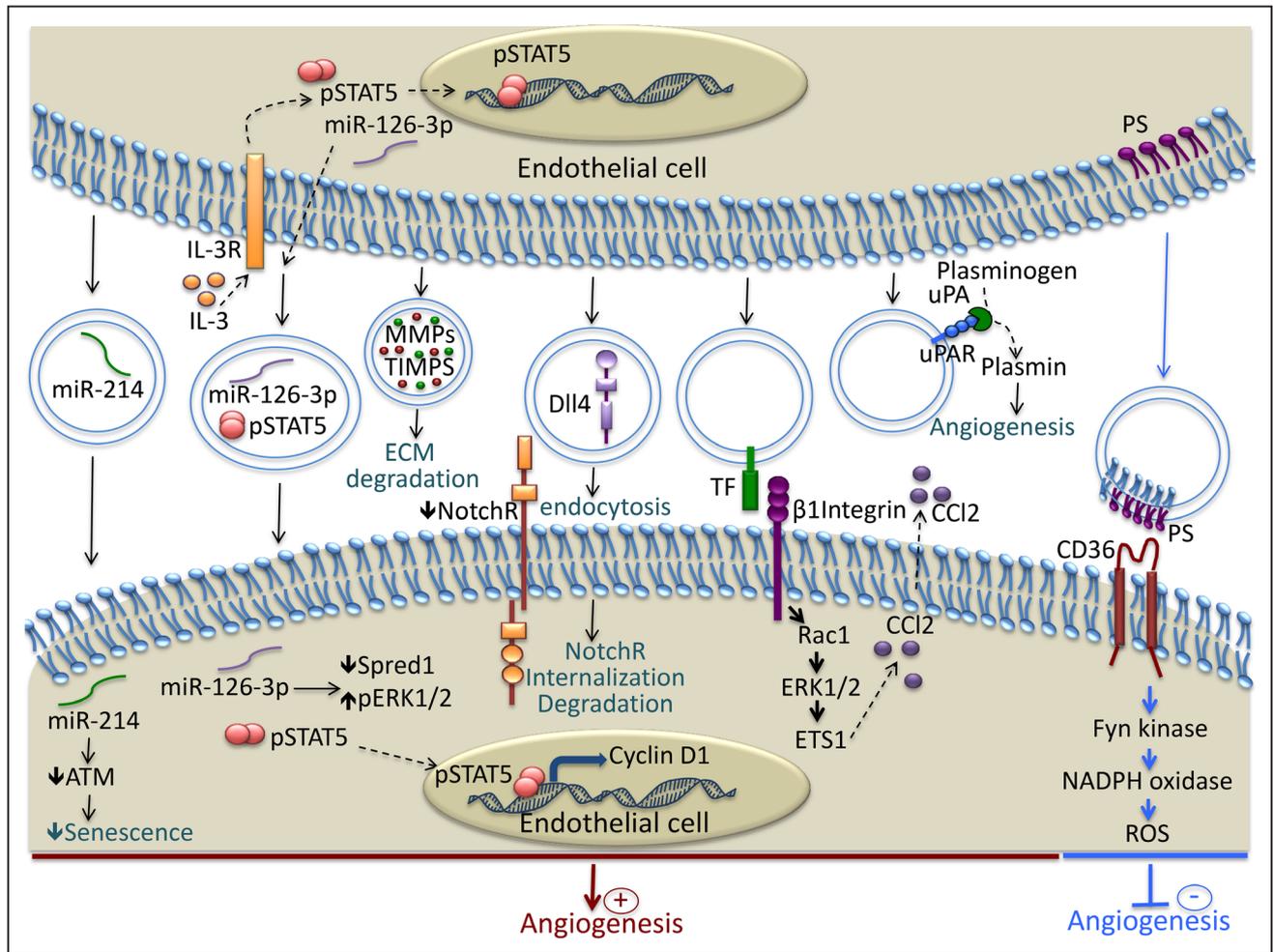
**Table 2. Main Mechanisms Involved in the Modulation of Angiogenesis by EVs**

Stimulation of Angiogenesis	Inhibition of Angiogenesis
Transfer of micro-RNAs: miR-126, <sup>61-63</sup> miR-214, <sup>64</sup> miR-296, <sup>63</sup> miR-125a, <sup>65</sup> miR-31, <sup>66</sup> and miR-150 <sup>67</sup>	CD36-dependent uptake of EVs <sup>68,69</sup> and induction of oxidative stress <sup>68-72</sup>
Transfer of proteins: VEGF, <sup>73,74</sup> FGF-2, <sup>74</sup> PDGF, <sup>74,75</sup> c-kit, <sup>76</sup> SCF, <sup>76</sup> RANTES, <sup>77</sup> CD40L, <sup>78</sup> CRP, <sup>79-81</sup> metalloproteinases, <sup>76,82</sup> uPA, <sup>83</sup> and Sonic hedgehog <sup>84-87</sup>	LDL receptor-mediated endocytosis <sup>88,89</sup>
Transfer of lipids: S1P <sup>90</sup>	
Activation of signaling pathways: PI3K, <sup>3,90</sup> ERK1/2, <sup>61,74,91-93</sup> Wnt4/ $\beta$ -catenin, <sup>94</sup> and NF- $\kappa$ B <sup>95,96</sup>	
Transfer of transcription factors: STAT3, <sup>95,96</sup> STAT5 <sup>61</sup>	

CRP indicates C-reactive protein; ERK1/2, extracellular signal-regulated kinase 1 and 2; EV, extracellular vesicle; FGF, fibroblast growth factor; LDL, low-density lipoprotein; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; RANTES, regulated on activation, normal T-cell-expressed and secreted; S1P, sphingosine-1-phosphate; SCF, stem cell factor; uPA, urokinase-type plasminogen activator; and VEGF, vascular endothelial growth factor.

### Endothelial Colony-Forming Cell-Derived EVs

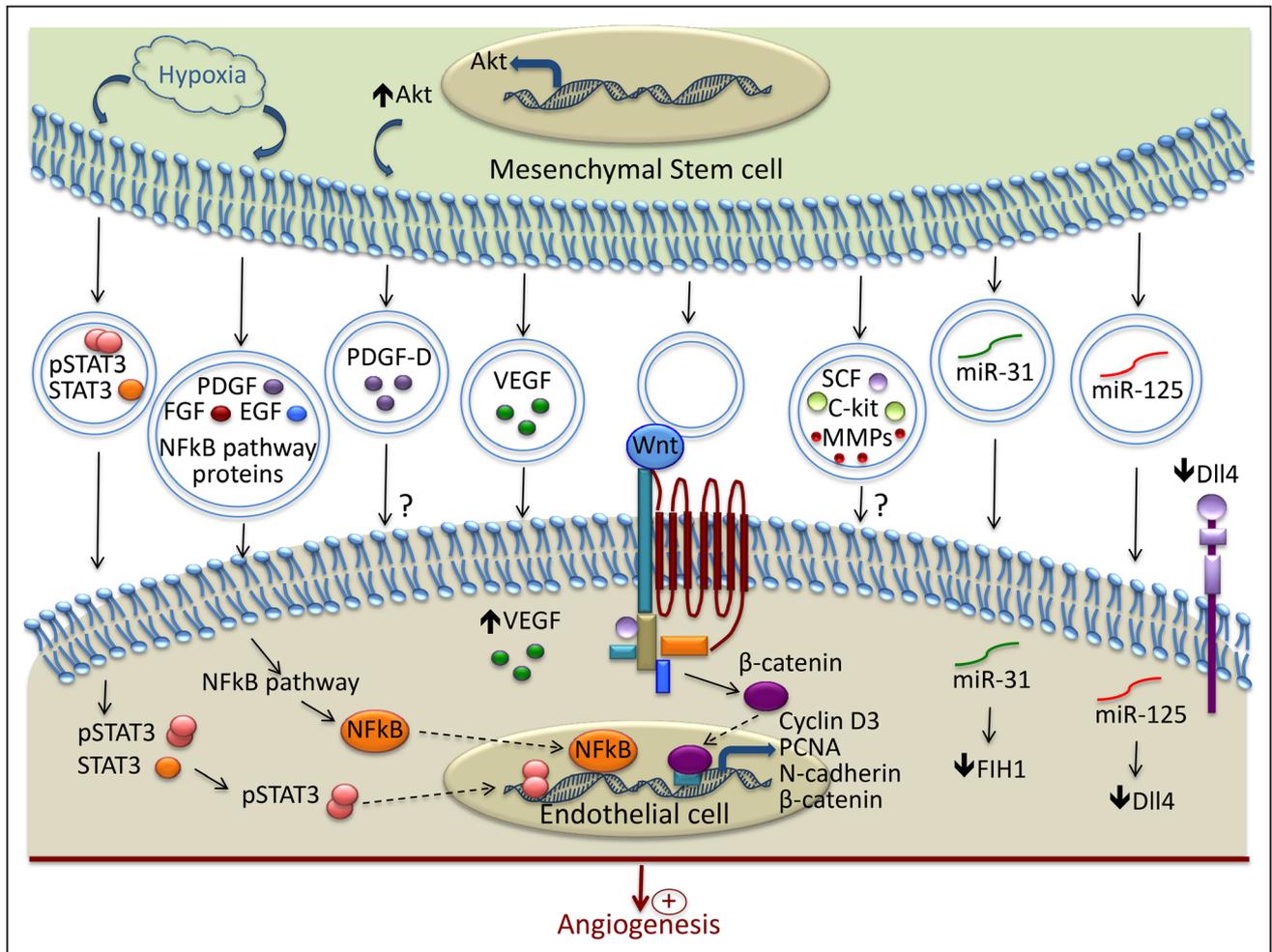
Since the discovery of endothelial progenitor cells (EPC) by Asahara et al<sup>100</sup> in 1997, there has been a considerable amount of debate surrounding the EPC identity and their functional role in postnatal neovascularization. Notably, the uptake of platelets microparticles and the subsequent platelet antigen acquisition by ex vivo cultured peripheral blood mononuclear cells has been recognized as a major confounding factor in the definition of the endothelial phenotype of circulating EPC.<sup>101</sup> By contrast, endothelial



**Figure 1. Mechanisms involved in the modulation of Angiogenesis by endothelial cell (EC)-derived extracellular vesicles (EVs).** EC release EVs rich in micro-RNA such as miR-214 and miR-126, that are transferred to recipient EC and induce proangiogenic signaling. EVs contain functional matrix metalloproteinases that facilitate angiogenesis through the degradation of components of the extracellular matrix. DII4 is transferred to EC by the EVs and induces Notch receptor internalization and tip cell formation. EVs bear, at their surface, a tissue factor that interacts with  $\beta 1$  integrin and induces Rac1-ERK1/2-ETS1 signaling, leading to the increased secretion of CCL2. EVs transport the complex uPA/uPAR, which stimulates angiogenesis through plasmin generation. The phosphatidylserine present on the surface of the EVs interacts with CD36 and induces Fyn kinase signaling, which leads to increased oxidative stress and the inhibition of angiogenesis. ATM indicates ataxia telangiectasia mutated; CCL2, chemokine c-c motif ligand 2; DII4, Delta-like 4; ECM, extracellular matrix; ERK1/2, extracellular signal-related kinase 1 and 2; ETS1, avian erythroblastosis virus E26 homolog-1; IL-3R, interleukin-3 receptor; MMPs, matrix metalloproteinases; NotchR, Notch receptor; PS, phosphatidylserine; Rac1, Ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; TF, tissue factor; TIMPS, tissue inhibitor of metalloproteinases; uPA, urokinase plasminogen activator; and uPAR, urokinase plasminogen activator receptor.

colony-forming cells (ECFC) have been recognized as genuine EPCs, displaying an unambiguous endothelial differentiation potential, proliferative activity, and a specific capacity to self-assemble into functional blood vessels *in vivo*.<sup>102</sup> Because these cells have higher vasculogenic potential compared with mature EC, EVs originating from ECFC have been proposed as potent mediators of proangiogenic signals. Several studies clearly demonstrated their capacity to stimulate blood vessel formation.<sup>63,103,104</sup> For instance, ECFC-derived EVs were shown to incorporate into EC via interaction with  $\alpha 4$  and  $\beta 1$  integrins, and they stimulated angiogenesis both *in vitro* and *in vivo* through the delivery of mRNA associated with eNOS and the PI3K/AKT signaling pathway.<sup>3</sup> In addition, ECFC-derived EVs prevented capillary rarefaction and subsequent tissue damage in a rat model of kidney ischemia-reperfusion injury.<sup>63</sup> The

renoprotective action of EVs was abolished by the specific depletion of miR-126 and miR-296, indicating their critical role in the stimulation of angiogenesis. Additional *in vitro* studies demonstrated that the EV internalization by hypoxic EC was mainly mediated by L-selectin, and it restored the expression of proangiogenic and antiapoptotic genes that were downregulated by hypoxia.<sup>63</sup> Similar mechanisms of miRNA delivery have been involved in the capacity of ECFC-EV to promote neovascularization and limit muscle damage after hindlimb ischemia or to enhance the survival of human islet transplants.<sup>103,104</sup> Recent studies reported that ECFC-derived exosomes promote cutaneous wound healing in diabetic rats by enhancing neovascularization in the wound sites. The exosomes mediated their proangiogenic potential by activating Erk1/2 signaling in EC and by stimulating the expression of several angiogenic molecules.<sup>92,93</sup>



**Figure 2. Mechanisms involved in the modulation of Angiogenesis by MSC-derived extracellular vesicles (EVs).** In response to hypoxia, mesenchymal stem/stromal cells release EVs containing active pSTAT3 and nuclear factor (NF)- $\kappa$ B pathway-associated proteins, which are transferred to recipient endothelial cell (EC) and promote the transcription of proangiogenic proteins. EVs contain and transfer several growth factors to EC (PDGF, FGF, EGF, VEGF, SCF, and c-kit). Wnt is present in the EVs and through interaction with its receptor, promotes the transcription of several molecules involved in angiogenesis. EVs stimulate vessel formation through the transfer of various micro-RNA. Among them, miR-31 acts by suppressing the factor inhibiting HIF-1 $\alpha$ , and miR-125 promotes tip cell specification by suppressing Delta-like 4 (DII4). ? indicates that the exact mechanisms of their transfer and the underlying signaling pathways are not completely described; EGF, epidermal growth factor; FGF, fibroblast growth factor; FIH, factor inhibiting HIF-1 $\alpha$ ; HIF, hypoxia inducible factor; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; SCF, stem cell factor; and VEGF, vascular endothelial growth factor.

### Mesenchymal Stem/Stromal Cell-derived EVs

Mesenchymal stem/stromal cells (MSCs) have potent proangiogenic properties that have been attributed to their secretion of paracrine factors. However, it was recently suggested that among soluble factors, EVs play an important role in intercellular communication and are involved in angiogenesis. Figure 2 summarizes the main mechanisms involved in the modulation of angiogenesis by MSC-derived EVs. The functionality of the MSC secretome is strongly influenced by the microenvironment. For example, on hypoxia stimulation, bone-marrow-derived MSC released EVs that can be incorporated by EC, stimulated *in vitro* tube formation, and promoted angiogenesis in a rat myocardial infarction model.<sup>105</sup> In addition, exosomes derived from MSC enhanced the tube formation of EC and stimulated neovascularization, leading to improved heart function after ischemic injury in a rat myocardial infarction model.<sup>105</sup> Recently, it was demonstrated that EVs isolated from bone-marrow-derived MSC increased

postischemic neuroangiogenesis in a way similar to that of MSC after focal cerebral ischemia in mice.<sup>106</sup> In line with this study, after traumatic brain injury in rats, the administration of exosomes derived from MSC significantly improved functional recovery by promoting endogenous angiogenesis.<sup>107</sup> The main mechanisms involved in the proangiogenic effect of MSC-derived exosomes in ischemic conditions involve the activation of the nuclear factor- $\kappa$ B pathway and the transfer of transcriptionally active STAT3 (Figure 2).<sup>95,96</sup> In addition, exosomes that were isolated from bone marrow MSC were able to reduce infarct size and enhance blood flow recovery by increasing the density of functional capillaries after myocardial infarction in rats.<sup>105</sup>

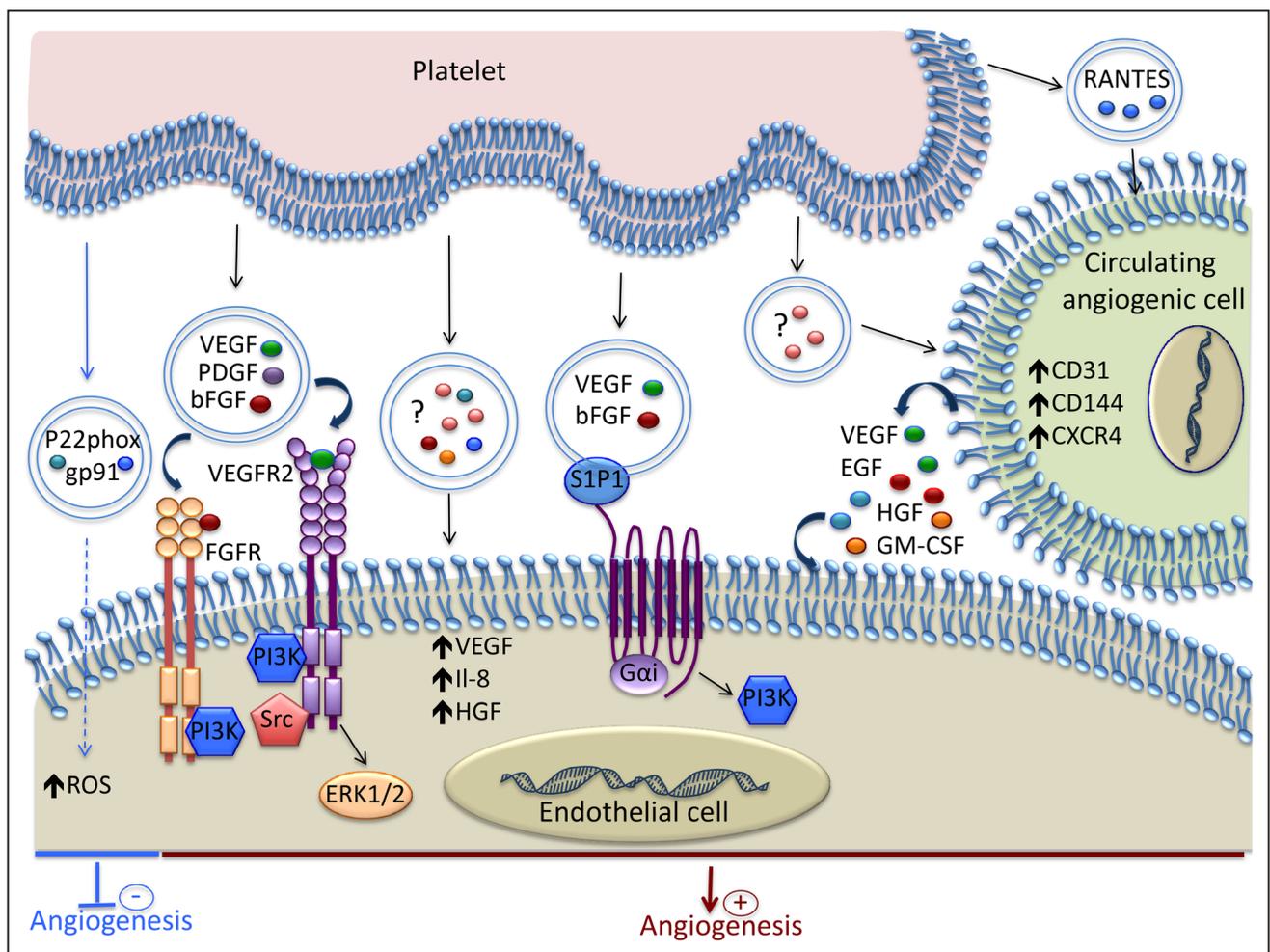
Another important source of MSC is the umbilical cord. A hypoxia treatment of umbilical cord-derived MSC increased their production of EVs with proangiogenic potential. These EVs enhanced *in vitro* capillary-like structure formation and further improved blood flow recovery in a rat hindlimb ischemia

model.<sup>108</sup> After unilateral kidney ischemia in rats, the intravenous injection of EVs derived from umbilical cord MSC had protective effects by increasing the capillary vessel density through the delivery of VEGF.<sup>73</sup> The exosomes released from Akt-overexpressing MSC derived from umbilical cord improved cardiac function after intravenous infusion in a rat model of acute myocardial infarction. In addition, the exosomes isolated from Akt-overexpressing MSC stimulated EC migration, proliferation, and tube-like formation in vitro, but they also increased the formation of blood vessels in vivo. This study identified PDGF-D (platelet-derived growth factor-D) as an important player in the exosome-mediated stimulation of angiogenesis.<sup>75</sup> The activation of the Wnt4/ $\beta$ -catenin pathway was reported as another mechanism involved in the proangiogenic effect of human umbilical cord MSC-derived exosomes (Figure 2).<sup>94</sup>

Exosomes that were isolated from adipose tissue-derived MSC were also shown to promote angiogenesis in vitro and

in vivo. This proangiogenic effect was because of the transfer of miR-125a that promoted tip cell specification through the direct suppression of  $\delta$ -like 4 (Figure 2).<sup>65</sup> The angiogenic potential of EVs can be greatly improved by modulating the culture conditions of MSC. Thus, PDGF treatment was shown to modulate EV protein composition, leading to an increased c-kit, SCF (stem cell factor), and metalloprotease content and, therefore, it enhanced their proangiogenic potential.<sup>76</sup> Preconditioning of the adipose-derived stem cells with an endothelial differentiation medium upregulated the release of microvesicles and their proangiogenic properties through the delivery of miR-31 and the suppression of factor inhibiting HIF-1 $\alpha$  (Figure 2).<sup>66</sup>

Induced pluripotent stem cells present an unlimited cellular source for MSC generation. Interestingly, it was reported that exosomes derived from induced pluripotent stem cells-differentiated MSC promoted angiogenesis in osteoporotic rats.<sup>109</sup>



**Figure 3. Mechanisms involved in the modulation of Angiogenesis by platelet-derived extracellular vesicles (EVs).** Platelet-derived EVs contain various growth factors and chemokines that induce proangiogenic signaling in endothelial cell (EC). Spingosine-1-phosphate (S1P1), present on the EV surface, induces PI3K activation and, together with VEGF and bFGF, promotes angiogenesis. The EVs released by platelets stimulate the proangiogenic potential of circulating angiogenic cells by increasing their expression of both membrane molecules and soluble factors. Platelet-derived EVs can inhibit angiogenesis by transferring the p22phox and gp91 subunits of NADPH oxidase and increasing the oxidative stress in EC. ? indicates that the exact content of the EVs is not reported; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; NADPH, nicotinamide adenine dinucleotide phosphate; PI3K, phosphoinositide 3-kinase; RANTES, regulated on activation, normal T-cell-expressed and secreted; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; and VEGFR, vascular endothelial growth factor receptor.

### Platelet-Derived EVs

In addition to their key role in hemostasis and thrombosis, platelets are involved in several biological processes such as inflammation, angiogenesis, and tissue regeneration. During the past 12 years, the role of platelet EVs in angiogenesis has been well documented. Figure 3 shows the main mechanisms involved in the modulation of angiogenesis by platelet-derived EVs. The amount of platelet microvesicles and their proteome are dependent on the stimulus used for their generation.<sup>110</sup> Thus, different pathological contexts lead to the release of microparticles with a specific protein content that will further determine the message delivered to the neighboring cells.

The role of platelet microparticles in angiogenesis was evidenced for the first time in 2004.<sup>90</sup> This study showed that the platelet microparticles isolated from healthy donors were capable of promoting proliferation, migration, and tube formation of human umbilical vein EC through the cooperative effect of VEGF, FGF-2, and lipid components, such as sphingosine-1-phosphate.<sup>90</sup> These effects were essentially mediated via the pertussis toxin-sensitive G protein and the PI3 kinase pathway (Figure 3).

The proangiogenic activity of platelet microparticles was further supported by a study showing that EPC cultures contain platelet microparticles with the capacity to stimulate endothelial tube formation *in vitro*.<sup>101</sup> This proangiogenic effect was attenuated after removing the microparticles by filtration or ultracentrifugation or by specifically inhibiting the formation of the platelet integrin complex  $\alpha\text{IIb}\beta\text{3}$ .

In a rat aortic ring model, platelet-derived microparticles exerted proangiogenic effects in a dose-dependent manner through the transfer of cytokines, such as VEGF, FGF-2, and PDGF, and the activation of PI3 kinase, src kinase, and ERK (Figure 3).<sup>74</sup> These results were supported by increased *in vitro* tube-like formation and *in vivo* angiogenesis using agarose beads that contained platelet microparticles. In addition, in a model of chronic myocardial ischemia in rats, platelet microparticles were shown to increase the number of functional capillaries after injection into the myocardium.<sup>74</sup> Platelet microvesicles were also documented as important mediators in tumor progression and angiogenesis in lung cancer through the upregulation of factors involved in tumor vascularization, such as interleukin-8, VEGF, and HGF (hepatocyte growth factor).<sup>111</sup>

It was also shown that platelet-derived EVs can have deleterious effects on EC. The exosomes isolated from septic patients contain the p22phox and gp91 subunits of NADPH oxidase and were able to produce reactive oxygen species (Figure 3).<sup>72</sup> The incubation of these exosomes with EC enhanced their apoptosis. Further studies showed that platelets exposed to lipopolysaccharide or NO, but not thrombin or tumor necrosis factor- $\alpha$ , generated exosomes similar to those recovered from septic patients, and they induced apoptosis in EC through redox signaling.<sup>112</sup>

It was also shown that platelet-derived microparticles stimulated endogenous stem cell repair mechanisms after middle cerebral artery occlusion in rats. The delivery of platelet microparticles increased angiogenesis and neurogenesis at the infarct zone in a dose-dependent manner.<sup>113</sup> These results were supported by another study that demonstrated the protective effect of platelet-derived microparticles against cerebral ischemic reperfusion injury.<sup>114</sup>

Platelet microparticles can also enhance the regenerative capacity of early outgrowth ECs by modulating their phenotype and secretome, as attested by the increased expression of CD31, VE-cadherin, and CXCR4, and the secretion of VEGF, EGF, HGF, and GM-CSF (granulocyte-macrophage colony-stimulating factor; Figure 3).<sup>115</sup> In addition, platelet microparticles increased the ability of early outgrowth ECs to stimulate endothelial migration, proliferation, tube formation, and importantly, endothelial regeneration after injury *in vivo*. In line with this study, the pretreatment of circulating angiogenic cells with platelet microparticles isolated from peripheral blood of atherosclerotic patients led to increased neovascularization in rats with hindlimb ischemia.<sup>77</sup> This proangiogenic effect was shown to be dependent on the release of RANTES by the platelet microparticles (Figure 3).

### Leukocyte-Derived EVs

Leukocyte EVs may originate from lymphocytes, monocyte/macrophages, or neutrophils, and they have been shown to actively participate in vascular homeostasis. Several studies, mostly focusing on lymphocytes and monocytes, emphasized that leukocyte EVs may also play a role in angiogenesis. Depending on the stimuli used for their generation, the EVs derived from lymphocytes can exert either pro- or antiangiogenic effects. For instance, lymphocytes undergoing activation and apoptosis release microvesicles with proangiogenic properties, whereas microparticles shed from apoptotic lymphocytes inhibit angiogenesis.<sup>69,84,85</sup>

The microparticles generated from T lymphocytes after treatment with phytohemagglutinin, phorbol ester, and actinomycin D express Sonic hedgehog and induce the formation of capillary-like structures *in vitro* through FAK activation and the upregulation of ICAM-1 (intercellular adhesion molecule-1), RhoA, and VEGF.<sup>84</sup> These microparticles expressing Sonic hedgehog favored neovascularization in a mouse model of hindlimb ischemia by activating the eNOS pathway and NO production in not only the ischemic area but also the aorta of mice.<sup>85</sup> The stimulation of NO production by lymphocyte microparticles carrying Sonic hedgehog also had a beneficial effect in restoring endothelial function in mouse models of angiotensin II-induced hypertension and myocardial ischemia/reperfusion injury.<sup>86,87</sup>

In contrast, actinomycin D treatment of lymphocytes in the absence of activation signals resulted in a microparticle release that inhibited angiogenesis.<sup>69</sup> Thus, in a model of *in vivo* corneal neovascularization and *in vitro* aortic rings, the microparticles generated after actinomycin D treatment suppressed angiogenesis through increased reactive oxygen species production, upregulation of CD36 on target EC and the consequent reduction of VEGFR2 and phospho-ERK levels.<sup>69</sup> In addition, the antiangiogenic effect of microparticles was shown to present an interesting therapeutic application in pathological angiogenesis, such as retinopathies and tumor angiogenesis.<sup>88,89</sup> The inhibition of angiogenesis by T-lymphocyte-derived microparticles was mediated by low-density lipoprotein receptor, as low-density lipoprotein receptor knockdown in target endothelial and tumor cells resulted in decreased microparticle uptake and abrogated the

antiangiogenic effect.<sup>88,89</sup> In the case of choroidal angiogenesis, it was demonstrated that the inhibitory effect of microparticles was dependent on the production of pigment epithelium-derived growth factor and nerve growth factor by retinal pigment epithelial cells and the activation of p75 neurotrophin receptor in choroidal microvascular cells.<sup>116</sup> Recently, the same group evidenced that on CD36-dependent uptake, lymphocyte microparticles can exert their antiangiogenic effect by altering the expression of several angiogenic factors in macrophages.<sup>117</sup>

The microvesicle derived from monocytes were also shown to promote angiogenesis both *in vitro* and *in vivo* through the transfer of miR-150 to EC.<sup>67</sup> THP-1 (human acute monocytic leukemia cell line)-derived EVs were also shown to generate monomeric CRP, which is highly angiogenic to vascular EC and therefore might impact on the process of vascularization.<sup>79–81</sup> In addition, CD40 ligand-positive microparticles, which were isolated from human atherosclerotic plaques and of mostly macrophage origin, were able to stimulate endothelial proliferation and angiogenesis, suggesting that microparticles could participate in intraplaque neovascularization and plaque vulnerability.<sup>78</sup>

### Erythrocyte-Derived EVs

In addition to their vital role in transporting oxygen to organs and tissues, erythrocytes were shown to participate in angiogenesis.<sup>118</sup> A recent study demonstrated that the production of sphingosine-1-phosphate by erythrocytes is essential for vascular stabilization, maturation, and remodeling during embryonic development.<sup>118</sup> The process of EV formation occurs during erythrocyte maturation and allows for the selective retention of certain proteins in the plasma membrane and the removal of noxious molecules, such as oxidized proteins.<sup>119</sup> Vesicle release is accelerated during erythrocyte senescence and is considered a part of apoptosis-like form in these cells.<sup>120</sup> Even though studies addressing the specific role of erythrocyte EVs in angiogenesis are still lacking, some studies suggest that erythrocyte-derived EVs may have a deleterious effect on EC, particularly in some pathological conditions, such as sickle cell disease and malaria. It was reported that EVs derived from malaria-infected erythrocytes altered EC gene expression and barrier properties through the transfer of miR-451a.<sup>121</sup> In addition, erythrocyte microparticles were significantly elevated in patients with sickle cell disease, and they expressed more sphingosine-1-phosphate than erythrocyte microparticles from healthy donors.<sup>122</sup> Sickle cell disease erythrocyte-derived MP are internalized by myeloid cells and promote proinflammatory cytokine production and EC adhesion in an ERK 1/2-ICAM-4-mediated fashion. These results suggest that circulating MP may contribute to the inflammation-rooted pathogenesis of the disease.<sup>122</sup> In addition, it was evidenced that erythrocyte vesiculation produced microparticles loaded with heme that could be further transferred to EC, inducing oxidative stress and apoptosis.<sup>123</sup> Heme-laden microparticles strongly reduced endothelium-dependent vasodilation and induced vasoconstrictions in kidneys.<sup>123</sup> These observations indicate that microparticles carrying heme may be a source of oxidant stress, contributing to endothelial dysfunction and vascular injury.

### Toward EV-Based Therapeutic Angiogenesis

Based on the various studies discussed above, EVs have emerged as a major form of intercellular communication,

playing important roles in angiogenesis and cardiovascular homeostasis. Their role in cellular signaling during vascular development, growth, and maturation has been extensively documented and has opened up new perspectives for their therapeutic application in regenerative medicine and tissue engineering as drug delivery vehicles modulating vascularization.

Since the early 2000s, EVs have been tested in clinical trials involving patients with melanoma,<sup>124</sup> nonsmall cell lung cancers,<sup>125,126</sup> colorectal cancer,<sup>126a</sup> and type I diabetes mellitus (NCT02138331). Although a small number of patients was included in the studies, these clinical trials demonstrated the feasibility and safety of EV administration in humans. Recently, a prospective clinical trial was initiated to evaluate the effect of autologous plasma-derived exosomes on wound healing in patients with intractable cutaneous ulcers (NCT02565264). This trial will provide important information about the feasibility and the efficiency of EV-based therapy in angiogenesis-related disorders.

Although the pro- and antiangiogenic effects of EVs from various sources have been reported, further *in vivo* studies are required to better understand the specific role of EVs in exchanging molecules between cell types. Several limitations need to be overcome to progress toward the use of EVs in the therapeutic modulation of angiogenesis. In particular, the standardization of the end product, quality control, and the associated costs remain major issues for EV-based therapies.

### Cellular Source and Conditions for EV Production

One of the key points to consider is the choice of the cellular source and the conditions for a suitable quantity of clinical grade EV production. Recent studies suggest that several stimuli and culture conditions play an important role in the biogenesis and secretion of EVs with proangiogenic properties.<sup>61,82,96,127</sup> In addition, it is important to evaluate how the patient's pathological environment impacts the EV properties and therapeutic potential. Even though most of the preclinical works supporting the capacity of EVs for therapeutic angiogenesis have been done with EVs from cells that were isolated from healthy donors, there are emerging data showing that the proangiogenic capacity of MSC-derived EVs are impaired in patients with cardiovascular risk factors.<sup>128</sup> Thus, donor-related variability may be responsible for the therapeutic differences observed among comparable EV fractions, requiring the definition of donor inclusion/exclusion criteria and potent markers to be able to anticipate the therapeutic effects of EVs *in vivo*.

### EV Concentration

One important question is what concentration of EVs should be used for the stimulation of angiogenesis without inducing any side effects? An overview of the literature shows that the effect of EVs on angiogenesis seems to be dose dependent. For instance, low amounts of endothelial microparticles ( $2 \times 10^3$  per well) were able to increase tube formation *in vitro*, whereas a high concentration had an inhibitory effect ( $2 \times 10^5$  per well).<sup>83</sup> Another study reported that endothelial microparticles inhibited angiogenesis in the cultured sections of hearts when concentrations higher than  $10^5$  microparticles/mL were used.<sup>99</sup> Platelet-derived microparticles exerted proangiogenic effects

in vitro when doses between 30 and 100  $\mu\text{g}$  of protein/mL were applied.<sup>74</sup> However, when platelet microparticles were used to stimulate vascularization in vivo, a much higher concentration (250  $\mu\text{g}/\text{mL}$ ) was necessary to stimulate postischemic revascularization of the myocardium.<sup>74</sup> In addition, it was reported that MSC-derived exosomes exerted the most potent angiogenic effect in vitro and in vivo at a concentration of 100  $\mu\text{g}/\text{mL}$ .<sup>65</sup> However, another study showed that a low concentration of MSC exosomes (1–10  $\mu\text{g}/\text{mL}$ ) was more effective for tube formation than a high concentration (100  $\mu\text{g}/\text{mL}$ ).<sup>95</sup>

All these observations demonstrate that the question on EV concentration remains controversial. It is difficult to address this question and compare the results from all these studies, as different culture conditions have been used for EV production and various methods have been used to quantify EVs.

Because of the small size of EVs and the technical limitations of nanosized particle analysis, EV quantification has been challenging to scientists. Several techniques have been used to quantify EVs, such as dynamic light scattering, nanoparticle tracking analysis, flow cytometry, and tunable resistive pulse sensing. However, a universal method for standardized EV quantification is still lacking. The studies mentioned above suggest that the optimal EV concentration for the stimulation of angiogenesis will depend on various factors, such as the cellular source of EVs, the stimulus used to induce EV release, EV content, the way of EV delivery in vivo, and also the pathological microenvironment. Determination of the EV concentration for the stimulation of angiogenesis is hampered not only by the various methods used for EV quantification but also by the different degrees of purity of the EV preparations.

### Purity of EV Preparations

Another important aspect to consider is the purity of the different EV populations and the characterization of their content and biological properties. Several techniques have been developed to isolate EVs, such as differential ultracentrifugation, polymer-based precipitation, density gradients, microfiltration, and size-exclusion-based approaches.<sup>129</sup> It is important to note that compared with ultracentrifugation and polymer-based precipitation, the development of flotation through density gradients, microfiltration, and size-exclusion chromatography for EV purification marked significant progress in the field.

A pure isolation of EVs from tissue culture supernatants and body fluids is hampered by the presence of macromolecular structures. For instance, the presence of bovine proteins and microvesicles in the culture media can impact the growth and phenotype of cultured cells, thereby influencing the quality and quantity of EVs produced in vitro.<sup>130</sup> Furthermore, the RNAs, proteins, and lipids carried by EVs that are present in the bovine serum can also affect the results involving the proteomic, transcriptomic, and functional analysis of EVs. Thus, it is necessary to eliminate the EVs carried by the bovine serum before cell culture. Filtration of the culture media using 0.1- $\mu\text{m}$  filters could help to eliminate microparticles, but not all the exosomes. Switching the cells to serum-free conditions during EV production could allow us to circumvent this problem; however, serum-free conditions may add additional stress to the cells and affect the nature and amount of secreted EVs.

Within the EV population, many distinct subtypes of vesicles exist. However, the currently used methods do not allow for their efficient separation and can negatively impact their integrity, function, and therapeutic potential. Therefore, the development of new, more selective isolation techniques is necessary to achieve good purity in each vesicle population and to determine, in a more precise way, the properties and biological functions of each of them. Most of the molecules responsible for the therapeutic effects of EVs still remain to be identified and extensively characterized. To further explore their potential, proteomic and mRNA/miRNA microarray analysis of each vesicle population may provide useful information.<sup>131–133</sup> Table 3 summarizes the main transcriptomic, proteomic, and lipidomic studies on the EVs that were derived from the cell types discussed in this review. In addition, the methods used for the isolation and purification of EVs varies considerably between different research groups and also varies depending on the tissue that they are extracted from. Indeed, considerable heterogeneity among EV preparations has been observed. Therefore, one of the most important challenges before applying EVs in therapeutics is the optimization and standardization of the methods of EV purification, enrichment, and characterization, which will allow increasing the homogeneity within EV preparations and will enable achieving good manufacturing practices.<sup>145</sup>

### EV Biodistribution After Administration

A better understanding of EV biodistribution after administration is an essential point for the safe successful clinical application of EVs in tissue regeneration. Although the substantial proangiogenic effects of EVs have been reported by several preclinical studies, our current knowledge of their biodistribution and clearance dynamics still remains limited. Several methods for EV labeling and tracking have been developed, such as reporter systems,<sup>146,147</sup> fluorescent dye labeling, loading with superparamagnetic iron oxide nanoparticles<sup>148</sup> or radioisotope labeling with clinically validated radio tracers.<sup>149</sup> Several studies independently reported the short half-life of exogenous EVs that were artificially introduced in the circulation because of their retention and uptake in organs. In vivo tracking studies showed that intravenously administered EVs derived from kidney embryonic cells are taken up mainly by the kidney, followed by the liver and lung, whereas some EVs were retained by the heart, brain and muscle, albeit in low amounts.<sup>146</sup> In addition, EVs derived from red blood cells showed increased uptake by the liver and bone, followed by the skin, muscle, spleen, kidney, and lung, whereas melanoma-derived EVs were mainly taken up by the lungs and spleen.<sup>8</sup> Thus, the cellular source and the route of administration are the key determinants of EV tissue distribution.<sup>150</sup> Therefore, establishing the best route of EV delivery in normal and pathological conditions may help improve the therapeutic efficiency of EVs. In addition, the efficiency of EV delivery could be improved by a better understanding of the mechanisms of EV uptake and interaction with the target cells, in particular, with the cells of the cardiovascular systems, as few studies are available.

Finally, further studies are necessary to evaluate the risk of adverse effects or side effects after EV administration. Although the EVs used in preclinical studies clearly lack the potential to

**Table 3. Transcriptomic, Proteomic, and Lipidomic Studies on EVs**

EV source	Isolation	Analysis	Year	References
Endothelial cells (HUVEC)	20 min 120g 90 min 16 000g	Next-generation sequencing of miRNAs	2012	Diehl et al <sup>53</sup>
	5 min 4000g 90 min 100 000g	Proteomic analysis (LC-MS/MS)	2005	Banfi et al <sup>134</sup>
	4 min 200g 1 h 100 000g	Proteomic analysis (LC-MS/MS)	2008	Peterson et al <sup>135</sup>
	5 min 200g 2 h 100 000g	Proteomic analysis (LC-MS/MS)	2013	Liu et al <sup>136</sup>
Endothelial progenitor cells	20 min 2000g 1 h 100 000g	Transcriptomic analysis (microarray)	2007	Deregibus et al <sup>3</sup>
	15 min 400g 5 min 12 500g 150 min 20 500g	Proteomic analysis (LC-MS/MS)	2009	Prokopi et al <sup>101</sup>
Mesenchymal stem cells	20 min 2000g 1 h 100 000g	Transcriptomic analysis (Microarray)	2009	Bruno et al <sup>137</sup>
	10 min 500g Concentration 1 kDa 1 h 15 000g 18 h 110 000g	Next-generation sequencing of miRNAs Proteomic analysis (LC-MS/MS) Lipidomic analysis	2014	Vallabhaneni et al <sup>138</sup>
	10 min 500g 15 min 800g Concentration 100 kDa Sucrose cushion 2 h 100 000g	Proteomic analysis (LC-MS/MS)	2012	Kim et al <sup>131</sup>
Platelets	15 min 710g 90 min 150 000g	Proteomic analysis (LC-MS/MS)	2005	Garcia et al <sup>139</sup>
	30 min 5000g 130 000g Gel filtration	Proteomic analysis (LC-MS/MS)	2009	Dean et al <sup>140</sup>
	1500g 1 h 100 000g	Proteomic analysis (LC-MS/MS)	2012	Shai et al <sup>110</sup>
Monocyte (THP-1)	20 min 120g 90 min 16 000g	Next-generation sequencing of miRNAs	2012	Diehl et al <sup>53</sup>
Macrophages	30 min 500g 20 min 16 500g Filtration 0.22 $\mu$ m 180 min 120 000g	Microarray	2011	Yang et al <sup>141</sup>
Erythrocytes	Microvesicles: 1550g; 40 000g Nanovesicles: 1550g; 40 000g; 100 000g	Proteomic analysis (LC-MS/MS)	2008	Bosman et al <sup>142</sup>
Neutrophils	10 min 3000g 1 h 100 000g	Proteomic analysis (LC-MS/MS)	2013	Dalli et al. <sup>143</sup>
Atherosclerotic plaques	15 min 500g 5 min 12 500g 150 min 20 500g	Proteomic analysis (LC-MS/MS)	2009	Mayr et al <sup>144</sup>

EV indicates extracellular vesicle; HUVEC, human umbilical vein endothelial cells; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; and THP-1, human acute monocytic leukemia cell line.

directly induce tumor growth, the proliferative and proangiogenic effects of EVs evoke the possibility that they could accelerate or participate in cancer progression. The procoagulant activity of EVs has been the subject of several studies, suggesting that another adverse effect associated with EV administration could be an increased risk of thrombosis. Moreover, because some EVs can both promote or inhibit angiogenesis,

preclinical models that allow us to study the involvement of endogenous EVs, in addition to those that are exogenously administered, are needed to better delineate the benefit/risk balance.

### Conclusion

In addition to their role as promising biomarkers in various pathological conditions, EVs are important mediators in

intercellular communication in normal physiology and disease. An increasing number of studies has documented the capacity of EVs to actively modulate the angiogenic programs in EC by acting on the key steps of vessel formation. Depending on the cellular source, the conditions in which they have been generated and their content, EVs may have either beneficial or detrimental effects on angiogenesis and tissue regeneration. EVs are released by various cell types, such as ECs, endothelial colony-forming cells, mesenchymal stem cells, platelets, or leucocytes, and are able to deliver powerful proangiogenic signals with subsequent beneficial effects in tissue repair. Specific conditions, such as hypoxia and angiogenic growth factor stimulation, favor the release of EVs with vasculogenic potential. However, some of the EVs produced by ECs, platelets, or lymphocytes can also exert an inhibitory activity on vessel growth, mainly by increasing the oxidative stress in target cells. Although the role of EVs in blood vessel formation is well documented and the main underlying mechanisms are identified, further studies are necessary to determine the precise role of each subpopulation of EVs. Indeed, one of the major challenges that the expanding field of EVs faces is the high diversity of vesicle subtypes and the complexity of specifically studying the characteristics, compositions and biological functions of each of them separately. Importantly, because some EVs can either promote or inhibit angiogenesis, animal models allowing us to study the specific involvement of endogenous EVs are needed to delineate when and how EVs can be protective.

A better understanding of EV biogenesis and the multifaceted functions of EVs that defines the specific phenotypic characteristics and active components carried by each subpopulation of EVs will allow us to standardize the process of EV preparation for therapeutic applications. Together with the validation of accurate technologies for the clinical grade manufacturing and quality control of EVs, such advances may provide a better appraisal of the benefit-risk ratio, sustaining the currently emerging therapeutic applications of EVs in regenerative medicine or in angiogenesis-related diseases.

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