Applications with Exosomes and Extracellular Vesicles in miRNA Research

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Introduction

Despite their discovery more than 35 years ago, knowledge of exosomes (and extracellular vesicles) and the role they play in the etiology of disease and normal cellular physiology remains in its infancy. This white paper discusses several key questions: What are they, why do they matter, and how do we measure them? This paper also provides a review of some recent investigative applications coupled to the use of NanoString® technology to profile microRNAs (miRNAs).

Initially, two back-to-back papers were published (Harding et al., 1983; Pan and Johnstone, 1983) describing a very bizarre phenomenon with some unexpected conclusions. The authors cultured immature red blood cells and reticulocytes and labeled them with transferrin receptors to trace the movement of transferrin receptors from the plasma membrane into the reticulocytes. Surprisingly, they observed that the labeled transferrin receptors were internalized within the reticulocytes and then repackaged into small (approximately 50 nm) vesicles inside them. These vesicles, originally thought to be extracellular and trafficked to lysosomes for destruction, were subsequently secreted out of the maturing blood reticulocytes into the extracellular space. Later in the year 1989, Johnstone et al. coined these vesicles "exosomes" (Johnstone et al., 1989). Exosomes belong to a large family of membrane vesicles referred to as extracellular vesicles (Figure 1), which generally include microvesicles (100-350 nm), apoptotic blebs (500-1000 nm), and exosomes (30-150 nm). They are derived from the luminal membrane of what is called multivesicular bodies, which are constitutively freed by fusion with the cell membrane and released to the extracellular space as exosomes. Since that original description, the term has been loosely used interchangeably under the general descriptive term "the extracellular vesicle" creating confusion in the field and, until recently, contributing to the skepticism surrounding this biological phenomena. For years, many considered the role of exosomes to be nothing more than a cellular trash can of sorts, whose job was to discard unwanted cellular components. Accordingly, these small vesicles remained overlooked and barely studied for the next decade (Guo et al., 2017). Over the past few years, however, evidence has begun to accumulate that these 'trash cans' also act as messengers, conveying important information to distant tissues.



FIGURE 1: Exosomes are small, lipid bilayer membrane vesicles (30-100 nm) derived from the luminal membrane of multivesicular bodies (MVBs). Reviewed in Guo et al., 2017.

Functions and Applications of Exosomes

Recent evidence suggests that exosomes participate in many functions. First, they are thought to provide a means of intercellular communication and of transmission for macromolecules between cells. Second, it is now known that they truly represent a biological box of delights containing surface receptors, membrane and soluble proteins, lipids, ribonucleic acids (mRNA, microRNA, tRNA, rRNA, small nucleolar RNA, small circular nucleolar RNA, piRNA, scaRNA, viral RNA, Y RNA, and long noncoding RNA) and DNA, all of which have been found to be contributing factors in the development of several diseases. Third, and of particular interest, they have been proposed to be useful vectors for drugs because they are composed of cell membranes (rather than synthetic polymers) and as such are better tolerated by the host (reviewed by Edgar J, 2016).



This brings us to recent data and renewed interest in their role in both physiological and disease processes (Figure 2). Exosomes can regulate the properties of target cells, which can be beneficial or harmful. Exosomes contribute to fundamental physiological processes, such as neuronal communication, antigen presentation, immune response, organ development, and reproductive performance. They also participate in many pathological disorders, including (but not limited to) cancer progression, cardiovascular disease, and inflammation; they even favor viral infection and prion dissemination. Given that exosomes can carry toxic damaged forms of aggregated proteins that are fated for destruction, they are also relevant to the progression of neurodegenerative diseases (reviewed by He et al., 2018). The plethora of peer-reviewed publications and the explosion of new exosome-based biotechnology companies focused on modifying and exploiting exosomes as biomarkers, vaccine/drug carriers, or novel therapeutics demonstrates the great interest of many researchers in this field.

Isolation and Identification of Exosomes

To date, exosomes can be found in multiple types of extracellular fluids, such as blood, urine, amniotic fluid, saliva, cerebrospinal fluid, and breast milk (reviewed in de la Torre Gomez et al., 2018). The challenge, however, is to identify, isolate, and quantify exosomes accurately, efficiently, and most importantly, selectively. The most commonly used protocol for isolation of exosomes is ultracentrifugation (UC); the final step of which is centrifugation at 100,000 × g for at least 70 min to pellet the small vesicles that correspond to exosomes. In addition, sucrose density gradients, ultrafiltration, high performance liquid chromatography-based protocols and immunoaffinitycapture methods (singly or combined with UC), can yield high enrichment and highly purified exosomes. In recent years, easy-to-use precipitation solutions, such as ExoQuick and Total Exosomes Isolation Reagent (TEI), have been utilized to precipitate particles. The procedure is convenient, technically simple, and saves time without the need for expensive equipment. However, these 'salting-out' methods are ultimately unable to resolve particle heterogeneity and are not specific for exosomes or other EVs. Such methods may thus lead to isolation of non-exosomal particles in addition to exosomes, leading to potentially spurious findings and consequently flawed conclusions (Figure 3, discussed in Coumans et al., 2017).

Once isolated, it should be standard practice to verify that one has indeed isolated exosomes and not cellular debris. To this end, there are now a variety of techniques that afford a researcher confidence in their ongoing exosomal research. Validation



FIGURE 2: Exosomal function in disease.



FIGURE 3: (A) In differential centrifugation, separation is based on size; large Extracellular Vesicles (gray) collect earlier at the bottom of the tube and at lower g forces than small EVs (green). While the soluble components are not affected by centrifugation, non-EV particles such as lipoproteins and protein aggregates may co-pellet with EVs. (B) In density-gradient centrifugation, separation is based on density and EVs will travel according to their equilibrium density. Non-EV particles such as lipoproteins may coelute with EVs due to similar density or interaction. The soluble components with a high density relative to the gradient will collect at the bottom of the tube. (C) Size-exclusion chromatography uses a porous matrix (dotted circles) that separates based on size. Soluble components and particles smaller than the size cutoff enter the porous matrix temporarily, whereas EVs and particles larger than the size cutoff do not enter the porous matrix. As a result, EVs and particles larger than the size cutoff elute before the soluble components and particles smaller than the size cutoff. (D) In ultrafiltration, soluble proteins and particles smaller than the size cutoff (approximately 105 kDa) are pushed through the filter while EVs are collected on the filter. (E) In immunocapture assays, EVs are captured based on their immunophenotype. EVs are captured using a monoclonal antibody (mAb) directed against an antigen exposed on the targeted (green) EVs only. (F) In precipitation, addition of a precipitating agent induces clumping of EVs, non-EV particles, and soluble proteins. The clumps will sediment, and sedimentation can be accelerated by centrifugation (Coumans et al., 2017)



processes typically include the use of transmission electron microscopy, nanoparticle tracking analysis (NTA), western blotting, and flow-based methodologies to ensure the recovery of cell-surface markers (Figure 4 and Table 1; Li et al., 2017, Wu et al., 2015). Figure 5 shows an overall summary of the isolation and validation workflow.

How can NanoString miRNA Panels Provide Further Insight Into the Role of Exosomes?

Exosomes contain mRNAs (usually highly fragmented) and miRNAs. These miRNAs can be detected in exosomes isolated from non-invasively obtained biofluids (such as urine and saliva), thus highlighting the potential advantages of exosomal miRNAs as novel biomarkers. miRNAs continue to be a very attractive area of research for many investigators. miRNAs were discovered over 30 years ago in *C. elegans* in Victor Ambros' lab and represent another form of RNA- in this case a 15-26 nt non-coding RNA molecule that is present in almost all animals, plants, and viruses (Lee et al., 1993). A list of all known miRNAs is curated in miRbase (www. mirbase.org). miRNAs are master regulators and participate in RNA silencing and post-transcriptional regulation of gene expression (reviewed in Krishnan et al., 2018). miRNAs can be released into microvesicles and exosomes, protein complexes, lipoproteins, and apoptotic bodies and can be isolated from any source, including biofluids such as serum,



Detection Methods		Quantification Methods	
Electron microscopy	 Direct evidence for the presence of EV Assessments of morphology and size No quantification of EV Need an expert in electron microscopy 	BCA protein assay	Easy protocol Low-cost method No specific information about EV concentration
Flow cytometry	 Detection of EV bigger than 300 nm Low Detection threshold (only analysis of large EV) 	ExoELISA (SBI)	Specific for exosome proteinsTechnical troubles. Unreliable
Western Blot	Detection of specific EV subset	NTA	Analysis of absolute concentration of particles Assessment of the particles size No distinguishment of EV from aggregated protein

TABLE 1: Workflowfor exosome isolation,purification, and validation.



plasma, urine, CSF, and saliva. NanoString offers miRNA panels for human, mouse and rat species which cover 92% of the observed signaling reads in miRbase v22. In addition, confidence levels, observed ratios, and expression analytics are provided to ensure that the content is weighted towards biologically relevant miRNAs that are actionable and clinically relevant. These panels are ideally suited for targeted discovery and validation experiments (Figure 6). The NanoString miRNA assays have enabled researchers to gain insight into potential biomarkers from exosomes in many different areas of research. Many of these publications can be found on our website and are also discussed herein.

Exosomal Biomarkers in Type-1 Diabetes Mellitus

The first publication comes from the lab of Dr. Camillo Ricordi, acknowledged by his peers as one of the world's leading scientists in cure-focused diabetes research and cell transplantation. Dr. Ricordi is known for inventing a machine that made it possible to isolate large numbers of islet cells (insulin-producing cells) from the human pancreas. He has also performed the first series of successful islet transplants in the clinic that reversed diabetes after the donor-purified islets were implanted into diabetes patients. In his recent publication, NanoString technology was used to determine if plasmaderived exosomes, enriched in specific miRNAs, could provide a disease-specific diagnostic signature allowing prediction





and monitoring of type-1 diabetes mellitus (T1DM) (Garcia Conteras et al., 2017). T1DM is the most severe form of diabetes mellitus and is triggered by environmental factors that result in autoimmune attack against insulin-producing cells localized in the pancreatic islet of Langerhans. This leads to a decrease in insulin synthesis resulting in hyperglycemic episodes in T1DM subjects. There is a lack of robust biomarkers in T1DM that allow early diagnosis and intervention that could prolong islet survival. In recent years, miRNA derived from exosomes have emerged as ideal candidate biomarkers because they are stable in plasma and appear to be tissue-specific. Figure 7 indicates the overall workflow of the study and sample processing. Validating the presence of true isolated exosomes was imperative. In this case, isolation and validation was performed via electron microscopy, NTA, and expression of known surface markers as well as by the use of a pico-chip and densitometry to ensure that the isolated exosomes contained the relevant small RNA fraction. NanoString miRNA assays were used to screen a total of 72 samples (both disease and control) for potential T1DM biomarkers among plasma derived exosomal miRNAs. Dr. Ricordi and coworkers identified seven miRNAs with statistically different expression between T1DM and healthy patients and further demonstrated that each of these miRNAs were involved in the progression of T1DM, further validating the potential of this unique signature.



FIGURE 6: The process of uncovering miRNA-based biomarkers for potential clinical use.



Role of Exosome-derived miRNAs in Tissue Regeneration

Mesenchymal stem cell (MSC)-derived exosomes are known to mediate tissue regeneration in a variety of diseases, including ischemic heart injury, liver fibrosis, and cerebrovascular disease. Despite an increasing number of studies reporting the therapeutic effects of MSC exosomes, the underlying molecular mechanisms and their miRNA complement are poorly characterized. In a recent study by the Nguyen lab in Buffalo, NY, a unique systems biology approach was used to profile and quantify the miRNA landscape in MSC exosomes. An accompanying bioinformatics approach was used to identify which pathways and networks were most likely to be affected by exosomal miRNAs. Angiogenesis-, cellular proliferation-, and fibrosis-based assays were used to further predict the regenerative effects of MSC exosomes (Ferguson et al., 2018). To confirm isolation of exosomes, Nguyen and coworkers examined the presence of the specific CD63 cell surface marker and performed size analysis using NTA and TEM. Profiling of MSC-derived exosomal miRNAs was then ranked by total counts from highest to lowest and was highly correlated amongst replicate samples. They found that the top 23 miRNAs accounted for approximately 80% of the entire exosomal miRNA content. Through seed sequence alignment, each of these 23 miRNAs was collectively predicted to target



FIGURE 7: Study workflow and sample processing. Plasma from T1DM (n = 36) and control subjects (n = 36) was collected. Exosomes were isolated by ultracentrifugation and characterized by TEM and NTA. Total exosome RNA was isolated and used for the miRNA microarray analysis (discovery set) or for qRT-PCR validation (validation set) (A). Plasma exosomes were analyzed under electron microscopy, revealing comparable morphology in T1DM and control subjects (B,C). Particle and size distribution of exosomes analyzed by NTA of T1DM and control subjects (D,E). Exosomal RNAs were assessed by Agilent RNA Pico Chip. Exosomal RNA samples contained no detectable 18S and 28S rRNAs (G). Validation of selected exosome protein expression by flow cytometry (control read peak) (F). Small RNA densitometry trace profiles were used to quantify and compare the relative abundance of various small RNAs in T1DM (H) and control subjects (I).



over 5000 genes with very high stringency, with a particular focus on targets contributing to vascularization, growth, fibrosis, and angiogenesis (Figure 8). This work ultimately helped define the MSC miRNA landscape, establish their biological functions on a system level, and provide a platform for further improving their intrinsic regenerative effects in the search for clinically viable exosome-based therapeutics.

The Interplay of Transcription Factors and Exosomes

Another study published in Nature by the Curtis Harris group at the NIH described the interplay between the p53 gene and exosomes (Cooks et al., 2018). Exosomes convey information to neighboring cells by delivering RNAs and proteins, thus affecting signaling pathways in various physiological and pathological conditions including cancer, where p53 plays a significant role. The production of exosomes and their molecular cargo are affected by external signals such as oxidative stress and ionizing radiation. Therefore, p53, a cellular stress responsive



FIGURE 8: (A) TEM image of MSC exosomes and analysis of exosome size by NTA. (B) Total reads of the top 50 miRNAs are shown. All miRNAs were normalized to positive and negative controls. miRNA profiling was performed using the NanoString platform and analyzed with nSolver Software 3. (C) Correlation of biological replicates of miRNA read counts of MSC exosomes. The Pearson correlation coefficient is 0.93. (D) The top 23 miRNAs account for 78.1% of total miRNAs present in MSC exosomes.



transcription factor, plays a major role in exosome machinery and release while under microenvironmental stress (Cooks et al., 2018). To contextualize this work, in most solid cancers a major component of the tumor stroma are macrophages, referred to as tumor-associated macrophages (TAMs). TAMs are mostly derived from peripheral blood monocytes recruited into the tumor mass. In recent years, TAMs have been extensively studied and proposed to be a significant contributing factor in tumor progression. The communication between tumor cells and macrophages was suggested to be mediated via exosomal transfer, where packaged proteins and miRNAs were reported to immunomodulate the macrophages at the receiving end. Harris and coworkers used the colorectal cancer cell lines HCT116 and DLD-1 (p53 isogenic panel of WT, null and mutp53), as these lines serve as useful tools to study gene effect either when knocked out (null) or mutated. Exosomes were isolated and verified with a variety of methods as indicated in this white paper and described in Figure 9. Isolated exosomes were profiled with the NanoString 800-plex human miRNA assay to determine differential expression between exosomes derived from WT and mutant cell lines. The results revealed a new potential biomarker role for miR1246 in cancer-promoting activity (Figure 9).



FIGURE 9: (A) Exosomes isolated from HCT116 cells harboring either WT, mutant (R248W), or no p53. Isolations were either filtered (0.22 µm) or kept unfiltered during the procedure. Subsequently, isolations were lysed and subjected to western blot analysis with the indicated antibodies for exosomal markers. Calnexin served as a marker for cellular contaminants. (B.C) Exosomes isolated from HCT116 cells underwent NTA to determine exosomal size distribution (B) and concentration (C). The exosome samples were compared with cell-free medium that underwent a similar isolation procedure (NEG). (D) Exosomes isolated from HCT116 cells were labeled with Syto RNAselect dye before incubation with macrophages for 24 or 48 h. Accumulation of exosome uptake was captured in time-lapse movies. Macrophage nuclei were labeled with DAPI and plasma membranes were labeled with CellMask farred. Bars=25 µm. (E) RNA was extracted from HCT116-derived exosomes and its integrity and quality were tested using a bioanalyzer system before being subjected to a miRNA expression assay and normalized to the 100 most abundant miRNAs. (F) A representative comparison displaying greater than 2-fold changes in miRs between HT29 cells either knocked down for mutp53 (Shp53) or not (ShCon). (G) Changes in expression of four prominent miRs that were observed to be significantly more abundant in mutp53 HCT116 and HT29 cells



An Optimized Method for Isolation of Exosomes from Urine

In the final publication (Gheinani et al., 2018), urine was used as a novel sample type for biomarker discovery. Urine contains a considerable number of proteins, including smallmolecule metabolites and urinary extracellular vesicles (uEVs). Therefore, urine would be an ideal body fluid for the diagnosis and monitoring of patients with upper and lower urinary tract diseases. However, the composition of urine can pose challenges for biomarker detection, as systemic circulating molecules excreted through the urinary track can contaminate exosomes. The environment of uEVs is less complex than bulk urine, making miRNA biomarker discovery more straightforward. Furthermore, miRNAs packaged in uEVs are protected against an environment with high RNAse content. RNAs are better preserved in urinary microvesicles than in urinary cell isolates, suggesting that microvesicles may protect RNA during urine passage. The technical complexity of the existing methods of uEV isolation and the growing number of commercially available products add a new source of variability. Comparisons of isolation methods often lack experimental characterization of the extracellular vesicles and their functions. The goal of this publication was to therefore optimize the methodology associated with the derivation of urinary exosomes. Briefly, isolated uEVs from urine samples of healthy donors were obtained from five different methods to determine the most optimal approach. This was followed by a comparison of uEV yield and size distribution, particle morphology, protein marker presence, and RNA content. Having selected the optimal conditions for uEV isolation, Gheinani and coworkers proceeded with miRNA characterization on the NanoString platform and validated the results by qPCR using Advanced TagMan miRNA assays. The validation of urinary exosomes following a differential centrifugation methodology is shown in Figure 10.

Mid-stream urine samples were collected and processed as described in the publication. The samples were pooled and processed, then divided into 5 × 50 ml fractions used for 1) ultracentrifugation (UC), 2) polyethylene glycol precipitation (PEG), 3) protein concentration and size-exclusion chromatography (C-SEC), 4) ultracentrifugation and SEC (UC-SEC), 5) polyethylene glycol precipitation and SEC (PEG-SEC). RNA samples were then derived directly from 50 ml of total urine or from uEVs isolated by UC-SEC from 50 ml of the same urine sample. miRNAs were then profiled using the NanoString nCounter platform. Results revealed by the NanoString assay and validated by qPCR showed that there were 18 miRNAs expressed in common between total urine and exosomal urine. The optimized UC-SEC procedure proposed in the study is therefore suitable for uEV purification and isolation of exosomal miRNA for biomarker discovery. Furthermore, the work also represents a novel unbiased and reproducible strategy for uEV isolation, content normalization, and miRNA cargo analysis that is suitable for further biomarker discovery studies.





FIGURE 10: Protein markers and RNA load of exosomes, purified using UC-SEC. (A) In a representative experiment, 20μ l of fractions 1, 4-19 and 45 were tested by western blotting with anti-CD81 antibody. NTA was used to count 50-150 nm particles in all SEC fractions, and the particle content of each lane is indicated. Uncropped images of 2 blots (9 samples each + Mw markers) are presented. (B) Particle and protein concentrations in 50 fractions of the UC-SEC. The data are the mean ± SEM of 3 independent experiments. (C) Exosomal markers CD9, CD63, CD81 and TSG101 were tested by western blotting. Shown are the UC pellet subsequently used for SEC, fractions 11 and 26 of the SEC, and a pool of all exosome-containing SEC fractions of a representative experiment. One membrane was cut and probed with anti-TSG101 + anti-CD81 (left) or anti-CD63 and anti-CD9 (right), and uncropped images were combined. (D) Total RNA isolated from TEU-2 cells, total urine and urinary exosomes from UC-SEC was analysed by NanoChip. Length of detected RNA species is indicated (nt). (E) Pearson correlation between urine contents (chemical composition and total RNA) and uEV parameters (exosome miRNA read count, protein content and particle number). Only Pearson correlations with p-value ≤ 0.05 are shown (blue for positive correlation), the intensity of blue colour corresponding to the degree of correlation. The data are diagnostic values for urine composition and RNA, protein and exosome concentrations of 6 total urine samples, processed by the optimized UC-SEC method.



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