

# Discovery of Double-Stranded Genomic DNA in Circulating Exosomes

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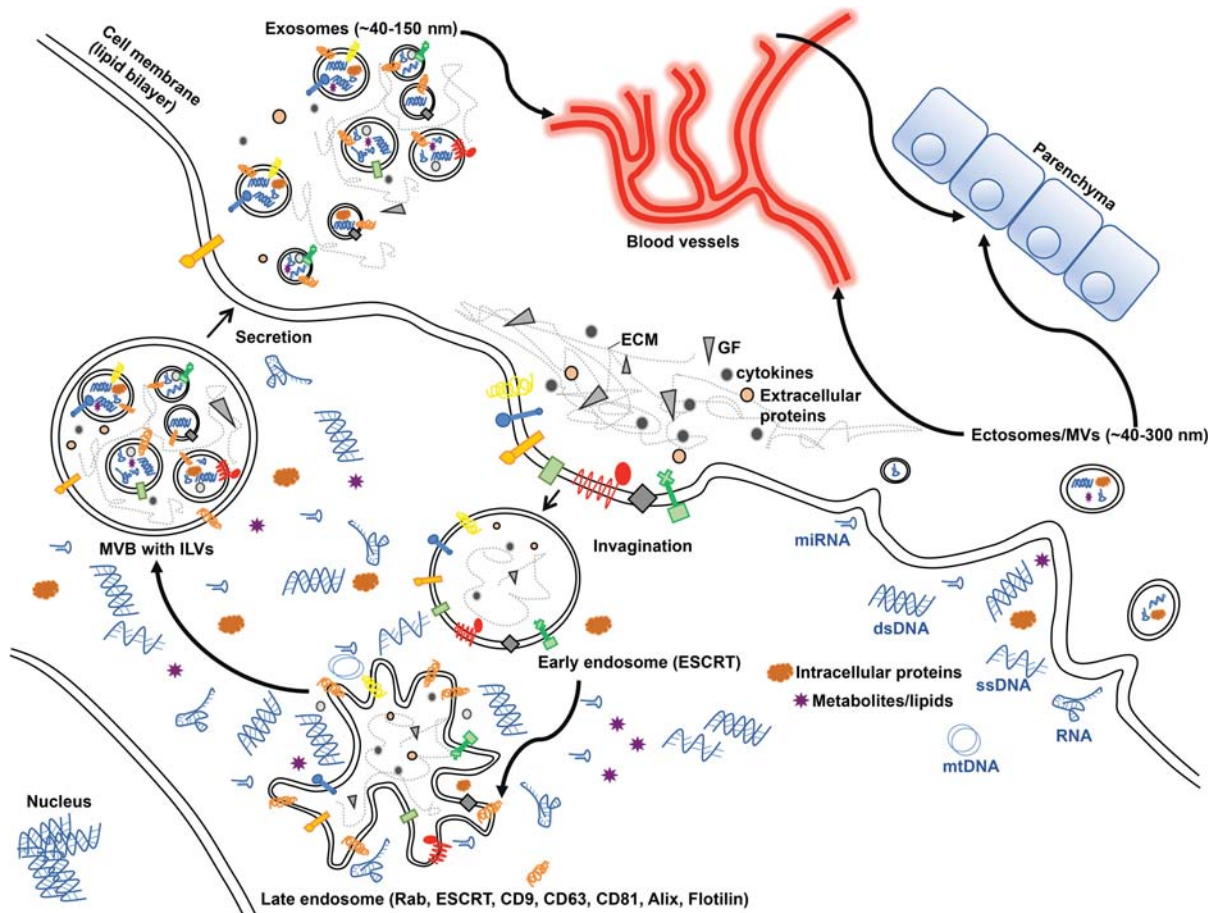
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It is becoming increasingly clear that small vesicles released from cells (extracellular vesicles [EVs]) represent a heterogeneous population implicated in cell-to-cell communication. The classifications and nomenclature of EVs are evolving as enrichment strategies and specific characteristics are being unraveled. At present, physical properties of EVs—namely, size, shape, and density—are often used to identify subpopulations of EVs. A distinct group of EVs, termed exosomes, largely defined by their small size (~40–150 nm) and proposed subcellular origin, has been extensively studied in several aspects of cancer biology. Exosomes are implicated in modulating behavior of cancer cells as well as the immune and angiogenic responses in tumors, possibly contributing to cancer progression locally and systemically. Most intriguingly, the nucleic acid content of exosomes has been proposed to play a role in oncogenic transformation and transfer of cancer-specific genome to promote cancer pathogenesis. Here, we specifically focus on the discovery of exosomal DNA, studies related to the origin of genomic DNA in exosomes, and its utility in cancer diagnosis and disease monitoring.

Exosomes are extracellular vesicles (EVs) released via fusion of multivesicular bodies with the plasma membrane of cells. The content of the multivesicular bodies (intraluminal vesicles [ILVs]) is released in the form of exosomes in the extracellular space (Colombo et al. 2014; Kalluri 2016). This propositioned biogenesis is in contrast with that of “ectosomes,” which emerge from the direct budding of the plasma membrane, giving rise to EVs of similar or greater size range when compared with exosomes (Fig. 1; Antonyak and Cerione 2015; Cocucci and Meldolesi 2015). Despite novel biology unraveling these processes, the exact molecular mechanism of exosome and ectosome formation is unknown. It remains challenging to definitively distinguish between exosomes and ectosomes, in particular when they share similar physical characteristics (size, shape, and density) and present with markers that can overlap between the two subpopulations. There are to date no definitive markers or processes to strictly enrich exosomes versus ectosomes, although proteins involved in their biogenesis are being rapidly unraveled (Colombo et al. 2014; Edgar 2016; Tkach and Thery 2016). The biogenesis of exosomes and ectosomes may play a role in defining their respective contents. Exosomes are implicated in intercellular exchange of macromolecules involving proteins, metabolites, lipids, mRNA, miRNA, lncRNA, viral RNA, mitochondrial, and chromosomal DNA (Kalluri 2016). Collectively, exosomes may present with a heterogeneous mixture of all cellular components (Fig. 1), raising an intriguing notion that exosomes may resemble “primordial”-like vesicles (Sinkovics 2015; Kalluri 2016). The evidence for the functional impact of cellular exchange

of exosomal content, such as signaling molecules, messenger RNA (mRNA), and microRNA (miRNA), implicates exosomes in widespread multiorgan communication. The functional outcome of such exchange may not be limited to neoplastic events and tumor growth but also involve embryonic/organ development and non-cancer-associated pathologies (Colombo et al. 2014; Edgar 2016; McGough and Vincent 2016; Tkach and Thery 2016).

Most results related to cell–cell communication mediated by exosomes, although intriguing, have largely been collected from experiments performed employing cell culture system or employing experimental designs wherein animals are given bolus administration of large quantities of exogenously prepared exosomes from cultured cells. It is critical to note that the physiological function of exosomes remains largely unknown. Several factors have been implicated in the release of exosomes, and the loss of the *tumor suppressor-activated pathway 6 (TSAP6)* gene in mice results in abnormal transferrin receptor down-regulation and reticulocyte maturation (Lespagnol et al. 2008)—processes that were first described in exosome biogenesis (Colombo et al. 2014; Edgar 2016). Interestingly, this process was linked to the DNA damage–induced TP53 pathway associated with exosome secretion (Lespagnol et al. 2008). It remains unknown whether such exosome secretion pathways are similar in all cell types. Emerging studies suggest distinct molecular mechanisms for exosome biogenesis, independent of the traditionally discussed endosomal sorting complexes required for transport (ESCRT)-dependent process (Stuffers et al. 2009).



**Figure 1.** Invagination of the cell plasma membrane leads to the formation of intercellular vesicles encapsulating extracellular components (extracellular matrix [ECM], extracellular proteins, cytokines, and growth factors [GFs]). The intracellular vesicles mature into the early and late endosomes, implicating the endosomal sorting complexes required for transport (ESCRT) machinery and Rab proteins. Proteins (CD9, CD63, CD81, Alix, Flotilin) that decorate the late endosomes are often used as exosome markers following their secretion. The formation of the multivesicular body (MVB) containing the intraluminal vesicles (ILVs) follows a secondary invagination of the late endosome membranes, trapping within newly forming ILVs components of the cytosol (metabolites, intracellular proteins, nucleic acids). Following fusion of the MVs with the plasma membrane, the ILVs are released and referred to as exosomes. The surface proteins on the released exosomes are conformed similarly to their presentation on the plasma membrane. The exosomes enter the circulation or surrounding parenchyma. Ectosomes or microvesicles (MVs), in contrast, emerge from the budding of the plasma membrane and are often larger in size when compared to exosomes. miRNA, microRNA; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; mtDNA, mitochondrial DNA.

In cancer, the biology and function of exosomes is still largely unknown, and some suggestive evidence today points to their role in promoting cancer (Kahlert and Kalluri 2013; Yu et al. 2015; Kalluri 2016). Cancer cell-derived exosomes may elicit phenotypic and molecular responses in recipient cells that result in the activation of signaling pathways that may promote cancer cell proliferation, survival, invasion, and resistance to therapy (Yu et al. 2015). Tumor-derived exosomes may also remodel the tumor microenvironment to promote tumor angiogenesis and impair the immune control of tumor growth (Kahlert and Kalluri 2013). The content of cancer cell-derived exosomes may reflect the content of their cell of origin, perpetuating potential transfer of specific oncogenic components to recipient cells (Fujita et al. 2016; Choi et al. 2017). Interestingly, exosomes may also serve as a modulator of cancer cell heterogeneity, possibly im-

pacting the populational equilibrium of diffuse B-cell lymphoma (Koch et al. 2014). Although these effects are largely attributed to signaling molecules and epigenetic regulators delivered into the recipient cells by exosomes, another intriguing mode of communication by exosomes entails the DNA present in them. Exosomes were reported to contain mitochondrial DNA (from culture supernatant of myoblasts [Guescini et al. 2010]) and chromosomal DNA (vide infra). Chromosomal DNA in exosomes was noted in cell culture supernatant as well as in human and mouse biological fluids, including blood, seminal fluid, and urine. Interestingly, the encapsulation of DNA within exosomes may confer enhanced stability to DNA when outside the cell (Jin et al. 2016). These discoveries have spurred a specific interest in exosomes DNA for use as liquid biopsies to facilitate the diagnosis and monitoring of cancer patients (Speicher and Pantel

2014; Wan et al. 2017; Yi et al. 2017). The exosomal DNA in the blood offers yet another level of complexity in the study of circulating DNA in cancer (Thierry et al. 2016) but also perhaps novel means to isolate cancer specific DNA, by possibly enriching for circulating exosomes derived from cancer cells (Kalluri 2016).

### IDENTIFICATION OF DNA IN THE EXOSOMES

Using whole-genome sequencing, Kahlert et al. (2014) provided definitive identification of large fragments of double-stranded genomic DNA (cumulatively encompassing all chromosomes) in the circulating exosomes from patients with pancreatic cancer and also in exosomes derived from cancer cells. Several groups have also identified double-stranded DNA fragments in the exosomes ranging from 100 b to 17 kb in size (Cai et al. 2013; Kahlert et al. 2014; Lee et al. 2014; Thakur et al. 2014). Cai et al. (2013) reported the detection of chromosomal and mitochondrial DNA in exosomes derived from healthy donor human plasma and culture supernatant of human primary vascular smooth muscle cells, HEK293 cells (human embryonic kidney cells) and K562 cells (human immortalized myelogenous leukemia cells) (Cai et al. 2013). In their studies, DNase treatment supported that the long fragments of double-stranded DNA were predominantly found within the exosomes rather than outside. They further showed that the exosome's DNA, upon transfer to the recipient cells, localizes to the nucleus, recruits nuclear factor  $\kappa$ B (NF- $\kappa$ B), and is transcribed. Further, exosome-derived DNA containing the BCR/ABL hybrid gene (from K562 cell supernatant) could be detected in normal recipient human neutrophils (Cai et al. 2013). Their results support the notion of functional horizontal transfer of DNA via exosomes (Cai et al. 2013, 2016). The detection of gene fusion in exosome's DNA sets the stage for further analyses of driver mutations in several cancer types.

### DETECTION OF DNA MUTATIONS IN EXOSOMES

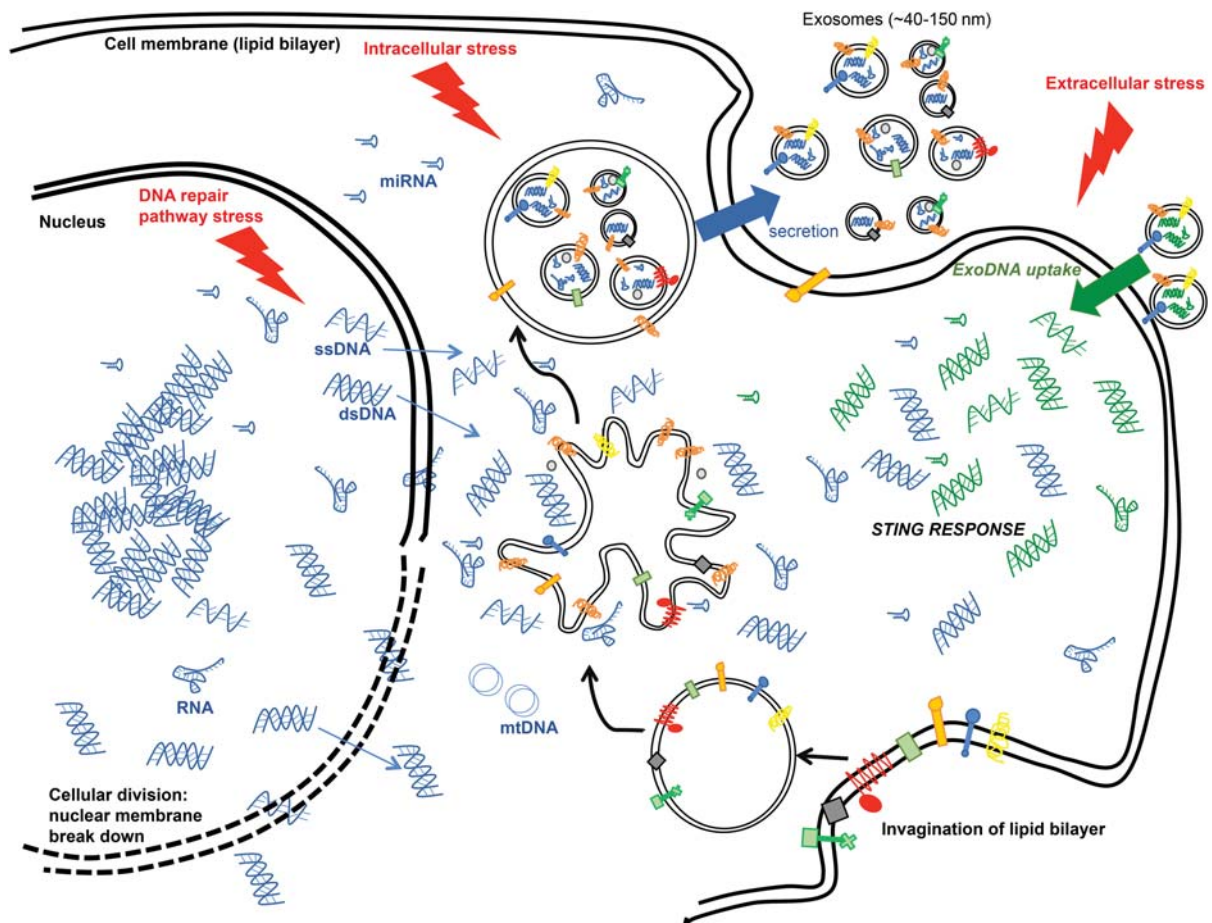
In the supernatants of pancreatic cancer cells and serum from pancreatic cancer patients, large fragments of double-stranded DNA were noted in exosomes, and Sanger sequencing analyses of polymerase chain reaction (PCR) amplicons revealed the presence of *KRAS* and *TP53* mutations (Kahlert et al. 2014). The double-stranded DNA in exosomes collected from prostate cancer cell supernatant also revealed mutations characteristic of the cell of origin (Lazaro-Ibanez et al. 2014), and double-stranded DNA was also identified in the plasma of prostate cancer patients (Lazaro-Ibanez et al. 2014) as well as in the seminal fluid of donors (Ronquist et al. 2012). The diagnostic value of exosome's DNA in liquid biopsies, including but not limited to blood, urine, and seminal fluid, remains to be determined because of the challenges associated with detection sensitivity and signal-to-noise

ratio owing to fragments of DNA from cancer cells (mutation) mixed with DNA from normal cells (no mutation). Nonetheless, detection of actionable mutations (in *NOTCH1* and *BRCA2*) in exosome's DNA from pancreatic cancer patients has been reported (San Lucas et al. 2016). The most common mutations in pancreatic cancer patients (*KRAS* and *TP53*) are readily detected in circulating exosomes DNA (Allenson et al. 2017; Yang et al. 2017). These studies, however, provide caution because detection of *KRAS* mutation from an exosome's DNA in healthy individuals confounds the diagnostic utility. The exosome's DNA in cancer may indeed represent a new treasure trove for monitoring changes in the mutational landscape (e.g., in response to therapy); however, this may only be feasible upon enrichment of cancer cell-derived exosomes from the blood (Kalluri 2016).

### BIOLOGICAL FUNCTION OF DNA IN THE EXOSOMES

The exact nature and relative abundance of the different species of DNA inside and outside of exosomes from cell culture supernatants and biological fluids is being actively investigated. It is likely that distinct types (single-stranded, double-stranded, mitochondrial; Fig. 2) and integrity of the exosome's DNA (fragment length, chromatin bound) are likely possible in the distinct type of exosomes based on their cell of origin. It is also plausible that the mechanisms enabling packaging of DNA within exosomes are cell type-specific and dynamically regulated. Analyses of exosomes from the supernatant of various cancer cell lines and non-cancer-associated fibroblast lines revealed exosomes DNA was more abundant in cancer cell-derived exosomes, and that double-stranded DNA was predominantly found within the exosomes (Kahlert et al. 2014; Thakur et al. 2014). DNase treatment of exosomes from cultured cell supernatants revealed that double-stranded DNA, rather than single-stranded DNA or RNA, was predominantly found inside exosomes, albeit DNA is also found outside exosomes (Kahlert et al. 2014; Thakur et al. 2014). This is in contrast with the reported single-stranded DNA in exosomes from the supernatant of medulloblastoma tumor cells (Balaj et al. 2011). Whole-genome sequence analyses revealed the entire coverage of the genome in exosomes and that there did not seem to be a notable bias for specific regions (Kahlert et al. 2014; Lee et al. 2014; Thakur et al. 2014). Using exosomes containing H-ras DNA sequence, derived from the culture supernatant of RAS-3 cells (tumorigenic c-H-ras transformed rat intestinal epithelial cells), the recipient RAT-1 cells (immortalized rat fibroblasts) showed transient H-ras DNA for up to 30 d following exposure to exosomes (Lee et al. 2014). Although this uptake did not transform RAT-1 cells, RAS-3-derived exosomes stimulated their proliferation (Lee et al. 2014).

*BRCA1*-KO human fibroblasts could be transformed into malignant cells when exposed to circulating exosomes from patients with breast, colorectal, pancreatic, and other



**Figure 2.** During the formation of intraluminal vesicles that will subsequently be released as exosomes, nucleic acids in the cytosol are encapsulated. These include messenger RNA (mRNA), microRNA (miRNA), mitochondrial DNA (mtDNA), and single-stranded and double-stranded chromosomal DNA (ssDNA and dsDNA, respectively). Increased cellular stress (extracellular stress, intracellular stress, and DNA repair pathway stress) may contribute to the increase in cytosolic nucleic acids that are shuttled into intraluminal vesicles (ILVs). Breakdown of the nuclear membrane during cellular division may also expose extraneous DNA to cytosolic compartments that would facilitate DNA uptake into ILVs. The increased content of cytosolic DNA, possibly via uptake of exosomal DNA, may elicit the stimulator of interferon genes (STING) pathway and stimulate an immune response.

cancer types (Hamam et al. 2016). Interestingly, studies using epidermal growth factor receptor (EGFR)-driven cancer cell lines subjected to EGFR kinase inhibitors yield an increase in the release of exosomes containing DNA (Montermini et al. 2015). Collectively these studies indicate that exosomal DNA cargo can be different but predominantly present as large fragments of double-stranded DNA. Moreover, DNA packaging may be influenced by exogenous agents acting on the cell of origin, yet with all regions of the genome appearing to be contained within a given pool of exosomes (billions). Further, the influence of exosomal DNA on the recipient cells may differ based on pre-existing genomic and microenvironmental stressors. The impact of an exosome's DNA may extend beyond cell-cell communications. Recent studies revealed that breast cancer cell-derived exosomal DNA, following treatment of the cancer cells with topoisomerase I inhibitor, may activate the stimulator of interferon genes (STING) pathway in the recipient dendritic cells (Fig. 2; Paludan and Bowie 2013; Kitai

et al. 2017). Whether these events are rate-limiting for antitumor immunity remains to be determined, but it certainly opens a new means to modulate tumor immunity via exosomal DNA.

## FUTURE DIRECTIONS

Among the fascinating, yet largely unanswered questions surrounding exosomal DNA are the mechanisms associated with chromosomal DNA packaging within ILVs, which eventually give rise to exosomes when released into the extracellular space. Because the biogenesis of ILVs could include the sampling of the cytosol, cytosolic chromosomal DNA likely can get encapsulated within the exosomes (Fig. 2). Cytosolic DNA accumulation in cancer cells may reflect their deregulated DNA repair pathways (Shen et al. 2015; Ho et al. 2016), and this could explain the higher abundance of exosomal DNA in cancer cells when compared with normal cells.

Alternatively, the different expression levels of cytosolic nucleases in distinct cell types, leading to enhanced cytosolic DNA content, could also account for different amounts of DNA within ILVs. Whether a correlation exists between cytosolic DNA content and abundance of exosomal DNA remains to be elucidated. Intriguingly, there may be a link between nuclear envelope breakdown in proliferating cells and vesicular packaging of aborted DNA replication forks (Fig. 2), although this hypothesis remains to be tested. The mechanism underlying the transfer of an exosome's DNA to its recipient cells is also largely unknown, although exosomal Connexin 43 (*GJA1* or Cx43) may enable docking and fusion of exosomes with the plasma membrane of Cx43 expressing recipient cells (Soares et al. 2015). The green fluorescent protein (GFP)-expressing plasmid incorporated into the Cx43<sup>+</sup> exosomes was more efficiently transcribed and translated when Cx43 was present on both exosomes and recipient cell surfaces (Soares et al. 2015). Interestingly, predicted DNA and RNA-binding motifs on Cx43 could suggest a role for Cx43 in exosome-mediated transfer of nucleic acids (Varela-Eirin et al. 2017).

In conclusion, many research groups validated the fascinating discovery that exosomes contain genomic double-stranded DNA. In the next decade, we will gain more knowledge with respect to the mechanism of how DNA enters the exosomes and how exosomal DNA can aid in diagnosis and therapy of cancer.

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