Emerging roles of exosomes during epithelial-mesenchymal transition and cancer progression

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a highly conserved process defined by the loss of epithelial characteristics, and acquisition of the mesenchymal phenotype. In addition to its central role in development, EMT has been implicated as a cellular process during tumourigenesis which facilitates tumour cell invasion and metastasis. The EMT process has been largely defined by signal transduction networks and transcriptional factors that activate mesenchymal-associated gene expression. Knowledge of secretome components that influence EMT including secreted proteins/ peptides and membrane-derived extracellular vesicles (EVs) (i.e., exosomes) has emerged. Here we review EV cargo associated with inducing the hallmarks of EMT and cancer progression, modulators of cell transformation, invasion/ migration, angiogenesis, and components involved in establishing the metastatic niche.

1. Introduction

Epithelial-mesenchymal transition (EMT) is a highly conserved morphogenic process defined by the abrogation of epithelial characteristics, and acquisition of a mesenchymal phenotype [1-3]. Accumulating evidence has highlighted a critical role of EMT-associated events during tumour progression and malignant transformation, and thereby endows the cancer cell with invasive and metastatic properties [4-7]. Although reciprocal crosstalk between numerous intracellular signalling pathways are known to regulate EMT [8], it is now emerging that extracellular modulators in the tumour microenvironment (TM) can influence tumour cell state and invasive potential [9-12]. The metastatic cascade consists of tumour cells undergoing detachment from the primary tumour site (suppression of cell-to-cell and cell–matrix adhesion), degradation and invasion of the local ECM, increased cell motility and penetration into the circulation for dissemination to distant organs [13, 14]. as After the metastatic cells have undergone colonisation, the secondary lesions often display an epithelial-like phenotype, suggesting that mesenchymal-epithelial transition (MET) may facilitate metastatic outgrowth [15, 16]. Furthermore, a subset of tumour cells can exhibit both epithelial and mesenchymal traits concurrently (referred to as partial EMT and the metastable phenotype [17, 18]). Tumour cells with partial EMT traits confer elevated metastatic competence enabling them to propagate in the dynamic TM [19]. The extracellular regulation of EMT is associated with phenotypic switching for tumour cell migration and invasion [20], further highlighting the importance of environmental cues to direct EMT and enhance metastasis [9, 21].

Tumour cells release soluble factors that modulate the TM and enhance cell transformation during EMT [22]. These diffusible factors (e.g., soluble proteins including growth factors, cytokines, chemokines) or cell-associated ligands/ receptors can mediate communication between cancer cells and surrounding stromal cells [13]. Importantly, these factors are involved in generating favourable cellular environments for metastatic niches [23, 24]. Intercellular crosstalk can be reciprocal, and can be mediated directly (e.g., gap junctions, membrane nanotubes, tunnelling nanotubes [25]) or by secretion of components such as extracellular matrix (ECM) proteins, enzymes, or paracrine signalling molecules such as growth factors and inflammatory cytokines (collectively referred to as the secretome) [24, 26, 27]. More recently, extracellular vesicles (EV) have emerged as another important mechanism in this paracrine signalling [28-30]. EVs are complex organelle-like lipid-bound

membranous structures released by most cell types, and provide an important molecular mechanism by which cells can influence local, neighbouring, and even distant cells and their environments.

Vesicle-based cell-cell communication by EVs, including exosomes (40-150 nm) and microvesicles (100-1000 nm), has been reported during EMT and cancer progression [31-36]. EVs containing disparate molecular cargo such as protein, mRNA, microRNA (miRNA), DNA and lipids, can be directly internalized by stromal cells and other cancer cells, and induce functional changes in these recipient cells [31, 37-39]. Recently, it was reported that positive feedback mechanisms can lead to increased/modulated EV production during tumourigenesis [40-42]. Such deregulation of exosome secretion pathways in tumour cells, in turn, can lead to an altered microenvironment, thereby promoting cancer progression. In this review we discuss the emerging role of EVs in promoting EMT during cancer progression, focussing on the functional capabilities of EVs in different hallmarks associated with cancer progression, including the transfer of oncogenic cargo, cell transformation, invasion/ migration, angiogenesis, and in establishing the metastatic niche.

2. Extracellular vesicles: Exosomes and microvesicles

Originally described as a mechanism to release cellular waste and toxins, there is now numerous reports of EVs as important mediators of extracellular signalling, via the direct membrane-transfer of their cargo [43]. EV uptake by recipient cells can reprogram signalling pathways to modulate the phenotype and function of the target cell [31, 44-48]. For example, mechanisms include transmembrane protein transfer to the plasma membrane to trigger signalling, oncoprotein transfer, transcriptional regulators transferred into the nucleus to regulate promoter activity, translational regulation mediated by mRNA/miRNA transfer, and DNA transfer for integration into the recipient cell genome [49].

The issue of EV annotation is a vexed one [50]. For example, some EVs are classified based on their cellular origin and/or biological function (exosomes, prostasomes, oncosomes, dexosomes, microparticles, promininosomes, argosomes, and exosome-like vesicles) or on their biogenesis, namely microvesicles (shed microvesicles) and exosomes. In contrast to heterogeneous microvesicles (100–>1000 nm), which are generated by budding from the plasma membrane, exosomes (30-150 nm) are derived from the endosomal pathway [51].

Microvesicles are enriched in phosphatidylserine, cell lineage markers, cell surface receptors, cholesterol-rich or specialized cell membrane microdomains (lipid-rafts), whereby the membrane composition of microvesicles reflects that of the parent cell more closely than the membrane composition of exosomes [52]. The biogenesis of microvesicles is regulated by a distinct set of molecular events including activation of AKT and acidic sphingomyelinase intracellular calcium flux variations, and enzymes involved in the maintenance of membrane phospholipid asymmetry [53-56]. Exosomes, in contrast, are usually smaller (30–150 nm), have a buoyant density of 1.09-1.19 g/mL, and form by the inward budding of the luminal membrane of endosomal multivesicular bodies (MVBs). Exosomes are abundant in tetraspanins (e.g., CD63, CD81), and their biogenesis is governed by several regulatory mechanisms, including elements of the endosomal sorting complex required for transport (ESCRT), Rab proteins (e.g., Rab11, Rab27, Rab35), syndecan-syntenin-Alix, p53/TSAP6 pathway, phospholipase D, ceramide, oligomerization and neutral sphingomyelinase 2 [51, 57-62]. Two distinct MVB pathways have been characterized [63], one that targets for lysosomal degradation, the other for trafficking to the plasma membrane where upon fusion with the plasma membrane, release their contents (exosomes) into the extracellular milieu. Distinct populations of exosomes have been reported for the highly polarized colon cancer tumour cell line LIM1863 that are consistent with apical and basolateral origin [64]. Although exosome internalisation by recipient cells has been reported to occur via multiple processes such as phagocytosis [65-67], clathrin-mediated endocytosis [68], macropinocytosis [69], receptor-mediated [70], and direct fusion [71], further studies are required to improve our understanding of these underlying mechanisms [32, 36, 46, 72, 73]. EVs provide a unique molecular platform to further explore cell communication, targeted cell selection, mechanisms of internalisation, intercellular reprogramming between distinct cell types, and also serve as a drug delivery system.

2.1 Exosome-mediated transfer of oncogenic cargo

2.1.1 DNA

In addition to mitochondrial DNA (mtDNA) and single-stranded DNA (ssDNA) [74, 75], tumour exosomes have also been shown to contain double-stranded DNA (dsDNA). Recently, Thakur and colleagues identified dsDNA of mutated *KRAS* and *p53* within exosomes derived from pancreatic cancer cells, and these mutations are the most frequent

mutations in human pancreatic cancer [39]. Interestingly, dsDNAs were shown to represent the largest proportion of exosomal DNAs (exoDNAs) [39]. ExoDNAs constitute similar mutations as the cell line from which they originate, such as *BRAF (V600E)* and *EGFR*, suggesting the potential role of exoDNAs as attractive biomarkers in detection and diagnosis of cancer.

2.1.2 mRNA

Exosomes containing mRNAs can be transferred and influence the translational profile of recipient cells and tumour progression [47, 76, 77] [72]. For example, Skog et al., showed that by incorporating mRNA for a reporter oncogenic protein *EGFRvIII* into EVs, these transcripts (3426 transcripts present at different levels in exosomes and their donor cells) could be delivered to, and translated by recipient cells [77]. Interestingly, these EVs also were enriched in angiogenic proteins and stimulated cell proliferation and tubule formation in recipient endothelial cells. A seminal observation was mutant/variant mRNAs and miRNAs signatures found in glioma cell line-derived EVs could be detected only in serum-derived EVs isolated from glioblastoma patients, but not in control patient serum. In another study, Hong et al. profiled the transcriptome of exosomes released from human colorectal cancer SW480 cells, and identified enrichment of cell cycle-related mRNAs that could stimulate endothelial cell proliferation, and facilitate tumour development [72].

2.1.3 miRNA

MiRNAs are short non-coding RNAs that suppress gene expression through mRNA destabilization, translational inhibition and mRNA degradation [78-80]. Encapsulation of miRNAs within exosomes can protect from degradation, potentially providing a clinical avenue to detect disease biomarkers [81]. Exosome-containing miRNAs have been implicated in cancer pathogenesis [82]. For example, microarray analysis has revealed the selective enrichment of let-7 miRNA family in exosomes derived from highly metastatic gastric cancer cells, in comparison to low metastatic gastric cells [83]. Given let-7 miRNA family has been demonstrated as a tumour suppressor targeting oncogenic Ras, this study proposes an exosome-mediated clearance mechanism whereby exosomes might permit highly invasive tumour cells to release let-7 family members from the cell, and thereby maintain their tumourigenic phenotype. In another study, Yang and colleagues demonstrated that IL-4-

activated tumour-associated macrophages release specific miRNAs (miR-223) via EVs, which when transferred to co-cultured breast cancer cells, enhanced their invasiveness through the Mef2c-β-catenin pathway [84]. In another study, comparative miRNA profiling of malignant U87 glioblastoma cells identified miR-1 as an orchestrator of EV function and tumour growth and invasion [85]. Pro-oncogenic effects (*in vivo* growth, neovascularization, and invasiveness) of glioblastoma tumour–derived exosomes were alleviated by miR-1, which directly targets Annexin A2 (ANXA2), an abundant exosomal protein. Further, the clinical relevance of miR-1/ANXA2 targeting in malignant glioblastoma, was shown by elevated ANXA2 mRNA and ANXA2 protein levels in patient malignant glioblastoma samples, and brain tumor samples, respectively.

3. Epithelial-mesenchymal transition (EMT) and cancer progression

The early events of metastatic dissemination are thought to be initiated by EMT-type processes in carcinoma cells [2, 9, 86-89]. Hallmarks of EMT include diminished expression of intercellular contact/adhesion components (e.g., E-cadherin), cell-matrix components, components involved in cell polarity, elevated expression of proteins involved in cytoskeleton remodelling (e.g., vimentin) and elevated expression of various proteases including matrix metalloproteinases (**Figure 1A**) [7, 20, 90-94].

3.1 Signalling pathways and transcriptional programs activated during EMT

Numerous signalling pathways have been identified and described to be functional during the EMT process, during stages of development, as well as tumour progression [20, 95] (Figure 1A). Transcriptional regulation by SNAIL, TWIST and zinc-finger E-box binding (ZEB) factors facilitate the activation of various signal transduction cascades that drive and maintain EMT [96]. In addition to the established EMT transcription factors, novel transcription factors that include forkhead box (FOX), GATA family, SRY (SOX) transcription factors are involved in regulating epithelial genes or polarity complexes as well as coordinate with SNAIL/SNAIL2 to drive EMT [97-103].

Long non-coding RNAs (lncRNAs, longer than 200 nt) and miRNAs have recently been reported to regulate the epithelial phenotype and tumour cell state during EMT and neoplastic transition [104-107]. Yuan et al., observed that lncRNA-activated by TGF-β upregulated

ZEB1 and ZEB2 and induced EMT and invasion in hepatocellular carcinoma (HCC) metastases [106]. This further resulted in organ colonization of disseminated tumour cells, autocrine induction of IL-11 and STAT3 signalling, and facilitated the invasion-metastasis cascade. lncRNAs have also been shown to regulate the transcription of Twist resulting in dysregulation of canonical signalling pathways such as Wnt, MAPK, JAK-STAT, TGF-β, mTOR, Hedgehog and p53 during EMT of MCF10A breast cancer cells [104].

Studying cell transformation, Park and colleagues examined miRNA expression in 60 cancer cell lines and identified the miR-200 miRNA family as an indicator of cells expressing E-cadherin but lacking vimentin [108]. In addition, the authors identified EMT transcriptional factors ZEB1 and ZEB2 as direct targets of miR-200. In another study Gregory and colleagues [109] reported a double-negative feedback loop between the miR-200 family and ZEB that mediated conversion in epithelial and mesenchymal plasticity. Interestingly, the authors also observed an autocrine TGF-β/ ZEB and miR-200 signal transduction cascade that modulated plasticity. Beltran et al, demonstrated that the expression of ZEB2 is modulated by a natural antisense transcript involved in the inhibition of a large intron in the 5'UTR, involved in the inhibition of ribosomal scanning [110]. The authors discovered increased levels of antisense transcripts bind to the 5'UTR that promote ribosomal scanning, leading to elevated levels of ZEB2 and EMT.

3.2 EMT regulation in the tumour microenvironment

The TM stroma includes an assortment of cell type including cancer-associated fibroblasts (CAFs), endothelial, epithelial, pericytes, macrophages, neutrophils and mesenchymal stem cells [111]. Dynamic interactions between cancer cells and constituents of the tumour stroma are known to facilitate EMT induction and drive metastatic progression. For example, Yu and colleagues showed that CAFs facilitate aggressive phenotypes of breast cancer cells through induction of EMT in a paracrine fashion mediated by TGF-β1 [112]. Given that CAFs could induce various morphogenetic and phenotypic changes in breast cancer subtypes, the authors proposed it as a common mechanism for acquiring metastatic potential in breast cancer cells with diverse biological traits. Multiple lines of evidence also highlight the influence that the stroma has on mediating EMT via secretion of various chemokines, growth factors and cytokines [9, 11, 21, 113]. Tuxhorn and colleagues investigated the stromal cell phenotype

and extracellular remodelling components during the progression of prostate cancer [114]. In comparison to normal prostate tissue, enhanced expression of vimentin, α-smooth muscle actin, tenascin, collagen 1, and fibroblast activation protein was observed in prostate cancer. Further, ADAMs and matrix metalloproteases that are secreted by myofibroblasts and immune cells can promote cytokine and growth factor activation and ECM remodelling [115, 116]. Indeed, diverse interactions between ECM proteins that include collagens, laminins, integrins and cathepsins arbitrate the transfer of signals that facilitate the determination of invasive potential, metastasis and influence tumour cell state.

3.3 Contribution of extracellular modulators during EMT

Given that cancer cells at the leading tumour edge can undergo EMT and initiate metastasis in response to signals from the microenvironment [2, 89], characterising and understanding the contribution of this extracellular region is an important element of EMT and cancer biology. Pioneering studies from Bissell and colleagues [117-120] demonstrated the critical role for cell–ECM interactions in normal breast epithelial differentiation and tumour cell morphogenesis. Shintani et al., [121] demonstrated exposure of epithelial cells to ECM components found in the mesenchymal compartment induced the loss of epithelial function. Type I collagen was shown to stimulate a mesenchymal phenotype including scattering and upregulation of N-cadherin in mammary epithelial cells. Additionally, α-smooth muscle actin expression and myofibroblast activation were suppressed by laminin [122, 123]. Other extracellular regulators that trigger EMT include matrix-degrading proteases, ECM components, and integrins [124, 125]. MMPs, cysteine proteases, and urokinase promote EMT not only by altering the extracellular milieu favouring cell migration via ECM degradation but also by liberating growth factors and cytokines stored in the ECM [126]. These changes in the ECM effect the mechanical environment of the cells and presumably lead to mechanical disruption of intercellular contacts [125]. Recently, lung cell metastasis has been shown to be regulated by miR-200 expression, which is responsive to TGF-β [127]. Metastatic tumour cells transited between epithelial and mesenchymal states, forming highly-polarized epithelial spheres in 3D culture and TGF-β-stimulation induced EMT [127]. Forced expression of miR-200 abrogated the capacity of tumour cells to undergo EMT-associated invasion and metastasis by conferred transcriptional features of metastasis-incompetent tumour cells.

To characterise extracellular proteins involved in mediating EMT, Mathias *et al*., [128, 129] analysed the secretome (soluble-secreted protein component) of oncogenic Ras-induced EMT in MDCK cells using a proteomic-profiling approach. These studies revealed diminution of components mediating cell-cell contact and cell-matrix adhesion (collagen XVII, IV, and laminin 5) epithelial cells that have undergone the EMT process and elevation of the levels of proteases and ECM constituents promoting cell migration (MMP-1, TIMP-1 kallikrein-6, -7, fibronectin, collagen I, fibulin-1, -3, biglycan, decorin, S100A4/ metastasin and SPARC) (**Figure 1B**). Collectively, these findings suggest that hierarchical regulation of a subset of extracellular effectors may coordinate a biological response during EMT that enhances cell motility [128]. The same group also investigated the contribution of EVs during EMT in the same cell model and observed profound protein differences (i.e., a reprogramming) in the EV proteome upon EMT (see section 4) [130, 131]. The functional consequences of the proteome component of EVs being reprogrammed (especially the unique enrichment of oncogenic factors, master transcriptional regulators (e.g., YBX1) and core splicing subunits in mesenchymal cells) is intriguing and warrants further investigation [130].

4. Functional contribution of EVs during the EMT process

EVs mediate intercellular communication and direct integral facets of carcinogenesis that include EMT, invasion, migration, angiogenesis, and metastasis [132]. Proteomic studies can provide a global assessment of protein expression levels and identify components that have the potential to drive signal transduction networks in the extracellular environment during cancer progression. For example, Garnier et al. reported that EMT induction in A431 mesenchymal-like cancer cells by epidermal growth factor receptor (EGFR) activation/ blockade of E-cadherin, led to elevated secretion of EVs containing EGFR and tissue factor, thereby transferring the pro-coagulant activity to endothelial cells, an accelerated event during cancer malignancy [133]. Proteomic analysis revealed that EMT in cancer cells resulted in a qualitative redistribution of EV cargo proteins, where enriched proteins were involved in cellular growth, cell-to-cell signalling, and cell movement [131]. Others have used quantitative proteomics to characterise exosomes released from non-metastatic and metastatic models [134, 135]. Interestingly, several of these studies report archetypical proteins observed across various EMT models that include increased vimentin and hepatoma-derived growth factor (HDGF) from metastatic cells, and expression of metastatic factors

such as MET, S100A8, S100A9, SCR, and TNIK. Tauro et al., compared the protein composition of exosomes released from MDCK cells with those from MDCK cells transformed with oncogenic-H-Ras (21D1 cells) [130]. Expression of epithelial markers such as E-cadherin and EpCAM were reduced in 21D1 exosomes, while mesenchymal markers (e.g. vimentin), key proteinases (e.g. MMP-1, -14, -19 and ADAM-10), integrins, tetraspanins (e.g. CD81, CD82 and CD151), EMT-related transcription factors (YBX1), and core splicing complex components were increased in 21D1 exosomes (**Figure 1C**). Importantly, several of these factors have been associated with conditioning the metastatic niche and facilitating tumourigenesis. Additionally, K-Ras has also been attributed to modulate exosome composition [136]. Exosomes released from mutant K-Ras cells contained higher levels of tumour-promoting proteins, including K-Ras, EGFR, SRC family kinases, and integrins. Internalisation of mutant K-Ras exosomes was shown to enhance 3D growth of wild-type K-Ras-expressing non-transformed cells. Collectively, these studies highlight exosomes from EGFR-activated and Ras-transformed cells are reprogrammed with factors which may be capable of maintaining their own EMT process or inducing EMT in recipient cells. Since EMT often correlates with the elevated tumour initiating capacity of cancer cells, it is possible that the accompanying changes in EV production may contribute to this process; for example, by conditioning the niche environment, influencing adjacent host cells [44] and regulating secondary metastatic regions [36].

4.1 Exosomes facilitate oncogenic cell transformation

Increasing focus has been placed on the transfer of oncogenic cargo through EVs, in particular exosomes, which have now been described to direct cell transformation and drive signal transduction cascades [137]. For example, exosomes have been shown to be capable of transferring the metastatic activity of highly metastatic Bl6–10 melanoma tumour cells to poorly metastatic F1 melanoma tumour cells *in vitro*. In murine models, lung metastatic colonies develop when F1 cells are injected in combination with exosomes from Bl6–10 exosomes, whereas mice injected solely with F1 cells develop no metastatic colonies [138]. The Rak lab showed that the truncated form of the EGFRvIII is transferred from glioma cancer cells by microvesicles, to glioma cells lacking this receptor, in a mechanism involving detergent-resistant membrane domains (lipid rafts) [137]. The transferred receptor is biologically active and results in transfer of oncogenic activity, including stimulation of the transforming signalling pathways (mitogen-activated protein kinase, AKT), and changes in

expression of EGFRvIII-regulated genes that include VEGF and p27. The authors propose that a horizontal dissemination of EGFRvIII and resulting change in oncogenic potential to tumourigenic cells could be EV-mediated. This study confers the ability of oncogenic protein/mRNA/miRNA-containing EVs may act as intercellular conduits that drive cell transformation.

In a recent study Cerione and colleagues demonstrated that microvesicles shed by human breast carcinoma (MDA-MB-231) and glioma (U87) cancer cells were capable of transferring the transformed characteristics of cancer cells (e.g., anchorage-independent growth and enhanced survival capability) to recipient normal fibroblasts and epithelial cells [139]. Interestingly, this process required the transfer of the protein cross-linking enzyme tissue transglutaminase (tTG), and crosslinking substrate fibronectin (FN). Functionally, both cross-linked FN and tTG were shown to cooperatively activate mitogenic signal transduction and induce transformation in the recipient fibroblasts. The study further revealed that the transformed phenotype requires continuous EV supplementation to maintain fibroblast transformation. In the context of the tumour microenvironment, release of cancer cell-derived EVs may provide the continuous supply of EVs required by recipient stroma and normal epithelia/ fibroblast cells to facilitate the progression of a transformed, and pro-tumourigenic phenotype [139, 140].

In a salient study by Wrana and colleagues, they report the involvement of stromal-derived exosomes in promoting protrusive activity, motility, and metastasis of breast cancer cells, via the activation of autocrine Wnt–planar cell polarity (Wnt-PCP) signalling [33]. The authors showed that the human breast cancer cells internalized fibroblast-derived exosomes, and repackaged exosome cargo with endogenous Wnt11, resulting in the activation of autocrine Wnt-PCP signalling. It was demonstrated that exosomal tetraspanin, CD81, is critical for exosome-stimulated cancer metastasis, dependent on autocrine Wnt11 produced in breast cancer cells [33]. These intriguing findings suggest that exosomes are agents of cross-talk between cancer and stromal cells to stimulate metastasis. These findings provide promising future research in the fields of tumour–stroma communication, exosome function, and Wnt-PCP signalling in cancer metastasis [141]. Further involvement of exosomes influencing Wnt signalling was revealed by Caplan and colleagues who reported exosome-mediated export of β-catenin and tetraspanins, CD9 and CD82, all of which are implicated in down-regulation of Wnt signalling [142]. Secretion of functionally-active Wnt proteins was shown for the first

time by Gross and collaborators, where exosomes from mammalian (HEK293) and *Drosophila* (Kc167) cells selectively packaged Wnt3A to activate Wnt signalling in target cells [143]. Together with the cargo receptor Evi/WIs, Wnts were transported through endosomal compartments in exosomes, a process that required the R-SNARE Ykt6. As essential morphogens during development, how Wnt proteins are trafficked across tissues has been a puzzling observation considering the innate lipid composition and specialized lipid raft microdomains of cell surface-derived EVs [144].

The capability of cancer-derived exosomes to modulate normal stromal fibroblasts was investigated by Webber and colleagues, where they screened a panel of cancer cell lines (mesothelioma, prostate, breast, bladder, and colorectal) and showed their differential ability to produce TGF-β-positive exosomes and regulate a cancer-altered stroma environment [35]. Tumour-derived exosomes with TGF-β expressed at the exosome surface in association with β-glycan, were able to induce elevated α-smooth muscle actin expression and other changes consistent with the process of fibroblast-myofibroblast differentiation [35]. Importantly, the cellular responses (such as mRNA induction) reflected differences between exosomal TGF-β and soluble-recombinant TGF-β. In another study, exosomes released from injured epithelial cells have been suggested to deliver TGF-β1 mRNA, and activate fibroblasts during hypoxia [145]. This functional exosome-mediated transfer initiates tissue repair/ regenerative responses through fibroblast proliferation, α-smooth muscle actin expression, F-actin expression, and type I collagen production. More recently, Webber et al., showed that prostate cancer cell-derived exosomes triggered TGF-β1-dependent fibroblast differentiation, to a distinctive myofibroblast phenotype, supporting angiogenesis *in vitro* and accelerating tumour growth *in vivo* [146]. Interestingly, myofibroblasts generated using soluble TGF-β1 were not pro-angiogenic or tumour-promoting, indicating the necessity for cancer exosomes as key regulators of stromal differentiation. Exosome-deficient cancer cells generated by silencing the exosome secretion regulator, Rab27a [57], abolished fibroblast differentiation and led to inhibition in stroma-assisted tumour growth *in vivo*. These studies implicate exosomes as a critical mode for stromal cell communication and regulator of tumour-associated stromal remodelling.

It is well established that that oncogenic Ras and TGF-β signalling cooperatively enhance EMT and tumour metastasis, as constitutive expression of Ras induces EMT, and this

phenotype is maintained by the autocrine production of TGF-β, induced by Ras [147, 148]. In addition to active TGF-β, Ras has also been identified in exosomes [130]. Further, the proteome profile of exosomes derived from isogenic human colorectal cancer cell lines, SW480 (primary) and SW620 (lymph node metastatic), revealed the presence of K-Ras, N-Ras and H-Ras within exosomes [134]. In particular, increased Ras expression was identified in exosomes released from metastatic colorectal cancer cells. Importantly, exosomes have been shown to be able to transfer mutant K-Ras from mutant K-Ras-expressing cells into recipient cells, leading to enhanced 3D growth of non-transformed cells [136]. This study demonstrates the role of exosomes in facilitating tumour niche development through horizontal delivery of tumour-promoting factors, including K-Ras, EGFR, SRC family kinases, and integrins. Notably, tumour suppressors (e.g. PTEN) have also been identified in exosomes, and shown to have functional activity. For example, exosomal-derived PTEN was shown to have phosphatase activity, and was able to antagonize PI3K signalling and cell proliferation in recipient mouse embryonic fibroblast cells [149]. Recently, Melo et al., demonstrated that cancer exosomes mediate significant transcriptome alterations in target cells via RISC-associated miRNAs [40]. Breast cancer-derived exosomes contain miRNAs (miR-10b and miR-21) associated with the RISC-Loading Complex (RLC) and in recipient MCF10A cells, displayed cell-independent capacity to process pre-miRNAs Dicer, AGO2, and TRBP into mature miRNAs. Exosomes derived from cells and sera of patients with breast cancer resulted in “oncogenic conversion”, whereby non-tumorigenic epithelial cells form tumours (colony formation capacity and *in vivo* mammary fat pad) in a Dicer-dependent manner.

4.2 Exosomes enhance cell migration and invasion

A central process in the metastatic cascade is cell invasion and migration, whereby disseminating tumour cells extravasate into distant sites and colonise secondary tissues and organs [150]. Recent evidence highlights the role of EVs, in particular exosomes, to stimulate invasion and migration in various cancer models [151]. For example, breast MCF-7-derived exosomes were shown to foster tumour growth, migration, and matrix degradation, via secretory Rab27b [152]. Further, human breast and colorectal cancer-derived exosomes contain full length, signalling-competent EGFR ligands [153]. The invasive potential of colorectal cancer cells was shown to be directly related to the concentration of exosomes containing the EGFR ligand, amphiregulin (AREG), suggesting that exosome-mediated

ligand transfer contributes to cancer invasiveness and metastasis. Exosomes from colon cancer cells with mutant *KRAS* exhibited both higher AREG levels and greater invasiveness. Recently, Atay and colleagues reported that exosomes are released by GIST cells *in vitro* and *in vivo* contained the oncogenic protein tyrosine kinase (KIT) [151]. These exosomes were shown to directly modify recipient cell morphology, and coordinate the transformation of progenitor smooth muscle cells to tumour-promoting cells. This was established via increased adhesion to ECM proteins, activation of intracellular pathways downstream of KIT, and expression of Interstitial Cell of Cajal–like markers that potentiated tumour cell invasion. In contrast, to facilitate cancer cell invasion and migration, tumour-released exosomes were found to modulate the ECM through secreting key proteinases. According to the proteomic analysis of exosomes released from MDCK and 21D1 (Ras-transformed MDCK cells) cells by Tauro et al., 21D1-exosomes were enriched with matrix metalloproteinases (MMP-1, -14 and -19), as well as ADAM 10 and ADAMTS1 [130]. MMPs are capable of degrading ECM components, such as gelatin fibronectin and collagen, and correlate with high grade of tumour invasion [154]. Likewise, ADAM-10 and ADAMTS1 promote cell invasion by proteolytically cleaving various cell adhesion molecules [155]. Also, MT1-MMP is secreted by exosomes in fibrosarcoma and melanoma cells, resulting in collagen degradation [156]. Further, activators of MMPs are also secreted by tumour-derived exosomes: exosomal-derived Hsp90 can activate MMP-2 and promote ECM remodelling [157], and exosomal-derived Hsp70 can promote cell invasion [158]. Oncogenic KIT-containing exosomes were described to induce the secretion of various MMPs, particularly MMP-1, generating a positive feedback mechanism between tumour and stromal cells to drive gastrointestinal tumour development [151]. Further studies are required to identify the mechanisms of tumour-derived exosome uptake in recipient cells, the molecular mechanisms involved in the production of MMP-1, and the ability to modulate continuous production and secretion of oncogenic exosomes from tumour cells.

4.3 Exosomes stimulate angiogenesis

Tumor growth and metastasis depend on angiogenesis triggered by secreting various growth factors and cytokines (e.g. VEGF) from tumour cells and the microenvironment [159]. The ability of tumour-derived EVs to induce angiogenesis in various cancers and tumour microenvironments has been well documented [29, 34, 36, 160-164]. For example, Hood et al. reported that melanoma-derived exosomes can stimulate endothelial cells and promote

endothelial spheroid formation, in a dose-dependent manner [36]. Recently, Kucharzewska et al. showed that exosomes constitute a potent regulator of hypoxia-dependent intercellular communication between malignant and vascular cells (endothelial cells, pericytes), suggesting an important mechanism of regulation during hypoxia-driven pro-angiogenic tumour responses [30]. Leandersson and colleagues identified the role of Wnt5a in the exosome-mediated secretion of pro-angiogenic and immunomodulatory factors that potentiate metastatic potential [160]. Given that elevated expression of Wnt5a in malignant melanoma is associated with formation of distant metastasis, the authors further identified a correlation between Wnt5a and the angiogenic marker ESAM, by gene analysis of primary malignant melanomas. Interestingly, endothelial cell branching was influenced by varying levels of Wnt5a in melanoma cells co-cultured with endothelial cells. The study indicates that EVs can regulate the capacity for tumour progression, rendering tumours more aggressive. Recent work by Asada and colleagues showed that exosomes comprising Delta-like 4 (Dll4) causes capillary endothelial tip cells to lose their filopodia [165]. Interestingly, transfer of Dll4 protein to distant tip cells was observed, with the application of Dll4 exosomes resulting in diminished sprout formation (i.e., capillary sprout retraction). Induction of Notch signalling in recipient cells correlated with treatment of endothelial cells with Dll4 exosomes. Therefore, EVs have functional roles to increase endothelial cell motility while suppressing their proliferation. Further, Sheldon et al. has shown that endothelial exosomes are involved in vascular development as they incorporate and transfer Dll4 protein to neighbouring endothelial cells, leading to an inhibition of Notch signalling and an increased capillary-like formation *in vitro* and *in vivo* [73]. This suggests that the Dll/Notch pathway doesn't require direct cell–cell contact, but exosomes can induce cell signalling to facilitate angiogenesis. Lee et al., showed that macrophage-derived exosomes attenuated endothelial cell migration though integrin trafficking, highlighting the important role of EVs associated with endothelial cell migration [166]. Ubiquitination of HUVEC integrin-β1 was enhanced in the presence of EVs, and exosome-mediated integrin degradation was inhibited by the lysosomal degradation inhibitor bafilomycin A. Further, integrin-dependent MAPK signalling and HUVEC migration was suppressed following treatment with exosomes, suggesting a role of exosome-mediated integrin trafficking as a novel regulatory mechanism of endothelial cell migration. Wang and colleagues reported that hypoxia potentiates EV release in breast cancer cells through the HIF-dependent expression of the GTPase Rab22A [167]. Rab22A was identified to co-localize with budding microvesicles at the plasma membrane, suggesting a role in their biogenesis. Further, Taraboletti et al. reported that matrix metalloproteinases (MMP-2, -9,

and MT1-MMP) within endothelial cell-derived EVs were functionally active, and led to endothelial cell invasion and capillary-like structure formation [168].

Recent evidence highlights exosomal miRNA in directing angiogenesis [34, 163, 169]. For example, Grange et al., showed that exosomes released from CD105-positive renal carcinoma cells stimulate angiogenesis, upregulate VEGF-A, MMP2 and MMP9 expression in pre-metastatic sites in the lung, and promote lung metastasis [34]. These tumour-derived exosomes release pro-angiogenic mRNAs and miRNAs that induce angiogenesis and promote formation of a pre-metastatic niche, contributing to prompting the angiogenic switch (a key hallmark of malignant tumour progression [170]). Van Balkom et al., identified miR-214, a key regulatory component involved in endothelial function and angiogenesis, in endothelial exosomes [163]. In addition, exosomes stimulated migration and angiogenesis in human and mouse recipient endothelial cells, whereas exosomes from miR-214-depleted endothelial cells failed to activate such processes. Exosomes containing miR-214 repressed the expression of ataxia telangiectasia mutated in recipient cells, thereby preventing senescence and facilitating the formation of blood vessels. Targeted reduction of miR-214 in exosome-producing endothelial cells also diminished the basal angiogeneic propensity of exosomes. Importantly, the ability of exosomes to promote metastasis and angiogenesis can be increased when exosomes are released under hypoxic conditions [161]. Hypoxic or re-oxygenated A431 carcinoma cells exhibited enhanced angiogenic and metastatic potential such as reduced cell-cell and cell-ECM adhesion, and increased invasiveness. Quantitative proteomics further revealed exosome-associated proteins such as Alix, tetraspanins, and proteins reported to facilitate angiogenesis and metastasis (e.g., angiogenin, VEGF, IL-1α, IL-3, GRO-α, and PDGF).

4.4 Exosomes reprogram the pre-metastatic niche and metastasis

Recent evidence suggests that prior to tumour cell dissemination, the primary tumour influences and modulates the TM, termed the pre-metastatic niche, to facilitate tumour cell colonisation and metastasis formation [23]. Exosomes have been described to directly regulate distinct oncogenic changes in ECM remodelling and recruitment of pro-tumourigenic factors at secondary metastatic sites [24, 32, 34, 36]. Primary tumour cells release a variety of soluble cytokines and growth factors that mobilize bone-marrow-derived cells (BMDCs) and recruit them to sites of secondary metastatic sites, creating a permissive

environment for tumour cells [171]. Exosomes derived from aggressive melanoma cells enhance the growth and metastasis of primary tumours, and program BMDCs at the pre--metastatic site to facilitate a pro-angiogenic phenotype [32]. Melanoma-derived exosomes injected intravenously preferentially colonised to sentinel lymph nodes, preparing niches conducive to the migration and growth of melanoma cells through the induction of molecular signals for primary melanoma cell recruitment, ECM deposition, inflammation to stimulate cell migration, and vascular proliferation and permeability. This effect was dependent on the receptor tyrosine kinase MET in exosomes known to be important in the migration, invasion, angiogenesis and mobilization of BMDCs. The authors showed that pro-metastatic exosomes indirectly increased the metastatic behaviour of primary melanoma cells through MET, and activated phospho-MET in BMDCs, resulting in a pro-vasculogenic phenotype whereby exosomes could mobilize to lungs or lymph nodes and modulate primary tumour cell angiogenesis, invasion and metastasis. Notably, reducing MET expression in tumour-derived exosomes diminished the pro-metastatic behaviour of BMDCs. Furthermore, the study identified a prognostic exosome signature of circulating BMDCs detected in patient blood which could accurately identify stage and metastatic outcome.

At sites of metastasis, tumour cells influence resident stromal cells and enable metastatic colonization and growth. Tumour-derived exosomal miR-105 has been attributed to disrupting the vascular endothelial barriers during early breast pre-metastatic niche formation, by targeting cellular tight junctions (as a key regulator of ZO-1) [172]. Overexpression of miR-105 in non-metastatic cancer cells induced metastasis and vascular permeability in distant organs (lung, liver, brain), whereas inhibition of miR-105 (anti-miR-105) in highly metastatic tumours alleviated these effects. Interestingly, circulating miR-105 derived from cancer patients was also shown important in regulating endothelial cells, causing disruption of vascular structures. Further, miR-105 can be detected in the circulation at the pre-metastatic stage, and its levels in the blood and tumour associated with ZO-1 expression and metastatic progression in early-stage breast cancer. Exosomal miRs have been shown to be important regulators of the tumour microenvironment, where miR-21 and miR-29a activated Toll-like receptor 7 and 8 in immune cells, triggering a pro-metastatic inflammatory response that may facilitate tumour growth and metastasis [82].

Rana and colleagues showed that tumour exosomes transfer miRNA to ‘educate’ selected host draining lymph nodes and lung tissue towards a pro-metastatic phenotype, and

modulating pre-metastatic organ cells [173]. Their study highlighted the role of exosomal cargo in preparing the pre-metastatic niche, where miRNA released from a metastatic tumour prepares pre-metastatic organ stroma cells for tumour cell hosting. Through the silencing of CD44v, miR-34a (tumour suppressor) was shown to exhibit diminished expression in exosomes, while metastasis-promoting miR-494 and miR-21 and apoptosis-regulating miR-24-1 were abundant. Metastatic-cell derived exosomes, recovered in draining lymph nodes following subcutaneous injection, preferentially were targeted and taken up by lymph node stroma cells (LnStr) and lung fibroblasts (LuFb). These internalised miRNA components modulate the stromal target cell, inducing a phenotype that supports tumour cell metastasis. Valencia and colleagues described the influence of miRNA on metastatic bone colonization [174]. Comparative transcriptomic profiling using an *in vivo* murine model of bone metastasis identified a repressed miRNA signature associated with high pro-metastatic activity. Interestingly, miR-192 was shown to markedly appease bone tissue (osseous) metastasis *in vivo*, non-cell-autonomously regulating invasiveness and metalloprotease activities, and impaired tumour-induced angiogenesis mediated by repression of pro-angiogenic IL-8, ICAM and CXCL1. Despite sharing the same seed sequence, only miR-192 and miR-215 induced similar invasive activity *in vitro* but not *in vivo*, the authors suggesting that other cell-specific factors may in fact be necessary, along with other miRNA target genes, to modulate cellular functions, including transformation. The cell-specific and context-specific manner of response of miR-192 is highlighted by the fact that no effects on cell growth kinetics have been identified in other cell types including HeLa or HEK293 cells upon miR-192 overexpression [175]. Because the multigenic regulatory network inducing such a repertoire of cellular functions was triggered directly by miR-192, the authors suggested targeting individual miRNAs as a potentially beneficial strategy to perturbing the metastatic process.

Exosomes that contribute to development of the pre-metastatic niche may also originate from the surrounding pre-metastatic environment, and condition the secondary site prior to arrival of tumour cells and/or tumour-derived exosomes. Exosomes, derived from cancer cells, have recently been shown to have an important role in pre-metastatic niche formation [32, 36, 176]. Further, stromal cells via exosomes have been attributed to formation of a pre-metastatic niche and promoting metastasis [33]. . Ono et al., identified exosomes from bone-marrow mesenchymal stem cells contain miR-23b (in addition to enriched miRs including miR-4657, -4506, -4758-5p, and -1182) that promote dormancy in metastatic breast cancer

cells [177]. Exosomal miR-23b was shown to promote dormancy through suppression of *MARCKS*, which encodes a protein that promotes cell cycling and motility. Interestingly, this is only one of many other suggested mechanisms that contribute to cell cycle suppression and dormancy in breast cancer cells. Increasing focus on exosomes, secreted from tumour- and stromal cells, is providing further insights into the regulation of the *in vivo* metastatic niche. As a key focus, EVs may mediate target specificity and biological activity in the metastatic organ, as they might selectively direct their fusion with specific target cells.

5. Concluding remarks

A key initiating step in tumour invasion involves EMT, during which cells lose epithelial markers and acquire mesenchymal traits dissociating them from neighbouring cells leading to a motile and invasive phenotype. Accumulating evidence highlights the critical role of EMT-like events during tumour progression and malignant transformation, endowing the incipient cancer cell with metastatic properties. This review presents the role of EVs as an emerging mechanism of intercellular communication by which cancer cells can facilitate tumourigenesis by promoting and maintaining cell transformation, invasion, migration, angiogenesis and metastasis. Exosomes emanating from cancer cells can alter the phenotype and biological behaviour of normal cells through intercellular trafficking of oncogenic material that includes DNA, mRNA, regulatory miRNAs and oncoproteins. Recent studies presented therein indicate that EVs mediate communication at primary and secondary tumour sites, with such contents having complex signalling potential to induce a pro-tumourigenic niche, direct organ tropism, and support cell transformation. In the field of EV biology and cancer progression, understanding the mode of cargo selection and regulation remains a significant area of interest. Further, intervention of mechanisms involved in the biogenesis, release, target cell recognition and internalisation of EVs and recipient cells will provide key insights into the biological effects of EVs, and the control of target cell signalling pathways in cancer progression.

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Figure legends

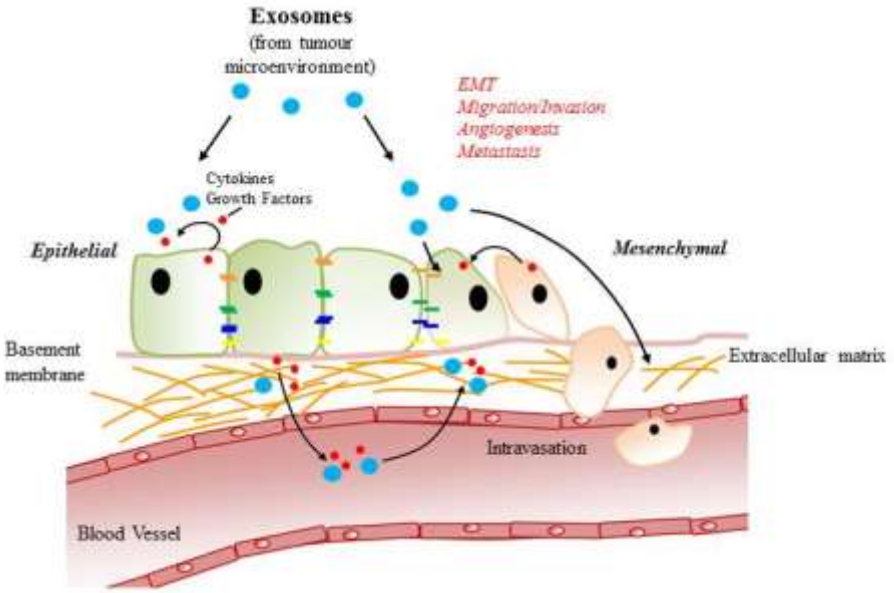
Figure 1. Molecular features and extracellular components regulating EMT. (A) Cellular characteristics of epithelial and mesenchymal cells observed during EMT. Epithelial traits (cell-cell adhesion, cell-ECM adhesion, apical-basal polarity) are lost in favour of mesenchymal characteristics (increased motility, migratory capacity, invasion, and cell individualisation). In addition, EMT includes the diminution of expression of epithelial markers (e.g., E-cadherin, cytokeratin-8, ZO-1), and enhanced expression of mesenchymal markers (e.g., N-cadherin, vimentin, FSP-1), as well as various transcription factors (including Snail, Slug, ZEB1/2, Twist1, FOXC2), miRNAs (including miR-200 family), and lncRNAs that induce signalling pathways orchestrating EMT (e.g., Wnt, MAPK, TGF-β, p53, mTOR). Further, contextual signals, such as TGF-β, WNT proteins, platelet-derived growth factors (PDGFs) and interleukin-6 (IL-6), arising from autocrine or paracrine signalling networks can activate intracellular signalling factors that influence the activation or maintenance of the EMT transcription factor network during an EMT. Recent evidence has shown that EMT contributes to the progression of solid tumours by permitting detachment of cells from their primary site and inducing a migratory phenotype, allowing cells to invade the local tissue and enter the lymphatic system or bloodstream for colonisation at distant sites. Recent experimental and clinical studies have improved our knowledge of this dynamic program and implicated EMT and its reverse program, mesenchymal–epithelial transition (MET), in the metastatic process. Exosomes and other extracellular secreted factors have been attributed to modulating and contributing to EMT in cancer progression. (B) Cells undergoing EMT have an altered extracellular proteome. Initiation of EMT requires external stimuli, including growth factors, and cytokines that activate intracellular signal transduction pathways to alter expression of downstream target genes. In epithelial cells, the secretome encompasses cell matrix and cell adhesion constituents (e.g., collagen XVII, IV, laminin 5) whilst in the mesenchymal secretome, proteases and ECM components (e.g. MMP-1, TIMP-1 kallikrein-6, -7, fibronectin) that facilitate enhanced cell migration and invasion are enriched. Cytokines and growth factors (e.g. TGF-β, EGF, HGF) are also differentially expressed during EMT. (C) EMT perturbs the ‘cargo’ released by exosomes and other EVs. Exosomes from epithelial cells contain cell adhesion/ cell matrix components (e.g., E-cadherin, EpCAM, Perlecan, Collagens, Laminins), and epithelial and cell polarity markers (e.g., CLDN3, CLDN4, MUC1). In contrast, mesenchymal cell exosomes are enriched with

proteases (e.g. MMP-1, ADAM-10), transcription factors (e.g., master transcriptional regulator YBX1, and Nucleolin), core splicing components (e.g., SF3B1, SF3B3, SFRS1, SRP20) and tetraspanins (e.g., CD81, CD82, CD151). Further, proteins associated with inducing cell signalling events (e.g. Wnt3A, H-Ras, K-Ras) and cytoskeletal remodelling (e.g., vimentin, ITGB1) have been identified to be enriched in mesenchymal cell-derived exosomes. Exosomes derived from cells undergoing EMT are reprogrammed with factors which may be capable of inducing EMT in recipient cells.

Figure 1

A

	Epithelial	Mesenchymal
Phenotype	Apical-basal polarity Uniform monolayer Cell adhesion (cell-cell and cell-matrix)	Loss of cell polarity (leading edge) Cytoskeletal rearrangement Cell individualisation (scattered) Loss of cell adhesion (cell-cell and cell-matrix) Ability for migration and invasion
Cellular Markers	E-cadherin, Cytokeratin-8, -18, -19, ZO-1, laminin-1, entactin, sydecan, desmoplakin, claudin-1, occludin, miR200 family	N-cadherin, FSP-1, vimentin, fibronectin, ZEB1/2, Snail, Slug, Twist, LEF-1, FOXC2, miR21



B

Extracellular Protein Changes	**Cell matrix** Laminin-4, -5, WISP-2, collagen type IV, **Cell-cell contact** Claudin-1, -4, -7, occludin, E-cadherin
	Cell matrix and cytoskeletal remodelling Fibulin-1, -3, -5, decorin, biglycan, collagen type I, vimentin, ezrin, radisixn **Integrins** α2β1, α3β1, αvβ3 integrin **Cytokines and growth factors** TGF-β, EGF, HGF, IGF-II, FGF-1, PDGF, Wnt-5a **Matricellular proteins** Osteopontin, tenascin C, SPARC, CTGF/CCN2 **Proteases and protease inhibitors** MT1-MMP, MMP-1, -3, -7, -9, -13, -28, KLK-3, -4, -6, -7, ADAM10, ADAM17, TIMP1, kallekrein-6, -7

C

EV/Exosome Protein Changes	**Cell adhesion and cell matrix** Collagen 12A1, laminins A4, B3, **Cell-cell contact** Thrombospondin-1, E-cadherin, EpCAM **Cell polarity** Mucin-1, claudin-3, -4
	Mesenchymal markers Vimentin **Proteases** MMP-1,-14, ,-19, ADAM10, ADMTS1, MT1-MMP **Transcription/Splicing factors** YBX1, SFRS1, SF3B1, SF3B3 **Cytosketetal remodelling** Integrins β1, α3, α6 **Cell signalling** Wnt-3a, -5a, -11, β-catenin, TGF-β, TNF-α, amphiregulin, DII4, EGFR, EGFRvIII, MET, H-Ras, K-Ras, N-Ras, KIT **Transcriptional regulation** *DNA – KRAS, p53* *mRNA* – EGFRvIII, TGF-β1 *miRNA* - Let-7, miR-1, -21, -24-1, -105, -141, -200, -203, -205, -214, -223, -494

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