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Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers

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Exosomes are tiny vesicles (30–150 nm) constantly secreted by all healthy and abnormal cells, and found in abundance in all body fluids. These vesicles, loaded with unique RNA and protein cargo, have a wide range of biological functions, including cell-to-cell communication and signalling. As such, exosomes hold tremendous potential as biomarkers and could lead to the development of minimally invasive diagnostics and next generation therapies within the next few years. Here, we describe the strategies for isolation of exosomes from human blood serum and urine, characterization of their RNA cargo by sequencing, and present the initial data on exosome labelling and uptake tracing in a cell culture model. The value of exosomes for clinical applications is discussed with an emphasis on their potential for diagnosing and treating neurodegenerative diseases and brain cancer.

1. Introduction

Exosomes are RNA and protein-containing small vesicles (30–150 nm) constantly secreted by all cells in culture and *in vivo*, in both a normal and disease state [1–3]. Blood, urine, cerebrospinal fluid (CSF), breast milk, ascites fluid, amniotic fluid, bile, semen, saliva and sputum all contain thousands to billions of exosomes per microlitre of sample. Depending on the cell or tissue of origin, many different roles and functions have been attributed to exosomes, for example: eradication of obsolete molecules, facilitation of the immune response, antigen presentation, programmed cell death, angiogenesis, inflammation, coagulation, dissemination of oncogenes from tumour cells and spread of pathogens such as prions and viruses from one cell to another [4,5]. More importantly, exosomes deliver macromolecular messages (RNA and protein) that enable cell-to-cell communication and signalling [1,6–9]. Interest in exosomes range from their function in the body to more practical applications such as their use in diagnostics and biomarker development based on analysis of their RNA and protein content.

In the past decade, considerable attention and copious resources have been dedicated to the discovery and validation of biomarkers for cancer and other diseases. An ideal biomarker should be easily assayed with minimally invasive or non-invasive medical procedures but possess high sensitivity and specificity. Presently, there is still a rather limited number of useful biomarkers available, most of which have been used in clinics without any substantial changes for several decades. Profiling of circulating RNAs, in particular miRNAs, have been used in a number of studies to identify novel and highly promising biomarkers for many pathologies including neurodegenerative diseases such as Alzheimer's and Parkinson's [10], brain injury [11] and presence of brain tumours [12]. Many of these molecules were determined to be associated with exosomes. As a consequence, within the research and medical communities, there is a pressing need for quick and easy methods of isolation of exosomes and their subsequent robust and sensitive analysis. Here, we describe strategies for isolation of exosomes from human blood serum and urine, characterization of their RNA

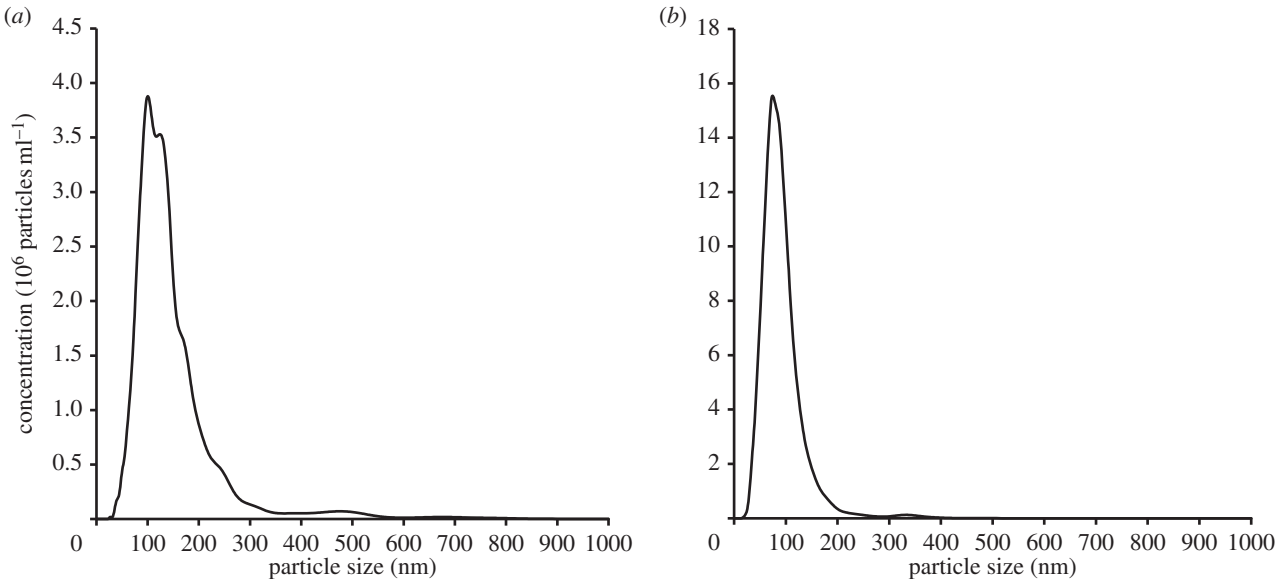

Figure 1. Size distribution and concentration of exosomes recovered from urine (*a*) and serum (*b*), using their specific Total exosome isolation reagents and protocol. Analysis performed on the NanoSight LM10 instrument.

cargo by sequencing on the Ion Torrent Personal Genome Machine (PGM) Sequencer, and initial data on exosome labelling and *in vivo* uptake tracing in a cell culture model. The value of exosomes for clinical applications is discussed with an emphasis on their potential for diagnosing and treating neurodegenerative diseases and brain cancer.

2. Results and discussion

(a) Exosome isolation and RNA recovery

We have developed five Total Exosome Isolation reagents that allow straightforward and reliable recovery of fully intact exosomes from all key body fluids and cell culture media, in a wide volume range, and are suitable for high-throughput applications. By tying up water molecules, the reagents force less-soluble components, such as nanovesicles, out of solution. To isolate exosomes, the reagent is added to a biological sample, and the mixture is incubated, at 4°C or ambient temperature, to allow precipitation, followed by sedimentation through standard centrifugation at 10 000*g*. The pellet containing the exosomes is then resuspended in phosphate buffered saline (PBS) or a similar buffer and the exosomes are ready for endpoint analysis such as sequencing, qRT-PCR, western blots or biological studies to explore functions, networks and pathways *in vivo*.

In this study, we focused on analysis of exosomes derived from blood serum and urine, as these body fluids are the most promising sources of biomarkers secreted by all organs. Exosomes were recovered from serum and urine of healthy human donors using the corresponding Total exosome isolation reagent. Sizing and quantification of exosomes was performed using the NanoSight LM10 instrument [13] and results are shown in figure 1. Over 95% of the nanoparticles recovered were smaller than 300 nm, most of them being in the size range of 30–150 nm characteristic of exosomes. A notable difference was the somewhat larger average size of urine-derived exosomes, versus serum, and the presence of a few 400–600 nm vesicles. Urine is known to contain Tamm–Horsfall protein (THP), which is capable of forming polymeric networks that could trap a fraction of the exosomes, resulting in their loss upon isolation, or alter their apparent size [14]. We have tested protocols with proteinase K digest and a dithiothreitol treatment step aimed at reducing THP [14], but did not find a significant improvement in terms of amount or size of the nanovesicles recovered with the reagent (data not shown).

On average, from 1 ml serum, approximately $1–3 \times 10^{12}$ exosomes were recovered, and from 1 ml urine, approximately $3–8 \times 10^{9}$ exosomes. Thus, exosome concentration in urine is substantially lower compared to serum. Fluid volume inputs (50 µl–10 ml) were tested, and exosome recovery was linear in this range. All samples were positive for CD63, a well-characterized exosomal marker [1,5], as confirmed by western blotting ([13]; data not shown).

Next, we proceeded with the isolation and subsequent analysis of the exosomal RNA. Using the total exosome RNA and protein isolation kit, developed specifically for this purpose, samples were put through an organic extraction followed by immobilization of RNA on glass-fibre filters to purify the total RNA [13]. Exosomes derived from 4 ml serum typically yielded approximately 2–10 ng of RNA; exosomes derived from 10 ml urine yielded approximately 2–4 ng RNA. It should be noted that whole serum and urine contain about 10-fold more RNA, but a substantial fraction is associated with proteins and vesicles other than exosomes (see Discussion). A minimal amount of DNA was associated with exosomes, as determined by 20 TaqMan assays for different targets (data not shown), demonstrating that the major cargo residing within the exosomes is RNA and protein. These data are in agreement with a number of earlier studies but runs in counter with a recent publication by Kahlert *et al.* [15] which reported a substantial amount of 10 kb and longer fragments of double-stranded genomic DNA associated with exosomes. The potential explanation is that the isolation protocol used in the above study recovered the fraction of larger exosomes probably along with some microvesicles, that can accommodate long DNA.

Exosomes have a diameter of 30–150 nm. Taking into account that without the lipid bilayer, the internal radius of exosomes is approximately 10–70 nm, the internal volume of

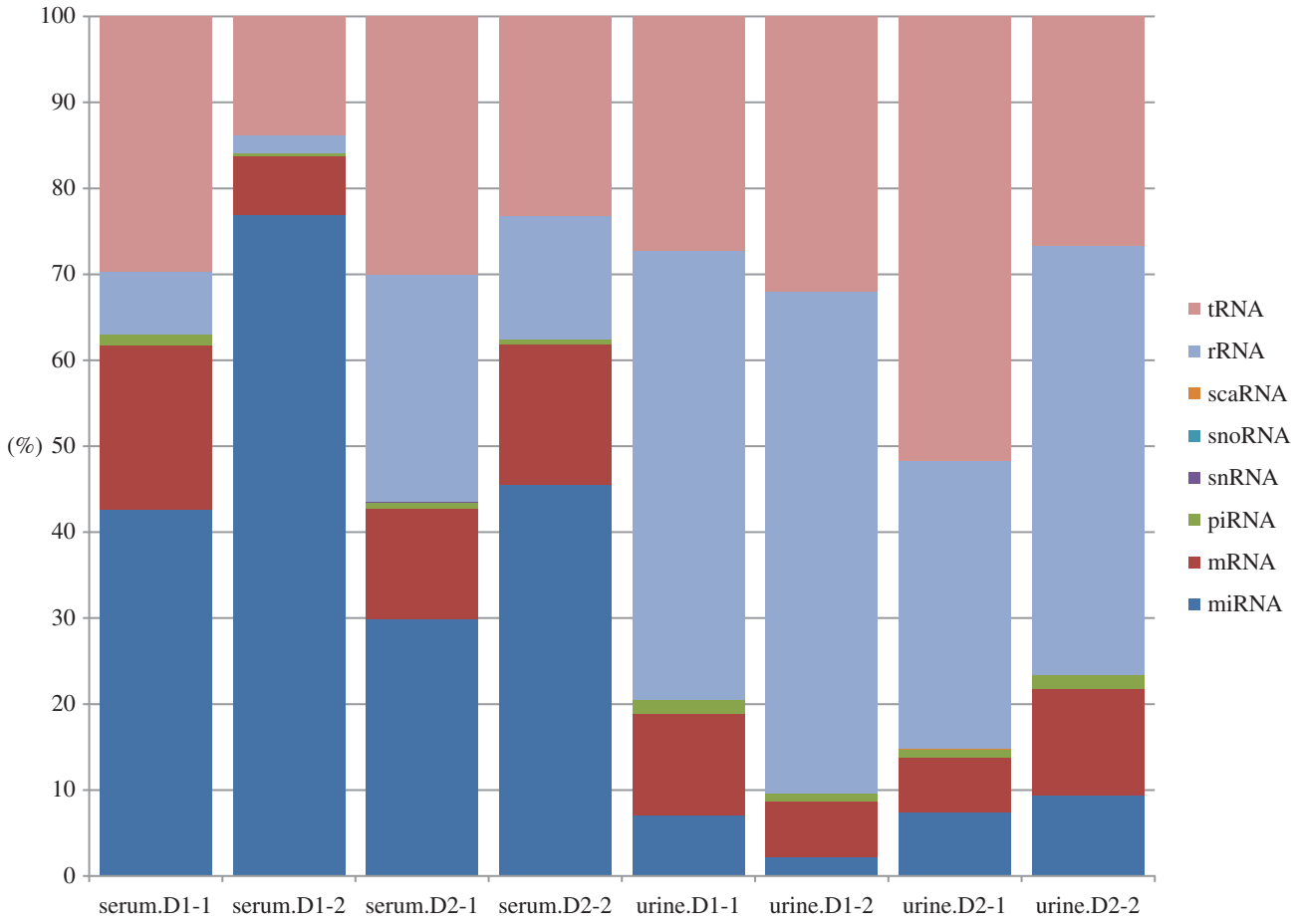

Figure 2. Sequencing results for exosomes isolated from human serum and urine. Two donors were used for each sample type (D1 and D2), and for each donor two libraries were prepared and sequenced individually. The PGM 318 chips hold over 11 million wells. For the samples derived from serum as well as urine, 9–10 million wells were loaded (75–90%) and sequenced. After subtracting the polyclonals, low-quality sequences and non-templated ion sphere particles, 5–6 million final readouts were obtained from each run. Of those reads, 90–98% was mapped following the earlier developed pipeline [16].

one exosome ($4/3\,\pi R^3$) is approximately 4×10^{-24}–1.5×10^{-21} m^3. The volume of one average 50 kDa protein or 100 nt RNA molecule is approximately 6×10^{-26} m^3. Thus, theoretically each exosome can accommodate approximately 70–25 000 small RNA or protein molecules. From the experiments, significantly lower numbers were obtained for RNA: an average serum-derived exosome seems to contain just one RNA molecule (or less). One potential explanation for this average number is that exosomes are a very heterogenous population of vesicles with different cargo and functions, and some of them might contain substantial number of RNA molecules, while others contain just the protein cargo and no RNA at all. Another very important thing to note is that despite the seemingly low RNA cargo per exosome, due to their extremely high numbers (up to 10^{12} particles per millilitre body fluid, occupying up to 0.1% volume), as a population, exosomes are definitely capable of inducing substantial biological effects.

(b) Analysis of exosomal RNA content by sequencing

Exosomal RNA recovered from serum- and urine-derived samples was further analysed by sequencing. Two donors were used for each sample type, and for each donor two libraries were prepared and sequenced individually. For the library construction, primer-binding flanking sequences were ligated to the RNA in the samples, followed by reverse transcription and polymerase chain reaction (PCR) amplification [16]. Once the libraries were constructed, they were sequenced using the Ion Torrent PGM instrument, with 318 chips, following standard protocols [16].

Using mapped sequence read counts, we quantified the RNA content of exosomes extracted from the serum and urine samples. As illustrated in figure 2, exosomes derived from both serum and urine contained extremely diverse RNA 'cargo'. As expected from previous research [1,6], a large number of miRNAs and mRNAs were detected. However, significant amounts of rRNA, tRNA, and to a lesser extent, other RNA types were discovered as well (piRNA, snRNA, snoRNA, scaRNA). There were clear differences in RNA species representation between serum- and urine-derived exosomes—the serum exosomes contained substantially more miRNA, while urine exosomes contained more ribosomal RNA.

When summarizing total counts per sample type, we calculated mean raw counts per RNA-Seq library and then ranked them from high to low for miRNA, mRNA, ncRNA and tRNA categories. The top 12 are listed in table 1. In this view, representation is not given as a normalized percentage but as ranks of individual transcripts by category and may be compared directly between exosomal samples. The biggest overlap between urine and serum exosomes was observed for tRNA: 10 out of the 12 top represented sequences were the

Table 1. RNA representation in serum- and urine-derived exosomes: top 12 RNA transcripts organized by RNA type. Values represent mean counts of mapped reads to reference transcripts.

miRNA			
miR_ID	**serum**	**miR_ID**	**urine**
hsa-mir-451a	9.040	hsa-mir-204	0.584
hsa-mir-223	4.739	hsa-mir-30a	0.470
hsa-mir-486	2.196	hsa-mir-10b	0.442
hsa-mir-320a	1.388	hsa-let-7b	0.352
hsa-mir-185	1.356	hsa-mir-10a	0.330
hsa-mir-205	1.102	hsa-mir-26a	0.321
hsa-mir-378a	0.999	hsa-mir-23b	0.231
hsa-mir-484	0.953	hsa-mir-21	0.221
hsa-mir-191	0.927	hsa-mir-125	0.221
hsa-mir-150	0.859	hsa-mir-99a	0.215
hsa-mir-145	0.832	hsa-let-7a-2	0.177
hsa-mir-21	0.777	hsa-mir-191	0.171
mRNA			
Gene_ID	**serum**	**Gene_ID**	**urine**
LOC728819	35.780	LOC728819	21.512
PPIAL4Fv1	0.604	PPIAL4Fv2	0.122
PPIAL4Fv2	0.513	ZNF467	0.070
TTLL2	0.048	PPIAL4Fv1	0.068
FNDC5	0.034	ANO9	0.060
PRR12	0.033	CDHR2	0.041
NSMCE1	0.032	SLC39A3	0.039
CT45A4	0.031	SLC8A2	0.025
ZNF880	0.027	SMARCB1	0.023
DNAJA4	0.026	ZNF324	0.016
C9orf106	0.026	OTUD6B	0.015
ACCSL	0.025	SACM1L	0.015
ncRNA			
ncRNA_ID	**serum**	**ncRNA_ID**	**urine**
SDC4P	1.449	SDC4P	5.294
GTF2IP1	0.890	PPY2	2.168
FAM138B	0.772	DLEU2L	1.699
CYCSP52	0.465	FAM138B	0.679
TTTY16	0.438	ECEL1P2	0.609
LOC100133050	0.402	GTF2IP1	0.372
NEURL3	0.397	RBMY3AP	0.352
GTF2IP2	0.383	SPCS2	0.327
LOH12CR2	0.366	C21orf49	0.320
FAM138D	0.347	XGPY2	0.319
FAM138E	0.300	C21orf54	0.316
TTTY19	0.274	LOC613037	0.296

(*Continued.*)

Table 1. (*Continued.*)

tRNA			
tRNA_ID	**serum**	**tRNA_ID**	**urine**
Asp-GAC	4.389	Gly-CCCv1	1.812
Gly-CCCv1	1.832	Gly-GGG	1.741
Gly-GGG	1.596	Glu-GAG	1.344
Gly-CCCv2	1.342	Gly-CCCv2	1.289
Glu-CTCv1	1.315	Glu-CTCv1	1.048
Glu-GAG	0.814	Val-CAC	0.889
Glu-GAA	0.805	Glu-GAA	0.800
Gly-GGC	0.710	Asp-GAC	0.707
Glu-CTC	0.487	Glu-CTCv2	0.690
Gly-GCCv1	0.474	Gly-GGC	0.672
Gly-GCCv2	0.462	Glu-CTC	0.641
Glu-CTCv2	0.460	Val-TAC	0.641

same; for ncRNA and mRNA, three of the 12 sequences were the same; for miRNA, two sequences were the same.

mRNA LOC728819 was present at very high levels in both urine- and serum-derived exosomes. While annotation of functional information in public databases for this target is sparse, it bears sequence homology to the C1GALT1-specific chaperone 1-like gene whose mutations have been associated with some nephropathies in case/control studies [17]. PPIAL4Fv2, or cyclophillin A, is also a highly represented coding transcript found in both urine and serum samples. The resulting protein of this transcript binds cyclosporin which could induce immunosuppression and also can interact with HIV-specific proteins.

Serum exosomes also contained high levels of Asp-GAC tRNA and miR-451a. Urine exosomes contained high levels of the SDC4P pseudogene; in fact, many of the most highly representative RNAs detected with this analysis are pseudogenes. Poliseno *et al*. [18] show that some pseudogenes appear to have a role in oncogene regulation by serving as decoys for the canonical coding transcript—in particular, the PTENP1 pseudogene acting as a miRNA decoy for PTEN. This raises the notion that SDC4P is perhaps shuttled to other cells via exosomes and functions as a miRNA decoy for SDC4. Interestingly, inactivation of SDC4 has been shown to inhibit immune response by limiting adhesion and motility of dendritic cells [19].

Using hierarchical clustering, we observed that miRNA clusters are easily distinguishable between urine and serum (figure 3). For instance, miR-183 is highly represented in serum relative to urine. Serum levels of miR-183 have been described in case/control studies where significantly lower levels of miR-183 are observed in stage IV non-small-cell lung carcinoma versus controls—making it a potential biomarker target for lung cancer [20]. In addition, a clinically aggressive medulloblastoma has been linked to the pleiotropic effects of the miR-183 cluster [21]. Conversely, miR-192 is highly represented in urine relative to serum. Loss of expression of this miRNA in kidney glomeruli has been linked to promote fibrogenesis in diabetic nephropathy. This suggests that the presence or lack of miR-192 in the urinary

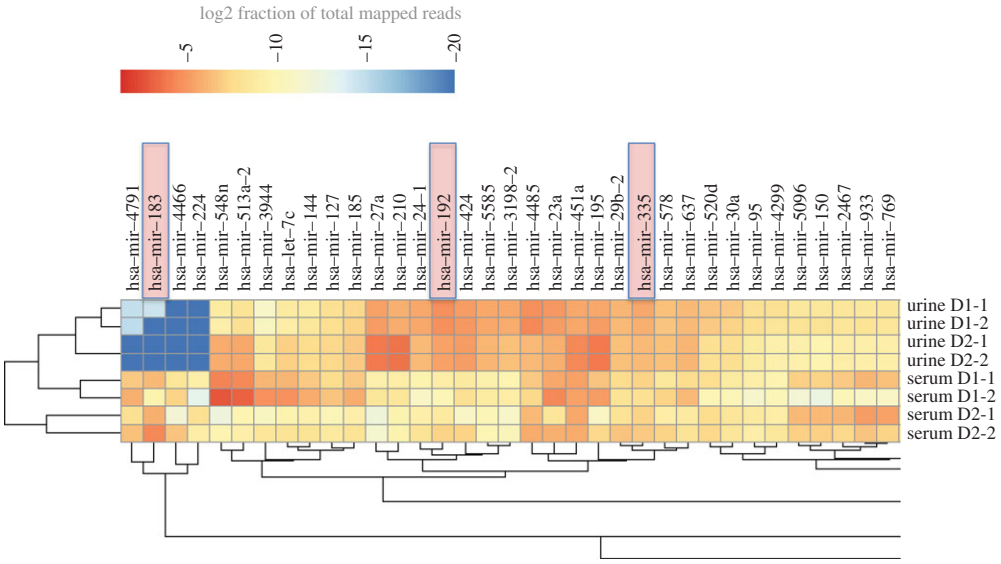

Figure 3. Heatmap with dendrogram depicting clustering results of miRNA representation across urine and serum samples types. Two donors were used for each sample type, and for each donor two technical replicates were performed. A hierarchical clustering was applied using the average linkage method and Euclidean distance. Values for the heatmap represent the log2-transformed fraction of total mapped reads being allocated to each miRNA per library. Light-red shaded boxes indicate potential miRNA markers described in the analysis section.

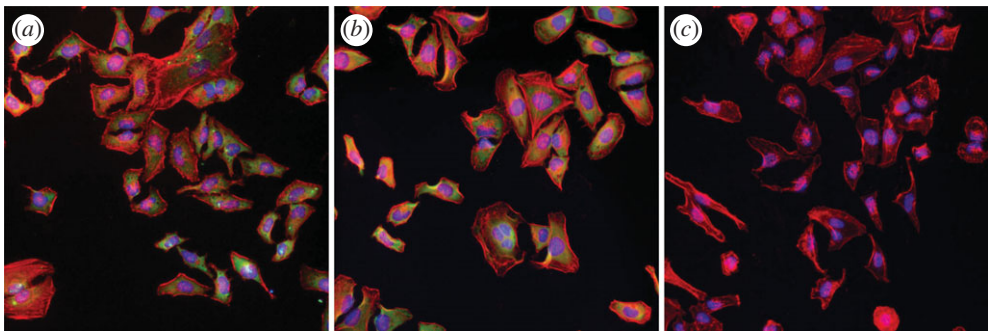

Figure 4. Uptake by HeLa cells of exosomes labelled with SYTO RNASelect stain. A FLoid Cell Imaging station was used. Red: Alexa Fluor 594; blue: DAPI; green: SYTO RNASelect stain. (*a*) Labelled exosomes added to cells, (*b*) dye only control and (*c*) no treatment control (cells only).

system serve as a biomarker for some types of nephropathies [22]. miR-335 is found at somewhat higher levels in serum-derived exosomes. Earlier research indicated that patients with high miR-335 expression gliomas had significantly shorter survival times than those with low miR-335 expression tumours [23].

Following the aforementioned workflow, RNA content of body fluid-derived exosomes may be characterized to identify reliable biomarkers, by comparing the RNA profiles from patients in various pathological states to healthy donors. Taking into account significant donor-to-donor variation, as well as variability between technical replicates, it is important to include a large number of donors in each cohort, and ideally have two to three replicates to ensure generation of reliable data. It is also crucial to use the same optimized workflow (from sample collection to exosome isolation to RNA extraction and sequencing) throughout the entire study.

(c) Tracing exosomal uptake in the *in vitro* system

As the field of exosome research has matured over the last few years, the focus has expanded from simply trying to understand what exosomes are to understanding the mechanisms of their formation, secretion, functions, trafficking and interaction with cells throughout the body. Development of new techniques for visualization and tracing of the exosomes is becoming increasingly important. Here, we describe a visualization method based on SYTO RNASelect cell stain as a way to label exosomes and trace their uptake into cells. Several alternative dyes were screened at the earlier stage, and the above mentioned dye was selected as the best option for labelling exosomal cargo (M Li *et al.* 2013, unpublished data).

The SYTO RNASelect green fluorescent cell stain is selective for RNA: it exhibits a bright green fluorescence when bound to RNA (absorption/emission maxima approximately 490/530 nm) with only a weak fluorescent signal when bound to DNA. Purified exosomes were labelled with SYTO RNASelect (which in less than 20 min crossed the membrane and stained the exosome cargo) and passed through Exosome spin columns to remove any unincorporated dye from the preparations. Efficiency of dye incorporation was determined by measuring the fluorescence of exosomes using the Qubit fluorometer and comparing to a non-labelled control (data not shown). Labelled exosomes were then added to recipient HeLa cells and incubated to allow uptake.

Cells were further analysed using fluorescent microscopy with the FLoid instrument (figure 4). Two controls were used in this experiment: (i) 'No treatment' control—cells that did not receive any labelled exosomes or dye, and (ii) 'Dye only' control—cells that received the dye by itself and no exosomes. As shown in the figure, there is a clear difference between the two controls and the samples with added labelled exosomes. Exosomes are seen in the form of bright green clusters of dye that stand out from the background and controls (due to the limited magnification allowed by the instrument (20×), individual exosomes cannot be seen). These clusters are localized in sub-cellular compartments, indicating efficient internalization of the exosomes, with the contents gradually released into the cytoplasm—a classic mechanism used for uptake of material into cells.

This experiment demonstrates that (i) the selected tools and protocols are suitable for labelling isolated exosomes and visualization in the *in vitro* system and (ii) exosomes are capable of efficiently crossing the cell membrane, and their RNA content appears to be delivered in the cytoplasm upon uptake. These types of studies are critical to further our understanding of how exosomes function as a delivery system in the human body, as well as how they are involved in metastasis formation. Moreover, this will allow the development of more effective methods of detection and treatment of cancer.

(d) Exosomes as a source of biomarkers

Exosomes are a fascinating group of small vesicles with sophisticated cargo and multiple functions which are only partially understood. From our present and past work with serum, plasma, urine and CSF, we found that exosomes derived from these bodily fluids contain substantial amounts of different RNA species such as miRNA, mRNA, rRNA, tRNA, scaRNA, snoRNA, snRNA and piRNA. With a subset of these RNA species, we have seen correlations reflecting the content of parental cells, whereas other RNA sequences are present at significantly different levels (lower or higher) compared with the parental cells [16]. This raises the possibility that the former could be used as biomarkers (enabling the liquid biopsy alternative), while the latter could serve as positive or negative exosomal markers.

In the last decade, RNA and proteins have emerged as next generation biomarkers for brain cancer, neurodegenerative diseases and a number of other disorders. Below are several examples of the latest studies highlighting utility of blood- and urine-derived RNA and protein analysis. Importantly, a substantial fraction of RNA and protein secreted into the body fluids is associated with exosomes, and upfront isolation of exosomes can significantly increase the sensitivity and potentially specificity of the biomarker analysis—versus profiling of the total RNA/protein population recovered from the whole body fluid.

Brain tumours are the most common type of solid cancer and the leading cause of death in children, thus there is an urgent need to develop tools to improve the patient outcome. A major difficulty in treating brain tumours is the lack of effective methods of identifying novel or recurrent disease. Smith *et al*. [12] reported a panel of urinary protein biomarkers (MMP-2, MMP-9, MMP-9/NGAL and VEGF) that predicts the presence of brain tumours. These biomarkers have shown correlation with the presence of disease, decrease with treatment, and can be tracked from source tissue to urine.

Neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases have been the subject of several miRNA profiling studies, that used the post-mortem brain tissue and body fluids of the patients (reviewed in [10]). The analysis shows altered expression of certain miRNAs, which suggests they may have a very important regulatory role by directly or indirectly affecting development and progression of these diseases and may be used as biomarkers.

Mild traumatic brain injury (TBI) is a head trauma resulting in a brief loss of consciousness and/or disorientation, the symptoms of which are usually not harmful but in certain incidences more escalated issues can arise. Currently, there is no reliable diagnostic method to determine whether a TBI has affected the brain and if so to what degree. A number of promising RNA and protein biomarkers from various body fluids have been identified that can help assess damage associated with concussive or sub-concussive blows to the head [11].

A growing number of companies including Exosome Diagnostics (http://www.exosomedx.com/), Exosome Sciences (http://www.exosomesciences.com/), Caris (http://www.carislifesciences.com/) and Hansa Bio Med (http://www.hansabiomed.eu/) are developing exosome-based diagnostics based on the analysis of their RNA and protein constituents. The initial tests for prostate cancer should be released later this year.

The most widely used body fluids for the isolation of biomarkers are blood serum and plasma because the blood is easily accessible, comes in contact with all organs and tissues and has a constant volume. CSF is produced in the choroid plexus of the brain and found in the brain and spine. Thus, it is probably best suited for accessing the brain 'content'. Collection of CSF samples still represents a major challenge as the procedure is rather sophisticated and expensive. Urine and saliva are increasingly becoming more popular options as they are easier to collect and have a high content of exosomes stemming from different organs and tissues. The difficulty, however, associated with these two fluids is that their exosomal concentration and overall composition varies largely depending on the liquid and food uptake while the blood and CSF volumes remain constant.

Exosomes are not the only entities found in body fluids that contain nucleic acid. 'Circulating' or 'extracellular' RNA is found within exosomes, microvesicles and in complexes with Ago2 and other proteins [24,25]. 'Circulating' DNA is contained within nucleosomes, in complexes with different proteins, within apoptotic bodies, and also bound to the cell surface [26]. All body fluids contain high levels of DNAses and RNAses and thus, any circulating nucleic acid is not 'free', but encapsulated in some microvesicles or bound to proteins to avoid instant degradation (proteins capable of binding nucleic acids include immunoglobulins, albumin, lactoferrin and fibronectin [27]). For this reason, it is extremely important to explore the potential of other extracellular vesicles and proteins, in addition to exosomes, for biomarker development. For some diseases, the value might be in exosomes. For others, in certain proteins or microvesicles. And, in some cases, the isolation and analysis of the total RNA from serum will provide exactly the same results as analysis of exosomes albeit within an easier workflow and at lower cost.

(e) Exosomes as therapeutics

The ability of exosomes to travel between cells and deliver their cargo in a highly specific and efficient manner makes them an appealing therapy option to treat brain disorders. For instance, treatment options for multiple sclerosis (MS) are limited and consist of immunosuppressors or agents to prevent immune infiltration of the brain. However, these therapies have potentially harmful side effects, and in fact do very little to promote myelin repair. It was recently reported that interferon gamma-stimulated dendritic cell-derived exosomes increased baseline myelination and improved recovery in a MS model by inducing demyelination [28]. Furthermore, when nasally administered to whole animals, these exosomes stimulated an increase in brain myelin.

Another group has demonstrated that exosomes derived from cultured cells can be harnessed for delivery of small interfering RNAs (siRNA) *in vivo* in a mouse brain [29]. Self-derived dendritic cells were used for exosome production in order to reduce immunogenicity. Targeting was achieved by engineering the dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide. Purified exosomes were loaded with synthetic siRNA by electroporation. Intravenously injected exosomes delivered GAPDH siRNA specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in a specific gene knockdown. The therapeutic potential of exosome-mediated siRNA delivery was demonstrated by the efficient mRNA and protein knockdown of BACE1, a therapeutic target in Alzheimer's disease, in wild-type mice.

Exosomes are essentially non-toxic and non-immunogenic natural delivery vesicles, capable of delivering any RNA and protein, including therapeutic molecules. It should be noted, however, that despite initial promising results there are still numerous challenges for exosomes as *in vivo* delivery vehicles, including large-scale production, successful 'loading' with therapeutic cargo (which is extremely inefficient at the moment according to our in house data and many other research groups), targeting to specific organ/tissue and efficient release of the cargo.

Another interesting approach was proposed by Aethlon Medical (http://www.aethlonmedical.com/). They are treating cancer and hepatitis C by depleting pathogenic exosomes and viral particles from blood of patients using the Hemopurifier medical device, which is essentially a large format flow-through canister with attached proprietary lectins. The same strategy presumably can be used to treat a broader range of diseases as well. The above data overall demonstrate that exosomes can be used in several ways towards development of the next generation therapies.

3. Conclusion

Owing to their unique RNA and protein cargo, largely reflecting the parental cell, exosomes appear to be an ideal source of biomarkers, which can be accessed with minimally invasive procedures. Furthermore, because they are shuttling this cargo from one cell to another in a highly specific and efficient fashion, exosomes also have a potential as next generation therapeutic vehicles that are capable of delivering macromolecular and small molecule drugs to the brain and other organs. The herein described tools and protocols for isolation of exosomes from human blood serum and urine, characterization of their RNA cargo by sequencing, as well as labelling and tracing exosomes *in vivo* will be extremely useful for researchers and medical specialists studying these ubiquitous and sophisticated nanovesicles.

4. Material and methods

(a) Isolation of exosomes from urine

Frozen urine samples were thawed in a water bath at room temperature until samples were completely liquid, then vortexed for 30–60 s to create a homogeneous sample and centrifuged *at* 2000*g* for 30 min to remove any cellular debris. The supernatant containing the cell-free urine was transferred to a fresh container and briefly held on ice until use. Next, each urine sample was combined with an equal volume of Total exosome isolation (from urine) reagent (Invitrogen) and mixed well by vortexing or inverting the tube until a homogeneous solution was formed. The urine volume range of 500 µl–10 ml was used depending on the downstream application. The samples were incubated at room temperature for 1 h, then centrifuged at 4°C at 10 000*g* for 1 h. The supernatant was aspirated and discarded, and the samples were re-spun for 5 min at room temperature at 10 000*g* to remove any excess reagent. Once all supernatant was removed, the exosome pellet was resuspended in PBS buffer (20–200 µl) and stored at 4°C short term (1–7 days) or −20°C for long term.

(b) Isolation of exosomes from serum

Frozen serum samples were thawed in a water bath at room temperature until samples were completely liquid, then centrifuged at 2000*g* for 30 min to remove any cellular debris. The supernatant containing the cell-free serum was transferred to a fresh container and briefly held on ice until use. Next, each serum sample was combined with 1/5th volume of Total exosome isolation (from serum) reagent (Invitrogen) and mixed well by vortexing or pipetting up and down until a homogeneous solution was formed. The serum volume range of 50 µl–5 ml was used depending on the downstream application. The samples were incubated at 4°C for 30 min, then centrifuged at room temperature at 10 000*g* for 10 min. The supernatant was aspirated and discarded, and the exosome pellet was resuspended in PBS buffer (20–500 µl), then stored at 4°C short term (1–7 days) or −20°C for long term.

(c) Fluorescent labelling of exosomal RNA and tracing exosomal uptake in the *in vitro* system

Exosomes were recovered from cell culture media or body fluid using the Total exosome isolation reagents. Exosomes (approx. 1×10^9–10^{10}) were resuspended in 100 µl of PBS and then combined with 1 µl of SYTO RNASelect stock solution (1 mM in DMSO). The samples were incubated at 37°C for 20 min (protected from light) and then passed through Exosome spin columns MW 3000 (Invitrogen) to remove any unincorporated dye from the labelled exosomes. Efficiency of the exosome labelling was analysed using the Qubit 2.0 fluorometer (488 nm excitation). For the *in vitro* tracing, the 8-well Chamber Slide System was prepared, with 10 000 HeLa cells/well, and the labelled exosomes were added to the 'recipient' cells. Samples were incubated at 37°C, for 30 min to 3 h, next fixed with 4% paraformaldehyde, at room temperature for 20 min, and then permeabilized with 0.1% Triton X-100, at room temperature for 3–5 min. Finally, cells were stained with Alexa Fluor 594, at room temperature for 20 min, and mounted in ProLong Gold Antifade Reagent with DAPI. Samples were analysed using the FLoid fluorescent microscope.

References

1. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. 2007 Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**, 654–659. (doi:10.1038/ncb1596)
2. Raposo G, Stoorvogel W. 2013 Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **4**, 373–383. (doi:10.1083/jcb.201211138)
3. Vlassov AV, Magdaleno S, Setterquist R, Conrad R. 2012 Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim. Biophys. Acta* **1820**, 940–948. (doi:10.1016/j.bbagen.2012.03.017)
4. Gyorgy B *et al*. 2011 Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell. Mol. Life Sci.* **68**, 2667–2688. (doi:10.1007/s00018-011-0689-3)
5. Simons M, Raposo G. 2009 Exosomes: vesicular carriers for intercellular communication. *Curr. Opin. Cell Biol.* **21**, 575–581. (doi:10.1016/j.ceb.2009.03.007)
6. Skog J *et al*. 2008 Glioblastoma microvesicles transport RNA and proteins that promote tumor growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10**, 1470–1476. (doi:10.1038/ncb1800)
7. Smythies J, Edelstein L. 2013 Transsynaptic modality codes in the brain: possible involvement of synchronized spike timing, microRNAs, exosomes and epigenetic processes. *Front. Integr. Neurosci.* **6**, 126. (doi:10.3389/fnint.2012.00126)
8. Taylor DD, Gercel-Taylor C. 2013 The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids. *Front. Genet.* **4**, 142. (doi:10.3389/fgene.2013.00142)
9. Xu D, Tahara H. 2013 The role of exosomes and microRNAs in senescence and aging. *Adv. Drug Deliv. Rev.* **65**, 368–375. (doi:10.1016/j.addr.2012.07.010)
10. Junn E, Mouradian MM. 2012 MicroRNAs in neurodegenerative diseases and their therapeutic potential. *Pharmacol. Ther.* **133**, 142–150. (doi:10.1016/j.pharmthera.2011.10.002)
11. Zetterberg H, Smith DH, Blennow K. 2013 Biomarkers of mild traumatic brain injury in cerebrospinal fluid and blood. *Nat. Rev. Neurol.* **9**, 201–210. (doi:10.1038/nrneurol.2013.9)
12. Smith ER, Zurakowski D, Saad A, Scott RM, Moses MA. 2008 Urinary biomarkers predict brain tumor presence and response to therapy. *Clin. Cancer Res.* **14**, 2378–2386. (doi:10.1158/1078-0432.CCR-07-1253)
13. Zeringer E, Li M, Barta T, Schageman J, Pedersen KW, Neurauter A, Magdaleno S, Setterquist R, Vlassov AV. 2013 Methods for the extraction and RNA profiling of exosomes. *World J. Methodol.* **3**, 11–18.
14. Fernandez-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. 2010 Tamm–Horsfall protein and urinary exosome isolation. *Kidney Int.* **77**, 736–742. (doi:10.1038/ki.2009.550)
15. Kahlert C *et al*. 2014 Identification of double stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J. Biol. Chem.* **289**, 3869–3875. (doi:10.1074/jbc.C113.532267)
16. Schageman J, Zeringer E, Li M, Barta T, Lea K, Gu J, Magdaleno S, Setterquist R, Vlassov AV. 2013 The complete exosome workflow solution: from isolation to characterization of RNA cargo. *BioMed Res. Int.* **2013**, 1–15. (doi:10.1155/2013/253957)
17. Bertinetto FE *et al*. 2012 Search for genetic association between IgA nephropathy and candidate genes selected by function or by gene mapping at loci IGAN2 and IGAN3. *Nephrol. Dial. Transplant.* **27**, 2328–2337. (doi:10.1093/ndt/gfr633)
18. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. 2010 A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* **465**, 1033–1038. (doi:10.1038/nature09144)
19. Bühligen J, Himmel M, Gebhardt C, Simon JC, Ziegler W, Averbeck M. 2010 Lysophosphatidylcholine-mediated functional inactivation of syndecan-4 results in decreased adhesion and motility of dendritic cells. *J. Cell Physiol.* **225**, 905–914. (doi:10.1002/jcp.22301)
20. Lin Q, Mao W, Shu Y, Lin F, Liu S, Shen H, Gao W, Li S, Shen D. 2012 A cluster of specified microRNAs in peripheral blood as biomarkers for metastatic non-small-cell lung cancer by stem-loop RT-PCR. *J. Cancer Res. Clin. Oncol.* **138**, 85–93. (doi:10.1007/s00432-011-1068-z)
21. Weeraratne SD *et al*. 2012 Pleiotropic effects of miR-183∼96∼182 converge to regulate cell survival, proliferation and migration in medulloblastoma. *Acta Neuropathol.* **123**, 539–552. (doi:10.1007/s00401-012-0969-5)
22. Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D. 2010 Loss of microRNA-192 promotes fibrogenesis in diabetic nephropathy. *J. Am. Soc. Nephrol.* **21**, 438–447. (doi:10.1681/ASN.2009050530)
23. Jiang J *et al*. 2012 Tumor microRNA-335 expression is associated with poor prognosis in human glioma. *Med. Oncol.* **29**, 3472–3477. (doi:10.1007/s12032-012-0259-z)
24. Arroyo JD *et al*. 2011 Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl Acad. Sci. USA* **108**, 5003–5008. (doi:10.1073/pnas.1019055108)
25. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. 2011 Characterization of extracellular circulating miRNA. *Nucleic Acids Res.* **39**, 7223–7233. (doi:10.1093/nar/gkr254)
26. Rykova EY, Morozkin ES, Ponomaryova AA, Loseva EM, Zaporozhchenko IA, Cherdyntseva NV, Vlassov VV, Laktionov PP. 2012 Cell-free and cell-bound circulating nucleic acid complexes: mechanisms of generation, concentration andcontent. *Expert Opin. Biol. Ther.* **12**, 141–153. (doi:10.1517/14712598.2012.673577)
27. Vlassov VV, Laktionov PP, Rykova EY. 2007 Extracellular nucleic acids. *Bioessays* **29**, 654–667. (doi:10.1002/bies.20604)
28. Pusic AD, Pusic KM, Clayton BLL, Kraig RP. 2014 IFNγ-stimulated dendritic cell exosomes as a potential therapeutic for remyelination. *Neuroimmunology* **266**, 12–23. (doi:10.1016/j.jneuroim.2013.10.014)
29. El-Andaloussi S, Lee Y, Lakhal-Littleton S, Li J, Seow Y, Gardiner C, Alvarez-Erviti L, Sargent IL, Wood MJ. 2012 Exosome-mediated delivery of siRNA *in vitro* and *in vivo*. *Nat. Protoc.* **7**, 2112–2126. (doi:10.1038/nprot.2012.131)