**Extracellular vesicles: their role in cancer biology and epithelial-mesenchymal transition**

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**Abbreviations**

ABCC1, ATP Binding Cassette Subfamily C Member 1

ADAM10, Disintegrin and metalloproteinase domain-containing protein 10

**ARF6, ADP-ribosylation factor 6**

BACH1, BTB Domain and CNC Homolog 1

BMDC, Bone marrow derived cell

DC, Differential centrifugation

DG, Density gradient

ECM, Extracellular matrix

EMT, Epithelial-mesenchymal transition

EV, Extracellular vesicle

LncRNA, Long non coding RNA

MDCK, Madin-Darby canine kidney

MDR, Multi drug resistance

MET, Mesenchymal-epithelial transition

miRNA, MicroRNA

mRNA, messenger RNA

MVB, Multi-vesicular body

PDPK1, 3-phosphoinositide-dependent protein kinase 1

SHIP1, Src homology 2 domain–containing inositol-5-phosphatase 1

SMV, Shed microvesicle

TGF-β, **Transforming growth factor beta**

**TIMP, Metalloproteinase inhibitor 1TM, Tumour microenvironment**

**VAMP3,** Vesicle-associated membrane protein 3

**VEGF, Vascular endothelial growth factor**

**VGF, neurosecretory protein VGF**

VLDLR, Very low-density lipoprotein receptor

YBX1, Y-box binding protein 1

**Abstract**

Cell-cell communication is critical across an assortment of physiological and pathological processes. Extracellular vesicles (EVs) represent an integral facet of inter-cellular communication largely through the transfer of functional cargo such as proteins, mRNAs, miRNAs, DNAs and lipids. EVs, especially exosomes and shed microvesicles, represent an important delivery medium in the tumour microenvironment through the reciprocal dissemination of signals between cancer and resident stromal cells to facilitate tumorigenesis and metastasis. An important step of the metastatic cascade is the reprogramming of cancer cells from an epithelial to mesenchymal phenotype (epithelial-mesenchymal transition, EMT), which is associated with increased aggressiveness, invasiveness, and metastatic potential. There is now increasing evidence demonstrating that EVs released by cells undergoing EMT are reprogrammed (protein and RNA content) during this process. This review summarises current knowledge of EV-mediated functional transfer of proteins and RNA species (mRNA, miRNA, lncRNA) between cells in cancer biology and the EMT process. An in-depth understanding of EVs associated with EMT, with emphasis on molecular composition (proteins and RNA species), will provide fundamental insights into cancer biology.

**1. Introduction**

Cell-cell communication is vital during development in a multiplicity of physiological and pathological processes [[1](#_ENREF_1)] that can be mediated by extracellular vesicles (EVs) [[2](#_ENREF_2), [3](#_ENREF_3)]. EVs are involved in membrane trafficking and the horizontal transfer of an assortment of constituents such proteins, RNA species (mRNA [[4](#_ENREF_4)], miRNA [[5](#_ENREF_5), [6](#_ENREF_6)], lncRNA [[7](#_ENREF_7)]), DNA [[8](#_ENREF_8), [9](#_ENREF_9)] and lipids [[10](#_ENREF_10)] to both local [[11](#_ENREF_11)] and distal cells [[12](#_ENREF_12), [13](#_ENREF_13)]. EVs are important mediators facilitating the acquisition of cancer-associated hallmarks in select recipient cells following their uptake [[14-17](#_ENREF_14)]. Specifically, in the tumour microenvironment (TM), EVs mediate heterotypic interactions between stromal and cancer cells to support fundamental cancer hallmarks such as evasion of growth suppressors, resisting cell apoptosis, sustained proliferative signalling, evasion of immune destruction, and induction of migration, invasion and angiogenesis [[18-23](#_ENREF_18)]. One of the important hallmarks of tumorigenesis is the process of epithelial-mesenchymal transition (EMT), a morphogenetic process that shifts the plasticity of epithelial cells in favour of a mesenchymal phenotype [[24](#_ENREF_24), [25](#_ENREF_25)]. EMT mediates motility and invasion of cancer cells to traverse across the extracellular matrix (ECM) and into the blood circulation to facilitate metastasis [[26-28](#_ENREF_26)].

 More recently, the role of EVs have become increasingly significant in mediating EMT-associated events, inducing the formation of pre-metastatic niches [[29](#_ENREF_29), [30](#_ENREF_30)] and facilitating metastasis [[22](#_ENREF_22)]. Here, we summarise the current knowledge of EV-mediated functional transfer of proteins and RNA species (e.g., mRNA, miRNA, lncRNA) between cells in cancer. We further discuss the emerging contribution of EVs during EMT. In this regard, the majority of experimental studies performed in the context of EMT and EVs have been focused on protein and RNA species. Experimental studies pertaining to EMT at the cellular level will not be covered in this review (see reviews [[31](#_ENREF_31), [32](#_ENREF_32)]).

**2. Defining Extracellular Vesicles**

EVs are membranous vesicles that are released by most cell types into the extracellular milieu [[33-35](#_ENREF_33)]. EVs carry diverse range of cargos that can exert pleotropic effects in recipient cells through distinct signalling cascades via autocrine, paracrine and juxtacrine feedback loops [[36](#_ENREF_36)]. EVs are broadly categorised into shed microvesicles (sMVs) and exosomes [[37](#_ENREF_37), [38](#_ENREF_38)]. Whilst sMVs are formed through the direct shedding/blebbing of the plasma membrane, exosomes are produced as intraluminal vesicles (ILVs) through the inward budding of the late endosome (referred to as multivesicular bodies (MVBs)) [[2](#_ENREF_2)]. MVBs then fuse with the plasma membrane and release their vesicular content as exosomes. Despite the delineation of various ESCRT-dependent and autonomous routes for EVs biogenesis, the precise molecular mechanisms governing these processes remain unclear [[2](#_ENREF_2), [39](#_ENREF_39), [40](#_ENREF_40)]. In this review, other lesser defined EV types such as apoptotic bodies [[41](#_ENREF_41)], migrasomes [[42](#_ENREF_42)] and oncosomes [[11](#_ENREF_11)] will not be discussed.

A large body of evidence highlights the ability of cells to release two predominant EV subtypes that can be differentiated via differential ultracentrifugation and vary in biophysical traits (e.g. size, buoyant density) and composition [[38](#_ENREF_38), [43-45](#_ENREF_43)]. In particular, these EV subpopulations constitute larger heterogeneous sMVs (sediment between ~10,000 – 14,000 *g*) ranging between ~100 to 1500 nm, and the smaller homogenous exosomes ranging between ~50 to 120 nm (sediment at ~100,000 *g*) [[46-48](#_ENREF_46)].

EV subtypes released from MDCK cells and oncogenic H-Ras transformed cells (21D1) display distinct differences in size; sMVs (~100-300 nm) are larger in size in comparison to exosomes (~50-120 nm) (Figure 1). Recently, Simpson and colleagues have identified and clearly demarcated that exosomes and sMVs isolated from human colon cancer cells (CRCs) are biochemically and functionally distinct, displaying 350 proteins uniquely identified in sMVs (e.g. KIF23, CSE1L, RACGAP1) in comparison to exosomes [[43](#_ENREF_43)]. Such studies are further supported by the isolation of highly-purified EV subtypes (exosomes and sMVs) from the same cellular origin are biochemically and functionally distinct [[45](#_ENREF_45), [49](#_ENREF_49)]. Evidently, these studies have to be extended to encompass other tissue-specific cell models in conjunction with *in vivo* validation (biofluids, biopsies) for use in clinical applications.

**3. Extracellular vesicle protein cargo important in cancer**

A well-established paradigm involves the ability of resident cell types to influence the cancer cell phenotype through a plethora of extracellular cues [[50](#_ENREF_50), [51](#_ENREF_51)]. In this regard, EVs form an integral facet of inter-cellular communication particularly through the transfer of functional cargo to recipient cells. Indeed, EVs serve as context-dependent cues that can modify the recipient cell microenvironment [[52](#_ENREF_52)]. By transporting functional cargo to multiple recipient cell types, EVs modulate various cellular events such as migration, invasion, angiogenesis and EMT in the TM [[15](#_ENREF_15), [22](#_ENREF_22)]. Recently, prior to metastatic spread, EVs have been shown to prime distant organs (future metastatic sites) towards a conducive microenvironment (i.e., pre-metastatic niche) [[12](#_ENREF_12), [13](#_ENREF_13), [30](#_ENREF_30), [53-55](#_ENREF_53)] that facilitates survival and outgrowth of incoming tumour cells (i.e., metastatic niche) [[29](#_ENREF_29)]. EVs encase fundamental protein constituents that exert pleotropic roles in the microenvironment of recipient cells (Table 1). The transfer of exosomal proteins can mediate receptor-ligand interactions and modulate growth signalling cascades in recipient cells [[56](#_ENREF_56), [57](#_ENREF_57)]. Notably, EV subtypes (exosomes and sMVs) are composed of a distinct repertoire of molecules [[38](#_ENREF_38)] and can exert diverse biological functionality within the recipient microenvironment [[36](#_ENREF_36), [43](#_ENREF_43)].

*3.1 Exosomal cargo*

Seminal research by Rak and colleagues has demonstrated that the oncogenic receptor EGFRvIII can be transferred between glioma cells through exosomes [[11](#_ENREF_11)]. Notably, MAPK and AKT signalling cascades were induced following treatment of EGFRvIII-positive exosomes and this effect was impeded by the use of pan-erb inhibitor, CI-1033 [[58](#_ENREF_58)] that blocks constitutively activated EGFRvIII and erbB signalling. Further, employing exosomes derived from a panel of cancer cells (e.g. prostate, mesothelioma, breast), Webber and colleagues determined that exosomes expressing high TGF-β levels induced α-SMA expression in fibroblasts [[59](#_ENREF_59)]. Betaglycan, a membrane anchored proteoglycan involved in binding to TGF-β, has been identified to be shed through pervandate and membrane type matrix metalloprotease -1 activity [[60](#_ENREF_60)]. By partially cleaving exosomal betaglycan through pervanadate treatment, diminished exosomal betaglycan expression led to repression of exosomal TGF-β levels and a corresponding decrease in the induction of α-SMA expression in fibroblasts. Importantly, this salient observation highlights the requirement of exosomal betaglycan in the tethering of TGF- onto the exosomal surface to mediate differentiation of fibroblasts. EVs are also able to confer oncogenic cell transforming properties (anchorage-independent growth and survival), as demonstrated by Antonyak et al., whom identified that the oncogenic transfer of tissue transglumtaminase and fibronectin to recipient cells that induced transformation and mitogenic signalling [[61](#_ENREF_61)]. Higginbotham and colleagues delineated exosome mediate EGFR ligand (AREG, EGF, TGF-α, HB-EGF) [[62](#_ENREF_62)] signalling in recipient cells [[63](#_ENREF_63)]. Importantly, AREG exosomes elevated the invasion of recipient breast cancer cells in comparison to TGF-α or HB-EGF exosomes. Moreover, these observations were recapitulated in exosomes isolated from DLD-1 CRCs (mutant KRAS) expressing enhanced AREG levels that exhibited a corresponding increase in invasion.

In the context of the pre-metastatic niche, Peinado and colleagues elucidated the role of melanoma-derived exosomes implicated in the education of bone marrow cells towards propagating tumour growth and metastasis [[12](#_ENREF_12)]. The receptor tyrosine kinase MET was identified to be required for pro-metastatic behaviour of primary tumours as a consequence of exosomal-mediated education of bone marrow progenitors. The expression of Rab family members (RAB1A, RAB5B, RAB7 and RAB 27A), a key family known to be involved in the biogenesis of exosomes [[64](#_ENREF_64), [65](#_ENREF_65)], were elevated in melanoma cells, with silencing of RAB27A resulting in reduced exosome production, tumour growth and metastatic potential. More recently, Hoshino et al., showed that exosomes from lung, liver and brain tumour cells selectively target with recipient cells and prepare the pre-metastatic niche in different regions (liver and lung) [[29](#_ENREF_29)]. In particular, targeting selective integrin’s α6β4 and αvβ5 attenuated exosome uptake to support lung and liver metastasis, indicating that exosomal integrins could be enlisted in the prediction of organ-specific metastasis.

The tumour suppressor PTEN was found to be secreted in exosomes and attenuated phosphorylation of AKT and proliferation in recipient cells [[66](#_ENREF_66)]. Strikingly, the recruitment of PTEN was found to be dependent on Ndfip1, an adaptor protein of the Nedd4 family and the selective packaging of PTEN in exosomes required ubiquitination of PTEN (lysine 13) by Nedd4-1. Luga et al., identified that fibroblasts secrete exosomes that induced Wnt-planar cell polarity (PCP) signalling protrusive activity and motility in breast cancer cells [[67](#_ENREF_67)]. In particular, injection of both breast cancer cells and fibroblasts augmented metastasis that was dependent upon PCP and CD81. In this regard, fibroblast exosomes were determined to enlist autocrine Wnt-PCP signalling to direct the invasive potential of breast cancer cells. Exosomes containing Delta-like 4 (Dll4), a Notch ligand induced Notch signalling in recipient endothelial cells and decreased sprout formation [[68](#_ENREF_68)]. In particular, Dll4-positive exosomes were able to traverse 3D collagen matrices and transfer Dll4 to distant tip cells resulting in filopodia and tip cell retraction, thereby ascribing a novel role for exosomal Dll4 in angiogenesis. Further, Beckler and colleagues identified the exosome-mediated transfer of mutant KRAS to recipient non-transformed CRCs led to induction of anchorage-independent growth [[69](#_ENREF_69)]. Moreover, proteome profiling of exosomes derived from mutant KRAS cells revealed enrichment of numerous oncogenic proteins, including various integrins, KRAS and Src tyrosine kinases.

The tissue inhibitors of metalloproteinases (TIMP) family of protease inhibitors is involved in the regulation of a broad spectrum of matrix metalloproteases (MMPs) that are selectively upregulated in various human cancers [[70](#_ENREF_70)]. Exosomes isolated from TIMP knockout fibroblasts stimulated cancer cell motility and stem cell markers [[71](#_ENREF_71)]. Notably, proteome profiling of exosomes derived from TIMP-knockout fibroblasts revealed ADAM10 upregulation, with exosomal ADAM10 shown to elevate aldehyde expression through Notch receptor activation. Critically, exosomes from human cancer-associated fibroblasts (CAFs) exhibited enrichment of ADAM10 and facilitated migration, RhoA activation and Notch signalling. Lyden and colleagues demonstrated that pancreatic ductal adenocarcinoma (PDAC)-derived exosomes induced TGF-β secretion in Kupffer cells and elevated fibronectin levels in hepatic stellate cells [[13](#_ENREF_13)]. Importantly, PDAC exosomes augmented liver metastatic burden in naïve mice, with macrophage migration inhibitor (MIF) shown to be upregulated in PDAC exosomes using proteome profiling. Of note, when MIF was inhibited, resulted in impeded liver metastatic burden. Prognostic value in identifying the development of PDAC liver metastasis was ascribed towards exosomal MIF, that was corroborated by the recognition of increased exosomal MIF from stage I PDAC patients. Recently, transfer of integrin αvβ6 via PC3-derived exosomes was identified to facilitate cell adhesion and migration in recipient cells [[72](#_ENREF_72)]. Exosomal integrin αvβ6 was identified to be transferred to integrin αvβ6-negative cells and found to be localised to the cell surface. Importantly, recipient cells conditioned with exosomes comprising integrin αvβ6 exhibited elevated migration in comparison to recipient cells treated with exosomes exhibiting RNAi-repressed integrin αvβ6, ascribing a role for exosomal integrin αvβ6 during cancer cell migration.

*3.2 sMV cargo*

An increasing emphasis has been placed towards defining the functional contribution of sMVs in recipient cells (Table 1). The recognition of sMVs as fundamental “delivery vehicles” that facilitate the acquisition and dissemination of multidrug resistance (MDR) represents a pivotal example. Bebaway and co-workers observed that P-glycoprotein (P-gp) could be transferred via sMVs from drug resistant to drug sensitive cells [[73](#_ENREF_73)]. Importantly, the cell surface P-gp in sMVs could be incorporated in recipient cell and drug accumulation assays further validated this functional transfer of P-gp. In line with this, sMV-mediated functional transfer of multidrug resistance-associated protein 1 (MRP1) was demonstrated in drug-sensitive leukaemia cells [[74](#_ENREF_74)]. Moreover, a kinetic difference in the sMV-mediated transfer of P-gp and MRP1 was observed. Notably, sMV-mediated transfer of P-gp-positive sMVs induced cellular reprogramming in recipient cells, thereby potentiating the acquisition of MDR.

Recently, Clancy and colleagues found that sorting of MT1-MMP to nascent sMVs was dependent upon on the association between VAMP3 and CD9 [[75](#_ENREF_75)]. RNAi-mediated silencing of VAMP3 depleted MT1-MMP expression in sMVs and abrogated invasion in collagen matrices. Importantly, sMVs derived from the ascites of ovarian cancer patients were enriched in VAMP3, MT1-MMP and ARF6 and exhibited localised proteolysis in degradation assays, further validating the biological role of VAMP3/MT1-MMP1 in sMVs. Both exosomes and sMVs purified from breast cancer cells were found to upregulate Wnt5a expression and induce invasion in macrophages [[76](#_ENREF_76)]. The selective packaging of Wnt5a into select EVs was identified to be partially dependent on Evi. EV-induced invasion was found to be impeded by Dickkopf-1, a Wnt inhibitor and RNAi-mediated silencing further decreased cell invasion, identifying a role for Wnt5a-positive EVs in modulating invasion.

The glycosylation status of tumour derived sMVs was determined by Menck and colleagues to be important for cell invasion [[77](#_ENREF_77)]. By employing deglycosylated sMVs, established by peptide N-glycosidase F (PNGaseF) targeting highly glycosylated EMMPRIN (HG-EMP), a specific role for HG-EMP was ascribed towards sMV-mediated invasion. Critically, EMMPRIN-positive sMVs promote invasion through the activation of the p38/MAPK signalling and sMV-induced invasive behaviour required HG-EMP. Recently, exposure of gemcitabine to human pancreatic cancer cells induced sMV release and conversely, inhibition of secretion sensitized cells to gemcitabine exposure [[78](#_ENREF_78)]. Furthermore, differences between drug resistant and sensitive pancreatic cancer cells were ascribed to the content of influx and efflux proteins in sMVs that permit retention of gemcitabine.

**4. Extracellular vesicle RNA cargo important in cancer**

Transcriptional regulation involves both protein coding RNAs (mRNAs) and non-coding RNAs (e.g. miRNA and lncRNA) and further represents an additional level of control during EMT [[79](#_ENREF_79)]. MiRNAs are small non-coding RNAs (18-24 nucleotides) that are involved in the post-transcriptional regulation of gene expression [[80](#_ENREF_80)]. In particular, mature miRNAs suppress the translation of their target mRNAs, through sequence specific interactions with the 3’ untranslated regions (UTRs) [[79](#_ENREF_79), [81](#_ENREF_81)]. By coordinating the expression of an assortment of protein coding genes, miRNAs exert pleotropic roles and are implicated in regulating critical cellular process such as differentiation, development and proliferation [[82](#_ENREF_82), [83](#_ENREF_83)]. Long non-coding RNAs (lncRNAs) (>200 nucleotides), similar to miRNAs, are involved in the regulation of cellular activities via interactions with DNA, RNA, and proteins [[84](#_ENREF_84), [85](#_ENREF_85)]. We review the accumulating evidence identifying the biological role of EV-derived RNA species (mRNA, lncRNA and miRNA) in recipient cells (Figure 2, Table 2).

*4.1 Messenger RNA (mRNA)*

Seminal research by Valadi et al., established that exosomes can transfer mouse mRNAs and miRNAs to recipient human mast cells [[86](#_ENREF_86)]. Furthermore, microarray analysis revealed the presence of 1300 mRNA transcripts in exosomes that represented ~8% of the total detected mRNAs in donor cells and conversely, exosomes contained 270 unique mRNAs. Skog and colleagues demonstrated that glioblastoma-derived exosomes can be taken up by endothelial cells and exosomal mRNA can be further translated [[4](#_ENREF_4)]. Isolation of exosomes from glioblastoma cells, transduced with a lentivirus vector encoding a secreted luciferase from Gaussia (Gluc), permitted incorporation of an mRNA for a reporter protein into exosomes. The continual synthesis of Gluc in recipient cells following exosome uptake, validated the functional transfer of exosomal mRNA. In addition to exerting autocrine effects on glioblastoma cells (i.e., proliferation), exosomal cargo were enriched in angiogenic-associated proteins and functionally, had the capacity to induce tube formation. Critically, prototypic mRNAs and miRNAs specific for gliomas were detected in microvesicles from glioblastoma patient sera.

Through the elegant use of the Cre-LoxP system, Zomer and co-workers interrogated EV uptake *in vivo,* by inducinga colour switch in recipient reporter positive cells that take up exosomes secreted from Cre-positive cells [[87](#_ENREF_87)]. Notably, tumour-derived exosomes were identified to be composed of mRNAs implicated in migration and metastasis and could be internalised by less malignant cells at both local and distal sites. These perturbed tumour cells exhibited enhanced migratory and metastatic behaviour as determined by intravital imaging. Further, glioma and cancer cells were engineered to express Cre recombinase and EVs isolated from these cells comprised Cre mRNA [[88](#_ENREF_88)]. Transplantation of these tumour cells into mice with a Cre reporter background led to the recognition of specific target cellssuch as leukocytes and myeloid derived suppressor cells. In this regard, Cre-lox based tracing of EV-mediated transfer can be enlisted to determine precise target cells *in vivo.*

*4.2 Long non coding RNA (lncRNA)*

Takahashi and colleagues demonstrated that the transfer of linc-RoR, a hypoxia responsive lncRNA, via exosomes to recipient cells augmented cell survival during hypoxia [[89](#_ENREF_89)]. In particular, linc-RoR was associated with miR-145, a linc-RoR target and HIF-1α signalling. Employing qRT-PCR based assays, the large intergenic non-coding RNA-VLDLR (linc-VLDLR) was determined to be significantly up-regulated in malignant human hepatocytes and enriched in EVs derived from these cells. In particular, lnc-VLDLR was observed to be transferred through exosomes and possessed the capacity to modulate chemotherapeutic response in recipient cells following exosomal transfer [[90](#_ENREF_90)]. Specifically, lnc-VLDLR was identified to be enriched in exosomes derived from malignant hepatocytes through lncRNA expression profiling. Exosome mediated transfer of lnc-VLDLR to recipient liver carcinoma cells attenuated chemotherapy-induced cell death and elevated lnc-VLDLR expression. This effect was further corroborated by the absence of lnc-VLDLR (RNAi) transfer in exosomes, resulting in altered recipient cell viability. Furthermore, an increase in ABCC1 expression was detected in EV treated recipient cells, postulating a mechanistic role for ABC transporters in the regulation of chemoresistance.

Liver cancer cells (CD90+) were identified to regulate endothelial cell behaviour via secretion of exosome lncRNA H19 [[91](#_ENREF_91)]. Employing a lncRNA qPCR array (LncProfiler), RNAs such as Air, Hotair, lincRNA-RoR, Hulc, and H19 related to heptacellular carcinoma were identified to be enriched in CD90+ liver cancer cells. Furthermore, lncRNA H19 was enriched in CD90+ liver exosomes and upregulated in recipient endothelial cells following exosome transfer, and overexpression of H19 enhanced angiogenesis (i.e., endothelial tube number and length). Assessment of serum exosomes from rheumatoid arthritis (RA) patients and blood nuclear cells by Song et al, led to the identification of an enrichment in the expression of lncRNA, Hotair [[92](#_ENREF_92)]. Critically, RA exosomes induced macrophage migration and diminished expression of Hotair was detected in differentiated osteoclast and rheumatoid synoviocytes, ascribing a potential role for Hotair as a diagnostic biomarker of RA. Recently, exosomes were found to be involved in the acquisition of sunitinib resistance, a significant challenge in the therapy of advanced renal cell carcinoma [[93](#_ENREF_93)]. In particular, bioactive lncARSR was identified to be selectively sorted into exosomes. Importantly, exosomes from resistant cells were found to transfer sunitinib resistance to sensitive cells through the competitive binding of miR-34/miR-449 that potentiates AXL and c-Met expression.

*4.3 MicroRNA (miRNA)*

Xin and co-workers observed that miR-133b level was enhanced in exosomes isolated from multipotent mesenchymal stromal cells (MSC) following exposure to ischemic tissue extracts subjected to occlusions [[94](#_ENREF_94)]. Importantly, miR-133b was specifically enriched in exosome-treated primary neurons and astrocytes. Furthermore, exosomes enriched in miR-133b induced elevated neurite length and branching in cultured neurones, thereby identifying the role of exosomes in mediating intercellular communication between MSCs and brain parenchymal cells. In the context of pre-metastatic niche formation, Liu and colleagues found that tumour-derived exosomal RNAs were able to support and facilitate lung pre-metastatic niche through TLR3 (Toll-like 3 receptor) [[93](#_ENREF_93)]. Specifically, tumour-derived exosomal RNAs activated TLR3 in lung epithelial cells to directly promote secretion of chemokines and neutrophil recruitment.

The Akao laboratory determined that CRC cells could communicate with endothelial cells via exosomes [[95](#_ENREF_95)]. Specifically, enhanced expression of miR-1246 and TGF-β was identified in exosomes derived from CRC cells and these CRC exosomes induced SMAD 1/5/8 signalling and potentiated angiogenic behaviour of endothelial cells. Through the use of an antagomiR targeting miR-1246, exosome induced angiogenesis was abrogated, attributing a specific role for exosomal miR-1246 in angiogenesis. The involvement of exosomes in facilitating the destruction of the blood-brain barrier was recently identified [[96](#_ENREF_96)]. Specifically, exosomal miRNA-181c induced ectopic actin filament organisation through its target gene, PDPK1. Exosomal miRNA-181c was also identified to be enhanced in the sera of brain metastasis patients. Remarkably, exosomes obtained from brain metastatic cancer cells augmented the brain metastatic capacity of breast cancer cells following systemic injection.

Recently, the regulation of the immune response through exosome mediated communication between immune cells was assessed [[5](#_ENREF_5)] (exosomal immune cell regulation in cancer reviewed [[23](#_ENREF_23)]). In particular, miR-155 and miR-146a were detected in exosomes and identified to exert opposing roles in facilitating endotoxin-induced inflammation in mice. Furthermore, the transfer of exosomal miR-155 reduced the expression levels of its target genes, including BACH1 and SHIP1. Critically, the use of exosomes derived from miR-155 knockout models nullified the functional transfer and exosomal miR-155 was found to potentiate inflammatory response (IL-6, TNF-α) *in vivo*. Cicero and colleagues found that keratinocytes, a principal component of the epidermis, employ exosomes to communicate with melanocytes [[97](#_ENREF_97)]. Notably, differential expression of miR-3196 was identified by miRNA profiling in the exosomes of irradiated and non-irradiated keratinocytes to determine their involvement in pigmentation. Furthermore, exosomes from keratinocytes transfected anti-miR-3196 exhibited a diminished potential to induce melanin synthesis in melanocytes, ascribing a direct role for exosomal miR-3196 in pigmentation. More recently, the Yu lab demonstrated that the microenvironment modulation of tumour suppressor PTEN by exosomal miRNA facilitates the progression of brain metastasis [[6](#_ENREF_6)]. In particular, tumour cells distinctively exhibit reduced PTEN expression following dissemination to the brain in comparison to other organs. Importantly, astrocyte-derived exosomes directed the transfer of PTEN-targeting miRNAs to metastatic cells. This salient observation was corroborated by the silencing of PTEN-targeting miRNAs and inhibition of astrocyte-derived exosome release that rescued PTEN silencing and repressed brain metastasis. In light of the plasticity of PTEN expression across different organs, the reprogramming of metastatic cells by exosomes describes an important signalling mechanism. Taken together, these observations substantiate the role of EVs as context-dependent cues that exert pleiotropic effects across various microenvironments.

**5. Epithelial-Mesenchymal Transition**

EMT represents a morphogenetic cellular program, initially identified to facilitate the formation of intricate embryonic structures during embryogenesis [[98-100](#_ENREF_98)]. EMT involves a shift in cell plasticity whereby epithelial cells lose traits such as apico-basolateral polarity and a cobblestone morphology in favour of a mesenchymal phenotype with elevated migratory and invasive potential [[25](#_ENREF_25), [101](#_ENREF_101)] (Figure 3). In this regard, EMT represents a multi-faceted program that is tightly controlled and coordinated by multiple pleotropic factors that target and influence downstream effector molecules and signal transduction pathways [[102](#_ENREF_102)]. In particular, the orchestrated interplay of inducers (e.g., HGF [[103](#_ENREF_103)], PDGF [[104](#_ENREF_104)]), transcriptional factors (e.g., Snail1 [[105](#_ENREF_105)], Twist [[106](#_ENREF_106)] and signalling pathways (e.g., TGF-β [[107](#_ENREF_107)] and Notch [[108](#_ENREF_108)]) facilitate the shift in plasticity.

Developing tumours recruit an assortment of normal resident cells that aid in the acquisition of cancer-associated hallmarks and facilitate metastatic progression [[1](#_ENREF_1)]. Metastasis represents a step-wise process whereby a localised primary cancer cells colonise distal secondary sites and develop metastatic lesions [[19](#_ENREF_19)]. Notably, plasticity of cancer cells occurs at various pivotal points (intravasation, extravasation) in the cascade and remains an important requirement for cells to become metastatic [[109](#_ENREF_109)]. The plasticity of cells is regulated through processes such as EMT and MET (mesenchymal-epithelial transition) [[26](#_ENREF_26), [110](#_ENREF_110), [111](#_ENREF_111)].

Simpson and colleagues have extensively assessed cellular and extracellular protein changes during EMT (secretome [[112](#_ENREF_112), [113](#_ENREF_113)], plasma membrane [[114](#_ENREF_114)], and exosomes [[115](#_ENREF_115)]) by employing the EMT model of MDCK (epithelial) cells and oncogenic H-Ras-transformed MDCK (21D1) cells. Specifically, comparative analysis of MDCK and 21D1 secretome revealed extensive remodelling of the ECM proteins that include diminished expression of basement membrane constituents (e.g., collagen type IV and laminin 5) and proteases (e.g., kallikreins-6,-7 and MMP1) [[112](#_ENREF_112)]. Proteomic analysis of exosomes revealed that epithelial markers such as E-cadherin and EpCAM were diminished in 21D1 exosomes, whilst mesenchymal markers (e.g., vimentin), proteinases (e.g., MMP-1), integrins, and transcription factors (e.g., YBX1) were increased in 21D1 exosomes. An in-depth understanding of the extracellular contribution during EMT is required, particularly relating to mechanisms of EV biogenesis, recipient cell uptake and target cell specificity. To this end, therapeutic windows can be exploited during EMT and metastasis, possibly through pharmacological intervention by targeting EV-associated biogenesis and secretion mechanisms.

**6. Extracellular Vesicles in Epithelial-Mesenchymal Transition Biology**

Accruing evidence highlights the emerging contribution of EVs released during EMT in facilitating the acquisition of cancer hallmarks. Various studies have identified oncogenic molecules and metastatic niche-associated factors (e.g. integrins, proteases) in EMT-derived EVs [[115](#_ENREF_115), [116](#_ENREF_116)]. Notably, EVs isolated from diverse cell models have been identified to induce EMT and cancer hallmarks in recipient cells [[22](#_ENREF_22), [117](#_ENREF_117)]. Given that components of the metastatic niche are closely related with EMT [[24](#_ENREF_24)], further understanding of the precise parameters governing the biology of these EVs would have significant implications for cancer biology. This section will discuss the capacity for EVs to be reprogrammed during EMT (protein and RNA content) and induce cancer-associated hallmarks in recipient cells (Figure 4).

*6.1 Cells Undergoing EMT Reprogram Extracellular Vesicle Protein Content*

EVs obtained from various EMT models have been found to be composed of cargo (e.g. protein and RNA) distinct from their parental counterparts (Table 3). For example, Garnier et al identified that human squamous carcinoma A431 and colon epithelial DLD-1 acquire mesenchymal traits following induction of EGFR coupled with the blockade of E-cadherin [[118](#_ENREF_118)]. Notably, elevated release of EVs was observed from mesenchymal cells in comparison to parental epithelial cells. Elevated expression of Tissue Factor (TF) was observed in mesenchymal EVs and increased transfer of mesenchymal microvesicles was further identified to be transferred to endothelial cells. Proteomic analysis of EVs obtained from EMT-induced A431 cancer cells resulted in the identification of profound qualitative differences in comparison to parental A431 epithelial EVs [[119](#_ENREF_119)]. Mesenchymal cell-derived exosomes were enriched in integrins (e.g., ITGB1, ITGA2) and cell junction associated proteins (e.g., JUP, DSG3) and pathways associated with cell growth, signalling and motility.

Employing an *in vivo* metastasis model of human bladder carcinoma cell line T24 and its two isogenic derivative cell lines SLT4 and FL3, Jeppesen et al purified exosomes and examined their membrane and luminal contents via quantitative proteomics [[120](#_ENREF_120)]. A salient observation of this analysis was the upregulation of EMT-associated proteins (e.g., VIM, HDGF) in membrane fractions and CSNK2A1 and ANXA2 in the luminal fractions of metastatic exosomes. EVs released by mesenchymal-like cells (MDA-MB-231, SKOV3 and APOCC) were distinctively able to induce endothelial cell activation and AKT phosphorylation in comparison to EVs secreted from epithelial-like cells (OVCAR3 and MCF7) [[121](#_ENREF_121)]. Elevated expression of angiogenic molecules such as PDGF, IL8 and angiogenin were identified in EVs from mesenchymal-like cells.

Global proteomic profiling of MDCK and 21D1 cell and derived exosomes led to the identification of prototypic expression of EMT hallmark proteins (E-cadherin, EpCAM and vimentin) in both cell and exosomal proteomes [[115](#_ENREF_115)]. In addition, 21D1 exosomes were enriched with several proteases (e.g., MMP-1, -14, -19, ADAM-10, and ADAMTS1) and integrin’s (e.g., ITGB1, ITGA3, and ITGA6). Notably, the unique presence of key transcriptional regulator (e.g., Y-box binding protein 1, YBX1) and splicing complex components (e.g., SF3B1, SF3B3, and SFRS1) were identified in mesenchymal 21D1 exosomes. Overexpression of YBX1, a pleiotropic transcriptional regulator in MDCK cells (MDCKYBX1) led to the induction of a partial EMT phenotype [[122](#_ENREF_122)]. In particular, MDCKYBX1 cells displayed cytosolic relocalisation of E-cadherin, increased cell scattering and anchorage-independent growth and formed vascularised tumour xenografts. Mass-spectrometry-based sequencing of the MDCKYBX1 secretome resulted in the identification of secreted factors known to enhance angiogenesis (e.g., CSF-1, NGF, VGF, ADAM9 and ADAM17). These observations identify YBX1 as a fundamental oncogenic enhancer that can promote angiogenesis. We also investigated the functional activity of exosomes derived from MDCKYBX1 cells in inducing angiogenesis [[123](#_ENREF_123)]. Unlike parental MDCK-derived exosomes, MDCKYBX1 exosomes significantly increased motility and tube formation capacity in endothelial cells. Comparative proteomics analysis revealed that MDCKYBX1 exosomes were enriched with Rac1 and PAK2 whilst 21D1 exosomes contained VEGF-associated proteins. As VEGF is a fundamental inducer of angiogenesis [[124](#_ENREF_124)], these proteins might potentially be involved in promoting various facets of angiogenesis such as endothelial cell proliferation, migration and infiltration. In this regard, exosomal Rac1/PAK2 can serve as angiogenic promoters that may function during the initial phases of metastasis, providing an avenue for communication with endothelial cells. Recently, Schekman and colleagues identified YBX1 was critical in the selective sorting of miRNAs in HEK293T-derived exosomes and involved in the binding and sorting of miR-223 [[125](#_ENREF_125)]. Given that miR-223 was found to be sequestered more efficiently than miR-190 into exosomes, it was suggested that the presence of a primary RNA sequence or a secondary structure stabilised by RNA-binding proteins that include YBX1, may be implicated in the selective RNA sorting.

Ectopic expression of the transmembrane glycoprotein podoplanin (PDPN) in MDCK cells was identified to induce EMT [[126](#_ENREF_126)]. Proteomic analysis of exosomes released by cells overexpressing PDPN (MDCKPDPN) revealed enhanced expression of various oncogenic proteins including semaporins (PLXNB2) and ephrins (EPHB2, EFNB1) involved in tumour angiogenesis, invasion and metastasis, and downregulation of cell adhesion associated molecules (e.g., EPCAM), catenins (e.g., CTNNA1, CTNNB1). The expression of PDPN induced the increased release of exosomes and sMVs that was further recapitulated in HN5 squamous carcinoma cells through an RNAi approach. Functionally, MDCKPDPN exosomes facilitated lymphatic vessel formation in a PDPN dependent manner corroborated by the use of neutralising monoclonal antibody targeting PDPN.

*6.2 Cells Undergoing EMT Reprogram Extracellular Vesicle RNA Content*

BM mesenchymal stromal cells (BM-MSCs) were identified to release exosomes that were transferred to multiple myeloma (MM) cells [[127](#_ENREF_127)]. In particular, MM BM-MSC derived exosomes exhibited elevated expression of oncogenic proteins, cell adhesion and cytokines in comparison to parental exosomes. The expression of tumour suppressor miR-15a was diminished in BM mesenchymal stromal exosomes. Exosomal miR-15a was identified to facilitate the distant cell communication, though EMT associated pathways. MiR-200 family members (miR-200a, miR-200b, miR-200c, miR-429 and miR-141) represent fundamental regulators of EMT that have been linked to influencing Zeb1 and Zeb2 expression [[127](#_ENREF_127)]. Le et al identified miR-200 in EVs obtained from metastatic mouse and breast cancer cells, and observed increased expression of miR-200 in the sera of mice with metastatic tumours [[128](#_ENREF_128)]. Moreover, miR-200 was identified to be transferred via metastatic breast cancer EVs to non-metastatic cells, accompanied by changes in gene expression and MET. Strikingly, EVs obtained from miR-200 expressing tumours facilitated metastasis of cells with lowered metastatic potential, thereby endowing them with the capacity to colonise distant tissues. Recently, Xiao et al demonstrated that exosomes obtained from melanoma cells were able to induce EMT in primary melanocytes [[129](#_ENREF_129)]. Importantly, EMT-derived exosomes mediated this through Let-7i, as evidenced through the use of a Let-7i mimic in invasion assays and immunofluorescence analyses. Moreover, induction of the MAPK pathway was observed following melanoma exosomes induced EMT and EMT-associated miRNAs (miR-191 and Let-7a) was found to be enriched in serum exosomes obtained from melanoma patients in comparison to non-melanoma subjects.

*6.3 Extracellular Vesicle Transfer Induces EMT in Target Cells*

The shift in plasticity of recipient cells towards a mesenchymal phenotype can be directed by EVs (Table 4). Linoleic acid, a polyunsaturated fatty acid is involved in regulating plasminogen activator inhibitor-1 and the proliferation, motility and invasive potential breast cancer cells [[130-132](#_ENREF_130)]. Exosomes isolated from MDA-MB-231 cells stimulated with linoleic acid induced a diminution in the expression of epithelial markers (e.g., E-cadherin) and increased expression of mesenchymal markers (e.g., vimentin, N-cadherin, Twist1, Snail1 and Snail2) in MCF-10A cells [[133](#_ENREF_133)]. Importantly, exosomes facilitated increased release of MMP-2 and MMP-9, invasion, motility and elevated NF-κB-DNA binding. Latent membrane protein 1 (LMP1), a fundamental oncogenic regulator of Epstein-Barr virus (EBV), facilitates tumorigenicity of invasive EBV malignancy and nasopharyngeal carcinoma. Aga et al., identified hypoxia-inducible factor-1α (HIF1α) in exosomes and that LMP1 increased HIF1α expression in exosomes [[134](#_ENREF_134)]. Treatment of EBV-negative cells with LMP1-positive exosomes enhanced migration motility of nasopharyngeal cells that correlated with an EMT phenotype. Importantly, exosome mediated transfer of HIF1α induced changes in the expression of E- and N-cadherins. Immunohistochemical analysis of nasopharyngeal cancer (NPC) tumour tissues further identify an association between LMP1 expression and exosome marker, CD63.

MMP13 is most often elevated across various tumours and was identified to overexpressed in NPC cells [[135](#_ENREF_135)]. Treatment of MMP13-containing exosomes onto NPC cells, increased the invasion and motility of those cells. Conversely, siRNA mediated repression of MMP13-containing exosomes reversed its effects on NPC cells. In addition, expression of prototypic EMT markers (E- and N cadherin) was significantly altered following treatment of NPC cells with MMP-13-containing exosomes and siRNA-repressed derivative.

Franzen et al., treated urothelial cells with bladder cancer exosomes and identified elevated expression of mesenchymal markers (α-SMA, S100A4) in comparison to untreated cells (PBS-conditioned cells) [[136](#_ENREF_136)]. Consistent with this, diminished expression of epithelial markers (E-cadherin and β-catenin) was identified in urothelial cells conditioned with bladder cancer exosomes. In addition, bladder cancer exosomes enhanced the invasion and migration of urothelial cells and the treatment of exosomes isolated from patient urine bladder samples induced the expression of mesenchymal markers in urothelial cells. A recent study by Qin et al, treated benign and malignant breast cancer cells with exosomes isolated from human breast milk (transitional, mature and wean samples) [[136](#_ENREF_136)]. Expression of TGF-β2 was significantly enhanced in breast milk exosomes during weaning and treatment of cancer and benign breast cells with these exosomes led to the induction of EMT. In particular, morphological, cytoskeletal changes coupled with enhanced expression of mesenchymal markers (α-SMA, F-actin and vimentin) was observed, postulating a role for high TGF-β2 expressing exosomes in influencing breast cancer.

**7. Concluding remarks**

EVs represent a fundamental delivery vehicle in the TM, largely through the horizontal transfer of signals (proteins and RNA species) between cancer and resident cells to regulate cancer progression and metastasis. Defining the contribution of EV subtypes during distinct phases of cancer is required. An in-depth understanding of EV biology is required in the context of functionality *in vitro* and importantly, recapitulated in *vivo* to establish clinical relevance. Recent reports reveal the role of EVs in regulating intercellular communication at both primary and secondary tumour sites, particularly by inducing the formation of the pre-metastatic niche and directing organ tropism. Importantly, targeting EVs released during disease and pathological processes, might serve as fundamental therapeutic avenues aimed at inhibiting various facets of EV biology such as biogenesis, release, cell uptake and/or targeting of specific EV components. By employing a systems biology-based strategy, future applications would involve interrogating cargo (e.g. proteins and RNA species) of EVs isolated from body fluids (e.g. blood, ascites, and urine*)* that could potentially unveil diagnostic and prognostic biomarkers. Consistent with the notion that EVs can transfer functionally-active cargo, future studies could enlist EVs in delivering therapeutic drugs to cancer cells. An in-depth understanding of EVs released during the EMT would provide key insights into the EMT process and therapeutic opportunities aimed at halting and limiting metastatic spread.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Figure legends**

**Figure 1: Cryo-electron microscopy of EV subtypes from MDCK, MDCKYBX1 and 21D1 cells.**

MDCK, MDCKYBX1 and 21D1 cells were cultured in DMEM (containing 10% FBS), washed with DMEM (0% FBS), and cultured in serum-free DMEM for 24 h. Conditioned medium was harvested and centrifuged (480 g, 5 min, 2000 g, 10 min) to sediment floating cells and remove cellular debris. The supernatant was centrifuged at 10,000 g, 30 min to isolate shed microvesicles (sMVs), and the resulting supernatant ultracentrifuged at 100,000 g, 1 h to pellet crude exosomes. Crude exosomes were layered onto OptiPrep density-gradient, and subjected to ultracentrifugation at 100,000 g for 18 h (for a detailed protocol refer [[137](#_ENREF_137)]). Aliquots of each purified exosome-specific fraction determined by immunoblotting and density, were subjected to cryo-electron microscopy. Electron micrographs of EV subtypes indicate a distinct variance in the size of sMVs (~100-300 nm) and purified exosomes (~50-120 nm) (Scale bar = 100 nm).

**Figure 2: Select EV cargo involved in functional transfer to recipient cells.**

EVs are implicated in the functional transfer of protein and RNA components that modulate that activity of recipient cells. Proteins that include (EGFRvIII [[11](#_ENREF_11)], AREG [[63](#_ENREF_63)], MET [[12](#_ENREF_12)]) are transferred to recipient cells via exosomes and influence migration, invasion and metastatic behaviour of recipient cells. RNA species such as mRNAs (CDC6 [[86](#_ENREF_86)], GLUC [[4](#_ENREF_4)], Cre [[87](#_ENREF_87)]), miRNA (miR-133 [[94](#_ENREF_94)], miR-1246 [[95](#_ENREF_95)], miR-181c[[96](#_ENREF_96)]) and lnc-RNA (Hotair [[92](#_ENREF_92)] and H19 [[91](#_ENREF_91)]) are involved in inducing biological activity (migration, angiogenesis) in recipient cells.

Figure 3: Epithelial-Mesenchymal transition (EMT).

EMT is a morphogenetic cellular process where cells lose epithelial traits in favour of mesenchymal features [[138](#_ENREF_138)]; diminished inter-cell adhesions (i.e., tight junctions, adherens junctions, desmosomes), loss of cell polarity, cytoskeletal rearrangement and increased invasive and migratory potential. Cells undergoing partial EMT exhibit both epithelial and mesenchymal features [[139](#_ENREF_139)]. Epithelial cells lose the round cobblestone like shape in contrast to mesenchymal cells that present with loss of apical and basolateral polarity and a spindle-like morphology. During this transition, select soluble secreted and extracellular vesicles are released from these cells to modulate their environment.

**Figure 4: Selected proteins identified in exosomes derived from EMT models.**

Selected exosomal proteins identified by proteomic profiling (mass spectrometry) from epithelial, partial EMT and mesenchymal models. These models include - epithelial cells (MDCK [[123](#_ENREF_123), [126](#_ENREF_126)], A431 [[119](#_ENREF_119)] , T24 [[120](#_ENREF_120)]) which indicate expression of proteins such as CDH1, EPCAM, AGRN and CTSB; partial EMT models (MDCKYBX1 [[123](#_ENREF_123)]), which reveal expression of RAC1, PAK2 and HRAS; and mesenchymal models (21D1 [[123](#_ENREF_123)], MDCKPDPN [[126](#_ENREF_126)], EMT-induced A431 [[119](#_ENREF_119)], FL3/SLT4 [[120](#_ENREF_120)]) indicate expression of VIM, EHD1, RAP2B and ANXA2.

**Table 1: Transfer of EV-derived functional proteins**

**Table 2: Transfer of exosome-derived functional RNA species**

**Table 3: EVs derived from EMT models**

**Table 4: Selected applications detailing exosome mediated EMT related changes in recipient cells**

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