

Membrane-Derived Extracellular Vesicles from Endothelial Progenitor Cells Activate Angiogenesis

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Abstract

The neoformation of blood vessels is a biological process involved in tissue homeostasis and repair as well as in pathologic conditions such as inflammatory diseases and cancer. Endothelial progenitor cells (EPCs) a cell population derived from the bone marrow and circulating in the blood stream, have been shown to take part to these processes. EPCs exert their effects mainly by the release of paracrine factors such as growth factors, cytokines and extracellular vesicles (EVs). EVs are small particles released by different types of activated cells by a membrane sorting process. Recent studies identified EVs as a new mechanism of cell-to-cell communication as they can mediate the exchange of receptors, proteins, bioactive lipids and nucleic acids between cells. EPC-derived EVs were shown to activate an angiogenic program in quiescent endothelial cells through an epigenetic reprogramming due to horizontal transfer of mRNA and microRNA. Whereas in the context of tumor neoangiogenesis this mechanism may be detrimental as it favors tumor vascularization and diffusion, in the context of regenerative medicine, EVs derived from EPCs can be exploited as potential therapeutic option to prevent ischemia-reperfusion injury.

Keywords

Endosomal sorting complex required for transport (ESCRT) • Endothelial progenitor cells and

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neoangiogenesis • EPC-derived EVs in tissue regeneration • Extracellular vesicles (EVs) • Multivesicular bodies (MVBs) • Pleiotropic functions • Tumor neoangiogenesis

Introduction

Neoangiogenesis is defined as the process that leads to the formation of new blood vessels in the body. Even though neoangiogenesis is a physiological process essential for tissue homeostasis and repair, the formation of new blood vessels is a common feature of several inflammatory diseases and cancer. Indeed, the neovascularization process is crucial for solid tumor growth and invasion as the vasculature provides metabolic support and access to the circulation, thus favoring metastasis. Tumor angiogenesis does not seem to depend exclusively on normal endothelial cell recruitment from the surrounding vascular network. Recent data suggest alternative strategies for tumor vascularization based on the distinct phenotype of intra-tumor endothelial cells. Indeed, it has been recently shown that tumor-derived endothelial cells are genetically unstable and are different from normal vessel endothelial cells at molecular and functional levels (Bussolati et al. 2011). The enhanced angiogenic properties of tumor endothelial cells have been related to the expression of embryonic genes and to the autocrine production of angiogenic growth factors. Recent studies suggested the potential role of stem cells in tumor angiogenesis and in the phenotypic switch of tumor-derived endothelial cells. Among bone marrow-derived stem cells, endothelial progenitor cells (EPCs) have been shown to localize within sites of vascular injury and within tumors exerting a pro-angiogenic effect mainly due to the release of paracrine factors (Bussolati et al. 2011).

Neoangiogenesis is a pivotal process also in the regeneration of tissues following ischemia-reperfusion injury (IRI). The regeneration of damaged endothelial cells is of particular interest in the field of cell and organ transplantation where the lack of revascularization is associated with primary or delayed graft function.

Endothelial Progenitor Cells and Neoangiogenesis

In the last years, the role of stem cells in regenerative therapies to heal injured tissues has been considerably expanding. In the adults, stem cells are critical for tissue self-renewal in the hematopoietic system, skin and intestine where an high cell turnover is required. On the other hand, a potential role of stem cells in tissue homeostasis has been described also in organs with a much lower rate of cell turnover such as kidney, lung, skeletal muscle and liver. Stem cells are characterized by their ability to self-renew and to differentiate into a variety of cell types. It has been shown that bone-marrow-derived stem cells have the ability to cross lineage boundaries making components of several tissues.

Recently, an endothelial committed type of progenitor cells, defined as EPCs provoked a great interest in regenerative medicine. EPCs, derived from the hematopoietic stem cell lineage, are produced in the bone marrow and subsequently released into the peripheral blood (Asahara et al. 1997).

EPCs are phenotypically identified by flow cytometry and are characterized by the expression of CD34, CD133 and/or VEGF receptor 2 (KDR). These molecules are shared by other hematopoietic stem/progenitor cells and the presence of hematopoietic contamination of EPCs is debated. Yoder et al. (2007) re-defined EPCs into two different cell populations: ECFCs (endothelial colony-forming cells) and CFU-EC (endothelial cell colony-forming units). ECFCs have endothelial (CD31, CD105, CD144, CD146, vWF, KDR and UEA-1) but not hematopoietic antigens, a robust proliferative potential and the ability to form secondary endothelial colony and vessels *in vivo*. Moreover, ECFCs possess a hierarchy of progenitor stages with different proliferative ability. CFU-ECs derive from hematopoietic stem cells, have both endothelial (CD31, vWF and UEA-1) and hematopoietic antigens (CD45, CD14), but with some myeloid progenitor activity and without ability to form secondary endothelial cell colony or vessels *in vivo*. Thus,

ECFCs are considered “true EPCs” whereas CFU-ECs are considered myeloid progenitors (Yoder et al. 2007). It has been shown that EPCs play a pivotal role in vascular homeostasis and repair, but these cells are also involved in tumor neoangiogenesis. EPCs have the potential to provide ongoing endothelial repair by homing to endothelial injury sites. Indeed, different studies showed that EPCs are recruited to injured vascular sites contributing to the preservation of endothelial integrity. EPC levels are lower in patients with different disease states such as coronary artery disease, diabetes, cerebral stroke and in particular chronic kidney disease. In these diseases, the reduction of EPC number seems due to the down-regulation of pathways responsible for EPC release from the bone marrow by exposure to risk factors rather than to EPC consumption.

Recently, it has been shown that EPCs are recruited in the kidney after IRI and that they are able to favour tissue repair by secretion of pro-angiogenic factors. It has been suggested that EPC paucity and dysfunction are the main mechanisms responsible for accelerated vascular injury in chronic kidney disease (CKD) patients. Moreover, the re-establishment of renal function after transplantation correlated with an increased number and function of circulating EPCs (Li et al. 2010).

EPC repair process is characterized by a series of specific and orchestrated steps: EPCs leave the bone marrow following gradients of growth factors and cytokines that are released into the circulation by injured endothelium and tissues (Schattelman et al. 2007). Once in the circulation, EPCs localize to sites of damage and promote vascular repair. Homing of circulating EPCs to sites of vascular injury is a complex process mainly directed by signalling via key CC- and CXC-chemokines, L-selectin and β -integrins. We previously demonstrated that EPCs exploit a mechanism typical of leukocyte adhesion for their localization at the sites of vascular injury, since L-selectin present on EPC surface can selectively bind to fucosylated oligosaccharide residues which are up-regulated following IRI (Biancone et al. 2004).

In the context of cancer, the recruitment of EPCs may be detrimental as they may favour

tumor vascularization. However, the role of EPCs in neo-vessel formation is debated. Whereas in some studies a direct contribution of EPCs to neoformed vessels has been suggested, other studies indicated that EPCs accumulate in the periphery of tumor and favour neoangiogenesis by paracrine mechanisms as they secrete a number of angiogenic factors (Lyden et al. 2001).

Among the soluble angiogenic factors, we recently demonstrated that EPCs released extracellular vesicles (EVs) able to activate an angiogenic program in quiescent endothelial cells by horizontal transfer of mRNA (Deregibus et al. 2007).

Whereas the pro-angiogenic and anti-apoptotic effects of EPC-derived EVs may be detrimental for tumor growth, they may find potential therapeutic applications in ischemic diseases and cell/organ transplantation. In fact, EVs released from EPCs protect the kidney from IRI and improve neoangiogenesis of human islets transplanted in SCID mice through a mechanism related to the epigenetic reprogramming of injured target cells based on the transfer of specific mRNA and microRNA subsets (Cantaluppi et al. 2012a, b).

Extracellular Vesicles (EVs)

Every cell needs to communicate with the environment and with other cells and uses different ways of signaling which can act in close proximity of cells or at long distance. In addition to the well-known cell-to-cell communication based on proteins, cells may relate each other by means of EVs. EVs are extruded from the cell membrane into the extra-cellular space by a universal process that is preserved in prokaryotes and eukaryotes (György et al. 2011). EVs can be isolated from medium of many *in vitro* cell cultures, including tumor cells and from several body fluids such as serum, plasma, urine, amniotic fluid, saliva, milk, bronchoalveolar fluid, etc.

Almost all cells release vesicles and these vesicles differ depending on the cell of origin and on the functional state of the cell from which they are produced (stimulated, apoptotic, tumoral, etc.)

(Simons and Raposo 2009). The classification and the nomenclature of these EVs are at the moment confusing and not well defined. Different names are present in the literature such as microvesicles, microparticles, exosomes, prostasomes, etc. and different protocols of vesicle purification are used. However, all these vesicles share the characteristic to be small and complex structures composed of cytosol surrounded by a lipid bilayer with hydrophilic soluble components resembling that of cell membrane. In addition, they expose many transmembrane proteins able to activate the target cell interacting with cell receptors and allowing recognition and internalization of EVs (Théry et al. 2009). EVs can be defined as carriers of bioactive molecules, since they are able to transfer biological active lipids, functional proteins and nucleic acids (mRNA and microRNA) to a recipient cell modifying its behavior. After their release into the extracellular space, EVs may undergo capture from neighboring cells by means of specific receptor-ligand interactions or enter the circulation and other biological fluids allowing a long distance transport of the bioactive molecules in them enclosed.

Biogenesis of EVs

Most of the studies in this field mainly concentrate on two major classes of EVs: microvesicles and exosomes. The EVs secreted by cells *in vitro* or *in vivo* are a blend of these two types and it is difficult to totally separate them with the actual procedures; therefore many of the studies do not discriminate the two groups of EVs. Microvesicles and exosomes differ not only for the size and density but also for the composition of their membrane, and the mechanism of their formation is not completely known. Microvesicles are a heterogeneous class of vesicles with size ranging from 100 to 1,000 nm and originating by direct blebbing and subsequent detaching from the plasma membrane of cells into the extracellular space in a process under the regulation of flippase, floppase, scramblase, calpain, and gelsolin enzymes involved in the membrane lipid sidedness (Mathivanan et al. 2010). Flippase and floppase generate and maintain membrane

phospholipid asymmetry; scramblase, by mixing the lipids between the two layers of the plasma membrane, promotes the collapse of the membrane asymmetry in a mechanism dependent on calcium concentration. In addition, an increased cytosolic calcium concentration, as it happens during cell activation, apoptosis and necrosis, may activate calpain facilitating membrane blebbing and the shedding of microvesicles from the cell membrane. The membrane of microvesicles contains components of membrane lipid rafts such as flotillin-1 and is enriched in cholesterol and sphingolipids with many saturated fatty acids that render the site less fluid than the adjacent membrane, and eligible for vesiculation (Mathivanan et al. 2010). Moreover, microvesicles may show high exposure of phosphatidylserine, and may express tissue factor and cell specific markers.

Exosomes are a homogenous class of vesicles of 30–120 nm in size and exhibit a characteristic protein and lipid composition which allows their identification. Some protein markers such as tumor susceptibility gene 101 (TSG101), Alix, CD9, CD63 and CD81 tetraspanins, and heat shock proteins (HSP60, HSP70, HSP90) are thought to be specific of exosomes, others are frequently present such as annexins and the small GTPases Rabs important for membrane trafficking and fusion; others are specific of the cell from which exosomes derive. The lipid composition of exosomes includes some components of lipid rafts such as cholesterol, ceramide, glycerophospholipids, and sphingolipids (Simons and Raposo 2009).

Exosome biogenesis is mostly unknown, but they are thought to originate from the endosomal compartment by internal budding and formation of intraluminal vesicles (ILVs) inside multivesicular bodies (MVBs). MVBs may have a dual destination: to reach the lysosomes, fuse their membrane with them and release the intraluminal vesicles into lysosomes undergoing degradation, or to reach the cell membrane and undergo release of ILVs into the extra-cellular space, by a process of exocytosis. These exocytic vesicles are named exosomes (Mathivanan et al. 2010).

The sorting and assembling of proteins into ILVs is a not yet defined process involving multiple

mechanisms. The finding of Alix, a protein associated with the endosomal sorting complex required for transport (ESCRT) machinery, in many exosomal membrane preparations, suggested the requirement of the ESCRT for the release of exosomes but, whilst the involvement of ESCRT multiprotein complexes in the mechanism of lysosomal degradation is well studied, more ambiguous is the ESCRT function in the process of exosomes biogenesis (Théry et al. 2001). However, a recent study provided evidence for the possibility that the transfer to exosomes from distinct subdomains on the endosomal membrane occurs independently from the ESCRT processing but rather depends on ceramide (Trajkovic et al. 2008).

The EVs derived from EPCs are most probably a mixture of exosomes and microvesicles shed from the cell surface. Transmission electron microscopy studies showed shedding of EVs from EPC surface. The majority of them have a size under 100 nm as judged by electron microscopy and ranged between 60 and 160 nm when evaluated by Nanosight. Molecules expressed on surface of EPC -derived EVs include $\alpha 4$ and $\beta 1$ integrins, CD34, L-selectin and CD154 (CD40-L) (Cantaluppi et al. 2012a). Conversely, they were negative for HLA class I and class II antigens and for markers of monocytes (CD14) and platelets (P-selectin, CD42b).

Pleiotropic Functions of EVs

After interaction with EVs the behavior of the recipient cell is modified by several mechanisms. EVs may directly stimulate target cells; for instance, the EVs bearing tissue factor may interact with P-selectin expressed on the surface of macrophages, polymorphonuclear neutrophils and platelets and activate these cells (Cocucci et al. 2008). EVs may transfer receptors and ligands between cells as shown for the adhesion molecule CD41 that can be shifted from platelets to endothelial cells (Barry et al. 1998). EVs may transfer biological active proteins as shown for EVs released from endotoxin-activated monocytes that transfer caspase-1 to vascular smooth muscle cells inducing apoptosis (Sarkar et al. 2009).

More recently, EVs were found to transfer genetic information as they contain a selection of mRNA and microRNA in association with ribonucleoproteins involved in the intracellular traffic of RNA (Valadi et al. 2007; Yuan et al. 2009; Collino et al. 2010). The horizontal transfer of mRNA and microRNA induces epigenetic reprogramming of the recipient cells (Ratajczak et al. 2006; Deregibus et al. 2007; Aliotta et al. 2010).

Role of EVs in Tumor Neoangiogenesis

The angiogenic shift is critical for tumor malignancy. Vascularization is needed to provide oxygen and nutrients for tumor growth and to allow its expansion and access to the circulation. For this purpose, tumor cells produce a number of factors that, acting on microenvironment, create favorable conditions for their growth. Among these factors, EVs play a critical role in inducing phenotypic changes in stroma cells including endothelial cells (Fig. 2.1). A number of studies provide evidences that tumor endothelial cell phenotype differs from normal endothelium (Bussolati et al. 2011). EVs released from tumor cells have the potential to induce these phenotype changes, as they have been shown to carry oncogene products, pro-angiogenic factors and nucleic acids involved in modulation of pro-angiogenic pathways. Indeed, tumor EVs have been shown to be involved in the activation of the angiogenic shift (Castellana et al. 2010). It has been shown that the activation of endothelial cells and fibroblasts that creates a favorable environment for tumor growth, can be mediated by EVs released from lung cancer cells (Wysoczynski and Ratajczak 2009). Tumor EVs were also shown to express on their surface matrix metalloproteinases (MMP) and extracellular MMP inducer involved in the extracellular matrix degradation needed for neoangiogenesis (Castellana et al. 2010). Al-Nedawi et al. (2009) demonstrated that glioma-derived EVs contain the oncogenic form of the epidermal growth factor receptor EGFRvIII, that can be horizontally transferred to endothelial cells promoting

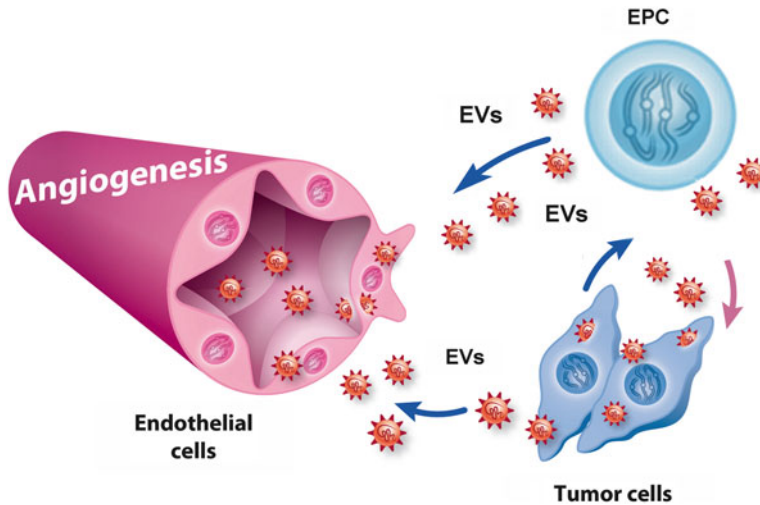


Fig. 2.1 Schematic representation of a bidirectional exchange of genetic information between EPC and tumor cell. The transfer of mRNA and microRNA delivered by EVs may lead to the formation of an environ-

ment favourable for tumor development. EPCs recruited within the tumors release EVs that may transfer molecules potentially involved in the stimulation of tumor angiogenesis

activation of EGFRvIII-regulated genes including the vascular endothelial growth factor (VEGF) gene. In turn, EVs derived from activated endothelial cells may propagate the angiogenic signal by transferring the Notch ligand Delta-like 4 (DII4) to quiescent endothelial cells (Sheldon et al. 2010). Millimaggi et al. (2007) showed that ovarian cancer-derived EVs express CD147/extracellular MMP inducer that activates an angiogenic program in endothelial cells. The critical role of CD147/extracellular MMP inducer shuttled by EVs was shown by using small interfering RNA against CD147 that abrogated the angiogenic activity of EVs. Tumor-derived EVs also convey tetraspanin 8 that favours endothelial cell activation and angiogenesis (Nazarenko et al. 2010). Moreover, cancer-derived EVs may reprogram normal endothelial cells by transferring genetic information from tumor cells to endothelium. The transfer of mRNA and microRNA via EVs has been shown in different experimental settings (Baj-Krzyworzeka et al. 2006; Ratajczak et al. 2006). Skog et al. (2008) showed the transfer of mRNA and microRNA associated to cell migration from glioblastoma to endothelial cells promoting angiogenesis. More recently, it has been shown that EVs shed from

tumors contain retro-transposon elements and amplified oncogene sequences (Balaj et al. 2011) and mitochondrial DNA that may be transferred to target cells (Guescini et al. 2010).

We recently demonstrated in the renal clear cell carcinoma that the EVs possessing the angiogenic activity are those released by tumor stem cells (Grange et al. 2011). These EVs, by creating a favorable endothelial environment in the lung, permit metastatic implantation of tumor cells.

In the context of tumor, the role of EPC is debated. Whereas some studies suggest a direct participation of EPC in the formation of new vessels, other studies suggest that they act by a paracrine mechanism (Bussolati et al. 2011). We found that EVs produced by EPC are able to activate quiescent normal endothelial cells as result of horizontal transfer of mRNA related to pro-angiogenic pathways (Fig. 2.1) (Deregibus et al. 2007). The transferred mRNAs are functional as EVs bearing GFP mRNA induce GFP protein synthesis in the recipient cells. In particular, EVs transfer to target endothelial cells mRNAs associated with the PI3K/AKT and eNOS signaling pathways. Protein expression and functional studies showed that PI3K and eNOS are one of the main effectors in this experimental setting.

EPC-Derived EVs in Tissue Regeneration After IRI

Different studies showed that ischemia-reperfusion induces acute and chronic vascular dysfunction characterized by loss of endothelium-dependent vasodilation. IRI generates high levels of free radicals that enhance endothelial dysfunction and may induce cell death (Lefer and Lefer 1993).

IRI is especially harmful in organ transplantation because it represents a key non-immunologic factor that influences early and late loss of graft function. It is well known that endothelial cell impairment and death associated with the accumulation of leukocytes and platelets causing microcirculatory disturbances and graft failure. Endothelial cell death after ischemic preservation is not only necrotic but also apoptotic. Endothelial cells are the main target of harmful mediators during cold ischemia and subsequent warm reperfusion injury. Reperfusion injury after cold storage induces apoptosis by caspase 3, 8, 9 activation. In particular, caspase activation depends primarily on triggering of Bax and Bak, the pro-apoptotic members of Bcl-2 family which are able to start a sequence of events leading to the release of cytochrome C from mitochondria into the cytosol. Caspase-3 is the final executor molecule, responsible for protein cleavage, thereby disabling cellular structure and repair processes (Kuwana and Newmeyer 2003).

The role of EPCs in neovascularization has also been studied in the model of pancreatic islet transplantation. Islet transplantation is a therapeutic option for the treatment of type I diabetes. In this context, bone marrow-derived stem cells have shown to exert a beneficial effect by enhancing neoangiogenesis and by modulating the allo- and autoimmune response. In particular, it has been shown that EPCs are able to specifically localize within the implanted islets and to chimerize with donor vessels. EPCs enhance transplanted islet revascularization with a better graft survival. Moreover, it has been shown that EPCs are recruited to the pancreas in response to islet injury, favouring neovascularization that may facilitate the recovery of injured beta-cells

improving islet allograft function (Zampetaki et al. 2008).

We have recently shown that EPCs may exert a paracrine effect on transplanted islets through the release of EVs. EPC-derived EVs are internalized in both endothelial and beta cells enhancing islet vascularisation in a model of xenotransplantation in SCID mice. Of interest, EPC-derived EVs favoured insulin secretion and survival by an anti-apoptotic and pro-angiogenic effect. These effects depend on the horizontal transfer mediated by EVs of specific mRNA and microRNA such as miR-126 and miR-296 (Cantaluppi et al. 2012b).

Another model in which we studied the effects of EPC-derived EVs is kidney IRI. Microvascular injury is a hallmark of kidney subjected to ischemia-reperfusion injury. Indeed, the presence of endothelial dysfunction is associated with an extension phase of renal damage often requiring long term dialysis. This is of particular importance in the setting of kidney transplantation where microvasculature derangement associated with tubular cell injury following ischemia-reperfusion injury are the main causes of the so called delayed graft function (DGF), a clinical syndrome characterized by acute dysfunction of grafted kidneys and defined as the need of dialysis in the first week after transplantation. Several on-going clinical trials and experimental studies showed the protective effect of bone marrow-derived stem cells in kidney regeneration after ischemic injury. EPCs are mobilized from bone marrow following kidney ischemia, form an intra-splenic niche and then migrate to the injured kidney promoting tissue regeneration *via* paracrine mechanisms. We found that EVs derived from EPCs mimic the effect of the cells by preventing acute kidney injury (Fig. 2.2). Indeed, EVs are internalized within endothelial cells of peritubular capillaries and tubular epithelial cells inducing tubular cell proliferation, apoptosis and leukocyte infiltration inhibition, thus leading to functional and morphologic protection. EPC-derived EVs are also able to interfere with the mechanisms of progression toward chronic kidney damage by inhibiting capillary rarefaction, glomerulosclerosis, and tubulointerstitial fibrosis

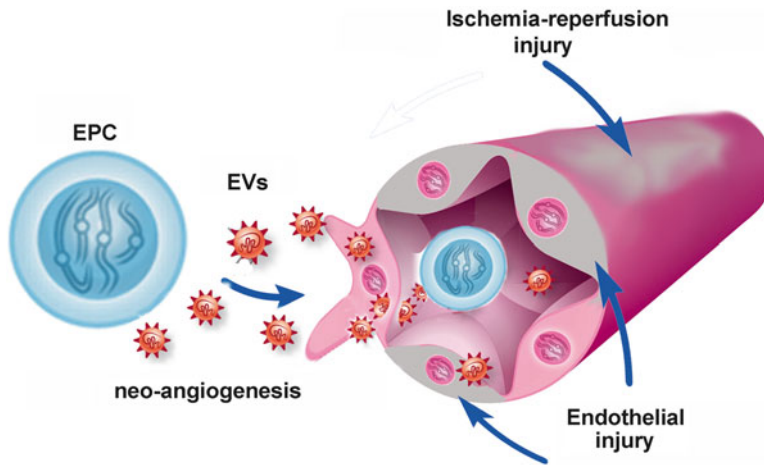


Fig. 2.2 Pro-angiogenic effect of EPC-derived EVs in injured ischemic tissues. EVs may deliver factors that restrain loss of endothelial cells thus limiting injury and that promote endothelial cell proliferation and angiogenesis

(Cantaluppi et al. 2012a). As described for islet transplantation, the renoprotective effect of EVs is due to transfer of mRNA and microRNA from EPCs to injured kidney endothelial and epithelial cells.

In conclusion, EVs emerged as a new mechanism of intercellular exchange of biological information. This may lead to phenotypical and functional changes in the recipient cells. In the context of tumor, EPC-derived EVs may be detrimental as they favor tumor vascularization. At variance, in diseases characterized by endothelial cell injury and loss of vascularization, EPC-derived EVs may find a therapeutic application. Indeed, they limit endothelial injury and stimulate neoangiogenesis.

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