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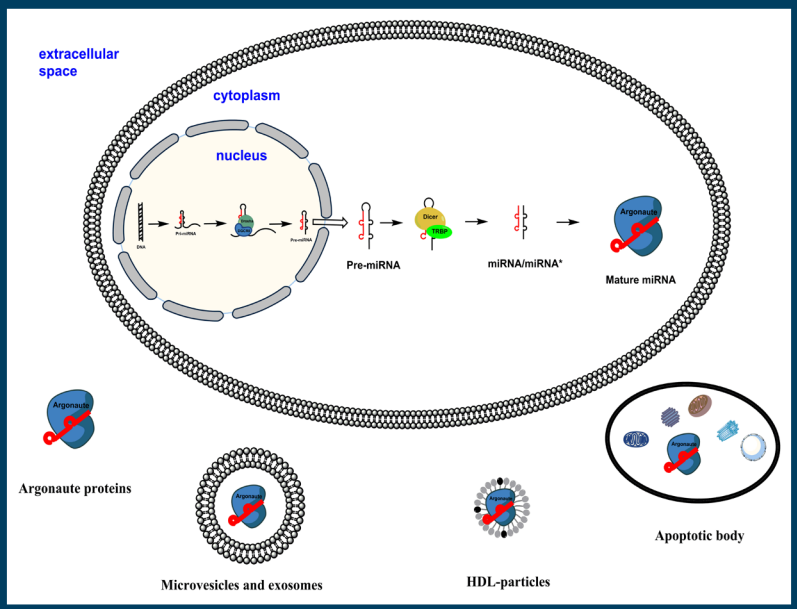

THE ORIGIN, FUNCTION AND DIAGNOSTIC POTENTIAL OF EXTRACELLULAR MICRORNA IN HUMAN BODY FLUIDS

Topic Editors
Andrey Turchinovich and William Cho

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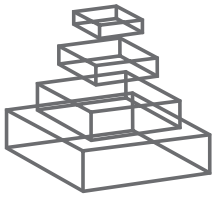

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THE ORIGIN, FUNCTION AND DIAGNOSTIC POTENTIAL OF EXTRACELLULAR MICRORNA IN HUMAN BODY FLUIDS

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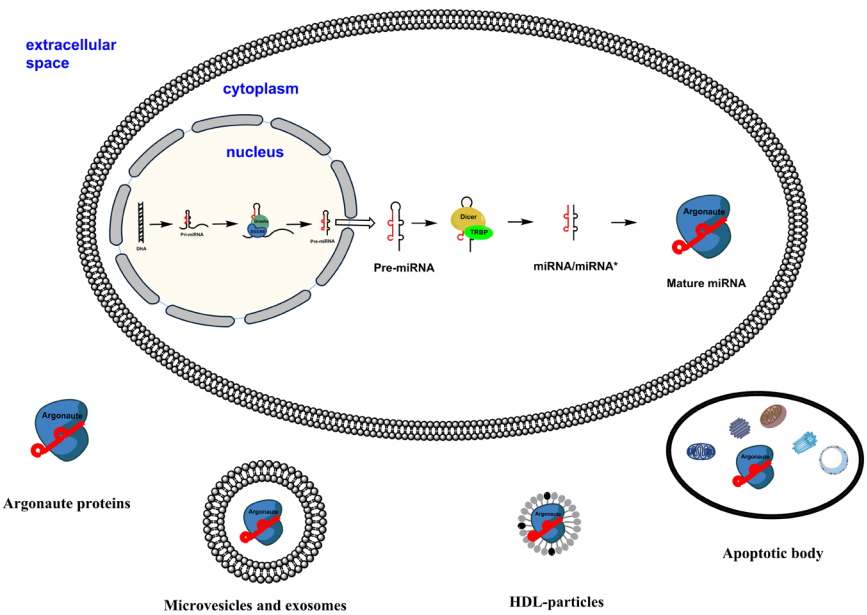

The biogenesis of miRNAs in the cell and types of extracellular miRNA. The biogenesis of miRNAs starts in the cell nucleus with generation of primary miRNAs (pri-miRNAs) transcripts. Pri-miRNAs are cleaved by microprocessor complex Drosha/DGCR8 into shorter miRNA precursors (pre-miRNA). The latter are transported to the cytoplasm and further cut by endonuclease Dicer into ~22 nt miRNA/miRNA* duplexes. Finally, one of the miRNA/miRNA* strands is incorporated into a protein of the Argonaute family (AGO1, AGO2, AGO3, or AGO4). The mature miRNA strand eventually serves as a guide for mRNA targeting resulting in either mRNA cleavage or translational interference. Extracellular miRNA can be either solely AGO protein-associated or additionally encapsulated into apoptotic bodies, microvesicles, and high-density lipoprotein (HDL) particles.

Short non-coding RNA molecules, microRNAs (miRNAs), post-transcriptionally regulate gene expression in living cells. In recent years, miRNAs have been found in a wide spectrum of mammalian body fluids including blood plasma, saliva, urine, milk, seminal plasma, tears and amniotic fluid as extracellular circulating nuclease-resistant entities. The changes in miRNA spectra observed in certain fluids correlated with various pathological conditions suggesting that extracellular miRNAs can serve as informative biomarkers for certain diseases including cancer. However, the mechanism of generation and a biological role of extracellular miRNAs remain unclear. The current theories regarding extracellular miRNA origin and function suggest that these miRNAs can be either non-specific 'by-products' of cellular activity and cell death or specifically released cell-cell signaling messengers.

The goal of this Research Topic is to bring together up-to-date knowledge about the extracellular miRNA and its role in disease diagnostics and, possibly, inter-cellular communication.

Table of Contents

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EDITORIAL
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The origin, function and diagnostic potential of extracellular microRNA in human body fluids

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Keywords: body fluids, diagnostic, cell communication, biological fluids

Over the past several years extracellular microRNAs (miRNAs) have been found in all mammalian body fluids such as blood plasma, saliva, urine, milk, seminal plasma, tears, and amniotic fluid as circulating entities capable of predicting the course of a wide range of diseases. Great progress has been made so far in understanding their existence. However, the mechanism of generation and the biological role of extracellular miRNAs remain questionable. Current theories regarding origin and function of extracellular miRNAs suggest that these miRNAs can be either non-specific "by-products" of cellular activity and cell death, or "on-purpose" released cell-cell signaling messengers.

The goal of this Research Topic is to bring together the accumulating knowledge about the extracellular miRNAs function and their utilities for disease diagnostics. We have collected 12 highly selected articles aiming to shed light on the origin and function of circulating miRNAs. We intended to transmit a balanced view on the topic by collecting both research and review articles from the leading scientists in the field of circulating nucleic acids. These papers provide either focused set of experiments or thorough literature analysis with the goal to defend certain concepts. Some researchers have expressed an opinion, strengthened by a number of evidence, that circulating miRNAs can be key mediators of various cell-cell communication processes. Other authors were defending an opinion that extracellular miRNAs can be merely by-products of non-specific cellular activity. While both theories can, in fact, be true, our aim was to secure that the both points of view are expressed.

Several papers touch on the critical methodological concerns associated with the analysis of miRNAs in biological fluids. Dr. Witwer and co-authors described an optimized protocol for isolation of miRNAs from liquid samples (Mcalexander et al., 2013). They showed that some commercial kits are superior to others for the recovery of miRNA from biological fluids. Dr. Cheng and colleagues have presented an excellent review of methods used for deep sequencing of small RNA species in the biological fluids (Cheng et al., 2013). The potential use of miRNA deep sequencing for biomarker discovery and its translation into clinical practice is also discussed. The reports of Dr. Kirschner and colleagues touches on the challenges in analysis, quantification and reporting of the data on the circulating nucleic acids (Kirschner et al., 2013a,b). They have demonstrated a significant impact of hemolysis, which frequently occurs during blood plasma collection, on extracellular circulating miRNA content. Thus, when investigating circulating miRNA in blood plasma and serum it is critical to avoid the hemolysis and to account for all possible artifacts derived from it. Especial branch of our topic is dedicated to the extracellular viral miRNAs. Thus, Dr. Lagana and co-authors provided an excellent state-of-the-art coverage toughing current knowledge and perspectives of the viral circulating miRNAs (Lagana et al., 2013). Virus-encoded miRNAs can be secreted by virus-infected cells and, possibly, be transferred to the recipient cells. The functional consequences of such transfer and the diagnostic potential of viral miRNAs are thoroughly discussed. Finally, Dr. Oliviery and co-authors provided some insights into inflammatory miRNAs in the circulation and their association with age-related diseases (Olivieri et al., 2013). Inflamma-miRs, a relatively small number of miRNAs that are involved in regulation of inflammation, and which mainly target the TLRs/NF-κB pathway, have been recently found in cell-free blood circulation. Interestingly, age-related inflammatory diseases are frequently accompanied by deregulation of most circulating inflamma-miRs in the plasma. Thus, circulating inflamma-miRs could have diagnostic/prognostic relevance in such diseases.

We hope that our Research Topic will raise further attention to this emerging field. Enormous potential of circulating nucleic acids for the prediction of a wide range of diseases is also mentioned. The spectrum of the disorders which can be diagnosed on their early stages using the analysis of circulating miRNA in human bio-fluids biopsies includes: prostate cancer, Alzheimer disease, cardiovascular diseases, viral infections, aging and age-related disorders, etc.

Despite a large number of promising studies, the field of extracellular nucleic acids is still in its infancy. Further advancement of

more sensitive and specific methods for detecting and analysis of circulating small RNAs is warranted for addressing the questions raised in this Research Topic. We envision that this Research Topic will attract bigger interest into the field of extracellular miRNAs and encourage the perusal of more in-depth investigations which are translating these molecules into biomarkers for disease diagnostics.

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The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids

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We have previously demonstrated that tumor cells release membranous structures into their extracellular environment, which are termed exosomes, microvesicles or extracellular vesicles depending on specific characteristics, including size, composition and biogenesis pathway. These cell-derived vesicles can exhibit an array of proteins, lipids and nucleic acids derived from the originating tumor. This review focuses of the transcriptome (RNA) of these extracellular vesicles. Based on current data, these vesicular components play essential roles as conveyers of intercellular communication and mediators of many of the pathological conditions associated with cancer development, progression and therapeutic failures. These extracellular vesicles express components responsible for angiogenesis promotion, stromal remodeling, signal pathway activation through growth factor/receptor transfer, chemoresistance, and genetic exchange. These tumor-derived extracellular vesicles not only to represent a central mediator of the tumor microenvironment, but their presence in the peripheral circulation may serve as a surrogate for tumor biopsies, enabling real-time diagnosis and disease monitoring.

Keywords: exosomes, microvesicles, transcriptome, microRNA, early diagnosis

BACKGROUND

The release of 50–200 nm sized membranous vesicles into biological fluids by viable tumor cells was initially described by us over three decades ago (Taylor and Doellgast, 1979) and has since been demonstrated multiple cell types and systems. *In vivo*, these nano-sized vesicles released by tumor cells accumulate in biologic fluids, including blood,urine, ascites, and pleural fluids (Taylor and Gercel-Taylor, 2011). The release and accumulation of these extracellular vesicles appear to be important in the malignant transformation process. Extracellular vesicles have been identified by various terms, including high molecular weight complexes, membrane fragments, exosomes, microvesicles, microparticles, and extracellular vesicles. While more recently restrictive definitions have been applied to these cell-derived vesicles, significant overlap (in terms of size, markers, cargoes, and function) exists between structures identified as exosomes and microvesicles. Within the circulation, it may not be possible to differentiate 50–100 nm "exosomes" from 50 to 200 nm "microvesicles." In many studies, uncharacterized cell-derived vesicles (in terms of markers or size) are termed "microvesicles," while numerous studies define "exosomes" solely based on density and the presence of the cell surface markers, tetraspanins. These overlaps in vesicle properties and terms suggest these distinctions may not be clear-cut. For these reasons, this review uses the term "extracellular vesicles" to include all 50–200 nm tumor-derived vesicles.

The "exosome" term was coined in 1981 for "exfoliated membrane vesicles with 5′-nucleotidase activity" (Trams et al., 1981). This term, "exosome," originated from the discovery of neoplastic cell line-derived exfoliated vesicles, which mirrored the 5′-nucleotidase activity of the parent cells (Trams et al., 1981). Several years later, vesicles release via the canonical pathway upon multi-vesicular endosome fusion with the cell surface was demonstrated in cultured sheep (Pan et al., 1983) and rat (Harding et al., 1983) reticulocytes. After purification by ultracentrifugation, the pelleted vesicles were found to contain the transferrin receptor that was also found in native reticulocytes (Johnstone et al., 1987). These reports proposed that this represented a mechanism for the elimination of specific cellular components as the reticulocytes matured and differentiated. These cell-derived vesicles were redefined as "exosomes" to differentiate them from "endosomes." The disparate natures of these studies are reflected in the various names that were proposed and which are still used to identify the cell surface-released and endocytic vesicles of different origins. It is of noted that these later studies (Harding et al., 1983; Pan et al., 1983; Johnstone et al., 1987) were exclusively *in vitro*.

The *in vivo* appearance of tumor-derived vesicles within the circulation was initially demonstrated in ovarian cancer patients (Taylor and Doellgast, 1979; Taylor et al., 1980, 1983). These gynecologic cancer patients exhibited intact extracellular vesicles within their peripheral circulation and malignant effusions (ascites and cyst fluids). These tumor-derived extracellular vesicles were found to express molecular markers that were generally linked with tumor plasma membranes, including placental type alkaline phosphatase and mdr-1 (Taylor et al., 1985, 1989; Taylor and Black, 1986); however, proteins not generally associated with plasma membranes, such as p53, GRP78 and nucleophosmin, were also identified with these circulating vesicles (Chinni et al., 1997; Manahan et al., 2001).

Extracellular vesicles have since been demonstrated to be released by a variety of non-cancerous cells, particularly cells of the immune system, including dendritic cells, macrophages,

B cells, T cells, and NK cells (Théry et al., 2009), as well as embryonic cells (Atay et al., 2011a,b). These extracellular vesicles have been demonstrated to be key mediators/regulators of normal immune responses (Whiteside, 2013). One can view tumors as a "cyber-terrorist" using these extracellular vesicles to elicit aberrant immune regulation. Extracellular vesicles released by the tumor may elicit a tolerogenic response and participate in other immune mechanisms, such as platelet activation, mast cell degranulation, germinal center reaction, and potential engulfment of apoptotic cells. The aberrant release of extracellular vesicles by tumors may allow them to circumvent these immunoregulatory antigen delivery pathways and evade immunosurveillance (Taylor and Black, 1986; Taylor and Gercel-Taylor, 2011).

EXTRACELLULAR VESICLES COMPOSITION AND CHARACTERIZATION

Over the past three decades, shed tumor-derived vesicles have been characterized for multiple human cancer types and they are not exact replicates of the plasma membrane or other membranous compartments of the originating tumor cells, but they represent "micromaps" with enhanced expression of tumor antigens, as well as other macromolecules, including major histocompatibility antigens (Taylor and Gercel-Taylor, 2011). Exosomes, vs. microvesicles, have been defined, based on size (30–100 nm lipid bilayer vesicles), density (1.12 g/ml for B cell derived to 1.19 g/ml for intestinal cell derived) and expression of specific biomarkers (including tetraspanins) (Théry et al., 2009). Extracellular vesicles can be viewed as cytoplasm enclosed in a lipid layer with the external domains of transmembrane proteins exposed to the extracellular environment in their normal cellular orientation. Electron microscopic studies have demonstrated the fusion of the limiting membrane of MVB with the plasma membrane as well as release of ILVs in different cell types of hematopoietic origin, such as Epstein-Barr virus (EBV)-transformed B cells (Zumaquero et al., 2010), mastocytes (Admyre et al., 2008), dendritic cells (Montecalvo et al., 2008), platelets (Rak, 2010), macrophages (Anand, 2010) and cells of non-hematopoietic origin like neurons and epithelial cells (Kesimer et al., 2009).

The exact mechanisms by which cells release exosomes/microvesicles remain unclear; however, the release is modulated by extracellular signals (Ostrowski et al., 2010). Three primary mechanisms have been proposed for the release of cellular components into the extracellular space: (1) exocytic fusion of multivesicular bodies (MVBs) resulting in "exosomes," (2) budding of vesicles directly from the plasma membranes resulting in shed "microvesicles" and (3) cell death leading to apoptotic bodies. The first two mechanisms are properties of viable cells and are energy requiring events. While most isolation protocols readily exclude apoptotic bodies, current methods do not differentiate extracellular vesicles from the endocytic pathway from shed "microvesicles" from the plasma membrane. As a result, most studies on these extracellular vesicle populations include a mixture of exosomes and microvesicles, which may confuse interpretation of biochemical data.

Since the formation of exosomes has an endocytic origin, this mechanism is a process of the endosomal pathway, including endocytic vesicles, early endosomes, late endosomes and lysosomes. The endocytic vesicles are formed through either clathrin- or non-clathrin-mediated endocytosis at the plasma membrane and are transported to early endosomes (Yu et al., 2006; Valapala and Vishwanatha, 2011). The late endosomes develop from early endosomes via acidification, changes in their protein content and their ability to fuse with vesicles or other cellular membranes. Early endosomes are localized near the outer margin of the cells and exhibit a tubular appearance, in contrast late endosomes are localized proximal to the nucleus and are spherical in shape. An essential step in MVB formation from late endosomes is reversed budding. In this step, limiting membranes of late endosomes "bud" into their lumen, resulting in a continuous enrichment of internal luminal vesicles (Yu et al., 2006). MVBs have been demonstrated to be involved in the exocytic fusion of their external membrane with the plasma membrane of the cell, resulting in release of their segregated vesicles to the extracellular space. Within the vesicles, two invaginations occur, such that the membrane orientation of proteins within the vesicles is the same as the plasma membrane of the cell. The release of large biomolecules through the plasma membrane can occur through the process termed exocytosis, which has regulatory and signaling functions. Exocytosis can be either constitutive (non-calcium-triggered) or regulated (calcium-triggered) (Yu et al., 2006; Valapala and Vishwanatha, 2011). Constitutive exocytosis occurs in all cells and serves to secrete extracellular matrix components or to incorporate newly-synthesized proteins into the plasma membrane following fusion with transport vesicles. Regulated exocytosis is critical to events, such as neurologic signaling, as synaptic vesicles fuse with the membrane at the synaptic cleft (Graner et al., 2009).

Extracellular vesicles isolated from the extracellular environment of tumors (such as from the peripheral circulation), either *in vitro* or *in vivo*, exhibit overlapping similarities in size (defined by dynamic light scattering), morphology (defined by electron microscopy), density (defined by of sucrose gradient centrifugation), and protein markers of both the endosomes and plasma membranes (defined by western immunoblotting and mass spectrometry) (Graner et al., 2009; Mathivanan and Simpson, 2009; Xiao et al., 2012). While many of the definitions are still used, we now recognize their flaws (**Table 1**). The apparent size and shape of exosomes appear to be artifacts of fixation and drying associated with electron microscopy. Principal markers of exosomes are tetraspanins, which as plasma membrane associated components are present on most vesicles, regardless for their origin. The importance of the endocytic pathway of vesicle formation has also been questioned as knock-out studies with Rab proteins only diminished vesicle release by ∼30% (based on exosomal protein) (Peinado et al., 2012).

We have compared the extracellular vesicle populations obtained from biologic fluids of ovarian cancer patients by both the technique described to isolate extracellular vesicles and our original chromatographic method isolating "microvesicles" (Taylor and Gercel-Taylor, 2005) (**Figure 1**). This comparative study demonstrated that these *in vivo* derived vesicles from both techniques isolated cup-shaped vesicles, with a density between 1.13 and 1.17 g/ml, a diameter between 50 and 150 nm, and

Table 1 | Glossary of extracellular vesicle terms.

Term	Current definition (Théry et al., 2009)	Deficiencies
Exosomes	Size: 50–100 nm Shape: Cup shaped Sedimentation: 100,000 × g Markers: Tetraspanins (CD63/CD9), Alix, TSG101, ESCRT Lipids: Cholesterol, sphingomyelin, ceramide, lipid rafts, phosphatidylserine Origin: Multivesicular endosomes	Size and shape may be altered by the fixation process Tetraspanins are also plasma membrane markers and are not specific for vesicles derived from MVB Knockout studies suggest MVB-derived vesicles represent only a portion of the 50–200 nm vesicles Lipid composition is also shared with "micromaps" of the plasma membrane
Microvesicles	Size: 100–1000 nm Shape: Irregular Sedimentation: 100,000 × g or 10,000 × g or 1200 × g (dependent on size) Markers: Intergins, selectins, CD40 ligand Lipids: Phosphatidylserine Origin: Plasma membrane	Most circulating vesicles fall in the range of 50–200 nm. *In vivo*, larger vesicles are generally not observed Impossible to distinguish 50–200 nm microvesicles from exosomes based on sedimentation Microvesicles derived from surface "micromaps" shared lipid and protein compositions with exosomes Tetraspanins can be detected on vesicles of all size ranges Microvesicle- "specific" markers were demonstrated on vesicles derived from B cells, thus these may not be relevant to those derived from other cell types
Apoptotic bodies	Size: 400–1000 nm Shape: Heterogeneous Sedimentation: 100,000 × g or 10,000 × g or 1200 × g Markers: Histones Lipids: Not determined Origin: Plasma membrane	Exosomes and microvesicles are the products of viable, healthy, proliferating cells, while apoptotic bodies are released as part of apoptotic cell death Exosomes and microvesicles exhibit high degrees of selectivity in cargoes, while apoptotic bodies contain debris of the dying cell

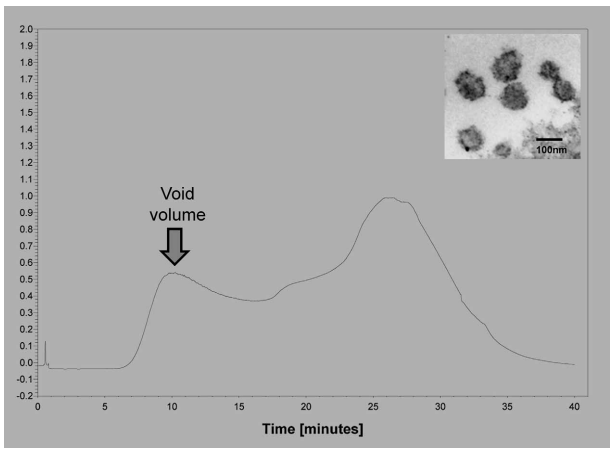

FIGURE 1 | Example of the isolation of extracellular vesicles from an ovarian cancer patients using size exclusion chromatography on Sepharose 2B. Insert shows an electron micrograph of the void volume material, demonstrating the presence of 50–150 nm vesicles.

expressing CD63, Alix, VPS35, galectin 3, HSP90, fibronectin, and placental alkaline phosphatase (Taylor and Gercel-Taylor, 2005; Taylor et al., 2011). While patient-derived circulating extracellular vesicles fit within the definition of exosomes, the contribution of these two populations is unclear. Since both populations exist within the peripheral circulation of cancer patients, this distinction between exosomes and shed microvesicles may not be critical to understand the biologic activities of these vesicles as they can interact with target cells of the host as a mixture. In a subsequent study comparing methods, we further described a more uniform vesicle size and superior recovery of vesicle components (proteins and RNA) by chromatography vs. centrifugation (Taylor et al., 2011). This advantage of the chromatographic approach may relate to the fact that vesicles are not subjected to shearing force associated with centrifugation.

The increased release of extracellular vesicles and their accumulation appear to be important in the malignant transformation process. Although shedding of extracellular vesicles occurs in other types of cells under specific physiological conditions, the accumulation of extracellular vesicles from tumor cells is augmented in biologic fluids. Recently, circulating vesicles of normal individuals, patients with benign ovarian disease and patients with ovarian cancer have been investigated using the Nanoparticle Tracking Analysis system (NanoSight) (Gercel-Taylor et al., 2012). The presence of circulating vesicular materials was demonstrated in all individuals; however, ovarian cancer patients exhibit ∼3–4-fold more vesicular material. In these cancer patients, the size range of these vesicles was between 50 and 250 nm, with the major peak at 98–99 nm.

CARGOES OF EXTRACELLULAR VESICLES

Extracellular vesicles contain proteins, non-coding RNAs, and mRNAs, and the exosomal lipid bilayer appears to protect these materials from degradation. While protein and RNA cargoes of extracellular vesicles vary depending on the originating cell, there are conserved proteins among extracellular vesicles from different cellular origins (Mathivanan and Simpson, 2009). The protein composition of extracellular vesicles has been extensively analyzed

by various techniques including Western blotting, fluorescence-activated cell sorting, immuno-electron microscopy and mass spectrometry. All extracellular vesicles exhibit cytoskeleton proteins (such as ezrin and actins), proteins associated with the MVB biogenesis (such as alix and TSG101), membrane transport and fusion proteins (e.g., annexins and Rab proteins), and tetraspanins (e.g., CD9, CD63, and CD81). A catalog of proteins, RNAs, and lipids assoicated with extracellular vesicles can be found at www.exocarta.org. Currently, ExoCarta lists entries for 13,333 proteins, 2375 mRNAs, 764 microRNA, and 194 lipids associated with extracellular vesicles.

We have analyzed the patient-derived exosomal proteome using ion trap mass spectrometry and identified 232 unique proteins. These proteins were classified as percent of the identified total proteins into molecular chaperones (8.5%), vesicle fusion (8.5%), cytoskeletal proteins and proteins involved in the assembly/disassembly of the cytoskeletal networks (17.6%), anionic and cationic ion transport channels (3.7%), proteins involved in lipid (6.9%), carbohydrate (3.2%) and amino-acids (2.1%) metabolisms, proteins involved in DNA replication (6.9%), mRNA splicing (5.3%), transcription/translation (5.3%), post-transcriptional protein modification (13.8%), and signal transduction (2.7%). Our studies demonstrated that cytosolic proteins were highly represented and we observed a diverse array of cytoskeletal constituents (actin, α-actinin-1, cofilin, filamin-A-B-C, tubulins, gelsolin, profilin-1, spectrin, symplekin, talin, vinculin, myosins). We identified that transmembrane proteins were also abundant, including multiple integrins (β1, α3, αv), intercellular adhesion molecule 1 (ICAM-1), and mucin-4. A variety of channels were observed, such as the voltage-dependent anion-selective channel protein 2 and 3, chloride intracellular channel protein 1, sodium/potassium-transporting ATPase subunit β-3, long of sodium/potassium-transporting ATPase subunit α-1, and transitional endoplasmic reticulum ATPase. In line with their endocytic origin, exosomal proteins belonging to the ESCRT complex that are important protein complexes involved in ubiquitin-dependent exosome biogenesis, have also observed (Doring et al., 2010). These ESCRT-associated proteins include vacuolar protein sorting-associated protein 35 (VPS-35), Alix, ubiquitin-like modifier-activating enzyme and ubiquitin carboxyl-terminal hydrolases. We demonstrated that proteins involved in membrane trafficking and fusion processes were enriched (annexin A2, A5, A6, clathrin heavy chain 1/2, coatomer subunit β, Rab1b, Rab2a, and Rab7a). A group of markers of endosomes and lysosomes were also detected (cathepsin-C, D, EH domain-containing protein 1 and β-hexoaminidase), and several chaperonnes were identified (HSP70, HSP90, HSC70, HSPA4, 8, 9, HSPA1A/B, HSPB1, HSP47, HSPA5, HSPβ1, HSPD1, HSP90AB1, B1, AA1; T-complex protein 1, endoplasmin, and protein disulfide-isomerase A3, A4, A6) (Graner et al., 2009; Mathivanan and Simpson, 2009).

EXTRACELLULAR VESICLE-ASSOCIATED RNA

The current hypothesis for the stability of circulating RNA is that they are released from cells in membranous vesicles. Recent data confirm that extracellular RNA can exist in four forms: free RNA, Argonaut 2-bound RNA, high-density lipoprotein-bound RNA and vesicle-associated RNA. This review focuses on RNA associated with extracellular vesicles. These extracellular vesicles are generated constitutively by most, but not all, cell types and contain both mRNAs and non-coding RNA. The ability of extracellular vesicles to transfer genetic information may facilitate cancer spread by delivering genetic material and oncogenic proteins. RNA profiles of extracellular vesicles differ from that of cellular RNA, since vesicles contain primarily small RNA, such as mRNA and microRNA, in the absence of ribosomal RNA (Skog et al., 2008; Taylor and Gercel-Taylor, 2008).

The presences of circulating RNAs have been extensively investigated, despite the presence of highly stable RNases, which should degrade any free RNA. The majority of the circulating RNAs have been defined as microRNAs based on the molecular weight (Mitchell et al., 2008). Studies also demonstrated that microRNAs not only have high stability in body fluids, but also survive in the unfavorable physiological conditions such as freeze-thawing, extreme variations in pH and long time at room temperature (Mitchell et al., 2008; Duggagupta et al., 2011; Chen et al., 2012). Whereas adding detergents, such as Triton X or SDS, to serum or plasma makes microRNAs easily degradation by RNases (Zhang et al., 2010). The results indicate there are at least two approaches responsible for the stability of extracellular microRNAs: be packaged in membrane-encapsulated vesicle and be protected by RNA-binding proteins.

The stability of extracellular microRNAs has been hypothesized to be due to the formation of the RNA-vesicle. During RISC disassembly in the cytoplasm, some microRNAs are found to be sorted into MVBs, which are commonly considered to form exosomes by fusion with the plasma membrane (Simons and Raposo, 2009). Both exosome and microvesicle can easily translocate across the cell membrane, which makes microRNAs enter recipient cells easily and mediate cell-to-cell communication.

Our studies have indicated that many of RNAs enriched in the extracellular vesicles may not be abundant, or even detectable, in the originating cell or highly expressed within the cell and low or absent within extracellular vesicles (Gercel-Taylor et al., 2013), indicating sorting of specific RNAs into extracellular vesicles. These released microRNAs can be classified in three categories based on the ratio between the amount of microRNA released from the cells and the amount retained in the cell (Pigati et al., 2010). The first group is selectively released microRNAs, which are characterized by being primarily released from tumor cells with relatively low concentrations remaining in the cell. In contrast, normal cells do not release appreciable quantities of these microRNAs (Pigati et al., 2010). An additional group of released microRNAs are those released in equal levels as they appear within the cell, termed neutrally released microRNA. These neutrally released microRNAs include miR16 and miR21, where the abundance in extracellular vesicles reflects increased abundance in the tumor cells. The selectivity of release of specific microRNAs differs depending on the cell type (Rabinowits et al., 2009; Pigati et al., 2010; Mittelbrunn et al., 2011). Selectivity appears to be influenced by malignant transformation. Breast and ovarian tumor cells have been demonstrated to release >99% of miR451 and miR1246 produced by the cells

(Pigati et al., 2010; Gercel-Taylor et al., 2013). These selectively released microRNAs have been linked to the malignant phenotype. MiR451 has been identified as a tumor suppressor, defining proliferation and cell polarity. miR451 has also been shown to induce chemosensitivity. miR1246 induces p53-dependent apoptosis triggered by DNA damage (Zhang et al., 2011). The changes in the release of cancer-related microRNAs may suggest a role for selective microRNA export in malignant transformation, and it may provide a cancer signature within the exported, circulating microRNA population.

While the mechanism of this selective sorting is unclear, some have postulated this selectivity relates to microRNA/RNA-induced silencing complex (RISC) components. Extracellular vesicles contain components of the microRNA/RISC, such as Argonaut 2, together with several RNA-binding proteins known to regulate RNA traffic between the nucleus and the cytoplasm. It can be therefore hypothesized that, during vesicle biogenesis, these RNA binding proteins regulate the accumulation of selected RNAs within extracellular vesicles. Studies on the transfer of reporter mRNAs and their translation into proteins, demonstrated both *in vitro* and *in vivo*, suggest that the mRNA delivered by extracellular vesicles is functional (El-Andaloussi et al., 2012; Tetta et al., 2013).

Extracellular vesicles derived from other tumors such as colorectal (Silva et al., 2012), lung (Rabinowits et al., 2009), and prostate cancer cells (Bryant et al., 2012) alter the phenotype of normal cells by transferring specific RNA subsets. In contrast, extracellular vesicles released from the surrounding cells may modify cancer cell gene expression (Bryant et al., 2012). Extracellular vesicles derived from cancer stem cells were shown to contain pro-angiogenic RNAs able to induce a pre-metastatic niche in the lungs, whereas those derived from differentiated cancer cells were not able to induce this niche and their mRNA and microRNA content differs (Grange et al., 2011). Extracellular vesicles from cancer stem cells contained miR29a, miR650, and miR151, all associated with tumor invasion and metastases, along with miR19b, miR29c, and miR151, known to be up-regulated in patients with renal carcinomas (Grange et al., 2011).

Extracellular vesicles have been isolated and analyzed from both normal healthy individuals and patients with various physiological conditions. We have previously shown that cancer patients (Gercel-Taylor et al., 2012) and pregnant women (Atay et al., 2011a) exhibit more extracellular vesicles in their blood compared to their normal, healthy counterparts. In pregnant women, the extracellular vesicles are thought to play a role in the maternal-fetal tolerance occurring during pregnancy, as it has been shown that placenta extracellular vesicles suppress T lymphocytes (Atay et al., 2011b).

Most investigations on small RNAs in exosomes have been limited to microRNA; however, next generation sequencing small RNAs in extracellular vesicles is expanding the populations identified. While intracellular microRNAs have been defined in many biological processes, identification of extracellular vesicle-associated microRNAs represents a non-invasive approach to investigate disease-specific microRNA and may provide a method for disease diagnosis (Duggagupta et al., 2011). To detect, analyze, and quantitate the RNA signatures of exosomes derived from biologic fluids, several approaches have been used, including microarrays, quantitative real-time PCR, and next-generation sequencing. The development of high detection sensitivity in next generation sequencing technologies has expanded the identification of the exosomal transcriptome, beyond miRNA. While most studies have focused on exosomal microRNAs, we now recognize the presence of numerous other small RNAs within these circulating exosomes, as well as fragments of larger RNAs (**Figure 2**). These exosomal small non-coding RNAs are <200 nucleotides in length (generally are 20–30 nt). There are three primary populations of small non-coding RNAs, including siRNAs, miRNAs, and piRNAs. Small non-coding RNAs have been shown to be key regulators in development, apoptosis, stem cell self-renewal, differentiation, and cell integrity maintenance. Piwi-interacting RNAs (piRNAs) are generated from intergenic elements, including transposable elements, through Dicer-independent pathways. These piRNAs function through the Piwi-Argonaute sub-family (AGO3, Aubergine, and Piwi), leading to silencing of transposable elements. A link between piRNAs and cancer has been

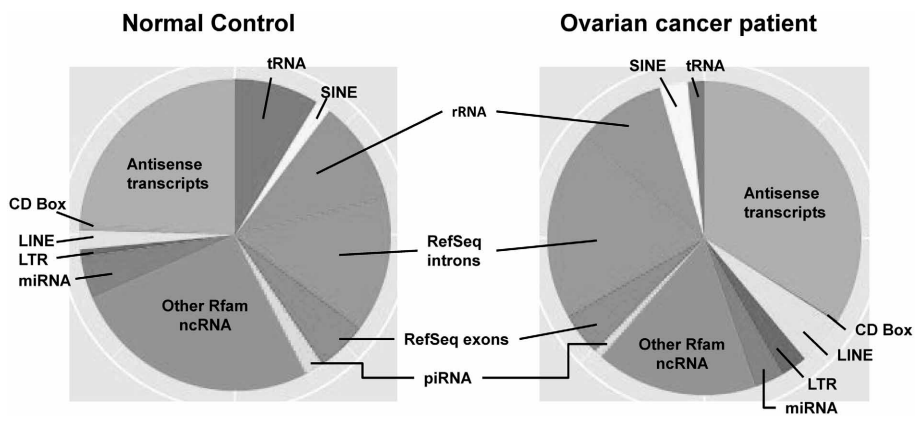

FIGURE 2 | The distribution of small RNAs derived from circulating extracellular vesicles of normal controls vs. ovarian cancer patients, based on next generation sequencing.

demonstrated in gastric cancers where two aberrantly expressed piRNAs, piRNA-651 and piRNA-823, were found in gastric tumor tissue vs. paired normal tissue (Cheng et al., 2011, 2012).

FUNCTIONS OF EXTRACELLULAR VESICLES

While some early studies implicated extracellular vesicles as "garbage bags" of the cells (Pan et al., 1983; Johnstone et al., 1987), the vesicles released from tumor cells have gained increasing recognition as "vehicles" for intercellular communication. Intercellular communication has been thought to be limited to cell-to-cell adhesion conduits (gap junctions) or secreted signals, such as hormones, neurotransmitters, and cytokines, released from cells and acting in an autocrine or paracrine manner. These extracellular vesicles interact with the plasma membrane of a recipient cell by ligand/receptor binding, fusion or internalization (or a combination of these, **Figure 3**). If the extracellular vesicles fuse with the target cell, they can transfer their cargo to that recipient cell. Due to the presence of cell-type specific adhesion molecules, extracellular vesicles can interact with specific cells and deliver their "cargoes," including bioactive lipids, cytokines, growth factors, receptors and genetic materials. In this manner, extracellular vesicles represent a pathway for intercellular transfer of information, similar to that observed with direct cell–cell contact, but that can function at distance. Extracellular vesicles provide stable conformational conditions for their protein content, conserve bioactivity of their proteins, improve bio-distribution and support an efficient interaction with target cells.

The complexity of extracellular vesicle associated bioactive macromolecules supports a critical role in generating the tumor microenvironment (Marhaba et al., 2008; Park et al., 2010). Extracellular vesicles can transfer specific proteins to target cells for the delivery of signaling pathways (Hong et al., 2009; Xiang et al., 2009). The presence of tumor-derived extracellular vesicles can increase matrix metalloproteinase (MMP) secretion and VEGF expression in target cells through the expression of pro-angiogenic molecules, such as members of the tetraspanin family, thereby promoting neo-angiogenesis even at secondary metastatic sites (Nazarenko et al., 2010). The released MMPs can digest the extracellular matrices where they arise. This degradation is enhanced when MMPs are co-released with exosome-associated extracellular MMP inducer (EMMPRIN) (Keller et al., 2009).

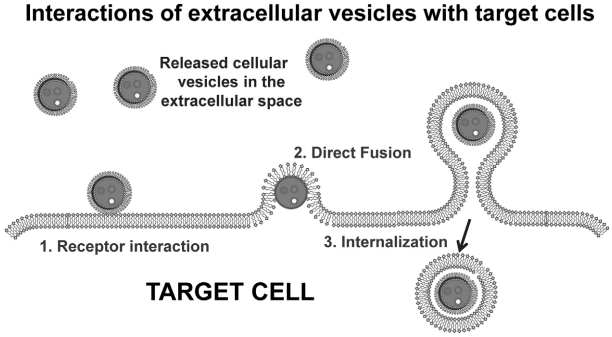

FIGURE 3 | Scheme of the potential mechanisms of interactions between an extracellular vesicle and a target cells. The interactions include receptor/ligand binding, fusion, and internalization.

Studies have shown that cancer ascites-derived extracellular vesicles carry extracellular matrix-remodeling enzymes, such as metalloproteinases 2 and 9 (MMP-2, MMP-9) (Dolo et al., 1999a,b), and urokinase plasminogen activator (Graves et al., 2004), leading to an increase in extracellular matrix degradation. The expression of matrix-remodeling enzymes increases the tumor's invasive phenotype and promotes metastasis. The presence of pro-angiogenic factors supports neovascularization of the developing tumor. A commonly identified cellular component of the tumor microenvironment is the monocyte/macrophage. Within the microenvironment, tumor-associated macrophages have been shown to assist in tumor progression by expressing cytokine/chemokine profiles that promote angiogenesis, stimulate tumor growth, and elicit immunosuppression by suppressing Th1 responses (Lewis and Pollard, 2006; Whiteside, 2013). The tumor microenvironment is characterized by pro-inflammatory profiles, including interleukin-1β (IL-1β). This profile is generally produced by infiltrating macrophages following interactions with tumors or their components. While we proposed that extracellular vesicles could "educate" macrophages to produce pro-inflammatory cytokines following internalization, we recently demonstrated that the induction of IL-1β was observed even when internalization of vesicles was blocked (Atay et al., 2011a,b). RGD peptides, which are used to block fibronectin binding to macrophage α5β1 integrin, were observed to abrogate vesicle-induction of IL-1β production and down-stream phosphorylation of Akt and c-Jun (Atay et al., 2013). This approach reveals the importance of receptor/ligand interactions in vesicle communication.

Extracellular vesicles can be internalized by recipient cells following receptor-ligand interactions and the varied assortment of bioactive molecules, derived from the cell of origin, such as proteins, bioactive lipids, and nucleic acids, can be transferred along with the proteins expressed on the vesicle surface. Extracellular vesicles may directly activate the recipient cell by acting as signaling complexes (Corrado et al., 2013; Ji et al., 2013). Extracellular vesicles derived from macrophages bind to platelets by means of the P-selectin glycoprotein ligand-1 expressed on their surface and extracellular vesicles from neutrophils expressing Mac-1 may induce platelet activation (Brouckova and Holada, 2009). Extracellular vesicles may also transfer receptors from one cell to another. Bystander B cells have been shown to rapidly acquire antigen receptors from activated B cells by membrane transfer with the resulting increase of a cell population presenting a specific antigen to CD4 T cells (Quah and O'Neill, 2005). Also, Fas ligand can be transferred from tumor cells by extracellular vesicles provoking activated T cell apoptosis (Teng et al., 2012). Moreover, extracellular vesicles may convey proteins to the cytoplasm of recipient cells, such as the cell death caspase-1 message conveyed by microvesicles derived from LPS-stimulated monocytes (Dubyak, 2012), or the tumor exosome-carried Notch ligand Delta-like 4 which inhibits Notch signaling, enhancing angiogenesis (Sheldon et al., 2010).

When released extracellular vesicles fuse with their target cells, they can transfer specific membrane components, including

receptors and ligands, which can express an activated phenotype. This transfer of receptors from extracellular vesicles to target cells was demonstrated by the observation that bystander B cells acquire antigen receptors from activated B cells by membrane transfer (McLellan, 2009). This transfer allows the amplified expansion of antigen-binding B cells with the ability to present specific antigens to CD4 T cells. Extracellular vesicles can transfer the adhesion molecule CD41 from platelets to endothelial cells or to tumor cells, conferring pro-adhesive properties to the target cell (Ratajczak et al., 2006). Exosome-mediated transfer of Fas ligand from tumor cells induces apoptosis of activated T cells enabling tumor immune escape (Ichim et al., 2008). Extracellular vesicles can also be protective for tumor cells by removing molecules, such as Fas or the membrane attack complex, from their membranes.

The horizontal transfer of macromolecules and their functional consequences has been demonstrated in human gliomas (Al-Nedawi et al., 2008, 2009a). In this model, only a fraction of the cells, exhibiting a transformed phenotype, expressed the truncated epidermal growth factor receptor, EGFRvIII, associated with dysregulaed tumor growth (Al-Nedawi et al., 2009b). Al-Nedawi et al. (2008) demonstrated transfer of the oncogenic EGFRvIII from human glioma cancer cells expressing the receptor to glioma cells without the EGFRvIII via the fusion of extracellular vesicles. After transfer, the glioma cells, lacking the receptor, were transformed to express EGFRvIII-regulated genes, including VEGF, Bcl-x_L, and p27 (Al-Nedawi et al., 2009a). Subsequent studies demonstrated that the oncogenic EGFRvIII from human squamous cell carcinoma cells was transferred via extracellular vesicles to tumor-associated endothelial cells to activate MAPK and Akt cell signaling pathways and promote endothelial VEGF expression (Al-Nedawi et al., 2009b).

Epigenetic changes have been frequently demonstrated in various tumors, resulting in regulation of gene transcription, altered proliferation, differentiation, and therapeutic resistance (Viswanathan et al., 2003; Camussi et al., 2010). Genetic information can be transferred through two proposed mechanisms: vertical gene transfer, gene exchange from parent to the next generation, and horizontal gene transfer, induced through, for example, bacteriophages or viruses. Since extracellular vesicles have been implicated as a potent source of macromolecule transfer to neighboring and distant cells, viruses and other pathogens appear to exploit this system. Extracellular vesicles are postulated to contribute to the spread of infective agents, such as human immunodeficiency virus type 1 (Lenassi et al., 2010). In macrophages receiving chemokine receptors, this can induce an increased risk of HIV infection together with resistance to apoptosis. The transfer of the chemokine (CXC motif) receptor 4 and the chemokine (CC motif) receptor 5 chemokine co-receptors for human immunodeficiency virus type I by released extracellular vesicles can enhance the entry of the virus into cell types other than the lymphohemopoietic lineage (Izquierdo-Useros et al., 2009). In addition to transferring receptors, extracellular vesicles can transfer viruses, contained within extracellular vesicles, by the "Trojan exosome hypothesis" involving direct delivery (Izquierdo-Useros et al., 2010).

Cell-derived extracellular vesicles represent another mechanism of horizontal gene transfer. Genomic instability may be mediated by horizontal transfer of tumor-derived materials via extracellular vesicles. Horizontal transfer of macromolecules, including RNA, proteins and lipids, via extracellular vesicles has been shown in a multiple tumor systems, including: gliomas, monocytes, mast cells, and T cells (Skog et al., 2008). Tumor-derived extracellular vesicles have been shown to be capable of transferring surface components (proteins and lipids) and RNAs to monocytes. Janowska-Wieczorek et al. (2005) demonstrated that extracellular vesicles derived from murine embryonic stem cells (ESCs) could induce epigenetic reprogramming of target cells. ESC-derived extracellular vesicles were shown to improve survival of hematopoietic stem/progenitor cells, to induce up-regulation of early pluripotent and early hematopoietic markers, and to induce phosphorylation of mitogen-activated protein kinase p42/44 and Akt. ESC-derived extracellular vesicles were shown to express mRNAs for several pluripotent transcription factors that can be delivered to target cells and translated to their corresponding proteins (Koh et al., 2010). As RNase-treatment inhibited their exosome-mediated biological effect, the involvement of mRNA in the observed biological effects was suggested. Yuan et al. (2010) have shown that in addition to mRNA, extracellular vesicles can transfer microRNA to target cells. They demonstrated that extracellular vesicles derived from ESCs contain abundant microRNA and that they can transfer a subset of microRNAs to mouse embryonic fibroblasts *in vitro*. Since microRNAs are regulators of protein translation, this observation raised the possibility that stem cells can alter the expression of genes in neighboring cells by transferring exosomal microRNAs. When shed vesicles fuse with their target cells, the portion of cytosol segregated within their lumen is discharged to and integrates with the cytosol of the target cell. Because this transfer can also include transmission of specific mRNAs, it can ultimately contribute to the epigenetic and proteomic properties of target cells.

Extracellular vesicles have been proposed to re-model and educate the host environment to generate a favorable niche for tumor growth, invasion and spread of metastasis (Peinado et al., 2012). Extracellular vesicles from lung cancer cells can activate the expression of pro-angiogenic factors, including IL-8, VEGF, LIF, oncostatin M, IL-11 and MMP 9 in adjacent stromal cells, "educating" the microenvironment to support lung cancer cell metastasis (Kucharzewska et al., 2013). Extracellular vesicles released by tumors have been shown to have immunosuppressive functions, including the suppression of both T lymphocytes and natural killer cell activation (Abusamra et al., 2005; Ashiru et al., 2010). It has also been suggested that tumor cell extracellular vesicles can migrate to the lymph nodes and condition them to become more favorable environments for metastasis (Rana et al., 2013). Recent studies demonstrate that the extracellular vesicles target specific organs and their presence can initiate organ tropism of metastases. It has been postulated that microRNAs delivered by extracellular vesicles play a role in immune system regulation. An extracellular vesicle-dependent exchange of microRNAs between APC and T cells occurs at the site of immune synapses (Gutiérrez-Vázquez et al., 2013), including the

exchange of genetic information between DCs and T cells through extracellular vesicles (Mittelbrunn et al., 2011).

It has been suggested that tumor cell progression could use multiple forms of extracellular vesicle-mediated communication to simultaneously affect multiple effector populations, based on release of tissue factors, immunoregulators and oncogenic molecules. Thus, the signals transferred to neighboring and distant cells via extracellular vesicles may mirror the transcriptional status of the parent cell, but due to the exosomal messenger RNA and microRNA being transferred, their consequences on the translational machinery of the target cells are extensive.

DIAGNOSTIC APPLICATION OF RNA WITHIN EXTRACELLULAR VESICLES

There are two critical roadblocks for successful long-term survival of patients with cancer. First, there are no clinically useable markers to identify early stage cancers in asymptomatic individuals or to differentiate benign from malignant disease. Second, there are no methods to evaluate the dynamic changes in tumors during therapy. Standard imaging approaches do not provide metrics of tumor-specific genetic/phenotypic changes and operative information is expensive, potentially morbid and limited by errors in topographic sampling. Lacking metrics, clinicians are left unable to target appropriate therapy to tumors in individual patients. This seriously compromises the development of novel effective therapies in addition to effective and improved use of current modalities. Circulating biomarkers have been proposed to be promising for the definitive diagnosis and monitoring treatment of various tumor types. Defining tumor-specific biomarkers has numerous advantages, such as diagnosing the disease, identifying processes that are difficult to image, predicting outcome by identifying patients at risk for therapeutic failure, defining tumor-specific molecular and pathological alterations for developing therapeutic targets and monitoring responses to acute interventions (Kim et al., 2013). Such circulating biomarkers could also serve to monitor disease progression and predicting risk of recurrence; however, circulating biomarkers are problematic and exhibit several critical issues. Free protein and nucleic acid biomarkers are extremely unstable in the circulation, thus to detect these, a high steady-state must be reached for detection, which is generally not observed except in late stage disease, minor changes over time (essential for monitoring) are difficult to quantify and these biomarkers are sensitive to sample handling. The use of exosome-associated biomarkers appears to be capable of circumventing these issues. These tumor-derived extracellular vesicles are extremely stable within the circulation, in the order of days (vs. minutes for soluble markers). In addition to serving as biomarkers of cancer, data demonstrate that these tumor-derived exosomes may mediate events associated with tumor progression and metastases.

Exosomes provide stable, disease-specific markers for detection, disease characterization, and predicting prognosis (Liang et al., 2013). Temporal changes in exosomal RNA profiles have been demonstrated to accurately predict disease recurrence and overall patient survival (Takeshita et al., 2013). The proteomic and genomic profiles of circulating exosomes provide a real-time monitor of therapeutic response, serving as a companion diagnostic. By correlating these circulating markers with the molecular characteristics and real-time clinical parameters, he has established the use of circulating exosomes as a "liquid biopsy." In 2008, we published the initial demonstration of circulating exosomal RNA for their diagnostic use (Taylor and Gercel-Taylor, 2008). Since that time, many studies have examined the diagnostic utility of profiling total circulating microRNA in specific pathologies; however, no study, to date, has defined circulating exosome microRNA signatures derived from a single cell type. The release of exosomal RNAs exhibit features for utility as diagnostic biomarkers, as they can be detected at early stages, are present in routinely obtained biologic fluids (blood, CSF, urine, and saliva), are specifically derived from the cancer tissue, and can be easily and accurately quantified. Studies have demonstrated that extracellular vesicles are enriched in tumor-derived bioactive molecules. The level of extracellular vesicles in the peripheral blood of healthy controls has been observed to be $\sim 10^{10}$/ ml of blood and may increase 3–4 fold in patients with cancer.

One issue for the use of extracellular vesicles as diagnostic markers is that they are also released by other cells associated with the peripheral blood, including lymphocytes, platelets, and endothelial cells. This has established the need for isolation of specific vesicles populations, To address this, in our initial study, extracellular vesicles of tumor origin were isolated from the blood of women with ovarian cancer using antibodies reactive with epithelial cell adhesion molecule (EpCAM). It was also shown that the level of circulating EpCAM-positive extracellular vesicles increased with disease progression (Taylor and Gercel-Taylor, 2008). Furthermore, eight microRNAs previously shown to be overexpressed in ovarian cancer were demonstrated to be present in both the tumors and the serum extracellular vesicles with a strong correlation in expression levels. This suggests that the exosomal RNA can be used to characterize the tumor without having to obtain biopsies of the tumor. A significant increase in the levels of the eight microRNAs was also seen in extracellular vesicles from patients with ovarian cancer, compared to the benign samples. This study demonstrated for the first time that the RNA in extracellular vesicles could be used as a diagnostic marker for cancer and importantly, it could be used to distinguish benign vs. malignant tumors (Taylor and Gercel-Taylor, 2008). A year later, similar results demonstrated that the microRNA in serum extracellular vesicles from lung cancer patients could be used as a diagnostic marker (Rabinowits et al., 2009). It was shown that patients with lung cancer also had more EpCAM-positive extracellular vesicles in their serum, compared to controls. The levels of 12 microRNAs previously found in lung tumor biopsies were detected in both the serum extracellular vesicles and the tumors. Again, a strong correlation in microRNA expression was found between these two sources (Rabinowits et al., 2009). These two studies showed that exosomal microRNAs, while exhibit some unique features, are generally representative of the tumor, suggesting that instead of biopsies, serum extracellular vesicles could be a relatively non-invasive route to profile a tumor.

The RNA content of serum extracellular vesicles from glioblastoma patients has also been investigated for its potential role as a biomarker. Skog et al. (2008) demonstrated mutated version of the epidermal growth factor receptor (EGFR) mRNA,

EGFRvIII, detected in 47% of the tumors in glioblastoma patients, could also be detected in serum-derived extracellular vesicles in 28% of the patients. This mRNA was not detected in the serum extracellular vesicles from healthy controls. After surgical removal of the tumor, the mRNA could no longer be detected in the patients' peripheral circulation, demonstrating the tumor as the source of the extracellular vesicles (Skog et al., 2008). This study showed that the vesicle-associated mRNA could serve as biomarkers, however, the correlation between mRNA expression in the extracellular vesicles and tumor was weaker than that observed in microRNA studies. Most critically, this study demonstrated that mutations in the tumor could be identified within RNA isolated from extracellular vesicles.

Extracellular vesicles are released by many cells, including as reticulocytes, dendritic cells, B cells, T cells, mast cells, macrophages, epithelial cells, and tumor cells (Gallo et al., 2012). Within mast cell lines, 121 microRNAs, including miR-1, miR-15, miR-16, miR-17, miR-18, miR-181, miR-375, lin-4 and let7, were demonstrated within their released extracellular vesicles (Valadi et al., 2007). Other microRNAs, such as miR223 expressed in IL-4-activated macrophages, miR451 in dendritic cells and miR335 in T cells, have been demonstrated in extracellular vesicles from normal cell types (Mittelbrunn et al., 2011; Yang et al., 2011). Based on these observations, most extracellular microRNAs within the peripheral circulation are primarily in extracellular vesicles (Gallo et al., 2012).

CONCLUSIONS

While the use of extracellular vesicles as biomarkers in the clinical setting remains years away, the findings presented within this review demonstrate their significant diagnostic potential. This overview of the RNA cargoes of extracellular vesicles reveals their ability to distinguish benign ovarian tumors from malignant disease. Further, we can obtain and analyze the microRNA profiles of lung and ovarian tumors prior to surgery, we can identify the mutation status of tumors based on a blood specimen, and we can predict patient survival and risk of recurrence.

In the 5 years since our initial demonstration of the diagnostic utility of RNA profiles from extracellular vesicles, new and sensitive techniques have been developed, including deep sequencing and focused microarrays and real time PCR, which can expand the information from transcriptome from the tumor-derived extracellular vesicles. The vesicle platform for diagnostics provides a multiplex approach to studying biomarkers relying on a single marker. This additional genetic information will extend the potential of using circulating vesicular RNAs as biomarkers in diagnostics, but provides biomarkers for with patient stratification, selection of personalized therapies, companion diagnostics and for monitoring therapeutic responses. As early detection is essential for improved patient survival, the utility of cargo analyses of extracellular vesicles will be particularly valuable in identifying asymptomatic patients and stratify patient populations. While microRNA expression profiles have been the primary RNA population investigated, current findings demonstrate the presence of other RNAs that may be important in malignant properties linked with progressive cancer. Current and future studies are addressing the ability of vesicle-associated RNAs to identify the risk for developing cancer, monitoring its progression, and predict its prognosis. The development of their vesicular RNA markers may also serve as valuable tools in companion diagnostics, for assessing clinical trials and defining therapeutic options.

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The complexity, function, and applications of RNA in circulation

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Blood carries a wide array of biomolecules, including nutrients, hormones, and molecules that are secreted by cells for specific biological functions. The recent finding of stable RNA of both endogenous and exogenous origin in circulation raises a number of questions and opens a broad, new field: exploring the origins, functions, and applications of these extracellular RNA molecules. These findings raise many important questions, including: what are the mechanisms of export and cellular uptake, what is the nature and source of their stability, what molecules do they interact with in the blood, and what are the possible biological functions of the circulating RNA? This review summarizes some key recent developments in circulating RNA research and discusses some of the open questions in the field.

Keywords: microRNA, exosomes, microvesicles, cell–cell communication, exogenous RNA

Coordination of the activities of different cells in the body is vital for multi-cellular organisms. For this purpose, at least three different types of intercellular communication systems have evolved. Physical interactions between cells through cell surface protein engagement like that between receptors and ligands are used to transmit signals between close, contacting cells (Schonbeck and Libby, 2001; Chillakuri et al., 2012). Gap junctions can also facilitate the transmission of molecular signals between cells (Valiunas et al., 2005; Wolvetang et al., 2007; Lim et al., 2011). Membrane nanotubes or cytonemes that extend up to 100 μm in length are physical connections that also allow communication between cells that are not immediately adjacent to each other (Gerdes and Carvalho, 2008). Multi-cellular organisms have also developed a network of circulating body fluids to deliver nutrients to cells, remove waste molecules, and transmit signals for "long-distance" cell–cell and organ–organ communication. The combination of the circulation system with central and peripheral neural networks, and other short and medium range communication processes, allows cells and organs in an organism to operate in close concert. Some circulating proteins or peptides are well-known for their roles as signaling molecules. These include hormones such as insulin, growth hormone, prolactin, and many other secreted proteins/peptides. In addition to these well-known examples, other biomolecules such as nucleic acids are also exported from cells and are present in various body fluids (Valadi et al., 2007; Weber et al., 2010), although their potential roles in intercellular signaling have not been confirmed. While the discovery of circulating extracellular nucleic acid is not new, having been described as early as 1948 (Mandel and Métais, 1948), there has been a renewed interest in the function and application of circulating nucleic acids due to several, diverse recent findings. These include the discoveries of fetal DNA in maternal blood (Lo and Chiu, 2012), tumor-derived DNA in circulation (Kohler et al., 2011) and stable regulatory non-coding RNA (ncRNA) in body fluids (Cortez et al., 2011).

RNA is generally considered to be an unstable molecule that is subject to degradation and turnover by ubiquitous RNase activity. However, intact RNAs of different types, including both protein-coding RNA (mRNA) and non-coding RNA (ncRNA) have been detected in circulation (Valadi et al., 2007). Some of these RNAs are likely the result of cell lysis through normal cell turnover and are in the process of degradation, reabsorption, or excretion (Turchinovich et al., 2011). However, the abundance and high stability of some of these RNA molecules, especially the short regulatory RNA, microRNA (miRNA), suggests a different scenario: the possibility of a cell–cell communication system through RNA-mediated signals (Lotvall and Valadi, 2007; Turchinovich et al., 2011; Montecalvo et al., 2012). Furthermore, recent studies have shown that exogenous RNA molecules can enter into circulation through the diet or other means, and can potentially be taken up by cells and thereby alter the cellular transcriptome (Semenov et al., 2012; Wang et al., 2012; Zhang et al., 2012a). Many questions remain unanswered, however, including: what are the sources of endogenous and exogenous RNA molecules in circulation, what is (are) the mechanism(s) by which these RNAs enter into circulation, how are they stabilized there, are they taken up by cells, and how are

they recognized for uptake by cells? Here we review several recent developments in the study of circulating RNAs and their potential functions and possible applications.

EXTRACELLULAR RNA IN BODY FLUIDS

Among all the circulating nucleic acids, miRNAs are probably the most extensively studied. These small molecules are short, ncRNAs involved in regulating the cellular transcriptome and proteome by destabilizing mRNA and/or attenuating protein translation (Filipowicz et al., 2008; Fabian et al., 2010). The interaction between miRNAs and their cognate mRNA targets is mediated by partial sequence complementarity between the two (Fabian et al., 2010). The RNA-induced silencing complex (RISC), which contains a number of proteins including members of the Argonaute family, the RNA recognition motif containing protein TNRC6B, putative DNA helicase MOV10, among others, is involved in the miRNA–mRNA interaction (Chendrimada et al., 2007).

MicroRNAs were first detected in the blood plasma and serum in 2008 (Chen et al., 2008; Chim et al., 2008; Hunter et al., 2008; Lawrie et al., 2008; Mitchell et al., 2008; Skog et al., 2008) and have subsequently been detected in many other different body fluids including urine, saliva, tears, and breast milk (Park et al., 2009; Hanke et al., 2010; Weber et al., 2010). The total amount and the concentration of individual miRNAs vary widely among the different fluid types (Weber et al., 2010). The potential of circulating miRNAs as biomarkers has been extensively investigated in recent years. miRNAs make good biomarker candidates for several reasons. They are stable in body fluids, which are easy to obtain from patients, they can be easily measured with high sensitivity because they are amplifiable, and some markers and sets of markers have shown profiles associated with specific pathologies. The changes in miRNA levels may be used as a diagnostic tool for several types of cancer (Mitchell et al., 2008; Taylor and Gercel-Taylor, 2008; Park et al., 2009; Hanke et al., 2010; Huang et al., 2010; Zhao et al., 2010; Moussay et al., 2011; Roth et al., 2011), cardiac damage (Ai et al., 2010; Corsten et al., 2010; Tijsen et al., 2010; Wang et al., 2010a), muscular injury and pathologies (Laterza et al., 2009; Mizuno et al., 2011; Vignier et al., 2013), diabetes (Chen et al., 2008; Erener et al., 2013), liver injury (Laterza et al., 2009; Wang et al., 2009, 2013; Starkey Lewis et al., 2011), among others.

The literature reporting correlations with human disease and pathologies has grown rapidly in the past few years. The number of papers, for example, on changes in circulating miRNAs in cases of myocardial infarction (MI) and cardiac diseases alone has recently exploded. We estimate that more than 30 reports on this specific topic alone have been published since 2009. Some of the circulating miRNAs identified in MI and cardiac diseases have been summarized in **Table 1**.

Efforts have also been made to use circulating miRNAs as a prognostic tool in response to therapeutic treatments (Ma et al., 2012). Furthermore, the possibility of using miRNAs as therapeutics themselves has been considered. It is a very exciting prospect if these molecules can be packaged and released into circulation as stable and perhaps even targeted packages. Applications in this area have taken different approaches. For example, creating synthetic miRNAs designed to target specific disease-associated genes, as used by Suckau et al. (2009) to treat heart failure. In this case, viral vectors containing short hairpin RNAs were administered intravenously in rats to reduce the level of a key regulator of cardiac calcium homeostasis, phospholamban (Suckau et al., 2009). The use of antagomirs to target miRNAs that are upregulated in pathological states has also been demonstrated. For example, an antagomir of miR-133 caused sustained cardiac hypertrophy in mice (Care et al., 2007); antagomirs targeting miR-1 and let-7f were successfully used in rats to extend neuroprotection after ischemic stroke (Selvamani et al., 2012); mice treated with high doses of an antagomir for miR-126 showed reduced ischemia-induced angiogenesis (van Solingen et al., 2009); and a miR-206 antagomir increased brain-derived neurotrophic factor levels and improved memory function in rats with Alzheimer's disease (Lee et al., 2012). Furthermore, a treatment for hepatitis C virus (HCV) chronic infection using an antagomir for the liver-specific miR-122 is currently in clinical trial (Janssen et al., 2013).

RNA EXPORT, PACKAGING, AND UPTAKE

While extracellular RNAs were first thought to be molecular debris released by cell lysis (Turchinovich et al., 2011), the difference in the spectrum of RNA within cultured cells and the external medium argues strongly for selective export of some fraction of the circulating RNAs (Wang et al., 2010b). It remains unclear how specific RNAs are targeted for export, and if some are simply released with the cytosol through bulk exocytosis. As free RNA molecules are very likely to be degraded outside the cell, it is generally assumed that circulating RNAs are packaged in some form to avoid RNase degradation. Lipid vesicles, such as exosomes or microvesicles are one type of packaging system used by secreted RNAs (Valadi et al., 2007; Camussi et al., 2011). Exosomes, which are approximately 30–100 nm in diameter, are formed by fusion of multivesicular bodies with the plasma membrane. Microvesicles are larger (100 nm–1 μm) and formed by blebbing of the plasma membrane. Both types of vesicles can contain a number of protein and RNA molecules, although the composition varies widely with the origin of the vesicle (Muller, 2012; Huang et al., 2013).

In addition to lipid vesicles, it has been demonstrated that miRNA in the extracellular environment can complex with high-density lipoproteins (HDL) and at least two RNA-binding proteins *in vivo*, Argonaute 1 (AGO1) and Argonaute 2 (AGO2), and with nucleophosmin 1 (NPM1) *in vitro* (Wang et al., 2010b; Arroyo et al., 2011; Turchinovich et al., 2011; Vickers et al., 2011; Turchinovich and Burwinkel, 2012; Wagner et al., 2013). Furthermore, the spectrum of miRNA associated with HDL depends on the health status of an individual (Vickers et al., 2011). However, it is not known if there are other RNA-binding proteins that form similar complexes to protect miRNAs or other types of RNA in circulation.

Besides miRNAs, lipid vesicles also contain a large number of protein-coding RNAs. Roughly 1300 different mRNA transcripts have been identified in lipid vesicles derived from human or mouse cell lines (Valadi et al., 2007). Interestingly, the mRNA composition and abundance in the exosomes differ from that of the original cell, which suggests the contents in the lipid vesicles are selectively packaged rather than included indiscriminately. The levels

Table 1 | Circulating miRNAs with biomarker potential to diagnose acute myocardial infarction (AMI).

Candidate miRNA(s)	Organism	Source	Sample size	Correlation	Reference
1, 133a, 133b, 499-5p	Human; mouse	Plasma	33 patients, 17 healthy; 4–5 AMI and control mice	Elevated levels distinguished cardiac damage patients from healthy subjects. Positive correlation with TnI[a]	D'Alessandra et al. (2010)
1	Human; rat	Serum	31 patients, 20 healthy; 8 AMI rats, 8 controls	High expression was associated with CK-MB[b]; positive correlation with myocardial infarct size	Cheng et al. (2010)
1	Human	Plasma	93 patients, 66 healthy	Up-regulation correlated with QRS[c] duration	Ai et al. (2010)
208a	Human	Plasma	33 patients, 30 healthy	Present only in patients and showed to be more specific and sensitive than TnI[a]	Wang et al. (2010a)
208b, 499	Human	Plasma	32 patients, 36 healthy	High levels correlated with TnT[d] and CPK[e]	Corsten et al. (2010)
499	Human	Plasma	9 patients, 10 healthy	Elevated expression correlated positively with CK-MB[b] activity	Adachi et al. (2010)
1, 133a	Human	Serum	29 patients, 42 non-AMI	Increased levels showed correlation with TnT[d]	Kuwabara et al. (2011)
133, 328	Human	Plasma, whole blood	51 patients, 28 healthy	High levels correlated with TnT[d]	Wang et al. (2011)
30c, 145, 1291, 663b	Human	Whole blood	20 patients, 20 non-AMI	Elevated levels of 30c and 145 correlated with TnT[d]; 1291 and 663b distinguished patients from healthy subjects	Meder et al. (2011)
208b, 499	Human	Plasma	510 patients, 87 healthy	Increased levels correlated with peak concentrations of CPK[e] and TnT[d]	Devaux et al. (2012)

[a]Cardiac troponin I; [b]creatine kinase muscle b; [c]QRS complex in an electrocardiogram; [d]cardiac troponin T; [e]creatine phosphokinase.

of circulating miRNAs as well as several mRNAs in circulation have been shown to be associated with stages and types of cancers (Sueoka et al., 2005; Mitchell et al., 2008; Taylor and Gercel-Taylor, 2008; Ohshima et al., 2010; Zhao et al., 2010; Zhang et al., 2012b), pregnancy (Zhong et al., 2008; Rajakumar et al., 2012; Wu et al., 2012; Zhao et al., 2012; Higashijima et al., 2013), and drug-induced liver injury (Miyamoto et al., 2008; Laterza et al., 2009; Wang et al., 2009, 2013; Bala et al., 2012; Su et al., 2012; Okubo et al., 2013).

The idea of using RNA as a mode of intercellular communication is particularly interesting for several reasons. First, RNA can carry information in a simple and efficient way (such as coding for proteins in mRNA), and, second, it can coordinate cellular activity in a fundamental and essential manner (such as microRNA-mediated transcriptome and proteome regulation). It has been demonstrated that cells can export miRNAs, which can be transferred to and are functional in recipient cells (Kosaka et al., 2010; Zhang et al., 2010; Hergenreider et al., 2012). However, it is inherently difficult to test definitively the functioning of an RNA-based communication system *in vivo*. Conditioned media exchange and co-culture experiments have been used to investigate cell–cell communications, and these methods have been adapted to investigate the possibility of RNA uptake by cells (Liu et al., 2010; Vencio et al., 2011; Yang et al., 2011). Some exosome-derived mRNAs have been shown to be functional in recipient cells (Valadi et al., 2007; Ismail et al., 2013). Furthermore, Ratajczak et al. (2006) showed that microvesicles derived from embryonic stem cells can enhance the survival and expansion of mouse hematopoietic progenitor cells, and are enriched in stem cell related transcripts including Oct-4, Rex-1, Nanog, SCL, and GATA-2. Reporter assays have shown that exosome-derived miRNAs can silence transcripts in recipient cells (Lotvall and Valadi, 2007; Valadi et al., 2007; Camussi et al., 2011; Kogure et al., 2011; Mittelbrunn et al., 2011). While evidence suggests that cultured cells can transfer packaged RNA molecules, it is difficult to demonstrate this as a general mechanism *in vivo*. One of the most important remaining biochemical questions is how the RNA in circulation is protected, packaged, and delivered to and recognized by their targets. The current lack of knowledge about these questions makes the study of the biological functions of circulating RNA both important and difficult.

Besides protein-coding RNAs and miRNAs, there are other more abundant types of regulatory RNA molecules, such as large intergenic ncRNAs (lincRNAs) and small nucleolar RNAs (snoRNAs) in extracellular vesicles (Huang et al., 2013). A recent characterization of neuronal exosomes found RNAs derived from repeat sequences, as well as mRNA and several types of small RNAs. While a majority of small RNA was tRNA (90%), other types of small RNAs including miRNA, snoRNA, and small cytoplasmic RNA (scRNA) were also detected (Bellingham et al., 2012). Since both mRNA and miRNA can be taken up and utilized by cells (see earlier paragraph), there is no reason to think that other types of RNA in circulation do not also have functional implications when they are taken up by cells. However, there is currently no direct experimental evidence to support this functional possibility for other types of RNA in circulation.

METHODS USED IN STUDYING RNA IN CIRCULATION

Microarray, qPCR (quantitative polymerase chain reaction), and sequencing-based approaches are the three major platforms that have been used for miRNA and mRNA profiling. All of these have been widely and successfully applied in assessing the spectra of mRNA and miRNA in cells and tissues. However, due to the relatively low concentration of RNA present in circulation, comprehensive measurement of any circulating RNA is difficult to do accurately and consistently. For miRNAs, the difficulties are compounded by the short lengths of the RNA molecules and the consequent sequence specificity of hybridization free energies. At the moment, the most commonly used method for miRNA profiles is qPCR because of its sensitivity and the limited number of known miRNAs (on the order of 1000 sequences). This method has significant drawbacks as well because of the specificity and differential amplification efficiency of the designed primers. This technique has also been applied to measure the level of specific mRNA sequences in circulation (Wang et al., 2013). Although microarrays have been the gold standard for transcriptome analysis in cells and tissues, the low concentration of RNA in circulation, and the sequence similarity of these short nucleic acid molecules make microarray analysis unsuitable in most cases. The development of next generation sequencing (NGS) has shown promise in measuring circulating RNAs. This platform not only solves some of the miRNA measurement problems related to short and highly similar sequences of miRNAs, but it can provide measurements on a broad spectrum of RNA in a single experiment. Because it does not rely on pre-designed probes or primers, NGS also allows the identification of novel RNA sequences, such as isomirs and exogenous RNAs in circulation (Lee et al., 2010; Wang et al., 2010a, 2012; Semenov et al., 2012). One difficulty with this method is the inherent bias in the preparation of the sequencing libraries. For this reason, absolute measurements of circulating RNA are very difficult, but when carefully done, comparative measurements can be reliable.

EXOGENOUS RNA IN CIRCULATION

Recent evidence, which we discuss below, suggests that RNAs in circulation are derived not only from cells within the human body, but also from foreign organisms and viruses (Meckes et al., 2010; Pegtel et al., 2010). It has been shown that exogenous RNAs find their way from the outside environment, through diet or from the complex human microbiome (Zhang et al., 2012a). Little is known about how exogenous RNAs are taken up by the gut epithelium from the environment, and there is only very limited and circumstantial evidence so far that these RNAs are functional in the body. However, in keeping with the substantial and growing body of evidence that some human circulating RNA are functional, the possibility that exogenous RNAs, found in the circulation can be taken up and exert specific functions in recipient cells, raises several interesting issues.

A recent report by Zhang et al. (2012a) indicated that exogenous miRNAs ingested from food (rice in this case) could enter into the human circulation and be taken up by and actually function in cells. They found that certain plant miRNAs, including miR-168a, an abundant miRNA in rice, is detectable in human plasma, as well as in plasma from other animals eating a diet containing plant

material. However, many questions such as how the RNA survives the cooking process and the harsh environment of the digestive tract, as well as how these biomolecules can pass through the gut epithelium, remain largely unanswered. Using Caco-2 cells transfected with synthetic rice miR-168a, Zhang et al. (2012a) found that transfected miR-168a can be packaged by cells into microvesicles and released into culture medium. Furthermore, their work suggests that the microvesicles containing exogenous miRNA can be taken up by cells and interact with endogenous transcripts both *in vitro* and *in vivo*. Mature miR-168a sequence has significant sequence homology with a liver-expressed transcript, low-density lipoprotein receptor adaptor protein 1 (LDLRAP1) transcript, raising the possibility that it can suppress the expression of LDLRAP1 through the action of the RISC complex. Indeed, mice fed a rice-containing diet have lower level of LDLRAP1 gene expression than mice fed a normal chow diet, suggesting that miR-168a derived from the rice can affect the level of an endogenous mouse transcript (Zhang et al., 2012a).

Recent work by our lab and others have systematically characterized the composition of RNAs in circulation and revealed that some of the circulating RNAs are derived from a variety of exogenous organisms, including the microbiome (Semenov et al., 2012; Wang et al., 2012, 2013). Using a map-and-remove strategy to analyze small RNA-seq data (after removing sequences mapped to human miRNAs, transcripts, and genomic sequences first), reveals that a significant portion of the total reads are of non-human origin. While some of these exogenous RNAs are derived from the diet, the majority of them are of either fungal or bacterial origins (Wang et al., 2012). The sequences detected implicate a wide range of bacterial phyla, including many already known to reside in the gut microbiome. Although there were not significant differences in the plasma RNA spectra between healthy individuals and those with colorectal cancer or ulcerative colitis, differences can be detected in the plasma of people eating different diets, such as a corn-based diet versus a rice-based diet (Wang et al., 2012).

While these results raise the interesting possibility that exogenous RNAs taken up from the environment can alter gene expression in the cells of an organism, many questions remain unanswered. How these RNAs pass through the body's epithelial lining barrier and enter the circulation, for example, is still completely unclear. Because of the relatively low levels of exogenous circulating RNA, it seems likely that if the packages containing exogenous RNAs exert a significant biological effect on the body's cells they must be effectively targeted in some way to specific cells or tissues.

Since some miRNAs are highly conserved in metazoans (Shi et al., 2012), the finding of diet derived exogenous RNA in circulation raises the interesting possibility that there are RNA-based processes that either induce or avert the development of diseases in humans arising from foreign RNA. For example, changing the levels of certain highly conserved miRNAs, such as miR-21, which has solid pro-proliferation activity (Sayed et al., 2008; Yao et al., 2009), and miR-150 and miR-146, which have strong association with inflammation activities (Sheedy and O'Neill, 2008; Sonkoly et al., 2008; Schmidt et al., 2009; Quinn and O'Neill, 2011; Zhong et al., 2012), by uptake of exogenous RNAs with similar activities may affect the initiation and progression of certain diseases.

Although promising both as biomarkers and potential functional impacts, such as for therapeutics, there is still a great deal to be learned about RNA in circulation. This newly recognized class of circulating molecules has the potential to be deeply involved in the symbiotic functioning of the human body and its microbiome. We currently know very little about the potential mechanisms of entry of RNAs into circulation, their mechanisms of action, their packaging and export process, and their targeting and uptake by cells. Technical improvements and standardization in measuring the levels of these RNAs as well as new model experimental systems are needed in order to explore the many facets of the transport, targeting, and function of these RNAs. In summary, the health impact of RNAs in circulation seems likely to be significant, but the field is just beginning to be explored and the investigation of the area of the quantitative characterization and specific biological functions of exogenous RNA is even earlier in its development.

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Circulating miRNAs: cell–cell communication function?

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Nuclease resistant extracellular miRNAs have been found in all known biological fluids. The biological function of extracellular miRNAs remains questionable; however, strong evidence suggests that these miRNAs can be more than just byproducts of cellular activity. Some extracellular miRNA species might carry cell–cell signaling function during various physiological and pathological processes. In this review, we discuss the state-of-the-art in the field of intercellular miRNA transport and highlight current theories regarding the origin and the biological function of extracellular miRNAs.

Keywords: miRNA, biofluids, cell communication, argonaute proteins, exosomes, microvesicles, HDL, biomarkers

INTRODUCTION

MicroRNAs (miRNAs), a subclass of short non-coding RNAs, are expressed in all eukaryotic cell types and mediate posttranscriptional regulation of gene expression (Ambros, 2004; Bartel, 2004). The action of miRNAs is mediated by its binding to the 3′-untranslated region (3′UTR) of the target mRNAs and thus regulating targeted mRNAs stability and protein synthesis (Ambros, 2004; Bartel, 2004). There have been more than 2000 different human miRNA species discovered so far and this amount is increasing (http://www.mirbase.org/). In humans, endogenous miRNAs regulate at least 30% of genes (Lewis et al., 2005) and, thus, coordinate key cellular processes including proliferation, DNA repair, differentiation, metabolism, and apoptosis (Ambros, 2004; Bartel, 2004; Croce and Calin, 2005). Deregulation of certain miRNAs' expression in the cell was consistently observed during various pathologies including cancers (Lu et al., 2005). Every miRNA has a unique nucleotide sequence and unique expression pattern in certain cell type (Lu et al., 2005; Landgraf et al., 2007).

Several years ago, significant amounts of miRNA were detected in all biological fluids including blood plasma, urine, tears, breast milk, amniotic fluid, cerebrospinal fluid, saliva, and semen [reviewed in Turchinovich et al. (2012)]. These extracellular circulating miRNAs are surprisingly stable and survive unfavorable physiological conditions such as extreme variations in pH, boiling, multiple freeze thaw cycles, and extended storage. In contrast to miRNAs, common RNA species like mRNA, rRNA, and tRNA are degraded within several seconds after being placed in nuclease rich extracellular environment (Chen et al., 2008; Turchinovich et al., 2011). In their pioneering work Valadi et al. reported that cells in culture transport intracellular miRNAs into the extracellular environment by exosomes (Valadi et al., 2007). This finding was confirmed in many subsequent reports and mechanisms of miRNA transfer have been suggested. In this review, we discuss the state-of-the-art in the field of intercellular miRNA transport, and particularly the mechanisms involved in this process. We will highlight actual theories regarding the origin and the biological function of extracellular circulating miRNAs in body fluids.

miRNA BIOGENESIS AND MODE OF ACTION

All miRNAs are originally generated in the cell nucleus as long primary miRNAs (pri-miRNAs) transcripts containing 5′cap and a 3′polyA tail (Lee et al., 2004). The pri-miRNAs are further cleaved by a microprocessor complex consisting of Drosha and DGCR8 proteins into ∼70 nt hairpin precursor miRNAs (pre-miRNAs) (Lee et al., 2003; Landthaler et al., 2004). On the next step pre-miRNAs are actively transported into the cytoplasm (Yi et al., 2003) where they cleaved into ∼22 bp miRNA/miRNA* duplexes by Dicer/TRBP enzyme complex (Zhang et al., 2002; Chendrimada et al., 2005). Finally, miRNA/miRNA* duplexes separate leaving one of strands associated with an Argonaute (AGO) protein (Okamura et al., 2004; Ender and Meister, 2010). This AGO associated "mature" miRNA strand sequence-specifically binds to complementary mRNAs, promoting their decay and inhibiting translation (**Figure 1**). Surprisingly, some miRNAs activated both translation and steady state levels of target mRNAs during cell cycle arrest in quiescent mammalian cells and Xenopus oocytes (Vasudevan et al., 2007; Truesdell et al., 2012)—a mechanism which is yet to be explained.

Four human AGO proteins (AGO1, AGO2, AGO3, and AGO4) have been described so far; all of them can mediate both translation repression of mRNA on ribosomes and mRNA decay in P-bodies. However, only AGO2 is capable of directly cleaving mRNA in the cytoplasm (Hock and Meister, 2008). AGO carrying

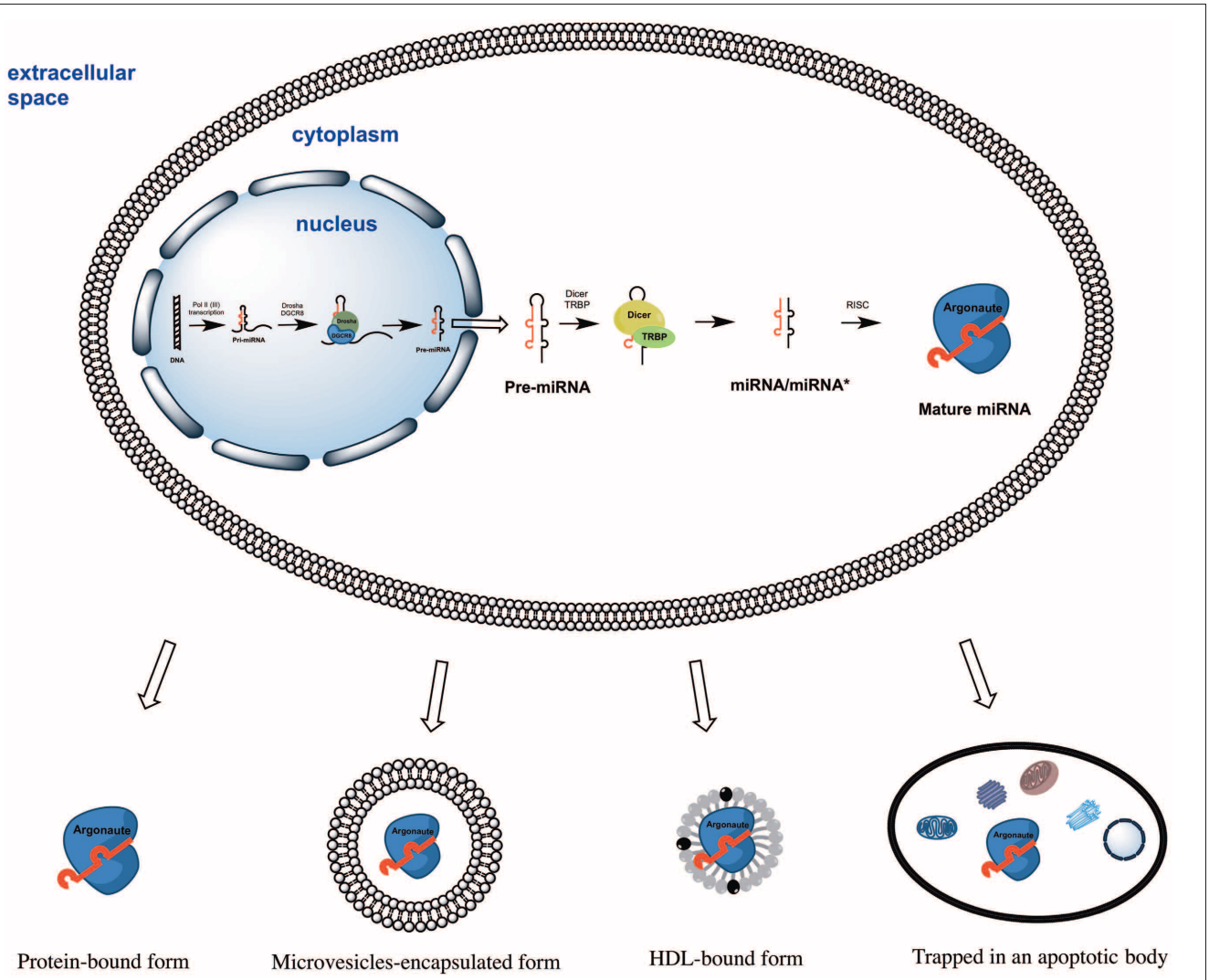

FIGURE 1 | The biogenesis of miRNAs starts in the cell nucleus with generation of primary miRNAs (pri-miRNAs) transcripts. Pri-miRNAs are cleaved by the microprocessor complex Drosha/DGCR8 into shorter miRNA precursors (pre-miRNA). The later are transported to the cytoplasm and further cut by the endonuclease Dicer into ~22 nt miRNA/miRNA* duplexes. Finally, one of the miRNA/miRNA* strands is incorporated into a protein of the Argonaute family (AGO1, AGO2, AGO3, or AGO4). The mature miRNA strand eventually serves as the guide for RISC-mediated mRNA targeting resulting in either mRNA cleavage or translational interference. Extracellular miRNA can be either solely AGO protein-associated or additionally encapsulated into apoptotic bodies, microvesicles, and HDL particles.

miRNA forms the RNA-induced silencing complex (RISC) by binding to GW182 via C-terminal domain of AGO protein (Lian et al., 2009; Braun et al., 2013). The RISC can be localized (1) diffusely in the cytoplasm; (2) in the dense cytoplasmic structures called GW- or P-bodies—the main localization for mRNAs which undergo decapping, deadenylation, and degradation (Kedersha et al., 2005). Importantly, only 1% of cytoplasmic AGO2 was found in P-bodies whereas the majority was diffusely distributed elsewhere in the cytoplasm (Leung and Sharp, 2013). When studying extracellular miRNA the researchers must take into account the particularities of miRNA biogenesis, its mode of action and localization in the cell. Importantly, neither mature miRNAs nor pre-miRNAs were ever found within the cells in non-protein bound forms.

EXTRACELLULAR miRNA

The pioneering observations that mature miRNAs are also present in cell-free blood plasma and serum was made in the year of 2008 by several independent research groups (Chen et al., 2008; Chim et al., 2008; Lawrie et al., 2008; Mitchell et al., 2008). Later, the existence of extracellular circulating miRNA in all other biological fluids was confirmed (Park et al., 2009; Hanke et al., 2010; Kosaka et al., 2010b; Weber et al., 2010). The mechanism which is responsible for the nuclease resistance of miRNA outside the cell remained enigmatic for quite a long period; however, the presence of miRNAs in the exosomes exported by cells in culture has been known before (Valadi et al., 2007). The theory that extracellular miRNA is protected by encapsulation into membrane-vesicles emerged after Hunter et al. detected miRNAs in peripheral blood

microvesicles (Hunter et al., 2008). Together with the evidence that exchange of miRNA (and also mRNA) between cells can be accomplished through exosome-mediated transfer (Valadi et al., 2007) the finding of Hunter and co-authors led to a revolutionary hypothesis—the existence intercellular and inter-organ communication system in the body by means of microvesicles (MVs) encapsulated miRNAs.

In 2011, the assumption that only membrane-vesicles encapsulated miRNAs are present in biological fluids was challenged by two independent research groups who demonstrated that 90–99% of extracellular miRNA are MVs-free and associated with proteins of the AGO family both in blood plasma/serum and cell culture media (Arroyo et al., 2011; Turchinovich et al., 2011). The remarkable stability of AGO2 protein in protease rich environment elegantly explained the resistance of associated miRNAs in nucleases containing biological fluids (Turchinovich et al., 2011).

Since then, accumulated reports have consistently shown that extracellular miRNAs can be shielded from RNAse degradation by: (1) packaging in apoptotic bodies, shedding vesicles and exosomes; or (2) solely by complexing with AGO proteins (reviewed in Cortez et al., 2011; Chen et al., 2012; Turchinovich et al., 2012) (**Figure 1**). Some miRNA species were also found in purified fractions of high-density lipoprotein (HDL) from human plasma (Vickers et al., 2011). The existence of HDL-associated miRNAs in the blood circulation have been recently confirmed by Dimmeler's group (Wagner et al., 2013), however, the analysed HDL-miRNAs constituted only minor proportion the total circulating miRNAs. Finally, synthetic miRNA can be protected from the degradation by RNAses when mixed with purified nucleophosmin 1 (NPM1) protein *in vitro* (Wang et al., 2010). Although NPM1 was indeed exported by cells in culture together with miRNA, neither intracellular nor extracellular miRNA association with NPM1 has been found *in vivo* (Wang et al., 2010; Turchinovich et al., 2011).

THE THEORY OF CELL–CELL COMMUNICATION VIA EXTRACELLULAR miRNA

The presence of miRNA in the extracellular environment ignited the hypotheses that cells selectively release miRNAs which mediate cell–cell signaling via paracrine or even endocrine routes (Valadi et al., 2007; Cortez et al., 2011; Chen et al., 2012). However, circulating miRNAs bound solely by AGO proteins are apparently non-specific remnants resulting from physiological activity of the cells and cell death (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012). Thus, both AGO2 protein and miRNAs remain stable for prolonged periods after the parental cells die. Furthermore, there are no indications of either active release of AGO-miRNA ribonucleoprotein complexes from cells or their uptake by recipient cells in mammals. The opinion that many extracellular miRNAs are released non-selectively after cell death also accords with the fact that upon toxicity in certain tissues the level of tissue-specific miRNAs in the blood increases (Laterza et al., 2009; Corsten et al., 2010; Lewis and Jopling, 2010; Zhang et al., 2010a; Pritchard et al., 2012).

At the same time, a number of independent research groups have demonstrated that extracellular miRNAs entrapped within apoptotic bodies and exosomes can be transferred to recipient cells, alter gene expression and mediate functional effects (Valadi et al., 2007; Skog et al., 2008; Kosaka et al., 2010a; Pegtel et al., 2010; Mittelbrunn et al., 2011; Montecalvo et al., 2012). Patterns of mRNAs in exosomes and their donor cells correlate poorly, suggesting specific sorting of miRNA "for export" (Valadi et al., 2007; Skog et al., 2008; Collino et al., 2010; Pigati et al., 2010; Mittelbrunn et al., 2011). The mechanism behind this sorting needs to be investigated in more detail, however, certain clues may lie within the fact that miRNAs, GW182 and AGO proteins co-localize in the compartments which are strongly linked with endosomes and multivesicular bodies (MVBs) (Gibbings et al., 2009). Because exosomes are formed in the MVB and also contain high levels of GW182, these observations may be important findings for the understanding of the loading of RNA into exosomes (Gibbings et al., 2009). It is feasible that AGO-bound miRNAs which reside in the MVBs become encapsulated randomly into the newly formed exosomes. The fact that different miRNAs might possess different decay kinetics could partially account for the fact that certain miRNAs were expressed at higher levels in extracellular MVs than in the parental cells (Bail et al., 2010; Krol et al., 2010). Another possible methodological bias which has to be addressed when comparing extracellular versus intracellular miRNA profiles include preferential loss of certain miRNAs during extraction from samples with very low RNA content (e.g., extracellular fluids) (Kim et al., 2012). Nevertheless, there is mounting evidence that cells selectively package certain miRNAs into MVs and actively secrete them. However the exact mechanisms of vesicular miRNAs sorting and secretion are yet to be discovered.

Collino and co-authors have demonstrated that MVs exported by human bone marrow derived mesenchymal stem cells (MSCs) and liver resident stem cells (HLSCs) indeed contained both miRNAs and AGO2 protein (Collino et al., 2010). Furthermore, selected patterns of miRNAs in MVs suggested their specific compartmentalization. Bioinformatics analysis revealed that MV-expressed miRNAs could be involved in organ development, cell survival, cell differentiation, and regulation of the immune system. The authors further showed that pre-treatment with the inhibitor of actin polymerization cytochalasin B significantly reduced the release of MVs from both MSCs and HLSCs (Collino et al., 2010).

At the same time several research groups have further demonstrated that exosomal miRNA is released via ceramide-dependent secretory pathway which is controlled by the enzyme of ceramide biosynthesis neutral sphingomyelinase (nSMase) (Kosaka et al., 2010a; Kogure et al., 2011; Mittelbrunn et al., 2011). nSMase mediates hydrolysis of sphingomyelin to form ceramide and is indispensable for budding of intracellular vesicles into the MVB (Trajkovic et al., 2008). Inhibition of nSMase2 with the small molecule compound GW4869 and the appropriate siRNA decreased both exosomes and miRNA secretion (Kosaka et al., 2010a). Consistently, ectopic overexpression of nSMase2 resulted in higher amounts of extracellular miRNAs (Kosaka et al., 2010a). An independent group of authors further demonstrated that inhibition of nSMase does not alter intracellular miRNA levels but reduces miRNA in secreted exosomes (Kogure et al., 2011). While

these data emphasize the importance of the MVBs and sphingomyelins for miRNA excretion, how exactly the selection and the loading of specific miRNA into exosomes occurs remains unknown. Finally, cell targeting has been hypothesized to be mediated by both exosomal surface proteins and receptors on the acceptor cells. The putative mechanisms of membrane vesicles uptake can be either direct membrane fusion or endocytosis (Thery et al., 2002; Cocucci et al., 2009; Simons and Raposo, 2009).

As it was mentioned before, some extracellular miRNA was co-purified with HDL from human blood (Vickers et al., 2011). HDL particles were able to deliver miRNAs to recipient cells and mediate direct targeting of mRNA reporters, while contrary to exosomes, cellular export of HDL associated miRNAs was negatively regulated by nSMase2. In addition, HDL mediated miRNA delivery was dependent on a cell surface HDL receptor SRBI, which binds HDL and mediates the uptake of cholesteryl ester from HDL. Because small RNAs can easily complex with zwitterionic liposomes it was hypothesized that HDL could simply bind to extracellular plasma miRNAs through divalent cation bridging (Vickers et al., 2011). This hypothesis, however, assumes the existence of naked mature miRNAs in the cell. Furthermore, targeting of mRNA by miRNA requires the latter to be associated with one of the AGO proteins. Importantly, neither formation of mature miRNAs nor their existence apart from AGO proteins has been found *in vivo*. It is, therefore, feasible that mature miRNAs in exosomes, MVs and HDL particles can be also bound to AGO proteins. Interestingly, recent evaluation of the HDL-bound miRNAs isolated from human blood revealed that the concentration of the most abundant HDL-bound miRNA miR-223 contributed to only 8% of the total miR-223 in the circulation (Wagner et al., 2013). Furthermore, no significant uptake of HDL-bound miRNAs was observed into endothelial cells, smooth muscle cells or peripheral blood mononuclear cells (Wagner et al., 2013).

EXTRACELLULAR miRNAs ASSOCIATED WITH MICROVESICLES

Two different types of extracellular MVs described so far are shedding vesicles and exosomes. Exosomes are 30–100 nm in size, formed within the MVBs and released upon fusion of MVBs with the plasma membrane (Thery et al., 2002). Unlike exosomes, shedding vesicles are formed by outward budding and fission of the plasma membrane and can vary in size from 0.1 to 1 μm (Cocucci et al., 2009). Both types of MVs contain various proteins, mRNAs and miRNAs in a proportion depending on the cell from which they originate (Simons and Raposo, 2009; Muralidharan-Chari et al., 2010). Due to the similar size of exosomes and small shedding vesicles, it is impossible to completely separate them using differential ultracentrifugation or other physical methods. It has to be mentioned that most current reports describing isolation of MVs-associated extracellular miRNA rely on using solely ultracentrifugation. As a result, such experiments inevitably characterize miRNAs in a mixed population of two MVs types. However, researchers often refer to the miRNA isolated from ultra-centrifuged MVs to as "exosomal" miRNA. To our knowledge, there are no reports describing specifically "shedding vesicles" miRNA or specifically "exosomal" miRNA.

Exosomes can have many cell-type specific functions which were attributed predominantly to exosomal surface proteins. For example, Fas ligand located on the surface of tumor exosomes induces apoptosis in T lymphocytes (Abusamra et al., 2005). The biological function of the exosomal RNA *in vivo* remains questionable. However, numerous experiments performed on cultured cells have demonstrated that exosomal miRNAs can affect gene expression in the recipient cells and mediate a physiological response. A growing body of evidence that miRNAs can play a role in intercellular communication suggests the paracrine function of miRNAs which are packed in extracellular MVs (reviewed in Cortez et al., 2011; Chen et al., 2012; Turchinovich et al., 2012). The Internet-based database, ExoCarta (http://www.exocarta.org/), currently lists 463 miRNAs which were found in exosomes from various cells. In addition, the phenomenon of cell-cell communication via extracellular miRNAs has been shown in multiple cell culture models (**Table 1**).

Collino and co-authors incubated murine tubular epithelial cells (mTEC) with MSC-derived MVs and confirmed the transport of selected miRNAs by qRT-PCR (Collino et al., 2010). The abundance of extracellular miRNAs in acceptor cell increased progressively and correlated with the extent of MV internalization. Additionally, incubation of mTEC with MSC-derived MVs resulted in the reduction of proteins known to be targeted by some of the enriched miRNAs found in MVs including: PTEN (targeted by miR-21), cyclin D1 (targeted by miR-100, miR-99a, and miR-223) and Bcl-2 (targeted by miR-34, miR-181b, and miR-16).

Microvesicular miRNA from macrophages have been shown to enhance the invasiveness of breast cancer cells in culture (Yang et al., 2011). Specifically, macrophages activated by treatments with IL-4 secreted exosomes packed with miR-223 and were able to promote migration of SKBR3 and MDA-MB-231 breast cancer cells in a transwell invasion assay. Blocking miR-223 with antisense oligonucleotides prevented the observed increase of invasion capacity. In addition, (1) miR-223 targeted Mef2c mRNA level was reduced in the exosome-treated cells, and (2) the expression of β-catenin in the nucleus increased. Based on their observations, the authors suggested that miR-223 was transferred from macrophages to breast cancer cells via exosomes where it affected the Mef2c-β-catenin pathway leading to invasiveness of the breast cancer cells. For the first time it was suggested that prevention of the exosomal communication between macrophages and breast cancer cells may help preventing cancer metastasis and being potential target for cancer therapy (Yang et al., 2011).

The capacity of exosomal miRNA to facilitate viral infection was reported by Pegtel and co-authors. After infection of B-lymphoblastoid cells with Epstein-Barr virus (EBV) the viral-specific miRNAs (EBV-miRNAs) were secreted via exosomes and affected the expression of EBV-miRNA target gene CXCL11 in co-cultured non-infected cells (Pegtel et al., 2010). Viral miRNAs were present in both B-cell and non-B-cell fractions isolated from infected patients, while viral DNA was restricted to the circulating B-cell population. This indicated that viral

Table 1 | Reports demonstrating cell–cell transfer of extracellular miRNA.

Donor cells	Acceptor cells	miRNA	Impact	References
MC/9, HMC-1	MC/9 and HMC-1	Multiple miRNAs	Not investigated	Valadi et al., 2007
THP-1	HMEC-1 cells	Overexpressed miR-150	Reduction of miR-150 target c-Myb Increase in HMEC-1 cells migration	Zhang et al., 2010b
EBV-infected B-cells	Dendritic cells, HeLa	Mature EBV-encoded miRNAs	Repression of EBV-miRNAs target CXCL11	Pegtel et al., 2010
COS-7, HEK 293	COS-7, HEK 293	Overexpressed luciferase siRNA	Repression of luciferase reporter gene	Kosaka et al., 2010a
COS-7	PC-3M (metastatic prostate cancer cells)	Overexpressed miR-146a	Decrease in proliferation Repression of miR-146a target ROCK1	Kosaka et al., 2010a
Mesenchymal stem cells	Tubular epithelial cells (mTEC)	Multiple miRNAs	Repression of PTEN, cyclin D1, Bcl-2 proteins	Collino et al., 2010
Macrophages	SKBR3 and MDAMB-231 breast cancer cells	Endogenous miR-223	Reduction of miR-223 targeted Mef2c mRNA Increased migration of SKBR3 and MDA-MB-231 cells	Yang et al., 2011
J77 T-cells, primary T-cells	Raji B cells (antigen presenting cells)	Overexpressed miR-335, endogenous miR-335, miR-92	Repression of miR-335 targeted 3′-UTR of SOX4 gene	Mittelbrunn et al., 2011
Hep3B	Hep3B	Endogenous miRNAs enriched in exosomes	Repression of putative miRNAs target TAK1	Kogure et al., 2011
Primary mesenchymal stromal cells	Primary astrocytes and neurons	Endogenous miR-133b	Increase in neurite outgrowth	Xin et al., 2012
Primary dendritic cells	Primary dendritic cells	Endogenous miR-451 and miR-148a	Repression of luciferase reporter	Montecalvo et al., 2012
HUVEC	Aortic smooth muscle cell	Endogenous miR-143/145	Repression of multiple miR-143/145 targets Protection against atherosclerotic lesion formation	Hergenreider et al., 2012

miRNAs transfer from infected to non-infected cells also occurs *in vivo*.

Another evidence for functional cell-to-cell miRNA transfer was found during investigation of the immune synapse formation. Mittelbrunn and co-authors showed that exosomes of T, B, and dendritic immune cells contained different miRNA repertoires. Furthermore, miRNAs were transported from T cells to antigen presenting cells unidirectionally and this transport was antigen-driven (Mittelbrunn et al., 2011). In addition, transferred miRNAs could modulate gene expression in recipient cells. The exosomal miRNA-based communication between different dendritic cells has also been reported, resulting in the repression of target mRNAs of acceptor dendritic cells (Montecalvo et al., 2012).

MVs produced by THP1 monocyte/macrophage cells have been shown to deliver FITC-labeled exogenous miR-150 to HMEC-1 endothelial cells in culture (Zhang et al., 2010b). In addition, the delivery of miR-150 correlated with the reduction of a validated miR-150 target c-Myb and was accompanied with an increase in HMEC-1 cells migration. Treatment of HMEC-1 cells with specific miR-150 inhibitor abrogated the observed increase in migration. While the effect was observed in cultured cells over-expressing miR-150, it remains unknown whether extracellular levels of endogenous miR-150 in body fluids is high enough to significantly affect gene expression in targeted cells *in vivo*. However, the observation that plasma MVs isolated from atherosclerotic patients contained elevated levels of miR-150 ignited a hypothesis that secreted

endogenous miR-150 may play a role in regulating endothelial cell migration.

Multipotent mesenchymal stromal cells are known to interact with brain parenchymal cells and promote their functional recovery. In the work of Xin et al., mesenchymal stromal cells exported miR-133b to the ipsilateral hemisphere. In addition, miR-133b was highly abundant in the primary cultures of neurons and astrocytes treated with exosome-enriched fraction released by mesenchymal stromal cells (Xin et al., 2012). Authors further showed that gap junction intercellular communication was important for the reported exosome-based miRNA transfer.

The elegant approach was used by Skog et al. to prove that exosomal RNA originating from glioblastoma tumor cells is taken up by recipient cells (Skog et al., 2008). The authors incorporated mRNA encoding luciferase reporter into exosomes and monitored luminescence of the recipient cells. Glioblastoma-derived MVs stimulated proliferation of a human glioma cell line enhancing further tumor progression. Besides, the authors demonstrated that serum MVs from glioblastoma patients contain mRNAs and miRNAs characteristic for gliomas and thus provide a potential diagnostic use.

Interestingly, the miRNAs from the let-7 family were found within the exosomes exported from the cultured metastatic gastric cancer cell line AZ-P7a but not from less metastatic cell lines (Ohshima et al., 2010). Because these miRNAs are known to be tumor-suppressive, the authors suggested that their elimination via exosomal export can maintain the oncogenic properties of the metastatic cells.

Hepatocellular carcinoma cells (HCC) have been shown to produce exosomes with specific mRNA, miRNA, and protein content (Kogure et al., 2011). The miRNAs highly enriched within HCC exosomes were predicted to target transforming growth factor β activated kinase-1 (TAK1), which contributes to local spread, intrahepatic metastases, or multifocal growth of this type of carcinoma cells (Kogure et al., 2011). Indeed, HCC-derived exosomes modulated TAK1 expression and enhanced transformed cell growth in recipient HCC in culture.

Another cancer-based model was based on human renal cancer stem cells. Grange and colleagues reported that a subset of tumor-initiating MSCs from human renal cell carcinoma released MVs which triggered angiogenesis and promoted the formation of a pre-metastatic niche. Importantly, cancer stem cell MVs contained miRNAs implicated in tumor progression and metastases, and conferred an angiogenic phenotype to normal human endothelial cells, stimulating their growth and vessel formation (Grange et al., 2011). However, it remains uninvestigated whether the miRNAs were responsible for the observed physiological impact.

Hergenreider and co-authors have found that extracellular vesicles mediate miRNA transfer from human endothelial cells to smooth muscle cell *in vitro*. Specifically, membrane vesicles secreted by shear-stressed cultured endothelial cells were enriched with miR-143/145 and modulated gene expression in co-cultured smooth muscle cells (Hergenreider et al., 2012). Moreover, miR-143/145-containing vesicles inhibited atherosclerotic lesion formation in the aorta in a mouse model suggesting a potential therapy against atherosclerosis.

EXTRACELLULAR miRNAs ASSOCIATED WITH OTHER CARRIERS

The products of cell apoptosis (or programmed cell death) are apoptotic bodies (AB) 1–2 μm in size (Kerr et al., 1972; Hengartner, 2000; Hristov et al., 2004). Together with exosomes and MVs, some researchers consider ABs as carriers of cell–cell communication information. Thus, both viral and chromosomal DNA can be transferred between somatic cells by uptake of the apoptotic bodies (Holmgren et al., 1999; Bergsmedh et al., 2001). Zernecke et al. has shown that ABs inhibit atherosclerosis progression when injected into the blood circulation. The authors also proposed that miR-126 encapsulated into ABs may be responsible for this protective effect via induction of the chemokine CXCL12 expression. Indeed ABs contained miR-126 and delivered miR-126 to recipient vascular cells (Zernecke et al., 2009). Furthermore, injections of miR-126 containing apoptotic bodies reduced manifestations of atherosclerosis in mice, while apoptotic bodies isolated from miR-126-deficient animals did not have such an effect. The protective effect was accompanied by elevated expression of CXCL12 in the carotid arteries. It has to be mentioned that in their experimental model the authors describe incubation of carotid arteries with relatively high concentrations of ABs *in vitro*. It remains to be tested whether physiological levels of ABs would affect gene expression in a similar manner.

A single report demonstrating that Hepatitis B subviral surface antigen particles (HBsAg) circulating in the Hepatitis B infected carriers contain both hepatocellular miRNAs and the AGO2 associated protein (Novellino et al., 2012). Interestingly, HBsAg associated miRNAs were liver-specific (miR-27a, miR-30b, miR-122, miR-126, and miR-145) as well as immune regulatory (miR-106b and miR-223). Computationally predicted target genes of HBsAg-associated miRNAs included molecular pathways of host-pathogen interactions.

Solely AGO protein-associated miRNA represents by far the largest class of extracellular miRNA (Arroyo et al., 2011; Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012). It was hypothesized that the AGO-ribonucleoprotein complexes are passively released by all cells after either necrotic or apoptotic death and remain stable in the extracellular space due to the high stability of the AGO proteins (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012). However, it cannot be completely excluded that certain cell membrane-associated channels or receptors mediate specific release of some AGO-miRNA complexes. Interestingly, in *C. elegans*, cellular uptake of dsRNA is mediated by a transmembane channel protein SID-1 (Feinberg and Hunter, 2003). In addition, SID-1 is capable of importing synthetic miRNA precursors and long hairpin molecules into the cell (Shih and Hunter, 2011). While the mammalian homologs of SID proteins do exist, it remains unclear whether they can uptake RNA from extracellular fluids (Duxbury et al., 2005; Wolfrum et al., 2007). Furthermore, it remains to be evaluated whether AGO-bound single stranded mature miRNA can be recognized by SID proteins in a similar manner as "naked" double stranded RNA.

Amazingly, two recent research reports suggest that extracellular miRNA may work in non-canonical ways. Specifically, either dead cell-released or exosomes secreted miRNAs can act as signaling molecules to mediate intercellular communication via binding to extracellular or intracellular Toll-like receptors (TLRs) (Fabbri et al., 2012; Lehmann et al., 2012). TLRs are a family of innate immune system receptors which recognize various molecular patterns of microbial pathogens and induce antimicrobial immune responses (Takeda et al., 2003; Blasius and Beutler, 2010). In 2001, Alexopoulou and co-authors first showed that dsRNA binds to mammalian TLR-3, consequently leading to the activation of NF-kappaB and the production of type I interferon response (Alexopoulou et al., 2001). Later, Kleinman and co-authors reported that cell surface TLR-3 mediates extracellular siRNA-induced inhibition of angiogenesis independently of siRNA sequence (Kleinman et al., 2008). The intracellular TLRs located within endolysosomal compartments can also bind both double stranded and single stranded nucleic acids derived from viruses and bacteria (Heil et al., 2004). Among the major effects of the activation of intracellular TLRs is the induction of cytokines essential for innate immune responses. In their work, Fabbri et al. showed that miR-21 and miR-29a secreted by tumor cells are capable of binding to murine TLR-7 and human TLR-8 in immune cells, triggering secretion of prometastatic inflammatory cytokines that ultimately may lead to tumor growth and metastasis (Fabbri et al., 2012). The authors also concluded that extracellular miRNAs could function as key regulators of the tumor microenvironment by acting as paracrine agonists of TLRs (Fabbri et al., 2012). The recent report of Lehmann and colleagues provided further evidence in favor of the unconventional role for the extracellular miRNAs (Lehmann et al., 2012). Intrathecal injection of extracellular let-7b into the cerebrospinal fluid of wild-type mice, but not TLR7 knockouts, resulted in activation of microglia/macrophages and neurodegeneration. Furthermore, susceptibility to let-7-induced toxicity was restored in neurons transfected with TLR7 by intrauterine electroporation of $Tlr7^{-/-}$ embryos. The authors also observed that: (1) dying neurons released let-7b *in vitro*; and (2) levels of let-7b were increased in CSF from patients with Alzheimer's disease (Lehmann et al., 2012). These results suggest that extracellular miRNAs can function as signaling via TLR-7 pathway and contribute to the spread of CNS damage.

In 2011, Vickers and colleagues reported that HDL complexes isolated from human blood plasma contain miRNA and could transmit this miRNA into other cells (Vickers et al., 2011). To examine whether miRNAs carried by HDL can alter gene expression in distant cells, HDL were isolated from hypercholesterolemia patients and healthy subjects. Treatment of human hepatocytes in culture with HDL derived from hypercholesterolemia subjects significantly increased the level of miR-105 in these cells, whereas HDL from healthy controls had no such effect. Further microarray analysis revealed that HDL from hypercholesterolemia patients induced profound alterations in mRNA expression including downregulation of multiple putative targets of miR-105 in cultured hepatocytes. Contrary to exosomes, cellular export of HDL-associated miRNAs was negatively regulated by nSMase2. In addition, HDL mediated miRNA delivery was dependent on a cell surface HDL receptor SRBI, which binds HDL and mediates the uptake of cholesterylester from HDL.

CONCLUSION AND FUTURE PERSPECTIVE OF THE FIELD

Despite a number of fascinating examples of intercellular communication via miRNA between cells in culture, the physiological significance of such paracrine or endocrine impact in the body is challenged by the fact that the vast majority of the extracellular miRNA are present in membrane-vesicle-free AGO protein-associated form. Furthermore, the concentration of miRNA in the biological fluids is drastically lower than in the surrounding cells and might be below the threshold for triggering any significant physiological effect *in vivo* (Turchinovich et al., 2011; Williams et al., 2013). Finally, so far extracellular miRNA trafficking was consistently shown: (1) only in cultured cells; and (2) only for several miRNAs.

In their recent report Tuschl group argues against a hormone-like effect of extracellular miRNA in the blood (Williams et al., 2013). Deep sequencing experiments revealed that the concentration of total miRNA in the plasma is within 100 fM range, and the concentration of any individual miRNA is only a fraction of this number. However, even the lowest level trace hormones in the blood are present at least in the picomolar concentration range. The action of hormones implies receptor-binding and multi-million amplification of the transmitted signal within the cell. Unlike hormones miRNAs require intracellular levels of greater than 1000 copies per cell to exert measureable activity on their mRNA targets (Williams et al., 2013). Based on these calculations the authors concluded that it is unlikely that miRNAs can function as hormones unless they bind to a sensitive miRNA receptor (Williams et al., 2013).

The paracrine mode of cell–cell signaling for extracellular miRNA appears to be more feasible. Indeed most, if not all, current reports describe evidence of rather short distance communication of cells via extracellular miRNA. Unlike average miRNA levels in a biological fluid, the local concentrations of extracellular miRNAs could suffice to secure the delivery of physiologically relevant amounts of miRNA from donor to neighboring acceptor cell. Recent evidence of interaction of miRNA with TLRs provided additional complexities to distinguish sequence-specific effects of extracellular miRNA on the targeted mRNAs expression in acceptor cells and non-specific response of the innate immune system (Fabbri et al., 2012; Lehmann et al., 2012).

Despite the fact that extracellular miRNA circulating in biofluids has many properties of promising biomarkers for various pathological conditions, the concept of miRNA mediated cell-cell signaling in vertebrates requires further validation. Among the central questions to be answered remains: (1) whether solely protein-bound extracellular miRNA can penetrate through the cell membrane and if so, which mechanisms are responsible; (2) whether concentrations of MVs-associated miRNAs are above the physiological limit to mediate any significant para- or endocrine signaling *in vivo*; (3) what are the mechanisms of selective export of miRNAs into extracellular space; (4) how many miRNAs out of the total extracellular pool participate in cell–cell signaling.

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Trash or Treasure: extracellular microRNAs and cell-to-cell communication

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Circulating RNAs in human body fluids are promising candidates for diagnostic purposes. However, the biological significance of circulating RNAs remains elusive. Recently, small non-coding RNAs, microRNAs (miRNAs), were isolated from multiple human body fluids, and these "circulating miRNAs" have been implicated as novel disease biomarkers. Concurrently, miRNAs were also identified in the extracellular space associated with extracellular vesicles (EVs), which are small membrane vesicles secreted from various types of cells. The function of these secreted miRNAs has been revealed in several papers. Circulating miRNAs have been experimentally found to be associated with EVs; however, other types of extracellular miRNAs were also described. This review discusses studies related to extracellular miRNAs, including circulating miRNAs and secreted miRNAs, to highlight the importance of studying not only secreted miRNAs, but also circulating miRNAs to determine the contribution of extracellular miRNAs especially in cancer development.

Keywords: circulating microRNA, exosomes, extracellular vesicles, extracellular microRNA, secretory microRNA, cell-to-cell communication

INTRODUCTION

Circulating RNAs have been isolated from human body fluids (Kamm and Smith, 1972; Fleischhacker and Schmidt, 2007). Javillier and Fabrykant (1931) reported the first discovery of circulating nucleic acids in 1931, before Watson and Crick (1953) reported the structure of DNA as a double helix. Furthermore, Mandel and Metais (1947) permitted ribonucleic acid and deoxyribonucleic acid to be separately measured. Since then, many researchers have attempted to use circulating RNA as disease biomarkers; however, the origins and meanings of circulating RNA are poorly understood.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate multiple phenomena, including development, organogenesis, and homeostasis (Ebert and Sharp, 2012). The mis-expression of miRNAs results in the onset of diseases, such as immune disease, cardiovascular disease, neurological disease, and cancer (Mendell and Olson, 2012). In 2007, the Lötvall group demonstrated that miRNAs were contained inside exosomes (Valadi et al., 2007), which are small membranous vesicles derived from the endosome (Raposo and Stoorvogel, 2013). Since the discovery of miRNAs in exosomes, several reports confirmed the existence of miRNAs in apoptotic bodies (Zernecke et al., 2009), high-/low-density lipoprotein (HDL/LDL; Vickers et al., 2011), and RNA-binding proteins (Arroyo et al., 2011; Turchinovich et al., 2011). Other studies have shown the existence of circulating miRNAs in human serum, including the serum from pregnant women (Chim et al., 2008) and cancer patients (Lawrie et al., 2008). Researchers have identified placental-specific miRNAs in the serum from pregnant women, which clearly disappeared after childbirth, indicating that circulating miRNAs reflect the status of the individual (Chim et al., 2008). Similarly, cancer-associated miRNAs were higher in the serum from cancer patients than in the serum from healthy individuals, indicating that circulating miRNAs can be used as biomarkers to monitor the existence of cancer cells in patients (Lawrie et al., 2008). These reports also demonstrated the stability of circulating miRNAs in the blood, despite the presence of large amounts of RNase (Reddi and Holland, 1976). Since the discovery of miRNAs in blood, many researchers have confirmed the existence of miRNA in a variety of other human body fluids, such as serum, plasma, saliva, breast milk, urine, and cerebrospinal fluid, among others (Kosaka et al., 2010a).

In this review, we chose miRNAs that were reported to have functions in cell–cell communication and also reported to be a potential biomarker, and we attempted to link the findings concerning secreted miRNAs used in cell–cell communication tools and circulating miRNAs used as biomarkers. This discussion may increase broad interests and improve the current understanding of the importance of extracellular miRNAs in cell–cell communication. We would like to discuss about the vesicles, such as exosomes, microvesicles, and apoptotic bodies (Bobrie et al., 2011; Raposo and Stoorvogel, 2013). The mean size of exosomes, 40–100 nm in diameter, corresponds to that of the internal vesicles of multivesicular bodies from which they originate. Exosomes contain enriched amounts of some specific markers, especially those of endosomal origin including CD63, CD81, CD9, major histocompatibility complex class II, and so on. On the other hand, the size of microvesicles varies between 50 nm and 1 μm in diameter and the microvesicles are generated by budding at the plasma membrane toward the outside of the cell. However, the term of microvesicles has also been used for exosome-like vesicles and clear distinction of exosome and microvesicles has not been established;

therefore, we will use "extracellular vesicle (EV)" in this review, according to the definition of the International Society for Extracellular Vesicles, when describing studies using ultracentrifugation to isolate EVs.

miRNAs IN EXTRACELLULAR VESICLES OR NON-VESICLE ASSOCIATED miRNAs

It has been shown that EVs, such as exosomes, microvesicles, and apoptotic bodies, contain miRNAs with functions that have been previously reported (Valadi et al., 2007; Zernecke et al., 2009). The existence of non-vesicle associated miRNAs has also been reported. These miRNAs bind to HDL/LDL (Vickers et al., 2011) or RNA-binding proteins, such as Argonaute 2 (Ago2) (Arroyo et al., 2011; Turchinovich et al., 2011) and Ago1 (Turchinovich and Burwinkel, 2012). Interestingly, Arroyo et al. (2011) reported that circulating miRNAs in plasma are predominantly coupled with Ago2. The liver-specific miRNA, miR-122 has been detected only in protein-associated fractions, suggesting that hepatocytes might release miR-122 through a protein carrier pathway. In addition, Turchinovich and Burwinkel (2012) showed that not only Ago2 but also Ago1-bound miRNAs has been identified in human blood plasma. Intriguingly, they also found that some miRNAs in the plasma did not derive from blood cells under normal conditions. Although the abundance of miRNAs associated with RNA-binding proteins has been recognized, the functions of these miRNAs in cell–cell communications have not been clarified.

miR-210

miR-210 is a hypoxia-inducible miRNA that is activated by the master regulator of hypoxic stress, hypoxia-inducible factor (HIF)-1alpha in a variety of cell types (Chan et al., 2012). This miRNA has been implicated in erythropoiesis (Kosaka et al., 2008), iron homeostasis (Yoshioka et al., 2012), angiogenesis (Fasanaro et al., 2008), and cancer (Huang et al., 2009), which are also conditions associated with hypoxic stress. This miRNA has also been implicated in the regulation of DNA repair pathways (Crosby et al., 2009). The function of miR-210 has been investigated, although its exact contribution to the cancer microenvironment has not been determined.

Recently, we observed that EVs isolated from metastatic breast cancer cells promote metastasis via the induction of angiogenesis in the tumor (Kosaka et al., 2013). We also showed that EVs contain multiple angiogenic miRNAs, and one of them, miR-210, is responsible for angiogenesis. Indeed, the addition of miR-210-enriched EVs induced the activation of endothelial cells *in vitro* (Kosaka et al., 2013). Moreover, miR-210 expression is known to be inversely correlated with a disease-free and overall survival in breast cancer (Camps et al., 2008). Intriguingly, circulating miR-210 in breast cancer patients has been reported. The expression of circulating miR-210 is significantly higher in plasma from circulating tumor cell (CTC)-positive metastatic breast cancer patients compared with that in plasma from CTC-negative metastatic breast cancer patients and controls (Madhavan et al., 2012). The use of CTC as a prognostic marker in metastatic breast cancer has been well documented (Lianidou and Markou, 2011); however, adequate detection methods are still needed. Thus, circulating miRNAs could be used to predict the status of patients with metastatic breast cancer instead of detecting CTC. Moreover, the indication of CTC is associated with bad prognosis for cancer patients, and circulating miR-210 might contribute to this phenomenon (Madhavan et al., 2012).

Interestingly, circulating miR-210 levels were significantly higher in individuals with residual disease than in those who achieved a pathologically complete response to trastuzumab (Jung et al., 2012), administered at baseline before patients received neoadjuvant chemotherapy, as a part of the standard treatment for patients with human epidermal growth factor receptor 2 (HER-2)-positive breast cancer. Indeed, circulating miR-210 was derived from tumor cells, as reduced levels of circulating miR-210 were observed in the serum of patients after surgery compared with that in serum from patients before surgery. Furthermore, miR-210 expression was also higher in patients whose cancer metastasized to the lymph nodes. These results suggest that circulating miR-210 can be used to predict and perhaps monitor responses to therapies involving the use of trastuzumab. Elevated levels of HIF-1alpha were also associated with HER-2 over-expression in invasive breast cancer (Yamamoto et al., 2008). Moreover, the induction of HER-2 signaling in breast cancer cells increases HIF-1alpha protein and vascular endothelial growth factor (VEGF) mRNA expression (Laughner et al., 2001).

Taken together, these results suggest that miR-210 contributes to cancer development through immediate effects on the cancer cells and the modulation of the cancer cell microenvironment, and when secreted into peripheral blood, circulating miR-210 can be detected to predict the status of cancer cells in the tumor (**Table 1**).

EBV miRNAs

Epstein–Barr virus (EBV) encodes miRNAs, which were first reported viral miRNAs in human. A recent study on EBV-infected normal and neoplastic tissues revealed that distinct EBV miRNA expression profiles are produced in various latency programs, and EBV miRNAs play key roles in maintaining EBV persistence through the inhibition of apoptosis and the suppression of the host immune response (Forte and Luftig, 2011).

Previously, Pegtel et al. (2010) observed that functional EBV miRNAs, secreted from EBV-infected cells, are transferred to uninfected recipient cells. These authors showed the miRNA-mediated repression of confirmed EBV target genes, including *CXCL11*. Importantly, in a co-culturing system, containing EBV-transformed lymphoblastic B cells (donor cells) and primary immature monocyte-derived dendritic cells (recipient cells), approximately 2×10^3 copies of EBV-miRNA BART1-5p were detected in a subset of the recipient cells after 24 h, and this level increased fourfold (nearly 8×10^3 copies) after an additional 24 h of co-culture. Moreover, these authors confirmed that the expression of *CXCL11* in recipient cells was down-regulated within 24 h co-culture, suggesting that the transfer of 2×10^3 copies of EBV-miRNA is sufficient to suppress miRNA-target genes in recipient cells. Surprisingly, EBV miRNAs were present in both B cell and non-B cell fractions in peripheral blood mononuclear cells obtained from patients with an increased EBV load, although EBV DNA was restricted to the circulating B cell population. These observations indicated that viral miRNAs are functional in non-infected cells after the transfer of virus miRNAs from infected cells

Table 1 | The miR-210 studies in the cells and in the extracellular space.

Location	Phenotype	Origin of miR-210 expression	Reference
Intracellular	Anti-apoptosis in erythroid cells	Erythroid cells	Kosaka et al. (2008)
Intracellular	Regulate iron homeostasis by targeting ISCU and TfR1	Breast cancer cells	Yoshioka et al. (2012)
Intracellular	Regulate response to hypoxia by suppressing Ephrin-A3	Endothelial cells	Fasanaro et al. (2008)
Intracellular	Regulating the hypoxic response of tumor cells and tumor growth	Renal cancer cells	Huang et al. (2009)
Intracellular	Promote genetic instability via suppression of RAD52	Cervical carcinoma cells and breast cancer cells	Crosby et al. (2009)
Extracellular (endothelial cells)	Promote metastasis via the induction of angiogenesis through EVs delivery	Metastatic breast cancer cells	Kosaka et al. (2013)
Extracellular (blood)	High expression in serum from patients who have trastuzumab-resistance cancer	Drug resistance breast cancer cells	Jung et al. (2012)
Extracellular (blood)	High expression in CTC-positive patient	Breast cancer cells	Huang et al. (2009)

EVs, extracellular vesicles; ISCU, iron–sulfur cluster scaffold; TfR1, transferrin receptor 1; CTCs, circulating tumor cells; EPO, erythropoietin.

to non-infected cells through EVs. As shown above, this study provided the quantitative information on the level of extracellular miRNAs, which is essential for research on exosomal miRNA-mediated cell–cell communication. Information, such as the level of exosomal miRNAs required to suppress target molecules in recipient cells, might improve the quality of research on exosomal miRNAs in cell-cell communications.

Nasopharyngeal carcinoma (NPC) is a human epithelial malignancy associated with EBV, and EBV miRNAs are abundantly found in NPC tumors (Lo et al., 2012). Interestingly, viral miRNAs are secreted into the extracellular space from NPC cells with secreted EVs (Gourzones et al., 2010). In addition, these miRNAs are not only detected in plasma samples from NPC xenografted nude mice, but also in plasma samples from NPC patients. Moreover, EBV miRNAs were significantly up-regulated in tumor tissues compared with non-tumor biopsies, and the distinct presence of EBV miRNAs in the serum of NPC patients has been positively correlated with the cellular copy numbers of EBV miRNAs (Wong et al., 2012). Taken together, these results indicated that the viral miRNAs secreted from NPC cells, are contained inside EVs, resulting in the high stability for diffusion from the tumor site to the peripheral blood.

Interestingly, non-infected cells harbor miRNAs from viruses, and this fact might be an important aspect to reconsider infectious diseases. In the case of NPC, several studies have shown the contribution of EBV miRNAs to cancer development (Lo et al., 2012), and circulating miRNAs might be useful for the evaluation of patient status (Gourzones et al., 2010; Wong et al., 2012). Considering the delivery of EBV miRNAs through EVs, it is important to characterize the roles of EBV miRNAs in "non-infected cells" during the development of NPC. Moreover, miRNAs have been identified in numerous virus types, such as herpes B virus, human cytomegalovirus, herpes simplex virus, and Kaposi's sarcoma-associated herpes virus, among others. Thus, it would be important to examine the roles for these viral miRNAs in non-infected cells. This information might broaden the current understanding of infectious diseases caused by virus miRNAs.

miR-21

miR-21 is a well-characterized miRNA that contributes to the development of cancer (Schetter et al., 2008; Medina et al., 2010), and the target genes for miR-21 have been identified as well-known tumor suppressor genes, such as PTEN (Meng et al., 2007) and PDCD4 (Asangani et al., 2008). Thus, it is natural to examine the expression of circulating miR-21 in the serum of cancer patients for diagnosis. Indeed, several reports have shown the increased expression of circulating miR-21 in the serum of cancer patients, including diffuse large B cell lymphoma (DLBCL; Lawrie et al., 2008), osteosarcoma (Ouyang et al., 2013), colorectal cancer (Kanaan et al., 2012), hepatocellular carcinoma (HCC; Zhou et al., 2011), gastric cancer (Li et al., 2012), head and neck squamous cell carcinoma (Hsu et al., 2012), esophageal squamous cell carcinoma (Komatsu et al., 2012), prostate cancer (Yaman Agaoglu et al., 2011), and glioblastoma (Skog et al., 2008).

Skog et al. (2008) previously reported that glioblastoma tumor cells release EVs containing mRNA, miRNA, and angiogenic proteins, and these EVs are taken up by normal host cells, such as brain microvascular endothelial cells. These authors also showed that miR-21 levels are elevated in serum EVs from glioblastoma patients compared with controls. Circulating miR-21 has been reported in the serum/plasma obtained from various cancer patients, although the contribution of miRNAs to cancer development through EVs has not been discerned. miR-21 acts as an oncogenic miRNA in various cancer cells and also regulates various phenotypes in the cancer cell microenvironment. Indeed, miR-21 is not only involved in cancer development but also participates in homeostasis (Niu et al., 2011); thus, understanding the contribution of miR-21 to the cellular microenvironment will increase the global understanding of animal development.

miR-21, associated with RNA-binding proteins, has also been detected in the culture supernatant from breast cancer cell

lines (Turchinovich et al., 2011) and serum from healthy donors (Arroyo et al., 2011), and the abundance of miR-21 in the extracellular space has been recognized as shown above. Thus, determining the biological significance for miR-21 binding to Ago2 might provide a better understanding of miRNA-associated cell–cell communication in cancer development.

miR-126

One of the earliest studies to show the transfer of miRNAs between the cells was revealed by the study of apoptotic bodies. In this study, the authors found that endothelial cell-derived apoptotic bodies contained miR-126 and these apoptotic bodies convey paracrine alarm signals to recipient vascular cells during atherosclerosis (Zernecke et al., 2009). In addition, another study also showed that secretory miR-126 was precipitated in the angiogenesis. The EVs from $CD34^+$ peripheral blood mononuclear cells exhibited proangiogenic properties via the transfer of miR-126 (Mocharla et al., 2013). Cantaluppi et al. (2012a) reported that EVs released from endothelial progenitor cells (EPCs) enhanced islet endothelial cell proliferation, migration, anti-apoptosis, and organization in vessel-like structures. They also found that EVs from EPCs contained the miR-126 and miR-296 and that these miRNAs contributed to the angiogenesis properties, suggesting that EVs from EPCs activate an angiogenic program in islet endothelium (Cantaluppi et al., 2012a). They also reported that miR-126 in EVs from EPCs contributed to the prevention of the ischemic acute injury in kidney by enhanced tubular cell proliferation, reduced apoptosis, and leukocyte infiltration (Cantaluppi et al., 2012b). In addition, EPC-derived EVs were able to induce neoangiogenesis and to enhance recovery in a hindlimb ischemia (Ranghino et al., 2012).

Although circulating miR-126 was enriched in systemic lupus erythematosus (Wang et al., 2012a), expression of circulating miR-126 was decreased in the breast cancer (Wang et al., 2010) and malignant mesothelioma (Tomasetti et al., 2012). Whereas there are only a few reports regarding the circulating miR-126, secretory miR-126 from cells has a great activity of endothelial cells activations as shown in above. Therefore, it is tempting to investigate the potential of miR-126 as biomarker in diseases which were caused by the abnormal angiogenesis.

miR-451

Kogure et al. (2011) showed a subset highly enriched miRNAs within EVs from HCC cells and identified a target of these miRNAs, transforming growth factor β activated kinase-1. Indeed, loss of this pathway resulted in the enhancement of transformed cell growth in recipient cells. One of the miRNAs that they identified in this study, miR-451, was found in the serum from patient with liver disease. Murakami et al. (2012) investigated the disease parameters in patients with chronic hepatitis C (CHC) by focusing on miRNAs isolated from EV-enriched fraction in serum. They successfully classified CHC and normal liver with 96.59% accuracy using the expression patterns of nine miRNAs including miR-451 (Murakami et al., 2012).

miR-223

Ismail et al. (2013) found that EVs from macrophage contained miR-223, and that this miR-223 was transported to target cells, including monocytes, endothelial cells, epithelial cells, and fibroblasts, and was functionally active. Macrophages are found in all tissues and they play roles in development, homeostasis, tissue repair, and immunity, and thus are therapeutic targets in many human diseases (Wynn et al., 2013). Indeed, an increased level of circulating miR-223 was found in serum/plasma from patients with gastric cancer (Li et al., 2012), non-small cell lung carcinoma (Sanfiorenzo et al., 2013), hepatitis B virus-related HCC (Zhou et al., 2011), NPC (Zeng et al., 2012), hypertension-induced heart failure (Dickinson et al., 2013), systemic lupus erythematosus, rheumatoid arthritis (Wang et al., 2012a), sepsis (Wang et al., 2012b), ischemic injury (Yu et al., 2009), and osteoarthritis (Okuhara et al., 2012). To date, origins of this circulating miR-223 have not been investigated yet; however, from the reports shown above, macrophage is probable candidate of origin for circulating miR-223. Interestingly, miR-223 is found not only in EVs but also in HDL (Vickers et al., 2011). In addition, miR-223 concentration in HDL was increased 3,780-fold with familial hypercholesterolemia when compared with controls. The HDL is involved in the transport of cholesterol from lipid-enriched macrophages of atherosclerotic arteries to the liver. Recently, Wagner et al. (2013) reported that miR-223 was detected at concentrations >10,000 copies/μg in HDL from healthy subjects. However, HDL-bound miR-223 contributed to only 8% of the total circulating miRNAs. In addition, a significant uptake of HDL-bound miRNAs into endothelial cells, smooth muscle cells, or peripheral blood mononuclear cells was not observed, suggesting that the lipoprotein-associated miR-223 does not regulate the function of the studied cells *in vitro*. Knowing the function of secretory miR-223 in macrophage homeostasis *in vivo* might lead to the development of not only the disease biomarker, but also the novel therapy against atherosclerosis.

miR-150

Zhang et al. (2010) demonstrated that miR-150 from monocytic cells were delivered into endothelial cell, and this miR-150 reduced its target gene, c-Myb, expression in endothelial cells, resulting in the enhancement of cell migration in endothelial cell both *in vitro* and *in vivo*. They also found that monocyte-secreted miR-150 promoted angiogenesis *in vivo* using tumor-implanted mice and ob/ob mice as models (Li et al., 2013). Intriguingly, the expression of miR-150 was higher in EVs isolated from the plasma of patients with atherosclerosis, and these EVs promoted endothelial cell migration compared to EVs from healthy donors (Zhang et al., 2010). A high level of circulating miR-150 was reported in several diseases including idiopathic childhood nephrotic syndrome (Luo et al., 2013), acute myeloid leukemia (Fayyad-Kazan et al., 2013), and so on. On the contrary, miR-150 serum concentrations upon admission were closely associated with intensive care unit (ICU) survival as well as long-term survival, and low miR-150 levels indicated an unfavorable prognosis (Roderburg et al., 2013).

SUMMARY AND PERSPECTIVES

In this review, we presented the results obtained from research on miRNAs to provide a better understanding of the relationship

between secreted miRNAs which contribute to cell–cell communication in cancer development, and circulating miRNAs which are used as disease biomarkers.

Recently, a novel concept for biomarkers, called "liquid biopsy," has been proposed (Forshew et al., 2012; Murtaza et al., 2013). Liquid biopsy would be useful for numerous diagnostic applications and avoid the need for tumor tissue biopsies. Current studies have shown that genomic alterations in solid cancer can be characterized through the massively parallel sequencing of circulating cell-free tumor DNA released from cancer cells into the plasma (Forshew et al., 2012; Murtaza et al., 2013). This suggests that circulating miRNAs are also good candidates for liquid biopsy, as the quantities and sequences of miRNAs convey information for diagnosis. Particularly, circulating miRNAs, which have been previously shown to function in cell–cell communication, might be good candidates for this application. Therefore, we emphasize that it is important to investigate the function of secretory miRNAs in cell–cell communication, and in parallel explore the usefulness of these molecules as biomarkers using animal models.

Much of the current research on circulating miRNAs for disease biomarkers does not describe the types of circulating miRNAs, such as EVs, microvesicles, HDL/LDLs, or RNA-binding proteins that are present in human body fluids. As previously discussed, focusing on a specific type of circulating miRNAs, such as exosomal miRNAs or miRNAs bound to RNA-binding proteins, might be useful as disease biomarkers compared with analyzing the total miRNA in human body fluids. Indeed, EV-enriched fractions isolated from patients with liver disease were useful for the determination of disease progression compared with the profiles obtained using total miRNA present in serum samples (Murakami et al., 2012). Therefore, it is essential that future studies concerning circulating miRNAs for diagnostic purposes should focus on the type of circulating miRNAs present in body fluids.

One of the crucial issues in research on cell–cell communication by secretory miRNAs is whether the secretory miRNAs which researcher identified are really physiologically functional enough or not. This issue might be revealed by showing the quantitative data of secretory miRNAs in more detail, such as the number of EVs, the number of miRNAs, and the number of cells. In addition, in the case of functional demonstration of secretory miRNAs, over-expression or knock-down of secretory miRNAs was performed; however, contamination of exogenous miRNAs, such as synthetic miRNAs, should be cared since the amount of those exogenous miRNAs are usually introduced in excess. The study on extracellular miRNAs has just begun. Thus, the researcher working on the EVs needs to take care of the physiological amount of those molecules in their research field.

Another crucial issue of extracellular miRNAs that how these miRNAs are secreted from cells and how these miRNAs work in the cells has not been answered yet, although recent reports proved the physiological and pathological importance of secretory miRNAs not only *in vitro* but also *in vivo*. We previously found that secretion of miRNAs from cells was regulated by neutral sphingomyelinase 2, which is known as a rate-limiting enzyme of ceramide biosynthesis and triggers secretion of EVs (Kosaka et al., 2010b). Although the molecules that are essential for EVs secretion has been reported, their contribution to miRNAs secretion has not been tested yet. One of the most important points for understanding of miRNAs secretion is the identification of a protein that binds to miRNAs in EVs. miRNAs are strongly bound to the Ago2 protein, which is a main component of the RNA-induced silencing complex (RISC), in the cells (Kim et al., 2009), but this molecule is not found in EVs (Gibbings et al., 2009). Meanwhile, knockdown of GW182, another main component of the RISC, reduced miRNA secretion via EVs. Interestingly, however, GW182 was not detected in the EVs from HEK293 (Yao et al., 2012). In contrast to the above report, GW182 can be found in EVs from monocyte, HeLa cells and *ex vivo*-derived dendritic cells (Gibbings et al., 2009). These paradoxical observations indicate that further experiments are required to elucidate whether there is a role for GW182 in miRNA secretion. Identification of proteins that are responsible for the transport of miRNAs from inner cells to inner EVs might reveal many of mysteries of secretory miRNAs in cell–cell communications.

Circulating RNA has been previously considered as "trash" from cells; however, we propose that this "trash" serves as a communication tool and should therefore be referred to as "treasure." Analyzing circulating miRNAs in human body fluids might provide a method for "listening" to the communication between cells, leading to the development of disease treatments based on the mechanisms of secreted miRNAs in cancer development.

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Comparison of methods for miRNA extraction from plasma and quantitative recovery of RNA from cerebrospinal fluid

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Interest in extracellular RNA (exRNA) has intensified as evidence accumulates that these molecules may be useful as indicators of a wide variety of biological conditions. To establish specific exRNA molecules as clinically relevant biomarkers, reproducible recovery from biological samples and reliable measurements of the isolated RNA are paramount. Toward these ends, careful and rigorous comparisons of technical procedures are needed at all steps from sample handling to RNA isolation to RNA measurement protocols. In the investigations described in this methods paper, RT-qPCR was used to examine the apparent recovery of specific endogenous miRNAs and a spiked-in synthetic RNA from blood plasma samples. RNA was isolated using several widely used RNA isolation kits, with or without the addition of glycogen as a carrier. Kits examined included total RNA isolation systems that have been commercially available for several years and commonly adapted for extraction of biofluid RNA, as well as more recently introduced biofluids-specific RNA methods. Our conclusions include the following: some RNA isolation methods appear to be superior to others for the recovery of RNA from biological fluids; addition of a carrier molecule seems to be beneficial for some but not all isolation methods; and quantitative recovery of RNA is observed from increasing volumes of cerebrospinal fluid.

Keywords: miRNA, biomarker, biofluid, RNA isolation, method, RT-qPCR, plasma, cerebrospinal fluid

INTRODUCTION

A leading goal of modern biomarker studies is to ascertain disease conditions and other biological states by examining molecules in easily obtained biological fluids. For example, the ability to use blood-based markers for diagnosis, location, and staging of cancer, or to predict outcome of several therapeutic options, would have several benefits, from early detection to obviating the need for invasive and expensive biopsies. While some biomarkers may simply be "bystanders" in the disease process, others might be actively involved and thus present targets for novel therapeutics (Kota et al., 2009; Cho, 2012; Lindow and Kauppinen, 2012). Of course, any successful marker or set of markers would need to be sufficiently stable during and following the isolation process to allow reliable detection and measurement. Among the many types of biomolecules currently under investigation as potential biomarkers are extracellular RNA (exRNA), including microRNA (miRNA) (Pritchard et al., 2012a). These short oligonucleotides are thought to be stable in extracellular fluids, protected from degradation by ubiquitous RNases (Mitchell et al., 2008) within or even on the surface of small particles.

Many questions about extracellular miRNAs are under active investigation. The identities of the various particles that carry and protect miRNAs, the frequency of miRNA association with different particle types, and the relative contributions of these particles to exRNA profiles in health and disease have yet to be firmly established (Witwer et al., 2013). The extent to which miRNAs are specifically or non-specifically associated with particle types is also largely unknown. Finally, the extent and mechanisms of function of extracellular miRNAs – e.g., in intercellular signaling – remain incompletely understood, although numerous studies have established that such functions exist (Valadi et al., 2007; Pegtel et al., 2010; Ismail et al., 2012). In the face of these many outstanding questions, the current consensus is that extracellular vesicles (Lotvall and Valadi, 2007), protein complexes (Arroyo et al., 2011; Turchinovich et al., 2011), and, possibly, additional particles (Turchinovich et al., 2012), contribute to the extracellular population of miRNAs. Furthermore, specific extracellular miRNAs or constellations of miRNAs appear to be co-regulated during disease: to give just a few of many examples, in cancers (Cho, 2011), acute retroviral infection (Witwer et al., 2011), and autoimmune disease (Murata et al., 2010).

The promise of extracellular miRNA biomarkers is tremendous, but several obstacles must be surmounted during ongoing, successful development of miRNA signatures of disease and other conditions. First, many high-profile preclinical studies in general cannot be replicated or reproduced (Ioannidis, 2005; Ioannidis et al., 2009); in the same way, many miRNA profiling studies are underpowered or analysis cannot be replicated because data are missing or procedures inadequately explained (Baggerly, 2013; Witwer, 2013). In some cases, then, better study design and reporting would be helpful. Second, lack of standardization is a challenge to much-needed comparisons of miRNA studies. Biological fluids contain small amounts of RNA relative to cells and tissues; if sample processing does not remove small cells and cell fragments, miRNAs from these particles may predominate in any "extracellular" miRNA profile (McDonald et al., 2011), just as

RNA from hemolysed samples may affect profiling (Kirschner et al., 2011; Pritchard et al., 2012b). A study of miRNAs in platelet-rich plasma – say, plasma that has been spun for 10 min at 800 × *g* – might simply reflect disease state-associated platelet abundance. Third, because of the aforementioned relative paucity of RNA in cell-free fluids as well as the presence of PCR inhibitors in biological fluids, efficient extraction methods are needed to ensure maximum recovery of RNA from low volumes of input fluid.

While several studies have described methods for RNA extraction from cell-free fluids (Tzimagiorgis et al., 2011; Debey-Pascher et al., 2012) or total blood (including cells) (Gaarz et al., 2010), relatively few studies have specifically compared the use of commercially available kits for RNA extraction from biofluids (Burgos et al., 2013) or constituents of biofluids (Eldh et al., 2012). We are unaware of any such studies employing recently released biofluids-specific RNA extraction methods from Qiagen and Exiqon. Here, we report comparisons of five commercially available kits/methods for RNA extraction from plasma; the effects of including or omitting glycogen as a carrier/co-precipitant; and the linear recovery of RNA from increasing volumes of plasma and cerebrospinal fluid (CSF) using a biofluids-specific method.

MATERIALS AND METHODS

ETHICS STATEMENT

De-identified plasma was from a healthy donor who provided informed consent in accordance with approval by the Johns Hopkins Institutional Review Board. CSF was from a healthy donor pigtailed macaque (*Macaca nemestrina*). Animal studies were approved by the Johns Hopkins University Institutional Animal Care and Use Committee and conducted in accordance with the Weatherall Report, the Guide for the Care and Use of Laboratory Animals, and the USDA Animal Welfare Act. Macaques are pair- or group-housed when possible and receive environmental enrichment including manipulanda, foraging, and opportunity to exhibit species-specific behavior. Animals are continually monitored for signs of distress. Ketamine is administered for blood or CSF draws.

REAGENTS

Paired stem-loop reverse transcription primers and hydrolysis probe/primer combinations for RT-qPCR were purchased from Life Technologies for the following miRNAs: endogenous mammalian miRs-16, -21, -34a, -126, and -150, and exogenous cel-miR-39. These miRNAs, along with most relatively abundant miRNAs, are conserved amongst primates (Brameier, 2010; Shao et al., 2010). RNA isolation kits were from Life Technologies (mirVana, AM1561), Exiqon (miRCURY RNA Isolation Kit – Cell and Plant, #300110; and miRCURY RNA Isolation Kit – Biofluids, #300112), and Qiagen (miRNeasy Serum/Plasma Kit, # 217184). TRIzol LS reagent was purchased from Life Technologies (#10296-010). TaqMan microRNA Reverse Transcription Kit (#4366596) and qPCR master mix (#4440047) were purchased from Life Technologies. iScript select cDNA synthesis kit (1708897) and SsoFast qPCR mix (1725232) were from BioRad. Additional reagents included glycogen (Ambion/Life Technologies, AM9510), synthetic cel-miR-39 (Integrated DNA Technologies – custom product synthesized to sequence from miRbase.org) (Mitchell et al., 2008), and highly pure diethyl pyrocarbonate (DEPC)-free and nuclease-free water (Qiagen, 129115).

RNA HANDLING AND STORAGE

We followed RNase-free protocols throughout all procedures up to qPCR setup (after RNA has been converted to cDNA). A laminar air flow cabinet dedicated to RNA work was used for RNA isolation and reverse transcription preparations. Surfaces (cabinet, racks, pipettes, centrifuge) were cleaned thoroughly with RNase-away prior to and after use. Pipettes and other portable objects to be used in the hood were exposed to UV light in a UV crosslinker for 2 min on each side. This was done to avoid introduction of contaminating nucleic acids and, potentially, proteins. We implemented double-gloving, regular glove changes, and wearing of Tyvek sleeves or clean lab coats. Certified RNase-free barrier pipette tips were used. Where possible, all reagents were certified RNase-free. Isolated RNA from all isolation methods was stored at −80°C.

All RT-qPCR reactions were performed with once-thawed RNA as substrate. RNA samples were thawed on ice, mixed gently, and centrifuged at 2500 × *g* for 5 min in a table-top centrifuge before use. This was done because some column-based clean-up methods introduced a fine white powder into the eluted sample, presumably from the filter materials. Although this material is likely inert, we pelleted it to avoid interference with downstream reactions. It may be preferable to pellet this material and transfer the clarified supernatant to a new RNase-free tube prior to initial freezing.

SAMPLE HANDLING

RNA isolations were performed from platelet-poor primate plasma that had been frozen (−80°C) and thawed once. Platelet-poor plasma was defined as plasma separated from blood cells less than 30 min following blood draw with a 1000 × *g* spin for 15 min at room temperature followed by a 2500 × *g* spin for 15 min at room temperature to remove most remaining platelets. Plasma was obtained from blood collected into ACD anticoagulant. All methods comparison experiments were conducted with aliquots of plasma from a single blood draw from one donor.

RNA ISOLATIONS

For all isolations, 5 or 10 pg synthetic cel-miR-39 per isolation was spiked into the respective lysis/denaturant buffer before combining with plasma. We note that synthetic RNA cannot be added directly to plasma or other biological substances because endogenous RNases degrade it immediately (Mitchell et al., 2008). Also, fresh cel-miR-39 dilutions were prepared for each set of isolation experiments since storing highly diluted RNA could result in substantial loss of material to container surfaces. Three micrograms highly pure glycogen (as indicated) per isolation was either omitted (negative) or added (positive) as a carrier/co-precipitant. We emphasize the importance of mixing samples well (including, where appropriate, vigorous vortexing for at least 1 min prior to centrifugation for phase separation). Screw-cap tubes are recommended to minimize losses during organic extraction methods.

Comparison of three RNA isolation methods, with and without glycogen

To compare mirVana, miRCURY Cell and Plant, and TRIzol LS isolation with mirVana column clean-up, triplicate isolations for each method were performed from 100 µl plasma each, both with and without glycogen (see above) for a total of 18 isolations. Manufacturer's protocols were followed for the Exiqon and Ambion/Life Technologies kits. For TRIzol LS/column clean-up, 100 µl plasma was diluted with 150 µl nuclease-free water, followed by addition of 750 µl TRIzol LS reagent. Samples were mixed and incubated at room temperature for 10 min. Two-hundred microliters chloroform was added with a glass pipette, followed by vortexing for 1 min and incubation at room temperature for 5 min. Phases were separated by centrifugation at 14,000 × *g* for 15 min in an Eppendorf 4514C centrifuge. Aqueous layer was removed, and 1.5 volumes EtOH were added. This mixture was applied to mirVana kit filter columns followed by wash and elution steps as per manufacturer's protocol. For this isolation experiment, the miRNAs measured were endogenous miR-16 and miR-21 and spiked-in cel-miR-39. miRNAs were measured by RT-qPCR in triplicate for each isolation. "Fold abundance" was calculated in comparison with the average of results for the three no-glycogen mirVana isolations.

Comparison of Exiqon miRCURY cell and plant kit and Exiqon miRCURY biofluids kit

Duplicate isolations from 100 µl plasma were done for each method. Glycogen was added for isolations using both methods; a no-glycogen condition was included only for the Biofluids Kit, for a total of six isolations. The manufacturer's protocols were followed. miRNAs measured included endogenous miRs-16, -34a, and -126, and spiked-in cel-miR-39. miRNAs were measured by RT-qPCR in triplicate for each isolation. "Fold abundance" was calculated in comparison with the average of results for the two glycogen-added miRCURY Cell and Plant isolations.

Exiqon biofluids versus Qiagen plasma/serum kits

Triplicate isolations were done for each method from 200 µl plasma. Glycogen was added for all isolations. Manufacturers' protocols were followed. Triplicate RT-qPCR reactions were performed with material from each isolation to measure endogenous miRs-16 and -150 and exogenous spike-in cel-miR-39. Fold abundance for the Qiagen isolations was calculated against the mean of results for the three Exiqon Biofluids isolations. Separately, for the Qiagen kit, different elution volumes (50 and 100 µl) were compared with the manufacturer-recommended 14 µl.

Cerebrospinal fluid RNA isolation

RNA was isolated by the Exiqon Biofluids method with addition of glycogen and spike-in to all isolations. About 50, 100, and 200 µl CSF were used as input volume. Duplicate isolations were performed for each input volume. Higher-abundance miRs-16 and -223 and lower-abundance miR-21 and let-7c were measured in triplicate reactions.

REVERSE TRANSCRIPTION AND REAL-TIME QUANTITATIVE PCR

Reverse transcription and RT-qPCR steps were performed largely according to the manufacturer's protocols, with any modifications as described previously.(Witwer et al., 2011, 2012) The stem-loop RT primer design allows specific amplification of mature miRNA; no significant differences are seen in DNase-treated versus untreated samples. Following reverse transcription, samples were diluted with 20 or 30 µl RNase-free water and were mixed, spun down, and stored at −20°C until use. The Applied Biosystems/Life Technologies recommended protocol for qPCR reaction setup was modified for a smaller volume of 10 µl per well before addition of sample, using the same reagent proportions as recommended by the manufacturer. To each well, 2 µl of diluted cDNA was added. Each plate was mixed gently, centrifuged, and loaded into the real-time machine. The manufacturer's amplification protocol was followed for 45 amplification cycles. Negative controls: no-template and/or no-reverse transcriptase control reactions were performed for selected isolations, substituting water for RNA or the reverse transcriptase enzyme. These reactions consistently failed to amplify or amplified only after 40 PCR cycles on average (data not shown). Reverse transcription and RT-qPCR reagents were from Life Technologies for all experiments except for the Exiqon Biofluids/Qiagen miRNeasy comparison, for which Bio-Rad reagents were used (we conducted a side-by-side comparison of the reagents and found no significant differences in results). Real-time detection systems were the iQ5 and CFX96 instruments (both from BioRad, Hercules, CA, USA).

DATA COLLECTION AND PROCESSING

To obtain Cq values, appropriate thresholds were drawn manually (iQ5) or automatically (CFX96) with checking and manual adjustment by the operator if needed. Mean Cq and standard deviation were calculated for each technical triplicate reaction for each miRNA. Mean Cq and standard deviation (for three isolations) or range (for two isolations) were determined for each type of isolation. Delta Cq values were calculated with reference to the chosen "control" condition for each isolation experiment, as specified above. From these values, fold abundance with respect to the control was calculated (Livak and Schmittgen, 2001). Mean fold abundance values and standard deviations were determined for the examined miRNAs.

RESULTS

Five RNA isolation methods/kits were compared for isolation of small RNA from human plasma. All but one method was tested in the presence and absence of glycogen as RNA carrier. Because assessment of different RNA isolation methods requires the use of a standard source sample, we conducted all isolations from plasma obtained from a single blood draw from a single donor. This plasma had been centrifuged twice to obtain platelet-poor plasma as described in methods. Aliquots of plasma had been frozen at −80°C and had not been thawed previously.

The low abundance of exRNA necessitates consideration of a carrier molecule. We chose glycogen as a carrier rather than complex exogenous RNA mixtures such as yeast tRNA or MS2 phage RNA. Little has been published on this topic, but there seem to be conflicting opinions on whether biological carrier RNAs might be responsible for non-specific hybridization or amplification in downstream analytics. Highly pure glycogen is an alternative carrier/co-precipitant that may avoid these unresolved questions

since it is inert and does not cause reported interference with downstream assays (Turchinovich et al., 2011; Turchinovich and Burwinkel, 2012). Still, since glycogen is purified from biological sources, chiefly bivalves, some commercial glycogen preparations may contain nucleic acids. As a result, UV-treated glycogen or an alternative co-precipitant, linear polyacrylamide (LPA), have been proposed (Bartram et al., 2009). In tests of the glycogen we used, however, we were unable to obtain specific signal for any of the mammalian miRNAs or other small RNAs examined (data not shown).

In addition to glycogen, we used a synthetic spiked-in cel-miR-39 RNA. It is important to remember that the spike-in is useful for assessment of recovery but cannot be used for biological normalization. In the experiments described here, no normalization was necessary, since the same plasma sample was used throughout. As noted in Section "Materials and Methods," fresh dilutions of cel-miR-39 stock were made for each of three successive isolation experiments; thus, normalization by spike-in between experiments would be inappropriate and was not done.

We first compared the Ambion mirVana kit [based on (Chomczynski and Sacchi, 1987)] with the Exiqon miRCURY Cell and Plant Kit and TRIzol LS extraction followed by mirVana column clean-up. Each of these three protocols was performed in triplicate with and without the use of glycogen as a carrier. The mirVana and TRIzol LS methods include a phase separation step, whereas the miRCURY method does not. Thus, the miRCURY protocol is faster, less technically demanding, and is likely subject to less technical variation, operator-dependent, and otherwise.

To assess performance of these kits, two endogenous miRNAs and the spike-in were measured in triplicate for each isolation replicate by RT-qPCR. (Please observe that we use the term "recovery" or "apparent recovery" to describe results, although from the RT-qPCR results it is not necessarily clear whether differences arise from different RNA recovery efficiencies, different inhibitor removal efficiencies, or both).

The Ambion mirVana kit yielded similar results with and without glycogen (**Figure 1**). Without glycogen, the Exiqon protocol showed slightly lower recovery than both mirVana kit protocols. Addition of glycogen to the Exiqon protocol significantly enhanced recovery by more than threefold compared with the no-glycogen Exiqon method ($p < 0.02$) or by 1.6-fold versus mirVana (trend, with $p < 0.1$). TRIzol isolation followed by column clean-up did not perform as well as the other methods in our hands (**Figure 1**). Surprisingly, the addition of glycogen appeared to exacerbate the low RNA recovery achieved by this method.

A new, biofluids-specific Exiqon kit became available in December, 2012. According to the manufacturer, this kit allowed better RNA recovery as well as PCR inhibitor removal in comparison with other methods. (It should be mentioned that RNA species less than 1000 nt in length are preferentially recovered.) The two protocols are similar in terms of time and operator skill requirements. We performed a second isolation experiment to compare the performance of this Biofluids kit (with and without glycogen) with the standard miRCURY kit (with glycogen). For this experiment, duplicate isolations were performed, followed by triplicate miRNA RT-qPCR reactions for each isolation replicate. Both

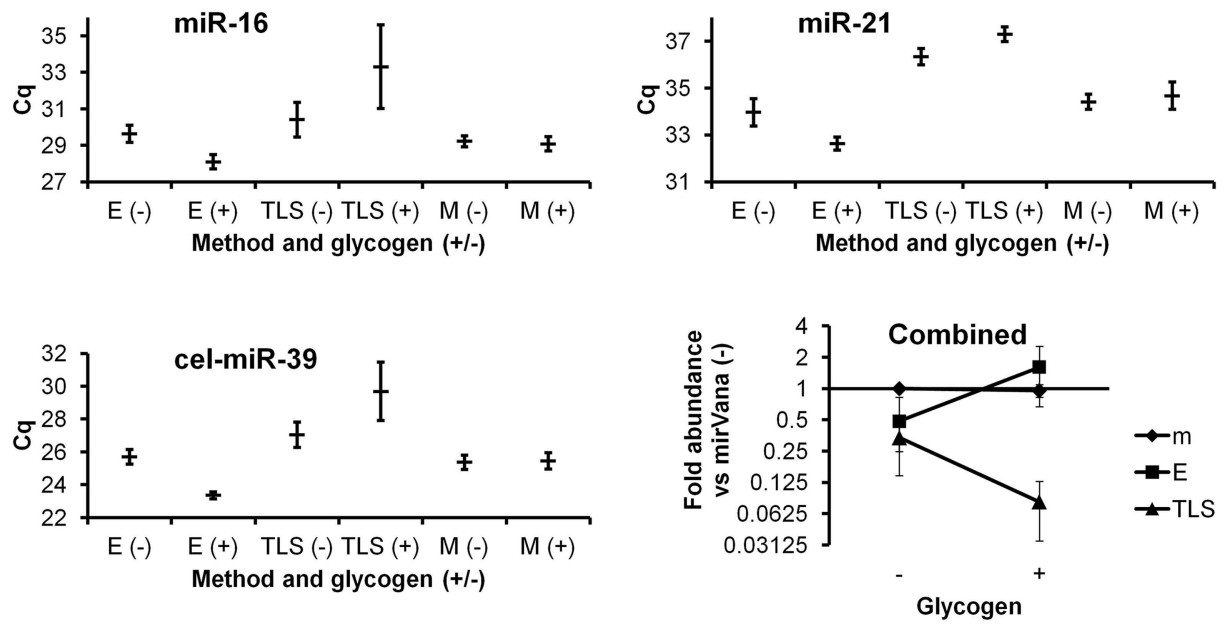

FIGURE 1 | Comparison of three RNA isolation methods, with and without glycogen. miRNAs from plasma RNA isolated in triplicate by the three indicated methods (E, Exiqon Cell and Plant kit; TLS, TRIzol LS; M, mirVana isolation kit), with and without glycogen, were assessed by RT-qPCR in triplicate. Results based on two endogenous and one spiked-in exogenous miRNA are shown. Raw Cq averages and standard deviations are presented for the higher-abundance miR-16, lower-abundance miR-21, and spiked-in cel-miR-39. The "combined" panel shows mean apparent combined abundance of miRNAs in samples isolated by the mirVana no-glycogen method set equal to one. Fold abundance (from deltaCq method) is displayed with standard deviation bars.

without and with glycogen, the Biofluids kit displayed significantly greater recovery than standard miRCURY with glycogen (**Figure 2**, $p < 0.02$ and $p < 0.01$, respectively), with large fold abundance differences averaging about six (without glycogen) or >10 (with glycogen). For qPCR Cq results by all methods, standard deviation of the technical triplicates was typically below 0.1 for high-abundance miRNAs. For the low-abundance miRNA miR-34a, high variability was observed.

In a third experiment, conducted with addition of glycogen for all isolations, the miRCURY Biofluids kit was compared with another biofluids-specific protocol: the Qiagen miRNeasy Serum/Plasma system. The Qiagen protocol includes a phase separation and is thus comparable to mirVana and TRIzol protocols in time and technique requirements. In contrast with the other methods we tested, the Qiagen protocol calls for a very small elution volume (14 µl), with the apparent intent of providing a more concentrated RNA sample.

Triplicate isolations were performed followed by triplicate RT-qPCR measurements of endogenous miRNAs and spiked-in cel-miR-39. As with all other experiments performed for this report, we measured miRNAs in 2 µl of purified RNA eluted as per the manufacturers' protocols. Both kits produced highly consistent results between isolation replicates. Results indicated similar apparent recovery by the two kits (**Figure 3**), albeit with slightly lower recover for the Qiagen kit. However, it must be considered that the results were obtained with 2 µl of elution volumes of 50 µl (Exiqon) versus 14 µl (Qiagen). Assuming that RNA was appropriately recovered with a 14-µl elution volume, i.e., that RNA was not left on the column, this might imply lower recovery by the Qiagen kit. Since we and others have previously observed with other isolation kits that smaller elution volume does not necessarily produce more concentrated RNA (Witwer et al., 2013), we performed an additional experiment to determine whether larger elution volume would recover additional RNA from the Qiagen columns. In this experiment, duplicate isolations were performed for 14, 50, and 100 µl elution volumes. Most of the RNA was in fact recovered by the 14-µl volume: practically all of a low-abundance RNA and ∼70–90% of high- to medium-abundance RNAs (data not shown). Thus, the apparent difference in recovery of the Exiqon Biofluids and Qiagen Plasma/Serum kits does not seem to be addressed by altering the elution volume for the latter.

To investigate quantitative recovery of RNA using the Exiqon Biofluids kit, we isolated RNA from 50, 100, and 200 µl of plasma and from the same volumes of *Macaca nemestrina* CSF from a single donor. The same amount of spike-in RNA was added to each isolation. In the case of plasma, endogenous miRNA had greater apparent recovery with increasing input volume, but the increase was not proportional from 100 to 200 µl input (**Figure 4**). Although we examined only three endogenous miRNAs, the lower-abundance miR-126 in particular displayed no apparent increase in recovery at 200 µl (**Figure 4**). Interestingly, cel-miR-39 was measured at a lower level in the 200-µl input samples, suggesting inefficient apparent recovery because of column clogging and/or concentration of inhibitors. In contrast, largely quantitative recovery was observed from increasing volumes of input CSF (**Figure 5**), a fluid that in health is relatively protein-poor compared with plasma. Spiked-in cel-miR-39 was recovered at the same level with each volume of input.

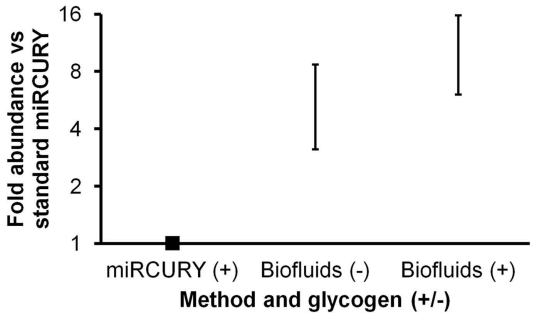

FIGURE 2 | Comparison of Exiqon miRCURY Cell and Plant and miRCURY Biofluids kits. Results of plasma miRNA RT-qPCR (technical triplicates) of duplicate isolations by the two Exiqon methods, with miRCURY Cell and Plant conducted with glycogen and Biofluids conducted both with and without glycogen. Fold abundance is relative to Exiqon miRCURY Cell and Plant. Bars represent range. Results of RT-qPCR for three endogenous and one spiked-in miRNA are shown. As noted in the text, the depicted differences versus Exiqon miRCURY Cell and Plant were significant.

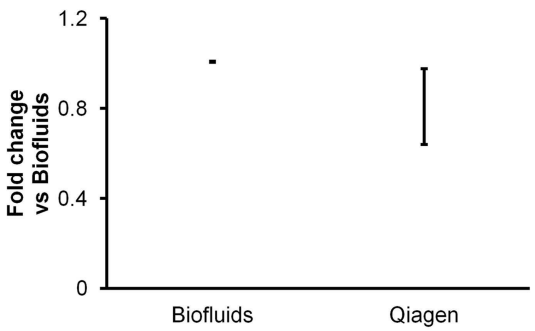

FIGURE 3 | Comparison of two biofluids-specific kits. Triplicate plasma RNA isolations with Exiqon Biofluids and Qiagen miRNeasy Serum/Plasma (glycogen added for both) were compared in triplicate RT-qPCR reactions for two endogenous and one spiked-in miRNA. Fold abundance is relative to Exiqon Biofluids. Note that for the Exiqon and Qiagen kits, the eluate volumes were 50 and 14 µl, respectively, as specified by the manufacturers' protocols, so the results are not necessarily directly comparable (see text).

DISCUSSION

Our results indicate that the Exiqon miRCURY Biofluids Kit outperforms other RNA isolation methods we tested, at least for isolation of small RNAs from plasma. This finding, furthermore, should be viewed in light of the relative ease of execution of the Exiqon method when compared with traditional phase-separation-based methods. Ease of use and short processing time are certainly appropriate considerations when results are not compromised.

Without glycogen, the standard Exiqon miRCURY slightly underperformed, and with glycogen, it slightly outperformed the Ambion mirVana kit. Both kits outperformed TRIzol LS with column clean-up in our hands. The new Exiqon miRCURY Biofluids kit appeared to be superior for small RNA isolation when compared with the standard miRCURY kit. With miRCURY Biofluids, all miRNAs were apparently recovered at higher quantities in the presence of glycogen, although the difference was not statistically

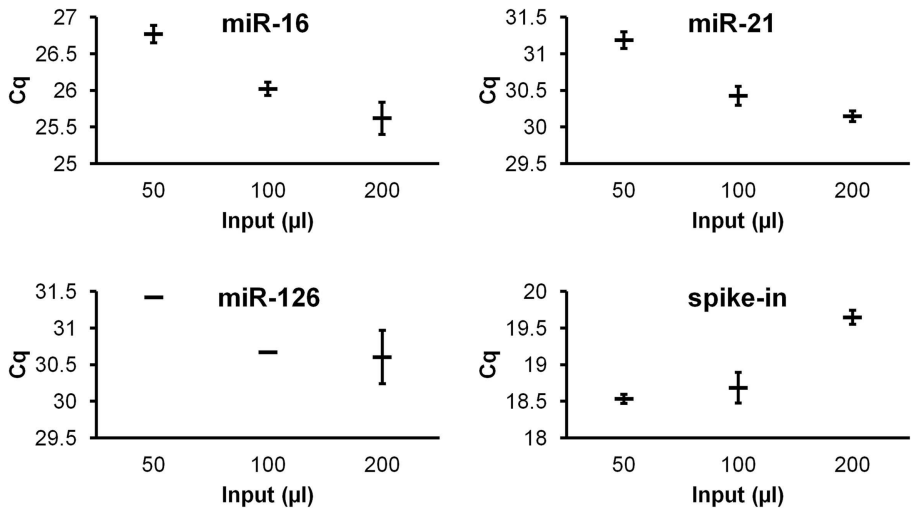

FIGURE 4 | Quantitative recovery of RNA from plasma does not extend to highest input volume. The Exiqon Biofluids kit was used to isolate RNA in duplicate isolations for each of the three indicated quantities of plasma. Range of Cq values for higher-abundance miR-16, lower-abundance miRs-21 and -126, and spiked-in cel-miR-39 (same input amount for each isolation) are shown.

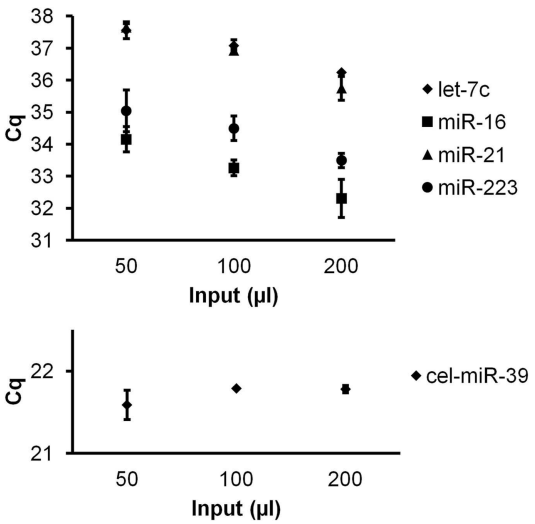

FIGURE 5 | Quantitative recovery of RNA from cerebrospinal fluid by Exiqon Biofluids. The Exiqon Biofluids kit was used to isolate RNA in duplicate isolations for each of three quantities of CSF. Linear response to input volume is shown for four endogenous miRNAs as indicated (measured in triplicate for each isolation), as well as spiked-in cel-miR-39 (same input amount for each isolation). Bars represent range of Cq values for the duplicate isolations.

significant at an alpha of 0.05. miRCURY Biofluids also provided better recovery than the Qiagen miRNeasy Serum/Plasma kit in our hands, considering the small elution volume from the Qiagen kit.

Although we found that the Qiagen Serum/Plasma kit allowed recovery of most of the on-column RNA with only 14 µl of eluant, it is not clear to us that using smaller elution volumes generally achieves greater RNA concentration. For some protocols and columns, smaller volumes of eluant may simply leave RNA on the column rather than providing greater concentration. An analogy that has been used previously (Eldh et al., 2012, International Society for Extracellular Vesicles Workshop in New York City) likened the column filter to a rag: to rinse it, the rag must be soaked completely (Witwer et al., 2013). Thus, we would recommend that any attempts to concentrate RNA in this manner be tested rigorously. Overall, additional optimization of existing methods is encouraged, since repeated extractions and temperature during isolation (Burgos et al., 2013), different carriers and spike-ins, and elution volumes are among the parameters that could be optimized for specific RNA isolation kits.

A conclusion from our experiments that we found particularly surprising is that TRIzol LS isolation followed by column clean-up did not perform well in our hands. We are uncertain as to why this method performed so poorly in our case or why the addition of glycogen appeared further to decrease yield, since this method or variations on it have been used with excellent results by others. The variability and low yield we report here is likely due to a separate issue from that reported by Kim et al. (2012b), who retracted their *Cell* article in 2012 after observing that standard TRIzol extraction resulted in apparently poor recovery of low-GC-content miRNAs from low-abundance samples. However, clarity on this point has yet to be achieved.

We submit that our results prompt at least three additional recommendations. First, based on our input volume experiments, it would appear that using 200 µl of plasma as input does not necessarily result in quantitatively greater recovery. This echoes previous findings (Kim et al., 2012a). In contrast, RNA was quantitatively recovered from the smallest to the largest input volume of CSF. Thus, it may be important to determine optimal input volume for each biofluid and isolation method. Second, we would like to emphasize the importance of co-isolation of all RNA samples that are that are to be compared within an experiment to

avoid introduction of batch effects (as we have noted previously) (Witwer et al., 2011, 2012; Sisk et al., 2012). Although raw Cq values for spike-in and endogenous miRNA measurements were highly consistent within each isolation experiment, they varied somewhat from experiment to experiment. For the spike-in, this may be due at least in part to the fact that we made a new dilution of spike-in for each isolation experiment from a highly concentrated stock. However, it is also clear that batch isolations (and batch RT reactions) should not be compared directly. Third, the Cq differences between replicate isolations, while often half a cycle or less, indicate that technical differences could contribute to apparent differences in miRNA expression, especially in studies with low "n." Many miRNA publications report differential expression of twofold or less. This underlines the importance of proper numbers of biological replicates and may also indicate that, where possible, multiple isolations from the same sample could increase rigor.

This study has several limitations, which may be helpful to review as they indicate opportunities for advances in the field. We measured a limited number of endogenous miRNAs in this study. It is possible that recovery may differ from one method to another on a miRNA-by-miRNA basis, similar to the differences reported by Kim et al. (2012b), in their retraction letter. This could be due to GC content, length, or other features. Assuming availability of appropriate resources, a miRNome-wide comparison of isolation methods might be highly useful. Also, we have not determined whether the differences we observe are due to inhibitors, to differential RNA recovery, or to some ratio of the two. Additional studies would be needed to make this determination. Many RNA isolation kits are available, along with many method modifications, and we have explored only a small portion of this methods space. We encourage other researchers to join us in comparing methods and working toward standardization as well as improvement of existing practices. Finally, we have examined only whole platelet-poor plasma in our methods comparisons. Other biofluids are also important potential sources of biomarkers, and comparatively little methodologic information is available for most biofluids [see, however (Burgos et al., 2013), published during review of this paper]. It is also possible that different isolation methods are better suited to recovering RNA from specific sub-populations of the various carrier particles that protect RNA in plasma and other biofluids. For example, isolation methods for exosomal RNA have been rigorously compared (Eldh et al., 2012). Similar studies for other RNA carriers would be informative and would help to establish whether different methods are needed, e.g., for isolation from whole plasma versus extracellular vesicles or protein complexes.

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The impact of hemolysis on cell-free microRNA biomarkers

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Cell-free microRNAs in plasma and serum have become a promising source of biomarkers for various diseases. Despite rapid progress in this field, there remains a lack of consensus regarding optimal quantification methods, reference genes, and quality control of samples. Recent studies have shown that hemolysis occurring during blood collection has substantial impact on the microRNA content in plasma/serum. To date, the impact of hemolysis has only been investigated for a limited number of microRNAs, mainly the red blood cell (RBC)-enriched miRs-16 and -451. In contrast, the effect of hemolysis on other microRNAs – in particular those proposed as biomarkers – has not been addressed. In this study we profiled the microRNA content of hemolyzed and non-hemolyzed plasma as well as RBCs to obtain a profile of microRNAs in the circulation affected or unaffected by hemolysis. Profiling by TaqMan Array Microfluidic Cards was used to compare three pairs of hemolyzed and non-hemolyzed plasma (with varying degrees of hemolysis) and one RBC sample. A total of 136 microRNAs were detectable in at least two of the samples, and of those 15 were at least twofold elevated in all three hemolyzed samples. This number increased to 88 microRNAs for the sample with the highest level of hemolysis, with all of these also detected in the RBC profile. Thus these microRNAs represent a large proportion of detectable microRNAs and those most likely to be affected by hemolysis. Several of the hemolysis-susceptible microRNAs (e.g., miRs-21, -106a, -92a, -17, -16) have also been previously proposed as plasma/serum biomarkers of disease, highlighting the importance of rigorous quality control of plasma/serum samples used for measurement of circulating microRNAs. As low-level hemolysis is a frequent occurrence during plasma/serum collection it is critical that this is taken into account in the measurement of any candidate circulating microRNA.

Keywords: cell-free microRNA, red blood cells, hemolysis, biomarker, quality control

INTRODUCTION

Since the discovery that microRNAs are not only present within cells, but can also be detected extracellularly in a variety of body fluids, a large number of studies have investigated the potential use of these cell-free microRNAs as diagnostic and/or prognostic biomarkers (Reid et al., 2011; Creemers et al., 2012; Mo et al., 2012). Unlike their longer mRNA counterparts which are prone to degradation in body fluids as a result of the high content of RNase particularly in blood, microRNAs are surprisingly stable. This increased stability of microRNAs can be attributed to: (i) their encapsulation into microvesicles such as exosomes and apoptotic bodies (Cortez and Calin, 2009; Kosaka et al., 2010) and (ii) their association with protein complexes such as argonaute 2 (Arroyo et al., 2011; Turchinovich et al., 2011) and high density lipoprotein (Vickers et al., 2011).

Being easily accessible and collected routinely as part of medical assessments, plasma and serum represent the most promising and best studied sources of cell-free microRNAs. While a large number of studies have aimed to identify microRNAs in plasma or serum that can serve as biomarkers for disease, there is still a lack of consensus regarding optimal quantification methods, appropriate reference genes as well as quality assurance and quality control of samples.

Of particular relevance to the identification of cell-free microRNA-based biomarkers in blood, a small number of studies have recently shown that rupturing of red blood cells (RBCs) occurring most often during blood collection or sample processing can have substantial impact on the levels of certain microRNAs detectable in plasma and serum (Kirschner et al., 2011; McDonald et al., 2011; Pritchard et al., 2012). These studies only investigated the effect of hemolysis on a very limited number of cell-free microRNAs, in particular miR-16 and miR-451, but serve as a first indication and warning that hemolysis can significantly alter the levels of microRNAs in plasma and serum. It is not surprising that the levels of miR-16 and miR-451 vary depending on the degree of hemolysis given they represent two of the most abundant microRNAs in RBCs (Bruchova et al., 2007; Vasilatou et al., 2010). However, our own data (Kirschner et al., 2011) and those of Pritchard et al. (2012) also revealed that levels of miR-92a, a microRNA proposed as a potential biomarker for ischemic heart disease (Fichtlscherer et al., 2010) and various cancer types (Tanaka et al., 2009; Huang et al., 2010; Ohyashiki et al., 2011)

are also affected by hemolysis. In addition, Blondal et al. (2013) have recently reported on 119 microRNAs measured in high quality and compromised (general blood cell contamination) serum samples. This study showed that in samples with blood cell contamination many of those 119 microRNAs significantly deviate from the mean expression levels observed in 381 high quality samples used as comparator. Together, these observations raise the possibility that other potential biomarkers could be equally impacted by hemolysis.

In the present study we profiled the microRNA content of non-hemolyzed and hemolyzed plasma as well as RBCs to identify those microRNA most likely to be affected by hemolysis.

MATERIALS AND METHODS

BLOOD COLLECTION

Blood was collected from consenting patients [with either malignant pleural mesothelioma (MPM) or coronary artery disease (CAD)] and healthy controls from the antecubital fossa using a butterfly device (21 G, BD Bioscience). A total of four 4 ml or three 10 ml K_3EDTA Vacutainer Plus Tubes (BD Biosciences) was taken in one collection. Written informed consent was obtained from all participants and the study was approved by the Human Research Ethics Committee at Concord Repatriation General and Royal Prince Alfred Hospitals, Sydney, Australia. Within 60 min of blood collection samples were subjected to centrifugation at 2500 *g* for 20 min at room temperature. Plasma supernatant was removed leaving at least a 500 µl layer behind to avoid disturbing the buffy coat layer. Samples were frozen as 500 µl aliquots and stored at −80˚C until further use. Purified RBCs from one healthy donor were obtained by separation of blood components using Ficoll-Paque PLUS according to the manufacturer's recommendations. The dilution series used in this study was the same as published previously (Kirschner et al., 2011). **Table 1** summarizes patient/volunteer demographics and identifies how samples were used in profiling and validation.

We observed that blood collection of single patients sometimes resulted in plasma with hemolysis occurring in one or two of the collection tubes while the plasma in the remaining tube(s) was non-hemolyzed. The hemolyzed sample of the matched pairs used in this study was the result observed after standard collection procedure, and was not induced chemically or physically.

Hemolysis in the dilution series was achieved by addition of lysed RBCs (freeze-thawed and mixed by continuous vortexing for 60 s) to non-hemolyzed plasma from the healthy volunteer. Serial dilution from this 2% RBC sample was performed to obtain the samples used in this study, as described (Kirschner et al., 2011). Two independent dilution series using plasma from two independent blood collections were prepared.

ASSESSMENT OF HEMOLYSIS

The level of hemolysis in plasma samples was assessed by spectrophotometry (NanoPhotometer P300, Implen) with readings at wavelengths scanning from 350 to 650 nm. The degree of hemolysis was determined based on the optical density at 414 nm (absorbance peak of free hemoglobin), with additional peaks occurring at 541 and 576 nm being indicative of very high levels of hemolysis. Samples were classified as being hemolyzed if the A_{414} reading exceeded a valued of 0.2 as we have previously shown that excluding samples with higher A_{414} significantly decreases the variability of miR-16 and miR-451 (microRNAs highly abundant in RBCs) within a sample series (Kirschner et al., 2011). Based on our experience non-hemolyzed samples present with an A_{414} reading between 0.14 and 0.18. See **Table 2** for A_{414} measurements of all samples used in this study.

RNA ISOLATION

Total RNA was isolated from 400 µl of plasma from six individuals for whom non-hemolyzed and hemolyzed plasma obtained from different collection tubes but the same blood collection was available. In addition RNA was isolated from 100 µl Ficoll-purified RBCs from the healthy donor and from 400 µl of plasma containing RBC dilution [5 points of a serial dilution series (Kirschner et al., 2011)]. RNA isolation was performed using the mirVana PARIS miRNA isolation Kit (Life Technologies) with small modifications. The RBC samples were mixed with 300 µl cell disruption buffer prior to further processing identical to the plasma samples. Following the denaturing step of the isolation process, 100 µg mussel glycogen (Roche) were added to each sample as carrier to enhance isolation efficiency. After separation and recovery of the aqueous phase, a second phenol–chloroform extraction of the aqueous phase was included to improve the removal of the high protein content of plasma. Following the column-purification part, RNA was eluted using 100 µl ultrapure H_2O (95˚C), resulting in a recovery of around 85 µl RNA. For each sample two independent RNA isolations were performed.

Table 1 | Patient/volunteer demographics and use of samples in this study.

Sample ID	Gender	Age	Diagnosis	Profiling	Validation
N1*	F	31	N/A	No	Yes
N2*	F	31	N/A	Yes	Yes
MPM1	M	64	Sarcomatoid malignant mesothelioma	Yes	Yes
CAD1	M	79	Stable coronary artery disease	Yes	Yes
CAD2	M	65	Stable coronary artery disease	No	Yes
CAD3	M	80	Stable coronary artery disease	No	Yes

**N1 and N2 are the same individual with samples obtained from two independent collections (2 months apart) which both resulted in collection of 1 hemolyzed and 1 non-hemolyzed plasma sample with different degrees hemolysis. Blood for the dilution series was collected from the same individual.*

Table 2 | Free hemoglobin measurements for matching sample pairs and dilution series.

Sample	A_{414}		Degree of hemolysis relative to non-hemolyzed
	Non-hemolyzed	Hemolyzed	
N1	0.174 ± 0.015	0.413 ± 0.062	2.36 ± 0.16
N2	0.153 ± 0.015	0.412 ± 0.039	2.68 ± 0.003
MPM1	0.124 ± 0.014	0.558 ± 0.01	4.53 ± 0.58
CAD1	0.184 ± 0.028	0.25 ± 0.025	1.37 ± 0.07
CAD2	0.127 ± 0.008	0.242 ± 0.012	1.90 ± 0.01
CAD3	0.143 ± 0.03	0.574 ± 0.129	4.01 ± 0.06
0.0% RBC	0.143 ± 0.013	N/A	N/A
0.008% RBC		0.184 ± 0.001	1.29 ± 0.13
0.016% RBC		0.245 ± 0.042	1.73 ± 0.45
0.031% RBC		0.321 ± 0.057	2.28 ± 0.61
0.0625% RBC		0.420 ± 0.036	2.96 ± 0.52
0.125% RBC		0.626 ± 0.019	4.39 ± 0.27

For those samples to be used for microRNA profiling 70 µl of the total RNA were further concentrated in order to increase the amount of RNA that could be reverse transcribed. These samples were mixed with 300 mM sodium acetate pH 5.2, 2.5 ng mussel glycogen, and 175 µl 100% Ethanol and incubated over night at −80°C. RNA was precipitated by centrifugation at 17000 *g* for 20 min at 4°C. Pellets were washed with 1 ml 75% Ethanol and re-precipitated at 17000 *g* for 10 min at 4°C. After removal of the supernatant RNA was air-dried for approximately 10 min (until the RNA pellet changed color from white to opaque) and then resuspended in 10 µl ultrapure H_2O. RNA concentration was assessed using the Qubit RNA Assay Kit (Life Technologies), however RNA concentrations for the plasma samples were below the limits of detection. All samples were stored at −80°C until further use.

TaqMAN ARRAY MICROFLUIDIC CARD microRNA PROFILING

MicroRNA profiling was performed using the TaqMan Array Human MicroRNA A+B Cards Set v3.0 together with the Megaplex™RT Primers, Human Pool Set v3.0 (Life Technologies) following the protocol for profiling without pre-amplification.

The profiling was performed twice using two independent isolations of matching non-hemolyzed and hemolyzed plasma pairs from three individuals. Each RNA sample was reverse transcribed using each the A and B Megaplex Primer Pools. Briefly, 3 µl precipitated RNA was mixed with 1× reaction buffer, 3 mM $MgCl_2$, 2 U RNase Inhibitor, 2.7 mM dNTPs, 1× Megaplex Primer Pool A or B, and 75 U MultiScribe Reverse Transcriptase under the following reaction conditions: 40 cycles of 2 min annealing at 16°C, complimentary DNA (cDNA) synthesis for 1 min at 42°C and 1 s at 50°C, followed by denaturing for 5 min at 85°C. The obtained cDNA was then stored at −20°C for use within 1 week.

Six microliters cDNA were mixed with 444 µl ultrapure H_2O and 450 µl 2× TaqMan Gene Expression Master Mix and 100 µl of this mix were loaded into each port of the corresponding Microfluidic Array card according to the manufacturer's instructions. Array cards were then run on a ViiA 7 Real-Time instrument (Life Technologies) with 2 min UDG incubation at 50°C and 10 min enzyme activation at 95°C followed by 40 cycles of 15 s denaturation at 95°C and 60 s annealing/elongation at 60°C.

Quantification cycle (Cq) values were determined using the ViiA 7 Software v1.2 applying a fixed threshold level of 0.05. Data were then analyzed using the $2^{-\Delta Cq}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) expressing levels of microRNAs in hemolyzed samples relative to those in non-hemolyzed samples without normalization to an endogenous control: relative expression = $2^{-((Cqtarget(Hemolyzed)) - (Cqtarget(Non\text{-}Hemolyzed)))}$. Alternatively, direct comparisons were performed at the level of raw Cq values. MicroRNAs with Cq value >35 in every sample analyzed were excluded from analysis.

INDIVIDUAL microRNA REVERSE TRANSCRIPTION REAL-TIME QUANTITATIVE PCR

Levels of selected microRNAs were assessed using TaqMan microRNA Assays. Reverse transcription (RT) was performed using microRNA-specific stem-loop RT primers and the MicroRNA RT Kit (both Life Technologies). A fixed volume of 1.67 µl total RNA was used as template in the RT reaction, and combined with 4 µl of an equimolar mix of 7 or 8 microRNA-specific primers (consisting of 31.25 nM of each specific primer), 1 nM dNTPs, 2.4 U RNase Inhibitor, 33 U MultiScribe reverse transcriptase, and RNase-free water in a total volume of 10 µl. RT primer mix 1 consisted of primers for miR-122 (ID 002245), -142-3p (ID 000464), -146a (ID 000468), -16 (ID 000391), -486-3p (ID 002093), -532-3p (ID 002355), -636 (ID 002088), and -886-5p (ID 002193), mix 2 contained primers for miR-1255B (ID 002801), -1274B (ID 002884), -15b (ID000390), -16, -451 (ID 001105), -625-3p (ID 002432), and RNU48 (001006), mix 3 consisted of miR-103 (ID 000439), -106a (ID 002169), -126 (ID 002228), -17 (ID 000393), -27a (ID 000408), -29a (ID 002112), and -92a (ID 000431), and mix 4 contained miR-155 (ID 002623), -16, -21 (ID 000397), 210 (ID 000512), -223 (002295), -31 (ID 002279), and -720 (ID 002895). Reaction conditions followed the manufacturer's instructions: annealing for 30 min at 16°C, followed by cDNA synthesis for 30 min at 42°C and denaturing for 5 min at 85°C. The resultant cDNA was diluted by addition of 57.8 µl water and used immediately in qPCR or stored at −20°C.

For real-time qPCR detection of microRNAs, 2.25 µl of the diluted cDNA were used as template in reactions containing 1× microRNA-specific TaqMan primers/probes (see above for assay IDs) in combination with 1× TaqMan GeneExpression MasterMix (both Life Technologies) according to the manufacturer's instruction in a total reaction volume of 10 µl, with the following reaction conditions: 2 min UDG incubation at 50°C and 10 min enzyme activation at 95°C followed by 40 cycles of 15 s denaturation at 95°C and 60 s annealing/elongation at 60°C. No-template samples were included as negative controls. Duplicate qPCR reactions were set up manually and run on a ViiA 7 Real-Time Instrument (Life Technologies). Cq values were determined using the ViiA 7 Software v1.2 applying a fixed threshold level of 0.05. In case of

matched pairs of non-hemolyzed and hemolyzed plasma, relative abundance in the hemolyzed samples as compared to the non-hemolyzed sample was calculated using the $2^{-\Delta Cq}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) as described above. Data for the dilution series are presented as raw Cq values for each microRNA.

All raw Cq data (average of duplicates) are provided in Tables 1 (profiling) and 2 (validation) in Supplementary Material. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) were followed for description of samples, RNA extraction, RT, qPCR protocol, and data analysis.

RESULTS

PROFILING MATCHED HEMOLYZED AND NON-HEMOLYZED PLASMA SAMPLES IDENTIFIES microRNAs AFFECTED BY HEMOLYSIS

In order to better understand the contribution of hemolysis to the levels of microRNAs in plasma, we profiled the microRNA content of three matched pairs of non-hemolyzed and hemolyzed plasma and one RBC sample from the healthy donor.

The number of microRNAs detected with a Cq < 35 in each sample varied between 91 and 194, as did the number of microRNAs affected by hemolysis (**Figure 1A**). We further filtered these microRNAs, identifying a total of 136 microRNAs that were detectable in at least 4 of the 6 plasma samples profiled and, with the exception of the liver-specific miR-122, all of these were also detected in the RBC sample. Relative expression levels of those 136 microRNAs in the hemolyzed sample as compared to the non-hemolyzed counterpart are pictured in **Figure 1B**. This shows that the majority of detectable microRNAs are elevated in hemolyzed samples (represented by red color), while only a handful of microRNAs are present at lower levels in hemolyzed plasma (blue). The heat map further shows that there is a general trend toward a correlation of higher levels of hemolysis-susceptible microRNAs with the degree of hemolysis.

Further analysis identified that of the microRNAs detected in plasma, 15 (11%, the center of the Venn diagram in **Figure 1C**) demonstrated >2-fold higher levels in the hemolyzed samples of all three pairs investigated. The number of elevated microRNAs in hemolyzed compared to non-hemolyzed plasma increased to 52 (38.2%) microRNAs when including those only elevated in two of the three pairs and to a total of 88 (64.7%) microRNAs being elevated >2-fold in at least one of the pairs. Those microRNAs identified as being significantly altered in only one of the pairs were generally also altered in the other pairs, but did not show increases above the selected threshold of at least twofold. Of the microRNAs detectable only 11 appeared to be truly unaffected by hemolysis and did not vary by more than ±1.5-fold in any of the investigated pairs. Our profiling studies also identified 3 microRNAs (miR-1255B, miR-636, miR-886-5p) and a small nucleolar RNA (snoRNA, RNU48) which were only present in RBCs and hemolyzed plasma. While additional microRNAs were found to be present only in RBCs and one or two of the hemolyzed samples, these were not subject to further validation due to Cqs being >35. **Figure 2** provides an overview of all microRNAs which increased in relative abundance by more than twofold following hemolysis as well as those unaffected by hemolysis.

CHANGES OF SELECTED microRNAs WITH INCREASING RBC CONTAMINATION

Our profiling data suggested that the levels of a number of microRNAs detected in plasma increased with hemolysis. To further investigate the contribution of RBC microRNAs to plasma levels, we measured the levels of selected microRNAs in a series of 5 RNA samples obtained from a serial dilution of RBCs in non-hemolyzed plasma (Kirschner et al., 2011) and six matched pairs of non-hemolyzed and hemolyzed plasma (the three pairs used for profiling +3 more). We quantified two microRNAs affected by hemolysis (miR-486-3p, miR-532-3p), three not affected by hemolysis (miR-1274B, miR-142-3p, miR-146a), and the four microRNAs/snoRNAs only present in RBCs and hemolyzed plasma (miR-1255B, miR-636, miR-886-5p, RNU48). In addition we included miR-16, miR-15b, and miR-451 as miRs previously shown to be affected by hemolysis as well as the liver-specific miR-122.

Using our RBC dilution series, we observed an increase in Cq values for miR-16, miR-451, and miR-15b with increasing RBC concentration (**Figure 3A**), consistent with previously published data (Kirschner et al., 2011; McDonald et al., 2011). In addition, we observed a considerable decrease in Cq (and therefore an increase in abundance) with increasing RBC contamination for the hemolysis-susceptible microRNAs miR-486-3p and miR-532-3p identified from the profiling (**Figure 3B**). Similarly, levels of miRs-1255B, -636, -886-5p, and RNU48, microRNAs/snoRNAs identified as being RBC- and hemolysis-specific, also increased with increasing RBC content (**Figure 3C**). In contrast levels of those microRNAs identified as being the most stable (miR-142-3p, miR-146a, and miR-1274B), as well as the liver-specific miR-122 did not change with increasing RBC concentration and showed only minor variability with <1.1 cycles between the highest and lowest Cq for any of those miRs (**Figure 3D**; **Table 3**).

When measuring levels of these microRNAs in matched pairs of non-hemolyzed and hemolyzed plasma, we found a similar pattern to that observed in the dilution series. The microRNAs shown to be influenced by RBC content of the sample – miR-16, -451, -15b, 486-3p, 532-3p, -886-5p, 636, -1255B, and RNU48 – were up to 13-times more abundant in the hemolyzed sample as compared to its non-hemolyzed counterpart (**Figure 4A**). In addition this increase in abundance was correlated with the degree of hemolysis observed for the corresponding sample. MicroRNAs identified as being unaffected by hemolysis showed only minor variation (<±1.5-fold difference) in relative expression between hemolyzed and non-hemolyzed plasma (with the exception of CAD3) (**Figure 4B**).

IMPACT OF HEMOLYSIS ON PROPOSED BIOMARKER CANDIDATES

Following validation of the microRNAs selected from our profiling studies, we investigated the effect of hemolysis on microRNAs previously proposed as biomarkers for disease (**Table 4**). These microRNAs included those for which the profiling suggested an influence of hemolysis (miR-103, -106a, 17, -21, -210, -27a, -31, -625-3p, -92a) as well as those unaffected by RBC contamination (miR-122, -126, -146a, -155, -223, -29a, -720). Measurement in the dilution series confirmed that microRNAs miR-106a, miR-17,

A

	N1	MPM1	CAD1	RBC
Total number detectable*	**194**	**149**	**91**	**348**
Up in hemolysed (>2-fold)	**78 (40.2 %)**	**71 (47.7 %)**	**49 (53.9 %)**	**N/A**
Up in hemolysed (1.5<x<2-fold)	**47 (24.2 %)**	**20 (13.4 %)**	**16 (17.6 %)**	**N/A**
No change (-1.5<x<1.5)	**56 (28.9 %)**	**44 (29.5 %)**	**23 (25.2 %)**	**N/A**
Down in hemolysed (-1.5>x>-2-fold)	**8 (4.1 %)**	**6 (4.0 %)**	**2 (2.2 %)**	**N/A**
Down in hemolysed (>-2-fold)	**5 (2.6 %)**	**8 (5.4 %)**	**1 (1.1 %)**	**N/A**

*MicroRNAs detectable are defined as Cq<35 in both the non-hemolysed and hemolysed sample in at least one of the two arrays performed.

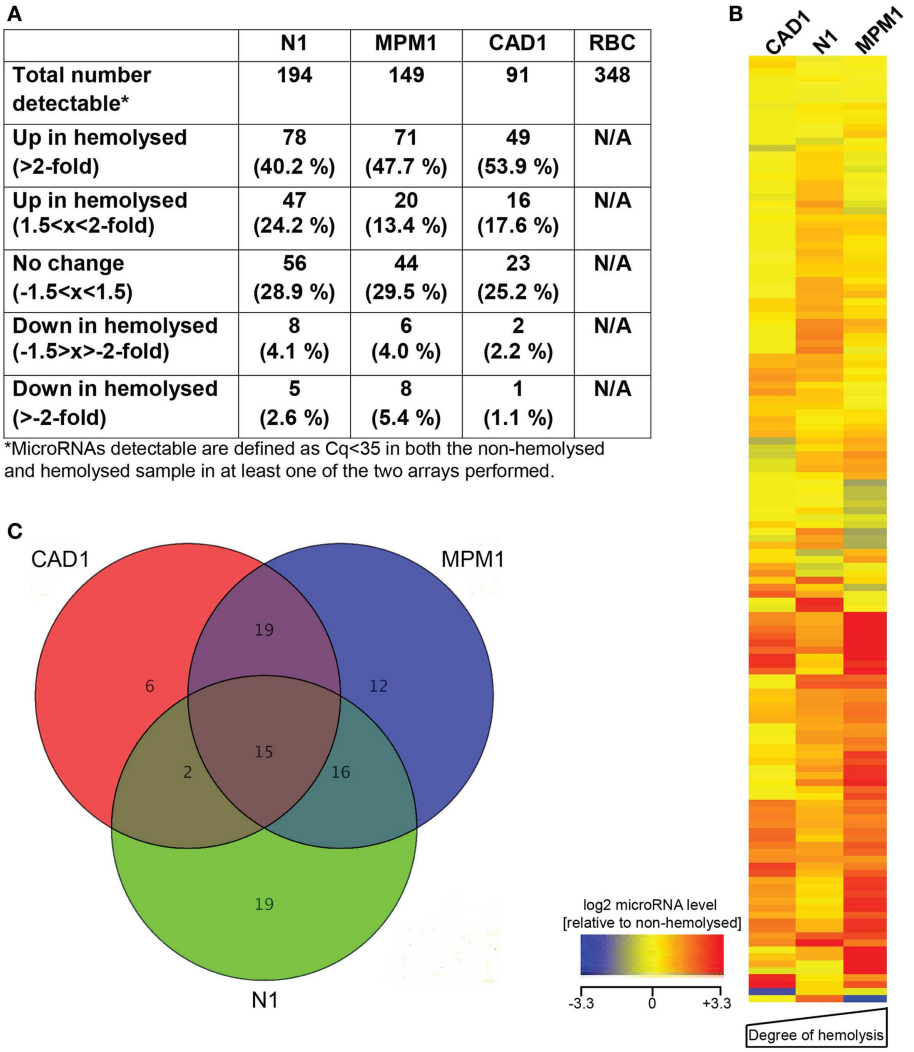

FIGURE 1 | MicroRNA profiling of hemolyzed and non-hemolyzed plasma. **(A)** Summary of each pairs microRNA profile, representing the total number of microRNAs detectable as well as numbers of microRNAs affected or unaffected by hemolysis in each pair. **(B)** Heatmap of relative abundance of 136 microRNAs in hemolyzed compared to non-hemolyzed plasma. Levels of microRNAs in hemolyzed samples are presented as log2 of the relative expression level (non-hemolyzed = 0), with ±3.3 being the equivalent of a ±10-fold difference in relative abundance. The microRNAs presented are those detectable in at least four out of the six plasma samples investigated. **(C)** Overlap of microRNAs increased >2-fold in each of the three pairs. Fifteen microRNAs (inner triangle) were >2-fold increased in all three investigated pairs, and an additional 37 microRNAs were >2-fold increased in at least two of the investigated pairs.

miR-92a, and miR-210 are significantly affected by the level of RBC contamination of the sample displaying decreases in Cq values of 1.5–2.4 cycles between 0 and 0.125% RBC contamination (**Figure 5A**). This was also confirmed when looking at the matched pairs in which those miRs were in most cases at least twofold higher in the hemolyzed sample (**Figure 5B**). In addition miR-21 seemed to be affected by high degrees of hemolysis (**Figure 5B**). Although miR-31 levels appear to be higher in hemolyzed compared to non-hemolyzed plasma, this variability could be a result of the low expression of this microRNA, with Cq values being >34 in all samples. None of the remaining microRNAs showed a correlation between Cq values and the degree of hemolysis (**Figures 5C,D**).

IDENTIFICATION OF microRNA SUBSETS DISPLAYING SIMILAR CHANGES DUE TO HEMOLYSIS

The clear influence of hemolysis on plasma microRNA content raises the question of whether hemolyzed samples can be included in biomarker discovery studies. To further address this issue we interrogated the profiling data in an attempt to identify groups of microRNAs with similar patterns of hemolysis-induced increase with the aim of identifying subsets of microRNA from which both endogenous controls and potential biomarkers could be derived. For the purpose of this part of the study we focused on the two samples exhibiting extensive hemolysis. Twenty-six microRNAs were increased by at least twofold, in these samples with increases following the degree of hemolysis (**Table 5**).

Similarly, we identified a second subset of 19 microRNAs which varied by <±1.5-fold (**Table 5**). Both groups include microRNAs previously proposed as biomarkers for disease. Although requiring further validation, these two subsets represent groups of microRNAs that may be used for selection of biomarker candidate(s) and endogenous control(s) potentially enabling hemolyzed samples to be included in biomarker discovery studies.

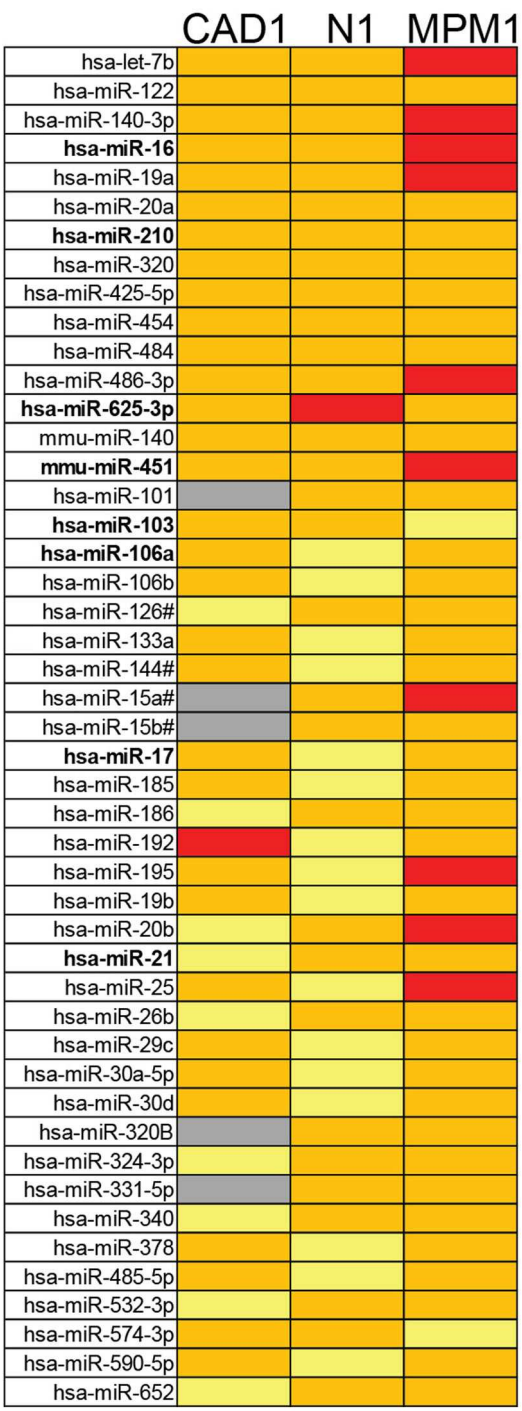

FIGURE 2 | Continued

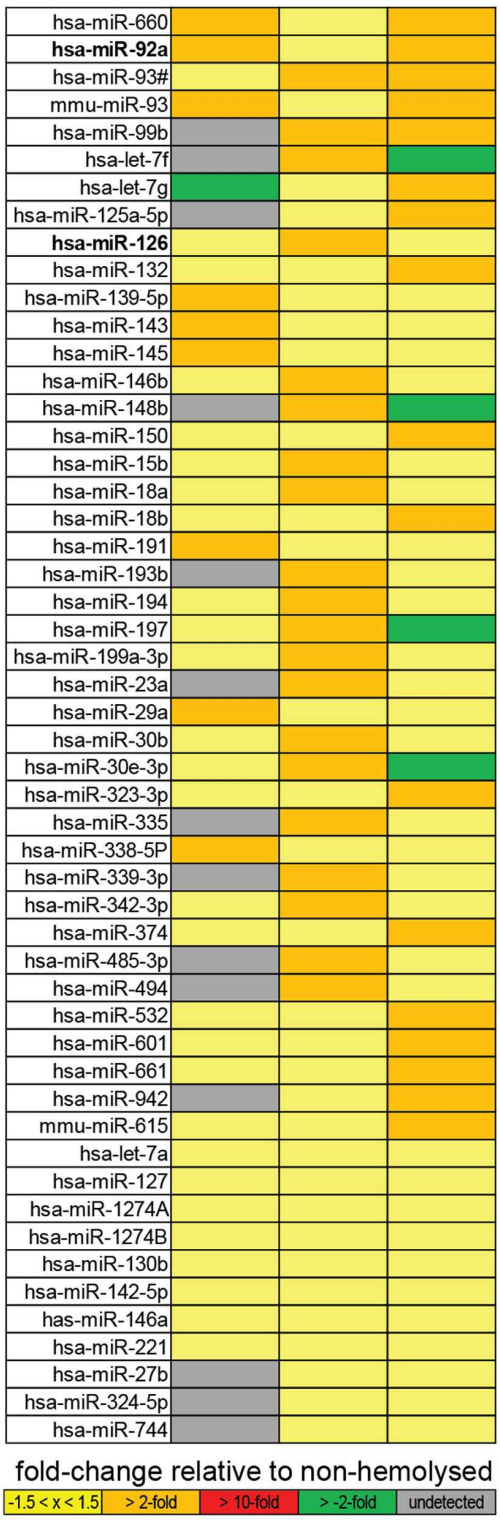

FIGURE 2 | Overview of microRNAs identified as being elevated in hemolyzed plasma or unaffected by hemolysis. The top 15 microRNAs are elevated in the hemolyzed sample of all three pairs, the following 37 microRNAs are elevated in two of the three pairs followed by the 36 microRNAs elevated in just one pair. The 11 microRNAs at the bottom of the list represent those with less than ±1.5-fold difference between the hemolyzed and non-hemolyzed sample.

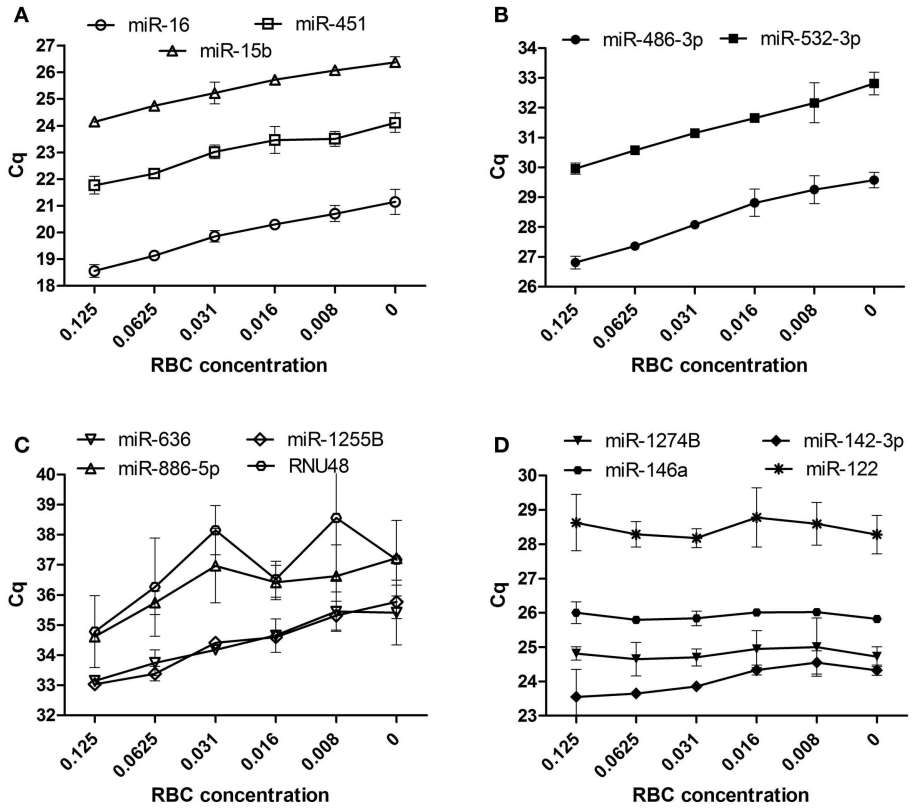

FIGURE 3 | RT-qPCR validation of candidates in dilution series of RBCs in plasma. **(A)** Previously identified hemolysis affected microRNAs **(B)** additional hemolysis affected microRNAs **(C)** RBC- and hemolyzed plasma specific microRNAs **(D)** unaffected microRNAs. Data are presented as raw Cq value ± SD obtained from measurements in RNA isolated from two independent dilution series.

Table 3 | Cq values of stable miRs in dilution series.

RBC concentration (%)	miR-1274B	miR-142-3p	miR-146a	miR-122
0.0	24.73 ± 0.29	24.33 ± 0.15	25.82 ± 0.06	28.28 ± 0.56
0.008	25.00 ± 0.85	24.55 ± 0.34	26.02 ± 0.08	28.59 ± 0.62
0.016	24.94 ± 0.54	24.34 ± 0.14	26.01 ± 0.08	28.78 ± 0.86
0.031	24.70 ± 0.24	23.86 ± 0.12	25.84 ± 0.21	28.18 ± 0.28
0.0625	24.65 ± 0.49	23.65 ± 0.08	25.80 ± 0.05	28.29 ± 0.37
0.125	24.82 ± 0.20	23.55 ± 0.80	26.00 ± 0.31	28.63 ± 0.82

DISCUSSION

Cell-free microRNAs are detectable in a variety of body fluids and have in recent years attracted a lot of attention due to their potential use as biomarkers for disease. The majority of studies have focused on plasma or serum, attractive because it is readily available, can be collected with minimal risk or discomfort and is routinely collected as part of clinical assessment of patients. As a result a large number of novel plasma or serum microRNA biomarkers have been suggested in the last 5 years (Brase et al., 2010; Reid et al., 2011; Creemers et al., 2012; Mo et al., 2012). Nevertheless, despite this rapid growth the field still suffers from a lack of standardized and detailed reporting methods.

The cellular origin of most microRNAs detectable in the circulation is as yet unknown. Studies comparing the microRNA profile of plasma or serum from healthy and diseased individuals (Chen et al., 2008; Kirschner et al., 2012) have shown that there is extensive overlap between the profiles suggesting that many of the microRNAs present in the circulation could play important roles in the normal functioning of the circulatory and immune system. Consequently, proposed markers for disease rarely seem to be tissue-specific, making their use as markers for specific conditions difficult. Examples of such microRNAs are miR-92a [proposed as a diagnostic marker for colorectal cancer (CRC) (Huang et al., 2010), and Non-Hodgkin's Lymphoma (Ohyashiki et al., 2011), as well as CAD (Fichtlscherer et al., 2010)] and miR-21 [e.g., in gastric cancer (Tsujiura et al., 2010; Li et al., 2012), CRC (Kanaan et al., 2012) and Non-small cell lung cancer (NSCLC) (Tang et al., 2013)]. Assessment of the true role of these microRNAs is further complicated by the high abundance of these and other microRNAs in blood cells, and by variations in blood cell counts that have been shown to have a significant impact on the levels detectable in plasma or serum (Duttagupta et al., 2011; Pritchard et al., 2012).

The influence of blood cell lysis on plasma and serum levels of microRNAs has until recently been neglected. While it had already been reported that lymphocytes and RBCs have specific microRNA content (Ramkissoon et al., 2006; Bruchova et al., 2007;

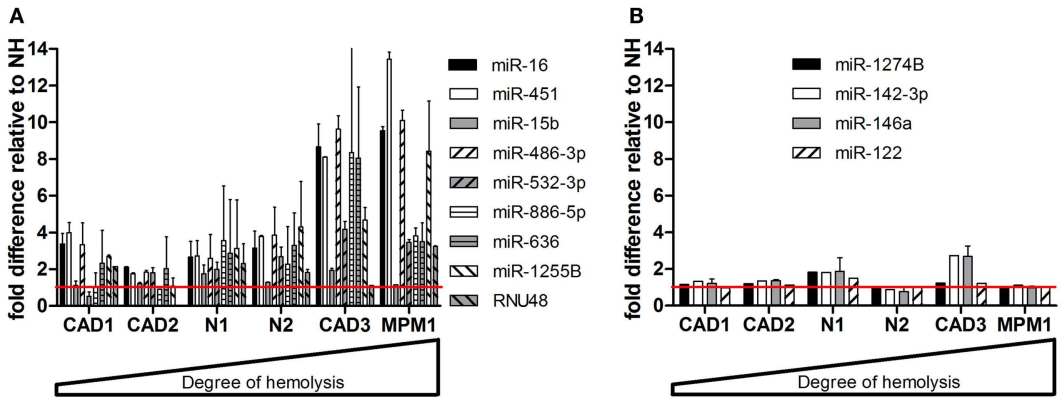

FIGURE 4 | Validation of microRNAs in matching pairs. **(A)** microRNAs changing with hemolysis **(B)** microRNAs not affected by hemolysis. Data are presented as relative microRNA level ± SD in the hemolyzed compared to the non-hemolyzed sample value obtained from measurements in RNA from two independent isolations per sample. The red line represents the relative expression of the corresponding non-hemolyzed samples.

Collino et al., 2010; Vasilatou et al., 2010), the overlap between these profiles and those in plasma were initially overlooked. In 2011, after 3 years of biomarker discovery studies, the first reports describing the effects of hemolysis occurring during blood collection or processing appeared, and suggested that this can have considerable impact on the levels of certain microRNAs detected in hemolyzed samples (Kirschner et al., 2011; McDonald et al., 2011; Pritchard et al., 2012). The three studies all identified miR-16 and miR-451 as being the most highly abundant microRNAs in RBCs, and found levels of these microRNAs in plasma to be most affected by hemolysis. In addition to the contribution of RBCs to the cell-free microRNA profile, Pritchard et al., also showed that platelet-derived microRNAs can significantly vary between samples. Most studies highlighting the problems associated with hemolysis have not yet gone beyond investigating small numbers of microRNAs (in particular miR-16 and miR-451), but two reports have already identified that miR-92a (a putative biomarker in several cancers) is heavily affected by hemolysis (Kirschner et al., 2011; Pritchard et al., 2012). In addition a recent study by Blondal et al. (2013) has compared levels of 119 microRNAs in high quality serum/plasma and compromised serum/plasma. This study has shown that the levels of many of those microRNAs deviate significantly from the average level observed in a large number of high quality samples when being measured in a compromised sample with high blood cell contamination. Furthermore, this study suggested the use of a ratio between a microRNA known to be highly variable with hemolysis (miR-451) and a microRNA found to be unaffected by hemolysis (miR-23a) as an indicator for hemolysis, especially in a situation where only RNA is available while the original plasma/serum sample is unavailable for assessment of hemolysis (Blondal et al., 2013).

To build on these observations and to further investigate the effect hemolysis has on proposed cell-free microRNA biomarker candidates we performed a more comprehensive comparison of the microRNA content in hemolyzed and non-hemolyzed plasma. This revealed up to 65% (88 of 136) of the microRNAs detectable in plasma to be elevated in hemolyzed samples (**Figure 2**). Among those were many microRNAs that have been previously proposed as biomarkers for various diseases (**Table 4**), and upon validation we demonstrate that in addition to miR-16, -451, and 92a, levels of miRs-106a, -17, -21, and -210 were susceptible to hemolysis (**Figures 4A,B**). This observation raises the question of whether microRNAs present in RBCs are suitable for use as biomarkers, even if they are often expressed at high levels in certain tissues or overexpressed in solid tumors. The enrichment of these microRNAs in RBCs complicates the interpretation of biomarker studies as it is difficult to discriminate between presence of a microRNA in the circulation due to controlled release from the tissue of origin (e.g., microvesicles), mechanical rupture of cells (e.g., ischemia) and the changes introduced by hemolysis.

Hemolysis may occur *in vivo* as part of the underlying disease process, or (more commonly) as a complication of the collection and processing of blood. Hematological malignancies such as chronic lymphocytic leukemia can result in autoimmune hemolysis (Rytting et al., 1996), while metastatic solid cancers can be associated with microangiopathic hemolysis and disseminated intravascular coagulation (Lohrmann et al., 1973; Rytting et al., 1996; Lechner and Obermeier, 2012). In addition, a number of drugs – in particular chemotherapeutic drugs – can influence the normal structure of RBCs (Dumez et al., 2004; Schauf et al., 2004), and render the cell vulnerable to damage as it passes through the microvasculature. This in turn can result in release of RBC intracellular contents, and therefore has the potential to increase RBC-enriched microRNAs in the plasma. However, these are all relatively rare events which in most patients would not be expected to contribute to a significant increase in RBC-related microRNAs. Besides the possibility of disease- or drug-associated hemolysis resulting in an increase in levels of RBC-enriched microRNAs, disease-related anemia has the potential to result in changes in the levels of potential biomarker microRNAs in plasma/serum. A number of studies have reported a decrease in levels of candidate microRNA biomarkers in cancer, which is somewhat counterintuitive. For example, reduced levels of the RBC-enriched

Table 4 | Proposed biomarker microRNAs.

miRNA	Disease	Regulation	Reference	Affected by hemolysis
miR-103	MPM	Down (compared to asbestos-exposed and healthy)	Weber et al. (2012)	No
	Pancreatic cancer (PC)	Up (compared to healthy)	Ren et al. (2012)	
miR-106a	Gastric cancer (GC)	Up (compared to healthy)	Tsujiura et al. (2010)	Yes
miR-122	Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC)	Up (compared to healthy)	Zhou et al. (2011)	No
	Hepatitis B	Up (compared to healthy)	Zhang et al. (2010)	
	Liver injury (mouse model)	Up (compared to no injury)	Zhang et al. (2010)	
miR-126	MPM	Down (compared to healthy and NSCLC)	Santarelli et al. (2011), Tomasetti et al. (2012)	No
	Abdominal aortic aneurysm (AAA)	Down (compared to healthy)	Kin et al. (2012)	
miR-146a	Esophagitis	Up (compared to healthy)	Lu et al. (2012a)	No
	PC	Down (compared to healthy)	Ali et al. (2010)	
miR-155	AAA	Down (compared to healthy)	Kin et al. (2012)	No
	Esophageal cancer	Down (compared to healthy)	Liu et al. (2012)	
	Breast cancer	Up (compared to healthy)	Lu et al. (2012b)	
miR-16	Traumatic brain injury	Up (compared to healthy)	Redell et al. (2010)	Yes
	Myelodysplastic syndrome	Up (compared to healthy)	Zuo et al. (2011)	
miR-17	Endometriosis	Down (compared to healthy)	Jia et al. (2013)	Yes
	GC	Up (in later stages)	Wang et al. (2012)	
	GC	Up (compared to healthy)	Tsujiura et al. (2010)	
miR-21	PC	Up (compared to healthy)	Ali et al. (2010)	Yes
	GC	Up (compared to healthy)	Tsujiura et al. (2010)	
	HBV-related HCC	Up (compared to healthy)	Zhou et al. (2011)	
	Esophagitis	Up (compared to healthy)	Lu et al. (2012a)	
miR-210	PC	Up (compared to healthy)	Wang et al. (2009)	Yes
	Kidney injury	Up (compared to healthy)	Lorenzen et al. (2011)	
miR-223	NSCLC	Down (in later stages)	Heegaard et al. (2012)	No
	AAA	Down (compared to healthy)	Kin et al. (2012)	
	Esophagitis	Up (compared to healthy)	Lu et al. (2012a)	
	HBV-related HCC	Down (compared to healthy)	Zhou et al. (2011)	
miR-27a	HBV-related HCC	Down (compared to healthy)	Zhou et al. (2011)	No
miR-29a	CRC	Up (compared to healthy)	Huang et al. (2010)	No
miR-31	Breast cancer	Up (compared to healthy)	Lu et al. (2012b)	No
miR-451	GC	Up (compared to healthy)	Konishi et al. (2012)	Yes
miR-625-3p	MPM	Up (compared to healthy and asbestosis)	Kirschner et al. (2012)	No
miR-720	Multiple myeloma	Up (compared to healthy)	Jones et al. (2012)	No
miR-92a	CAD	Down (compared to healthy)	Fichtlscherer et al. (2010)	Yes
	Non-Hodgkin's lymphoma	Down (compared to healthy)	Ohyashiki et al. (2011)	
	Multiple myeloma	Down (compared to healthy)	Yoshizawa et al. (2012)	
	CRC	Up (compared to healthy)	Huang et al. (2010)	

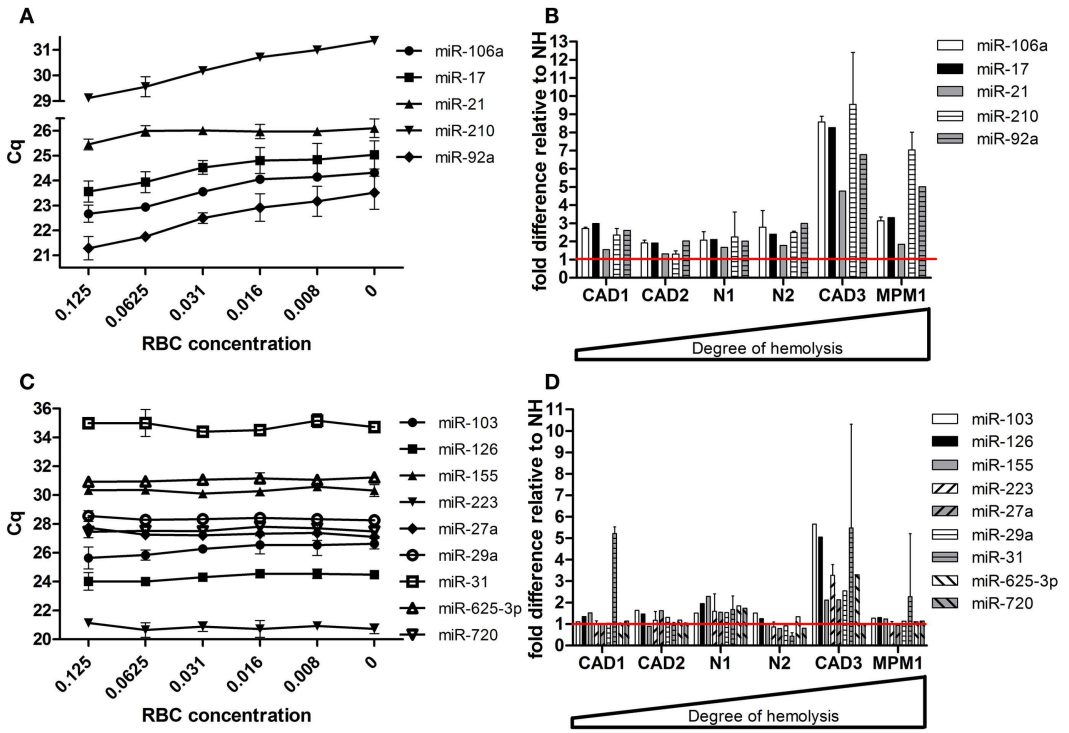

FIGURE 5 | Effect of hemolysis on biomarker candidates. Five proposed biomarker candidates were identified to be changing with increased hemolysis in **(A)** the RBC dilution series and **(B)** the six matching pairs of hemolyzed and non-hemolyzed plasma. Another nine proposed biomarkers remained unaffected by increasing hemolysis in both the dilution series **(C)** and the matched pairs **(D)**. The red line represents the relative expression of the corresponding non-hemolyzed samples. Data are presented as raw Cq value ± SD **(A,C)** or relative microRNA level ± SD **(B,D)**.

miR-92a were reported to have diagnostic potential in hematological malignancies, while levels of this microRNA are increased in the corresponding malignant cells (Tanaka et al., 2009; Ohyashiki et al., 2011; Yoshizawa et al., 2012). In addition miR-126, another microRNA present at high levels in RBCs was found to be reduced in plasma from MPM patients (Santarelli et al., 2011; Tomasetti et al., 2012), a disease in which anemia is a relatively frequent occurrence. It is tempting to speculate that this somewhat surprising discrepancy between plasma and cell levels of miR-92a in leukemia, and the decrease of miR-126 in MPM, results from reduced levels of RBCs as a result of cancer-related anemia in those patients.

More commonly hemolysis occurs during blood collection and sample processing. Hemolysis has been reported in up to 42.6% of general medical hospital inpatients (free hemoglobin >0.5 g/l), but only 5.6% when using visual inspection (Lippi et al., 2009; Hawkins, 2010). Causes of sample hemolysis include venous stasis, phlebotomist experience and site of collection (Lippi et al., 2005; Hawkins, 2010). Using spectrophotometry we have detected significant hemolysis in approximately 15% of collected samples (often in the absence of color change in plasma/serum visible to the eye). Hemolysis may be an under-reported and important contributor to changes in microRNA levels in plasma or serum. The relative importance of a technical cause of hemolysis should be considered in the context of two different scenarios: biomarker discovery and diagnostic testing. In the case of studies aiming to identify microRNAs as diagnostic or prognostic markers for a disease, the effect of hemolysis may be limited as one would expect that hemolysis occurring during blood taking or plasma processing would affect equal numbers of samples in both case and control groups. Thus, variations introduced by *in vitro* hemolysis should therefore be present in any sample series investigated and not influence the identification of potential biomarkers. However, the large variations in abundance of microRNA biomarkers reported in a number of studies might be a result of the inclusion of hemolyzed samples. This speculation is supported by our own experience; when excluding hemolyzed plasma from our sample series we find a significant reduction in variability in levels of RBC-microRNA (Kirschner et al., 2011) and also see a lower variability in the levels of potential diagnostic markers (Kirschner et al., 2012) than those reported by others (Ng et al., 2009; Heneghan et al., 2010; Huang et al., 2010; Devaux et al., 2012). Together, these observations suggest that a quantification of hemolysis in samples should be included in any study of a potential biomarker candidate.

Although hemolysis is likely to represent only a minor confounding factor in biomarker discovery studies, the situation is different once a microRNA is actually confirmed as a marker for a specific disease and used as a diagnostic/prognostic tool. In this setting, where for instance a single sample is analyzed to determine presence or absence of disease, hemolysis can significantly impact on the result, especially if the microRNA used as marker

Table 5 | Subsets of biomarker-endogenous control candidates.

microRNAs changing with hemolysis			microRNAs unaffected by hemolysis		
microRNA	Relative fold increase H vs. NH		microRNA	Relative fold difference H vs. NH	
	N1	MPM1		N1	MPM1
let-7b	2.12 ± 0.43	14.06 ± 9.83	let-7a	1.33 ± 1.3	1.25 ± 0.71
miR-126#	2.70 ± 1.03	2.87 ± 1.00	let-7d	1.49 ± 0.77	0.97 ± 0.78
miR-140-3p	2.60 ± 0.70	10.96 ± 3.69	miR-1260	1.37 ± 1.14	1.23 ± 0.52
miR-15a#	2.92 ± 1.96	2.00 ± 1.10	miR-127	1.29 ± 0.68	0.66 ± 0.86
miR-15b#	5.40 ± 4.17	4.96 ± 2.04	miR-1274A	1.14 ± 0.27	1.20 ± 0.59
miR-16	3.55 ± 0.87	10.18 ± 0.38	miR-1274B	1.49 ± 0.39	0.87 ± 0.06
miR-193b	5.26 ± 3.39	6.16 ± 6.00	miR-130b	1.00 ± 0.38	1.10 ± 0.33
miR-194	18.14 ± 14.34	2.86 ± 2.21	miR-142-5p	1.50 ± 1.04	1.30 ± 0.43
miR-20a	2.10 ± 0.83	3.83 ± 0.37	miR-143	0.89 ± 0.47	1.09 ± 0.32
miR-20b	2.46 ± 1.80	19.24 ± 3.30	miR-146a	1.29 ± 0.14	1.49 ± 0.87
miR-21	2.57 ± 0.47	2.90 ± 0.22	miR-221	1.35 ± 0.58	1.50 ± 0.93
miR-210	2.50 ± 1.01	26.93 ± 7.48	miR-222	1.26 ± 0.45	1.38 ± 0.06
miR-26b	2.56 ± 0.48	3.58 ± 1.29	miR-27b	1.49 ± 1.40	1.27 ± 0.21
miR-320	2.25 ± 0.13	3.32 ± 0.01	miR-324-5p	1.20 ± 0.04	1.08 ± 0.90
miR-320B	2.28 ± 0.96	3.66 ± 3.50	miR-338-5P	0.76 ± 0.06	1.41 ± 0.77
miR-324-3p	3.30 ± 1.51	9.12 ± 6.61	miR-339-5p	1.29 ± 0.94	1.04 ± 0.16
miR-331-5p	3.54 ± 3.40	22.65 ± 19.81	miR-340#	0.94 ± 0.38	0.8 ± 0.53
miR-340	2.63 ± 1.43	3.71 ± 1.20	miR-425#	1.10 ± 0.83	0.96 ± 0.02
miR-425-5p	2.44 ± 0.90	4.75 ± 3.74	miR-744	0.71 ± 0.21	1.01 ± 0.31
miR-454	4.62 ± 1.19	6.15 ± 3.64			
miR-484	2.11 ± 0.12	3.13 ± 0.09			
miR-486-3p	2.51 ± 1.32	14.20 ± 5.57			
miR-532-3p	2.50 ± 2.05	3.75 ± 2.71			
miR-652	3.08 ± 2.52	3.45 ± 0.43			
miR-140	2.17 ± 0.18	3.20 ± 0.56			
miR-451	2.38 ± 0.84	15.27 ± 1.78			

is hemolysis-susceptible. As the clinical application of microRNAs as diagnostic/prognostic markers is the ultimate aim of the discovery studies currently performed, it is essential that these potential confounders are taken into consideration, and hemolysis needs to be quantified as part of the quality control process. While appropriate methods for normalization of microRNA expression data [generally obtained through real-time quantitative PCR (RT-qPCR)] could potentially overcome the problems associated with hemolysis-related variability, the normalization of plasma/serum microRNA data has yet to be standardized. The problem of normalization has been addressed in many studies as it is often not possible to accurately quantify RNA concentration, with the result that using equal amounts of RNA as input into the reactions is mostly impossible (Brase et al., 2010; Reid et al., 2011; Zampetaki and Mayr, 2012). A frequently used alternative is to spike-in known amounts of exogenous microRNAs (such as *C. elegans* miRs) during the isolation process (Mitchell et al., 2008). This approach certainly has the potential to account for differences in RNA isolation efficiency between different samples, but it is not able to account for differences introduced by hemolysis. Based on the profiling data in the present study, we propose the possibility of selecting a biomarker and an appropriate endogenous control from subsets of microRNAs affected equally by hemolysis. Our pilot study identified two potential subsets, one with microRNAs unaffected by hemolysis and one with hemolysis-susceptible microRNAs. Such an approach can serve as a starting point for further in-depth validation in a much larger sample set.

In summary, our data provide further evidence that hemolysis has a substantial impact on a large number of cell-free microRNAs in plasma and serum, many of which are being investigated as potential biomarkers of disease. These data suggest that future investigations of cell-free microRNA biomarkers should carefully assess hemolysis and the effect it has on a biomarker candidate.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Non-Coding_RNA/10.3389/fgene.2013.00094/abstract

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Cell-free microRNAs: potential biomarkers in need of standardized reporting

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MicroRNAs are abundantly present and surprisingly stable in multiple biological fluids. These findings have been followed by numerous reverse transcription real-time quantitative PCR (RT-qPCR)-based reports revealing the clinical potential of using microRNA levels in body fluids as a biomarker of disease. Despite a rapidly increasing body of literature, the field has failed to adopt a set of standardized criteria for reporting the methodology used in the quantification of cell-free microRNAs. Not only do many studies based on RT-qPCR fail to address the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) criteria but frequently there is also a distinct lack of detail in descriptions of sample source and RNA isolation. As a direct result, it is often impossible to compare the results of different studies, which in turn, hinders progress in the field. To address this point, we propose a simple set of criteria to be used in conjunction with MIQE to reveal the true potential of cell-free microRNAs as biomarkers.

Keywords: cell-free microRNA, isolation, quantification, reporting, standard

INTRODUCTION

In recent years, a number of studies have shown that cell-free microRNAs are readily detectable in body fluids, such as plasma or serum. The surprising stability of cell-free microRNAs in these body fluids has been attributed to encapsulation of microRNAs in microvesicles such as exosomes (Cortez and Calin, 2009; Kosaka et al., 2010), and association with protein complexes, like high density lipoproteins (Vickers et al., 2011) and argonaute 2 (Arroyo et al., 2011; Turchinovich et al., 2011). These findings led to numerous studies aiming at the identification of microRNAs, mainly in plasma and serum, as potential diagnostic and/or prognostic markers for a variety of diseases (Reid et al., 2011; Creemers et al., 2012; Mo et al., 2012). However, although most studies are using reverse transcription real-time quantitative PCR (RT-qPCR) for detection of microRNAs, there is still a lack of consensus regarding isolation procedures, optimal quantification methods, reference genes, and quality control of samples. In addition, most studies fail to provide sufficient detail on the methods used for RNA isolation, quality control of samples, and do not report according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) criteria (Bustin et al., 2009). As a result, comparisons between studies from different laboratories are almost impossible, and it is not surprising that different studies using profiling strategies to identify plasma/serum biomarkers for the same disease fail to identify the same microRNAs.

In order to overcome these problems, we propose a simple set of reporting criteria to be used in conjunction with MIQE reporting guidelines (Bustin et al., 2009). The following sections outline the steps undertaken to quantify cell-free microRNAs together with important aspects to be considered in each step. We limit the microRNA quantification section to RT-qPCR as this is the most commonly used method for microRNA detection, and for simplicity we focus on plasma and serum, but many aspects will apply equally to other quantification methods (such as microarray) or other biological fluids. Finally, we provide lists of important points to be included when reporting investigation of cell-free microRNAs.

SAMPLE COLLECTION

The first critical step in the process of assessing levels of cell-free microRNAs in plasma and serum is the collection and handling of the sample. Both serum and plasma are suitable for assessment of cell-free microRNAs, but when using plasma samples ethylenediaminetetraacetic acid (EDTA) or citrate are the preferred anticoagulants as heparin is known to inhibit both reverse transcription and qPCR steps (Kroh et al., 2010). The use of more than one type of collection tube within a study is generally discouraged as the resulting microRNA profiles can vary between different tubes, making comparisons inaccurate (Kroh et al., 2010). Reports on cell-free microRNAs should therefore always include details regarding the blood collection procedure, type of blood collection tube, and processing of the sample.

Essential information to be provided:

- Collection site, caliber, and type of needle/cannula
- Type and size of collection tube (including manufacturer)
- Time between collection and further processing
- In case of serum, time allowed for clotting
- Conditions of sample processing (centrifugation speed and time)
- Storage condition of samples

QUALITY CONTROL OF SAMPLES

One of the most critical points in the process of quantifying cell-free microRNAs is that of quality assurance and quality control (QA/QC). While being neglected in the early stages of cell-free microRNA research, a small number of studies have now shown

that the contamination with microRNAs released from circulating blood cells represents one of the major sources of microRNAs detectable in plasma or serum (Kirschner et al., 2011; McDonald et al., 2011; Pritchard et al., 2012). The most common source of blood cell derived microRNAs are red blood cells (RBCs) and two studies have shown that hemolysis occurring during sample collection or processing can significantly alter the levels of certain RBC-enriched microRNAs, such as miR-16 and miR-451 (Kirschner et al., 2011; McDonald et al., 2011). In addition, microRNAs released from platelets can also alter the microRNA profile (Pritchard et al., 2012). These changes caused by rupturing of blood cells result in variation in microRNA levels independent of the presence or absence of a certain disease state. While not yet investigated in detail, one can confidently assume that other microRNAs, including those previously being proposed as potential biomarkers for disease can be affected in a similar way to, e.g., miR-16 and miR-451. Measuring the degree of hemolysis, a major source of blood cell microRNAs in the fluid component, can be easily achieved by assessing the presence of free hemoglobin through simple measurement of the absorbance at 414 nm (the absorbance maximum of free hemoglobin) in the initial plasma or serum sample using a standard spectrophotometer (Wong et al., 2006). Data on the degree of hemolysis of samples should always be provided, and applying a cut-off level of free hemoglobin to determine if samples are usable for further analysis, such as an OD_{414} of 0.2 as we have suggested (Kirschner et al., 2011), should always be considered. In addition, like for formalin-fixed paraffin-embedded (FFPE) tissue samples (Szafranska et al., 2008), the age of a sample will certainly play a role in respect to the quality of RNA that can be obtained (Grasedieck et al., 2012). Both storage condition and sample age should therefore be provided.

RNA ISOLATION

The isolation of RNA represents another critical step in order to obtain a sample of acceptable quality for further investigation. The most commonly used methods for RNA isolation from body fluids are (i) column-based purification with mostly mirVana PARIS and Qiagen (miRNeasy Serum/Plasma Kit) or (ii) non-column purification using reagents such as TRIzol LS or QIAzol. While no study has yet shown a direct comparison between column-based and non-column purification, a study in cell lines suggests that care must be taken regarding the choice of isolation method. Kim et al. (2012) have shown that when isolating RNA from cells using a non-column method the number of cells seems to not only affect the efficiency of RNA isolation in general, but also result in a selective loss of microRNAs with low guanine–cytosine (GC) content when isolating from a small cell number, hence when handling samples with low RNA concentration. Plasma and serum are low RNA concentration samples, raising the possibility that preferential isolation of a subset of microRNAs might occur too when using non-column purification. In addition, data from our own studies generally comparing TRIzol LS and mirVana PARIS isolation efficiencies have shown that while TRIzol LS purification results in higher levels of total RNA recovered from plasma samples (**Figure 1A**), mirVana PARIS is superior in isolating small RNAs as shown for both moderately to highly abundant endogenous microRNAs (**Figure 1B**), and exogenous spike-ins (**Figure 1C**). Nevertheless, as no formal comparison for larger numbers of microRNAs has been performed so far for plasma and serum, no firm conclusions can be drawn regarding the best isolation method for these sample types.

Regardless of the relative merits of the various options for RNA isolation, studies investigating cell-free microRNAs should always provide sufficient details on the isolation method used, and these should be:

- Sample volume from which RNA was isolated
- Reagent or kit used for RNA isolation
- Whether a carrier (glycogen, tRNA, etc.) was used, the concentration of any such reagent and at which step of the isolation they have been added
- Whether and in which concentration any spike-ins such as synthetic *C. elegans* microRNAs have been used and at which step of the isolation process those have been added
- The volume used for resuspension or elution of the final RNA sample.

RNA QUANTIFICATION

Quantification of RNA isolated from dilute samples such as plasma/serum can be very difficult (Kroh et al., 2010). Even when using a carrier to enhance the isolation efficiency, attempts to measure RNA concentration by standard spectrophotometry often fail due to the detection limit of this method (generally around 4–10 ng/μl). Alternatively fluorometric quantitation can be used, however, especially when using serum the concentration of the obtained samples can still be too low for accurate quantitation. Using larger sample input into the isolation process may overcome this problem. However, large amounts of sample are not always available and most studies have shown that microRNAs are readily detectable when isolating from 500 μl or less of plasma/serum (Kroh et al., 2010).

As a consequence of these difficulties in quantifying the RNA concentration of dilute solutions, it is in many cases not possible to use equal amounts (e.g., nanogram) of template RNA for each sample in the subsequent reverse transcription step. Often the only alternative is to use equal input volume of RNA (Mitchell et al., 2008), but this approach requires the availability of an alternative normalization method to account for differences in input RNA concentration. The most commonly used approaches here are the use of an exogenous spike-in microRNA of known quantity (Mitchell et al., 2008) or the use of endogenous control microRNAs which do not vary significantly between samples obtained from study and control subjects (Kirschner et al., 2011). However, both these strategies are associated with further problems which are discussed in more detail below.

REVERSE TRANSCRIPTION REAL-TIME QUANTITATIVE PCR

A number of different technologies are available for RT-qPCR quantification of microRNAs. The most commonly used technologies are the microRNA-specific stem-loop reverse transcription in combination with TaqMan qPCR primer/probe assays (Life Technologies) or the combination of poly-A-tailing in the reverse transcription step followed by either locked-nucleic-acid (LNA)-enhanced forward and reverse primers (Exiqon) or standard SYBR green forward and reverse primers (Qiagen). Each technology

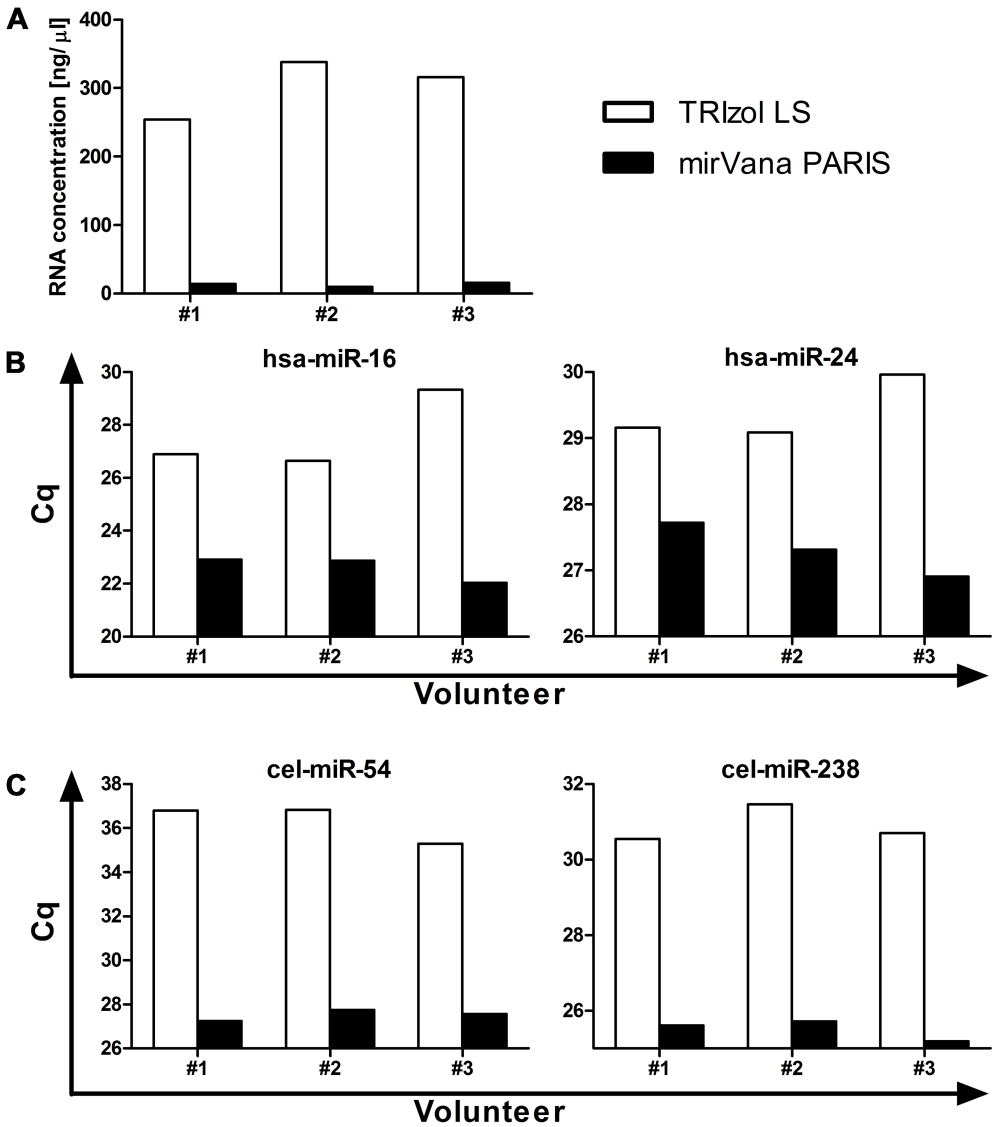

FIGURE 1 | Comparison between TRIzol LS and mirVana PARIS microRNA isolation efficiency. Total RNA was isolated from 500 µl plasma from three healthy volunteers and total RNA concentration **(A)**, levels of moderately to highly abundant endogenous microRNAs **(B)**, and of exogenous spike-ins **(C)** were assessed. RT-qPCR data are presented as raw Cq values.

provides advantages and disadvantages, but the superiority of one system over the other has not been proven (Zampetaki and Mayr, 2012). When reporting on RT-qPCR for cell-free microRNAs it is therefore essential to provide sufficient detail (following the MIQE guidelines; Bustin et al., 2009) to allow the reader to understand and reproduce published data using the same technology.

The minimum details that should be provided are:

- Primer sequences or exact assay IDs/catalog numbers when using proprietary assays
- Amount of RNA used as input into reverse transcription, or volume of eluate
- Reverse transcription procedure (poly-A-tailing or stem-loop) including all reagents and concentrations used, and reaction conditions (even if as per manufacturer)
- Details of instrument used to perform reverse transcription
- Whether preamplification was done, and if so how
- Dilution of cDNA and amount of cDNA used in qPCR
- All reagents and the concentrations used for qPCR as well as reaction conditions
- Details on instrument used to run qPCR and methods used to determine threshold (details on software used to analyze qPCR data/determine threshold)

NORMALIZATION OF RT-qPCR DATA

The final and perhaps most critical step when quantifying cell-free microRNAs is the quantification or normalization method used. With the amount of RNA isolated from plasma or serum being very low and even with the use of carrier reagents during

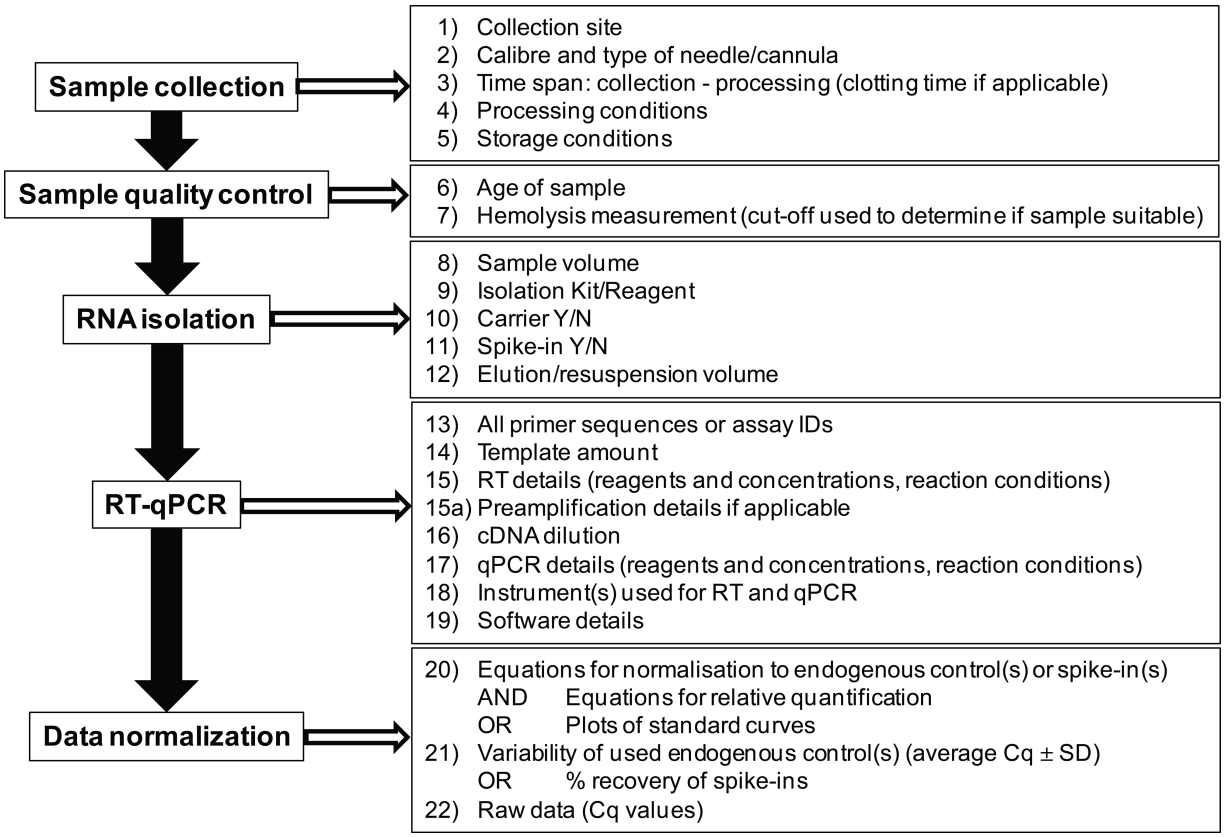

FIGURE 2 | Summary of details that should be provided when reporting on cell-free microRNAs.

RNA isolation often below the threshold of detection (at least for standard spectrophotometry), it is frequently not possible to normalize the concentration of RNA used for reverse transcription. Equal volumes of input RNA are therefore often used instead of a fixed concentration; however this approach introduces the variable associated with comparing samples of unknown RNA concentration.

The major strategies proposed to overcome these problems are (i) the use of endogenous control microRNAs for normalization (Kirschner et al., 2011), (ii) the use of known quantities of exogenous microRNAs (mainly *C. elegans* miRs) that are spiked-in to compensate for differences in isolation efficiency (Mitchell et al., 2008; Kroh et al., 2010), and (iii) absolute quantification based on standard curves generated from synthetic microRNAs.

While an absolute quantification would most likely be the preferred measurement for a clinical setting, the variability in RNA input into the reverse transcription still leaves the problem of normalization between different samples.

Measurement of microRNAs spiked-in during the process of RNA isolation has the potential to account for the variability in initial RNA concentration. However, these spiked-in oligos do not account for variability caused by ruptured blood cells (Kirschner et al., 2011; McDonald et al., 2011; Pritchard et al., 2012). As discussed above, the quality of a sample, in particular the degree of hemolysis can have substantial impact on the levels of microRNAs measured in plasma or serum and should therefore always be taken into consideration. Spiked-in microRNAs are not suited to be used as a second quality control step, like miR-16 or miR-451 are, and one should therefore be careful when using only those as normalization controls for qPCR data. Nevertheless, if spiked-in oligos are used then more information should be provided, including some indication of recovery (%) and variation between samples.

Endogenous microRNAs such as miR-16 and miR-451 are among the most abundant microRNAs present in plasma. These microRNAs represent suitable candidates for normalization of qPCR data. However, as it is not known yet if other microRNAs are also affected by hemolysis, one cannot use miR-16 and miR-451 to adjust for varying degrees of hemolysis in different samples. A detailed analysis of the microRNA profiles in non-hemolyzed and hemolyzed samples is required to determine which microRNAs are affected by rupturing of RBCs. This exercise may help us to define subsets of microRNAs that are affected by hemolysis in the same way, thereby allowing a combination of endogenous control and biomarkers microRNA(s) to be selected. Independent of the outstanding problems associated with the sample quality's effect on cell-free microRNAs, an additional problem with many studies currently being published is a lack of detail regarding the exact ways of normalization and calculation of relative abundance levels of microRNAs.

Studies reporting on cell-free microRNAs should provide the following details:

- Whether a fixed volume or a fixed RNA concentration was used as input into reverse transcription
- Whether a spiked-in microRNA(s) was used, and if so how many and the exact concentration added during the isolation procedure
- If spiked-in microRNAs were used, at least the average recovery of those should be reported
- In case of normalization to a spiked-in microRNA or endogenous control, details on the normalization procedure should be provided (equations used)
- In case of reporting relative expression in one group as compared to another group, details on the calculations should be provided ($2^{-\Delta\Delta Cq}$; Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) is often not detailed enough – how exactly was $\Delta\Delta$Cq calculated?)
- Raw data should be provided

CONCLUSION/PERSPECTIVE

Since their discovery, cell-free microRNAs have been quickly introduced as potential biomarkers. However, it is important to bear in mind that the field of cell-free microRNA research is still in its infancy and that a lack of standardized reporting is making comparisons between studies as well as reproducibility of published data in independent sample series virtually impossible. While we have not solved all the problems associated with quantification of cell-free microRNAs, such as the most appropriate normalization strategies or whether samples with hemolysis can/should be used for analysis, the field would certainly benefit from a standardized way of reporting as summarized in **Figure 2**. Standardized reporting will allow researchers to better understand the data obtained in different laboratories and to use the same methods for potential follow-up or validation studies. Single discovery studies are unlikely to result in the discovery of novel biomarkers for clinical use and it is important to provide all the details of the methodology used to allow further validation by independent investigators. Only in this way the field is enabled to advance eventually leading to the implementation of novel microRNA-based diagnostic/prognostic tests.

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The detection of microRNA associated with Alzheimer's disease in biological fluids using next-generation sequencing technologies

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Diagnostic tools for neurodegenerative diseases such as Alzheimer's disease (AD) currently involve subjective neuropsychological testing and specialized brain imaging techniques. While definitive diagnosis requires a pathological brain evaluation at autopsy, neurodegenerative changes are believed to begin years before the clinical presentation of cognitive decline. Therefore, there is an essential need for reliable biomarkers to aid in the early detection of disease in order to implement preventative strategies. microRNAs (miRNA) are small non-coding RNA species that are involved in post-transcriptional gene regulation. Expression levels of miRNAs have potential as diagnostic biomarkers as they are known to circulate and tissue specific profiles can be identified in a number of bodily fluids such as plasma, CSF and urine. Recent developments in deep sequencing technology present a viable approach to develop biomarker discovery pipelines in order to profile miRNA signatures in bodily fluids specific to neurodegenerative diseases. Here we review the potential use of miRNA deep sequencing in biomarker identification from biological fluids and its translation into clinical practice.

Keywords: microRNA, biological fluids, exosomes, Alzheimer's disease, deep sequencing

INTRODUCTION

The pathophysiological process of neurodegenerative disorders such as Alzheimer's disease (AD) begins well before the diagnosis of clinical dementia. AD is characterized pathologically by the presence of insoluble plaques and tangles composed of beta-amyloid (Aβ) formed by sequential amyloid precursor protein (APP) proteolysis and hyperphosphorlyated Tau (pTau) proteins (Hardy and Allsop, 1991; Walsh and Selkoe, 2004; Cole and Vassar, 2008). The accumulation of Aβ has been the major focus of AD research and has been shown to interfere with long term potentiation which is required for neuronal signaling, and is implicated in pro-apoptotic signaling leading to neuronal loss (Chapman et al., 1999; Roth, 2001). The majority of AD patients are asymptomatic during the pre-clinical stages of the pathological process which is believed to be a period of approximately 17 years (Villemagne et al., 2013). Therefore, early diagnosis of AD is required before or during the pre-clinical phase in order that therapeutic intervention, or the use of disease modifying drugs, can be administered.

The main biomarker targets currently employed for AD diagnosis are measurements of Aβ and pTau (Hansson et al., 2010; Prvulovic and Hampel, 2011; Watt et al., 2011; Rosenmann, 2012). MicroRNA (miRNA) are a class of non-coding RNA of approximately 22 nucleotides in length, and are known to regulate post-translational transcription. Expression profiling of miRNA levels represents a new class of potential biomarkers that are currently being investigated for the diagnosis of a number of diseases (Skog et al., 2008; Taylor and Gercel-Taylor, 2008; Baraniskin et al., 2012b; Geekiyanage et al., 2012; Jones et al., 2012). miRNAs are derived from RNA hairpins comprising of precursor miRNA and processed by endoribonucleases (Dicer and Drosha) to form mature miRNA fragment (Krol et al., 2010). The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) which binds to complementary sites in the 3′ untranslated region of their mRNA targets resulting in down-regulation of gene expression (He and Hannon, 2004). They can be secreted into biological fluids where they can be detected and profiled using methods including quantitative real-time PCR (qRT-PCR), microarrays, and more recently by deep sequencing technologies.

Here, we review the current literature to highlight the diagnostic potential to screen for neurodegeneration using gene expression profiling in biological fluids. In particular, we have focused on the potential of profiling miRNA expression associated with AD and evaluate the current deep sequencing platforms suitable for biomarker discovery including the implementation into clinical diagnostic laboratories.

BRAIN-ASSOCIATED MICRORNAS AND THEIR DETECTION IN BIOLOGICAL FLUIDS

It has been revealed that the highest expression of tissue specific miRNA is found in the brain (Babak et al., 2004; Sempere et al., 2004; Schonrock et al., 2010). The significance of miRNA and their conclusive biological functions were gradually discovered using knockout mouse models (Schaefer et al., 2007). For example, Dicer knockout mice have been shown to deregulate

miRNA processing, leading to defects in neuronal development and underdevelopment of the brain, demonstrating a role for miRNA in neurogenesis (Schaefer et al., 2007; Kawase-Koga et al., 2009; Huang et al., 2010). The first reports translating early miRNA studies to the brain observed a number of brain enriched miRNA such as miR-9, miR-29a, miR-125, miR-128, miR-134, and miR-137 (**Table 1**). With respect to AD, a number of deregulated miRNA have been identified to correlate with disease, including miR-9, miR-20a, and miR-132 (Makeyev et al., 2007; Cogswell et al., 2008; Hebert et al., 2009, 2012). While, synthetic miRNA precursors, miR-20a, miR-17-5p, and miR-106b, when co-transfected in HeLa cell lines, inhibited APP protein translation (Hebert et al., 2009). Highly abundant and brain enriched miRNAs found to be deregulated in AD models (human and mice models) are summarized in **Table 1**.

Extracellular miRNA originating from specific tissues such as the brain and cancerous tissues can be released into biological fluids for example, Cerebral spinal fluid (CSF) (Cogswell et al., 2008; Baraniskin et al., 2012b), blood (Hunter et al., 2008; Baraniskin et al., 2011), saliva (Patel et al., 2011; Ogawa et al., 2013) and urine (Gilad et al., 2008; Qi et al., 2012). Despite the presence of high RNase activity, circulating miRNAs are protected from degradation by binding to RNA binding proteins such as lipoproteins (Arroyo et al., 2011; Vickers and Remaley, 2012) or contained in membrane derived microvesicles, in particular, exosomes (Mitchell et al., 2008; Arroyo et al., 2011). Exosomes are formed within multi-vesicular bodies (MVBs) in the endosomal system, which co-ordinates the transport of cargo between the plasma membrane, trans-Golgi network (TGN) and lysosomal system [reviewed in (Bellingham et al., 2012b)]. Exosomes can function as an intercellular delivery mechanism by sending miRNA cargo to initiate cell-to-cell communication as they shuttle between neighboring and distant cells (Valadi et al., 2007).

Profiles of deregulated miRNA isolated from peripheral blood (Jin et al., 2008) and serum (Skog et al., 2008; Taylor and Gercel-Taylor, 2008; Edbauer et al., 2010) have been demonstrated and suggest they have diagnostic potential for human diseases such as cancer. For example, the pathology of various cancers and miRNA originating from the site of metastasis correlate positively in both plasma and serum (Skog et al., 2008; Tsujiura et al., 2010). The use of miRNA expression levels as biomarkers can be applied to other human diseases and has not been thoroughly investigated in neurodegenerative diseases such as AD. There are some limitations to this approach, in particular the transport of brain specific miRNA through the blood brain barrier (BBB) into the circulation system of which the current mechanism is unknown. The BBB serves as a tight control point that has specialized molecular machinery to regulate the transport of nutrients and macromolecules, while ensuring viruses and bacteria do not cross the barrier (Begley and Brightman, 2003). On the contrary, under normal conditions, Aβ is cleared from the brain and transported across the BBB mediated by low-density lipoprotein receptor-related protein (Tanzi et al., 2004), suggesting the possibility of other neurodegenerative disease markers crossing the BBB.

There are two possible mechanisms by which miRNAs are able to transport through the BBB. Firstly, a number of neurological diseases such as Multiple Sclerosis and meningitis are well known conditions that weaken and eventually disrupt the BBB, consequently permitting a non-specific release of cellular factors and nucleic acids (Begley and Brightman, 2003). Moreover, thinning and perforations of the vascular basement membrane have been observed in post-mortem brains of late-stage AD patients (Blennow et al., 1990; Berzin et al., 2000; Zipser et al., 2007). Secondly, exosomes and microvesicles may play an important role as carriers of miRNA across the endothelial cellular layers of the BBB in order to communicate between the brain and distant organs via biological fluids (Haqqani et al., 2013). A proposed mechanism involves the transcytosis of extracellular vesicles, such as exosomes, across endothelial cells of the BBB by receptor-mediated endocytosis and releasing exosomal contents into circulation. As a result, the contents of the vesicles can be used as biomarkers reflective of the brain (Haqqani et al., 2013). miRNAs found highly abundant in the brain have been detected in human biological fluids such as plasma, urine, and CSF (summarized in **Table 1**).

DETECTION OF microRNA IN BLOOD

Blood is a highly reliable specimen used for diagnostic testing, with the majority of blood tests being minimally invasive. The cellular components of blood (red blood cells, white blood cells and platelets) provide a rich source of RNA species suitable for biomarker analysis. The most abundant source of miRNAs in blood are found in white blood cells (WBCs). The analysis of miRNA in cellular components of the blood may provide an understanding into the indirect causes of neurodegeneration or indeed reveal information on the pathogenesis of sporadic AD (Schipper et al., 2007; Xu et al., 2007). However, as the brain derived miRNA signal is essentially diluted in the circulating blood, there lies a greater signal-to-noise ratio.

In order to detect disease-specific miRNA profiles, the analysis of plasma and serum (cell-free) samples is most commonly performed (Chen et al., 2008; Pareek et al., 2011; Turchinovich et al., 2011), whereby a smaller but specific pool of miRNAs can be detected. The appeal in detecting circulating miRNA profiles is the potential of capturing the intracellular cross-talk between neighboring and distant cells in the body (Valadi et al., 2007; Turchinovich et al., 2011; Jones et al., 2012). Only a handful of studies have profiled miRNA biomarkers in AD patients using plasma and serum samples. The expression levels of brain-enriched miRNAs (miR-137, miR-9, miR-29a, and miR-29b) have been found to be significantly down regulated in plasma collected from probable AD patients (Geekiyanage et al., 2012). In another study, miR-128 and miR-134 were found to be highly abundant in the brain, and were also detected in cases of mild cognitive impairment (MCI), which is an early form of AD (Sheinerman et al., 2012). Furthermore, it is possible to isolate exosomes from plasma and serum to profile exosomal-specific miRNA by differential ultracentrifugation (Skog et al., 2008; Taylor and Gercel-Taylor, 2008).

DETECTION OF microRNA IN CSF

Identifying circulating miRNA biomarkers in blood represents a clinical advantage for early disease diagnosis however, differential miRNA expression may not accurately reflect miRNA

Table 1 | Highly abundant miRNAs that are deregulated in AD and detected in biological fluids.

miRNA	Homeostatic functions	References	Species	Deregulated in AD	References	Species	Detected in biological fluids	References	Species
miR-9	Neuronal differentiation	Saunders et al., 2010; Shibata et al., 2011; Tan et al., 2012	Mouse	Down regulated in the presence of Aβ	Cogswell et al., 2008; Schonrock et al., 2010, 2012	Human	Plasma, urine, CSF	Melkonyan et al., 2008; Alexandrov et al., 2012; Geekiyanage et al., 2012; Patz et al., 2013	Human
miR-29 (a/b)	Neuronal maturation and apoptosis	Kole et al., 2011	Mouse	Down regulation affects BACE1 and Aβ levels	Hebert et al., 2008	Human, mouse	Plasma, urine, CSF	Geekiyanage et al., 2012; Wang et al., 2012b; Patz et al., 2013	Human
miR-124	Neuronal differentiation	Smirnova et al., 2005; Caltagarone et al., 2007; Hebert et al., 2008	Mouse, chick embryo, human	Targets BACE1	Fang et al., 2012	Rat (PC12)	Urine, CSF	Melkonyan et al., 2008; Patz et al., 2013	Human
miR-128	Neuronal differentiation and development	Smirnova et al., 2005; Schonrock et al., 2010	Mouse	Deregulated upon Reactive Oxygen Species neuronal stress	Lukiw and Pogue, 2007	Human	Plasma, urine, CSF	Melkonyan et al., 2008; Alexandrov et al., 2012; Sheinerman et al., 2012; Patz et al., 2013	Human
miR-134	Synaptic development, maturation and/or plasticity	Gao et al., 2010	Mouse	Detected in MCI human blood plasma	Sheinerman et al., 2012	Human	Plasma, urine, CSF	Melkonyan et al., 2008; Sheinerman et al., 2012; Pacifici et al., 2013; Patz et al., 2013	Human
miR-137	Neuronal maturation and dendritic morphogenesis during development	Taylor and Gercel-Taylor, 2008; Szulwach et al., 2010; Fang et al., 2012	Human, mouse, rat (PC12)	Affects Aβ generation	Szulwach et al., 2010; Geekiyanage and Chan, 2011	Mouse, human	Plasma, urine, CSF	Melkonyan et al., 2008; Geekiyanage et al., 2012; Pacifici et al., 2013	Human

deregulation in neuronal tissues subject to neurodegenerative disease. CSF is a clear biological fluid produced in the choroid plexus of the brain, and circulates though the inner ventricular system, across the BBB and is absorbed into the bloodstream. CSF represents a more suitable and relevant source of material for diagnosis of central nervous system (CNS) disorders. CSF is obtained by lumbar puncture and has been shown to contain circulating miRNAs that have been utilized in several studies for miRNA profiling of neurological and neurodegenerative disorders; including AD (Cogswell et al., 2008; Alexandrov et al., 2012; Lehmann et al., 2012), schizophrenia (Gallego et al., 2012), Multiple Sclerosis (Haghikia et al., 2012), HIV-encephalitis (Pacifici et al., 2013), traumatic brain injury (Balakathiresan et al., 2012; Patz et al., 2013) and various cancers of the CNS (Baraniskin et al., 2012a,b; Teplyuk et al., 2012).

Studies of CSF from AD patients have used either a combination of miRNA microarrays (Alexandrov et al., 2012; Lukiw et al., 2012), multiplex miRNA qPCR assay (Cogswell et al., 2008) or a target candidate miRNA approach (Lehmann et al., 2012) to identify differentially expressed miRNA. In these studies, no correlation was observed between independent research groups or when validated in corresponding tissues samples extracted from AD patients (Cogswell et al., 2008). Using microarrays and qPCR validation, miR-9, miR-146a and miR-155 were found to be significantly up-regulated in AD patient CSF compared to age-matched controls (Alexandrov et al., 2012; Lukiw et al., 2012). However, these miRNAs were not identified in an independent study in which 60 miRNAs were found to be deregulated in CSF and corresponding brain tissue from AD patients (Cogswell et al., 2008). Likewise, candidate miRNA let-7b found increased in AD CSF (Lehmann et al., 2012) was not significantly altered in previous studies (Cogswell et al., 2008; Alexandrov et al., 2012; Lukiw et al., 2012). These observations highlight the need for a uniform approach to miRNA profiling for disease diagnosis. Collection of brain tissue, CSF, and peripheral blood samples in the same subjects would be advantageous however, this approach is challenging due the difficulty in recruiting study participants willing to undergo multiple invasive procedures.

DETECTION OF microRNA IN URINE

Clinically, urine is collected non-invasively for biomarker discovery and diagnostic purposes. The procedure for urine collection is relatively time- and cost-efficient compared with other clinical samples such as blood and CSF. This has led to an increase in miRNA biomarker studies examining urine samples to screen for disease biomarkers (Weber et al., 2010; Bryant et al., 2012; Wang et al., 2012a). Circulating extracellular miRNAs can be delivered to renal epithelial cells and released into the urine bound to RNA-binding proteins or packaged into microvesicles such as exosomes (Weber et al., 2010). The urine sediment, including whole cells, cell debris and polymerized protein, is able to be separated from whole urine using low-speed centrifugation (Wang et al., 2010, 2012a). Many studies exploit the urine cellular sediment obtained from low speed centrifugation to analyse miRNA implicated in prostate and bladder cancers (Wang et al., 2010, 2012c). Bryant et al. have reported several deregulated miRNAs associated with prostate cancer which were validated in serum, plasma and urine (Bryant et al., 2012). Analysis of urinary miRNA from the cell sediment may not be suitable for neurodegenerative diseases, as it is rich with cells or cell debris of hematologic origin, renal epithelial origin and urothelial origin in addition to microorganisms such as bacteria and yeast (Koss and Sherman, 1984; Wang et al., 2010). Fewer studies have analysed cell-free urine to isolate miRNAs, mainly because it may contain miRNAs from organs of the body outside of the excretory system. Exosomes can be purified from urine using a differential ultracentrifugation method which is the most widely applied technique (Alvarez et al., 2012). Future advances in methodologies to improve sensitivity and accuracy in profiling urinary miRNA biomarkers, in particular from using exosomes, would be of great value to investigate whether miRNA can be detected in urine.

Many studies using human cell lines differentiated with all-trans-retinoic acid, cultured primary neurons, astrocytes and brain sections from mice and human models highlight the significance of miRNA in neurodegeneration (Sempere et al., 2004; Smirnova et al., 2005; Alvarez et al., 2012). Overall, the potential to detect miRNA in biological fluids, in particular those highly expressed in the brain, is well supported by research published in the literature (summarized in **Table 1**). The majority of experimental methods have used Northern blotting, qPCR and microarrays though these methodologies are not suitable for biomarker discovery or mass screening programs. Researchers are now developing high-throughput, cost effective strategies to improve the sensitivity and specificity of for miRNA diagnostics in biological fluids. One of these technologies is deep sequencing.

THE USE OF DEEP SEQUENCING TECHNOLOGY TO SCREEN FOR MICRORNA BIOMARKERS

Implementing deep sequencing technology represents a powerful and innovative approach to discover differentially expressed miRNAs in neurodegenerative diseases (Brase et al., 2010; Debey-Pascher et al., 2012). The advantage of using deep sequencing, unlike traditional Sanger sequencing, lies upon the capability to simultaneously process millions of independent sequencing events. This offers billions of nucleotide information within a single experiment (Shendure et al., 2004; Church, 2006). Deep sequencing experiments enable comprehensive analyses of large amounts of sequence data, resulting in dramatically accelerated research compared to traditional labor-intensive efforts and is a powerful approach to determine accurate encoded-information from nucleotide fragments (Hall, 2007; Shendure and Ji, 2008; Tucker et al., 2009).

LARGE-SCALE DEEP SEQUENCING PLATFORMS

Detecting low abundance or differentially expressed circulating miRNA signatures in biological fluids requires a large-scale and high-throughput platform. The large-scale sequencing instruments presently available are the 454 Genome Sequencer (GS) FLX+ system from 454 Life Sciences, 5500 Sequencing by Oligo Ligation Detection (SOLiD) system from Applied Biosystems (Life Technologies), HiSeq 2000 system from Illumina and the recently introduced Ion Proton from Ion Torrent (Life Technologies). Each platform employs different sequencing chemistries for data generation (summarized in **Table 2**). All

Table 2 | Specifications of current "Next-Generation" Deep Sequencing platforms[†].

Sequencing platform	Read length (bases)[#]	Throughput / time per run	Accuracy (%)	Advantages	Disadvantages	Cost per run
Illumina HiSeq™ 2000	35–100	100–600 Gb / 2–11 days	98.0	Ultra high throughput High capacity of multi-plexing	Short read assembly may miss large structural variations Signal interference among neighbouring clusters Homopolymer errors	$$$
Applied Biosystems 5500 SOLiD™	35–75	120 Gb / 7–14 days	99.9	Ultra high throughput Two-base coding (higher accuracy) High capacity of multi-plexing	Short read assembly may miss large structural variations Long run time Signal interference among neighbouring clusters Signal degradation	$$$
Roche (454 Life Sciences) GS FLX Titanium	700–1000	0.7 Gb / 0.35–0.42 days	99.9	Long read assembly allows detection of large structural variations Short run time	Lower throughput Homopolymer errors Signal interference among neighbouring clusters	$$
Ion Torrent Ion Proton™ PI PII	100–400 100–400	10 Gb / 4 h 30 Gb / 4 h	98.5	Fast run time Highly scalable (different chips available) Low cost	Newest to the market	$/$$
Ion Torrent Ion PGM™ 314 chip 316 chip 318 chip	100–400 100–400 100–400	0.01 Gb / 1 h 0.1 Gb / 2 h 1 Gb / 3 h	98.5	Highly scalable (different chips available) Low cost Fast run time	Homopolymer errors	$
Illumina MiSeq™	35–150	1.5 Gb / 27 h	99.2	Well-proven sequencing technology Fully automated workflow Low cost Fast run time	Low abundance of amplified template	$
Roche (454 Life Sciences) 454 GS Junior	250–400	0.035 Gb / 8 h	99.0	Long read length Relatively fast run time	Lower throughput Homopolymer errors	$

[#] *Average read length depends on specific sample and genomic characteristics.*

[†] *Specifications for all platforms are derived from company websites.*

454 systems adopt a principle of pyrosequencing which, is based on the detection of pyrophosphate molecules during nucleotide incorporation and the intensity of signals produced by chemiluminescence (Ahmadian et al., 2006; Rothberg and Leamon, 2008). SOLiD utilizes ligation-based chemistry with dye-labeled probes, involving rounds of oligonucleotide ligation extension and two-base encoding detection (Pandey et al., 2008). Illumina systems rely on a sequencing-by-synthesis (SBS) approach involving cycles of nucleotide incorporation and use of reversible dye terminators (Fuller et al., 2009). Ion Proton also employs SBS chemistry however, it measures signals through the production of hydrogen ions resulting from the process of nucleotide replication on a chip containing an array of semiconductor sensors (Rothberg et al., 2011). Despite the various sequencing chemistries, all large-scale deep sequencing platforms are capable of generating up to 3 billion sequencing reads, with an output of 0.7–600 gigabases (Gb), quickly and efficiently (**Table 2**). To date, many unbiased miRNA biomarkers have been discovered through large-scale deep sequencing techniques (Maes et al., 2009; Cortez et al., 2011; Etheridge et al., 2011; Bellingham et al., 2012a). However, deep sequencing of small sample sizes via large-scale platforms will be very expensive if the capacity of each run is not fully utilized

(Wall et al., 2009). Furthermore, the advantages of these large-scale platforms are immediately offset by the reagent costs, in the thousands of dollars per sequencing run, and run times of up to 14 days, which are usually constrained in small laboratories and particularly in diagnostics (Morozova and Marra, 2008). In order to facilitate access of deep sequencing for the majority of laboratories, the recent launch of small-scale benchtop deep sequencers has offered a cheaper alternative to sequence genomes with greater speed, ∼2 h run time, and lower cost compared to large-scale deep sequencer (Eyre et al., 2012).

BENCHTOP DEEP SEQUENCING PLATFORMS

The smaller benchtop deep sequencing instruments allow the possibility of small-scale biomarker discovery. Commonly used small-scale sequencing instruments are 454 GS Junior from 454 Life Sciences, MiSeq from Illumina, Ion Personal Genome Machine (PGM) from Ion Torrent (summarized in **Table 2**). The employment of benchtop sequencers offers an alternative option for low number of samples in a cohort (Kumar and Webster, 2011) and present additional advantages with their convenience of storage and simple procedures in sample preparation (Hutchison, 2007; Eyre et al., 2012; Wilson, 2012). Loman et al. and Quail et al. sequenced bacterial genomes, using laser-printer sized benchtop platforms capable of generating usable sequences with fast turnaround time in a straightforward workflow and low running costs. In early 2010, the release of 454 GS Junior instrument substituted the larger 454 GS FLX instrument for small-scale sequencing. It employs similar template amplification and pyrosequencing-based approaches as of the 454 higher-scale sequencer (Margulies et al., 2005). Illumina and Ion Torrent also provide for large and small-scale projects with the introduction of Illumina MiSeq and Ion PGM, smaller versions of the Illumina HiSeq and Ion Proton, respectively. These exploit the existing platform-specific sequencing chemistry suitable for smaller scale projects (Bentley et al., 2008). All benchtop sequencers are as competitive as their respective large-scale platforms as they are able to generate millions of reads in their output (Loman et al., 2012a; Quail et al., 2012). Although both 454 JS Junior and Illumina MiSeq utilize years-proven sequencing strategies, the Ion PGM is a useful addition to the current deep sequencing platforms by adding scalability (i.e., different chips to allow different scale of studies to be sequenced cost-efficiently) and lower instrument cost (Defrancesco and Subbaraman, 2011; Scholz et al., 2011; Kodama et al., 2012; Vogel et al., 2012). The output of sequencing reactions ranges from 10-, 100-, to 1000-million reads due to the availability of three different chips used for sequencing (e.g., 314-, 316-, and 318-chips). The signal detection of each nucleotide in Ion Torrent system depends on a sensitive pH measurement, thus eliminating the requirement for modified chemiluminesecent reporters and expensive detector devices (Pareek et al., 2011). In essence, benchtop deep sequencing technologies provide an affordable way to produce accurate throughput with sufficient sequencing coverage. Notably, Ion Proton can be classified as benchtop sequencer as it is currently the only instrument promised to provide shorter sequencing time than other large-scale systems and hundred folds more data output than other bench-top sequencers. Once these advantages are tested in future studies, Ion Proton could have its own niche bridging between small-scale and large-scale for routine full genome screening.

BIOMARKER DISCOVERY TO CLINICAL PRACTICE

Typically, the data generated from benchtop deep sequencing instruments (e.g., high assembly coverage by 454 GS Junior due to long read length) are generally sufficient to obtain disease specific profiles from individual samples (Loman et al., 2012b). Ultimately, the choice of platform will depend on its performance metrics (i.e., read length, accuracy and data output) to complement the type of study being undertaken. For clinical practice, platforms such as the Ion PGM and MiSeq offer the best value for money, more flexibility, accuracy, adequate throughput and coverage depths for study of miRNA.

To incorporate miRNA deep sequencing for clinical practice, a simple and standardized workflow for the routine biomarker and diagnostic screening needs to be defined (Barzon et al., 2011; Natrajan and Reis, 2011; Radford et al., 2012; Rizzo and Buck, 2012). A typical workflow involved in performing miRNA-sequencing from sample preparation to data analysis is outlined in **Figure 1**. The sequencing workflow consists of the following steps: (i) miRNA isolation; (ii) Library preparation and enrichment of templates containing size selected libraries of the appropriate fragment length; (iii) and Sequencing reaction. Isolation of low abundance miRNA present in body fluids (e.g., blood and urine) can be obtained by the use of RNA isolation columns that specifically enrich for miRNA (Mitchell et al., 2008; Arroyo et al., 2011). Preparation of isolated miRNA for sequencing includes the assessment of quality (i.e., RNA integrity number; RIN), profiling and quantification of small RNA species, in particular miRNA (Ozsolak and Milos, 2010). Preparation of sample library often requires barcoding, which involves ligation of different adaptors of identifiable sequences to either end of each sample. This method is to increase capacity as it allows multiplexing of samples in the same sequencing reaction (Chen et al., 2012). Template preparation for sequencing and the sequencing reaction is platform specific as seen in **Table 2**.

Another approach to increase throughput is by capturing and sequencing disease specific targets known as sequence enrichment. In clinical practice, the factors to consider while sequencing for an enriched population of targets may involve the size of the region of interest, capture efficiency of interest region, average read depth coverage of the targeted region, distribution of coverage and its sensitivity and specificity (Mamanova et al., 2010). The quality control involved would require a set of positive and negative control references to establish a standard depth and quality percentage of targeted regions for determining the accuracy of enrichment (Harismendy et al., 2009; Teer et al., 2010). Furthermore, reference controls eliminate any experimental problems which may arise from sequence variations that affect hybridization to biotinylated probes during enrichment, causing low capture efficiency (Schwartz et al., 2011). Hence, quality control measures should be routinely assessed before proceeding to analyse samples for a diagnosis to eliminate false positives which may be caused by poor miRNA quality or technical artefacts

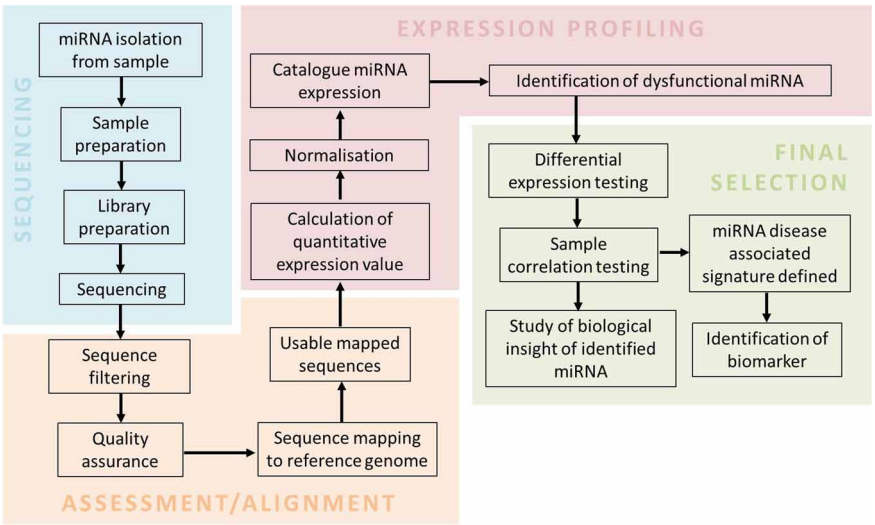

FIGURE 1 | A miRNA-sequencing workflow. The workflow consists of sample preparation, sequencing, assessment and alignment, expression profiling and final selection. Sequencing involves sample preparation of isolated miRNAs to generate a library for the sequencing reaction. Assessment and alignment requires bioinformatics tools to generate usable mapped sequences. Final selection of miRNAs related to the disease are filtered by sophisticated statistical tools.

(Fehniger et al., 2010; Lee et al., 2010). Furthermore, instruments that provide a fast turn-around time will be suitable in diagnostic laboratories in order to produce diagnostic reports in a timely manner (Tucker et al., 2009). In clinical practice, a high throughput strategy may involve using deep sequencing platforms to screen for AD biomarkers by indexing a large number of patient samples and pooling samples into one sequencing run. Indexing patient samples and batch testing in a diagnostic laboratory would be a cost-effective and practical approach to handle large population screening.

All deep sequencing instruments produce a massive quantity of raw data that requires extensive computational tools to process the information (Zhang et al., 2011). Open-source tools and in-house (Perl) scripts are available for handling large quantities of sequencing data by providing an integrated and streamlined analysis workflow (Nix et al., 2010). A fundamental workflow for post-analytic miRNA data analysis (**Figure 1**) consists of four fundamental steps: (i) Sequence assessment; (ii) Sequence alignment; (iii) Profiling of miRNA associated with neurodegenerative disease; (iv) and Final selection. This workflow requires databases and tools such as FastQC (quality control check), miRbase/miRandola (miRNA database), bowtie (short read aligner) and EdgeR/DESeq (normalization) (Horner et al., 2010; Kozomara and Griffiths-Jones, 2011; Russo et al., 2012). In order to make data analysis more accessible to the diagnostic end-user, there are a number of commercially available software packages that aim to make analysis uncomplicated by providing user-friendly graphical interfaces (Richter and Sexton, 2009). This includes Partek Genomics Suite, CLC Genomics Workbench, Ingenuity Systems and platform-specific software (e.g., Ion Torrent Suite and Illumina GenomeStudio). These commercial software tools have less flexibility and scalability in terms of their parameters' settings, but are suitable for small-scale studies to establish miRNA profiles and perform differential expression analysis in order to detect disease specific miRNA (Meldrum et al., 2011).

CONCLUSIONS

Early diagnosis of AD is critical as it is hypothesized that the pathology of the disease occurs up to 20 years before cognitive decline. Many groups have searched for protein biomarkers in blood, plasma, serum and CSF have not yielded a reliable, sensitive, and specific candidate biomarker marker for AD diagnosis. Due to the sensitivity of deep sequencing, it is possible to detect genetic modifications in disease, in particular deregulated miRNA. The possibilities of profiling miRNA in CSF, urine, blood, plasma and serum have been explored with some successes in cancer and may be applicable to AD and other neurodegenerative disorders. In addition, the genetic information contained in circulating exosomes may provide a highly specific readout of disease and should be further investigated. Deep sequencing of miRNA together with high-throughput validation methods will complement diagnostic testing and represents a vital step toward developing a cost-effective, non-invasive and low risk diagnostic test to detect the onset and monitor various stages of AD. Furthermore, the development of a diagnostic test comprising of a profile of RNA biomarkers associated with AD, and has potential for other neurodegenerative diseases such as Parkinson's, Prion and Huntington's diseases.

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Cardiovascular extracellular microRNAs: emerging diagnostic markers and mechanisms of cell-to-cell RNA communication

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Cardiovascular diseases are a leading cause of morbidity and mortality in Western societies. It is now well established that microRNAs (miRNAs) are determinant regulators in various medical conditions including cardiovascular diseases. The recent discovery that miRNAs, while associated with different carriers, can be exported out of the cell, has triggered a renewed interest to analyze the potential to use extracellular miRNAs as tools for diagnostic and therapeutic studies. Circulating miRNAs in biological fluids present a technological advantage compared to current diagnostic tools by virtue of their remarkable stability and relative ease of detection rendering them ideal tools for non-invasive and rapid diagnosis. Extracellular miRNAs also represent a novel form of inter-cellular communication by transferring genetic information from a donor cell to a recipient cell. This review briefly summarizes recent insights in the origin, function and diagnostic potential of extracellular miRNAs by focusing on a select number of cardiovascular diseases.

Keywords: extracellular microRNA, inter-cellular communications, biomarkers, cardiovascular diseases

INTRODUCTION

Cardiovascular disease is a global health problem. Of all 60 million cases of deaths from all causes worldwide in 2005, an estimated 18 million were due to cardiovascular diseases, three times more than caused by infectious diseases including HIV/AIDS, tuberculosis, and malaria combined (World Health Organization, 2013). Current clinical diagnostics fail to identify early changes of adverse cardiac or vascular remodeling, forcing clinicians to wait for these cardiovascular disorders to become clinically evident before initiating intervention. Additionally, treatment efficacy cannot be reliably assessed in individual patients, in part as many interventions are merely treating symptoms (e.g., diuretics). Ideally, one would not assess intervention success based on survival or hospitalization, but build in intermediate end-points that can reliably assess therapeutic benefit. Thus, for the cardiovascular field, there is a need to identify intermediate diagnostic measures that monitor subtle biological changes in the heart or vasculature that directly reflect and predict adverse changes before they become clinically apparent.

To achieve the goal of early diagnosis and treatment, microRNAs (miRNAs) could play an unexpected role. MiRNAs are a group of non-coding regulatory RNAs of about 22 nucleotides that control gene expression at the post-transcriptional level (Bartel, 2004) and act as crucial regulators of most physiological and pathological processes. Indeed, dysregulation of intracellular miRNA expression has been linked to many clinically relevant cardiovascular conditions (Small and Olson, 2011; Da Costa Martins and De Windt, 2012; Gladka et al., 2012; van Empel et al., 2012). Unexpectedly, the recent discovery of circulating miRNAs has opened the possibility to study this class of biologically active agents as modes of inter-cellular information flow as well as biomarkers of disease. Here, we present an overview of the different carriers associated with extracellular miRNAs that render them stable in biological fluids, present the current level of understanding of their role in cell-to-cell communication and give an overview about the clinical utility of extracellular miRNAs as putative biomarkers for cardiovascular disease entities.

VEHICLES THAT STABILIZE EXTRACELLULAR miRNAs

The first accounts of extracellular miRNA biomarkers were described in serum of lymphoma patients (Lawrie et al., 2008) and in plasma and serum of prostate cancer patients (Mitchell et al., 2008). Subsequently, it became evident that miRNAs can be exported from cells, and found in most extracellular biological fluids including plasma, serum, saliva, urine, tears, and breast milk (Chim et al., 2008; Weber et al., 2010; Boon and Vickers, 2013). Extracellular miRNAs are unexpectedly stable, and must be shielded from degradation, as naked RNA is readily targeted by exonucleases that are abundantly present in various extracellular fluids (Kamm and Smith, 1972). Indeed, miRNAs are packaged in microparticles (exosomes, microvesicles, and apoptotic bodies; Valadi et al., 2007; Hunter et al., 2008; Zernecke et al., 2009) or by their association with RNA-binding proteins including Argonaute 2 (Ago2; Arroyo et al., 2011) or lipoprotein complexes such as high-density lipoprotein (HDL; Kamm and Smith, 1972; Vickers et al., 2011; **Figure 1**).

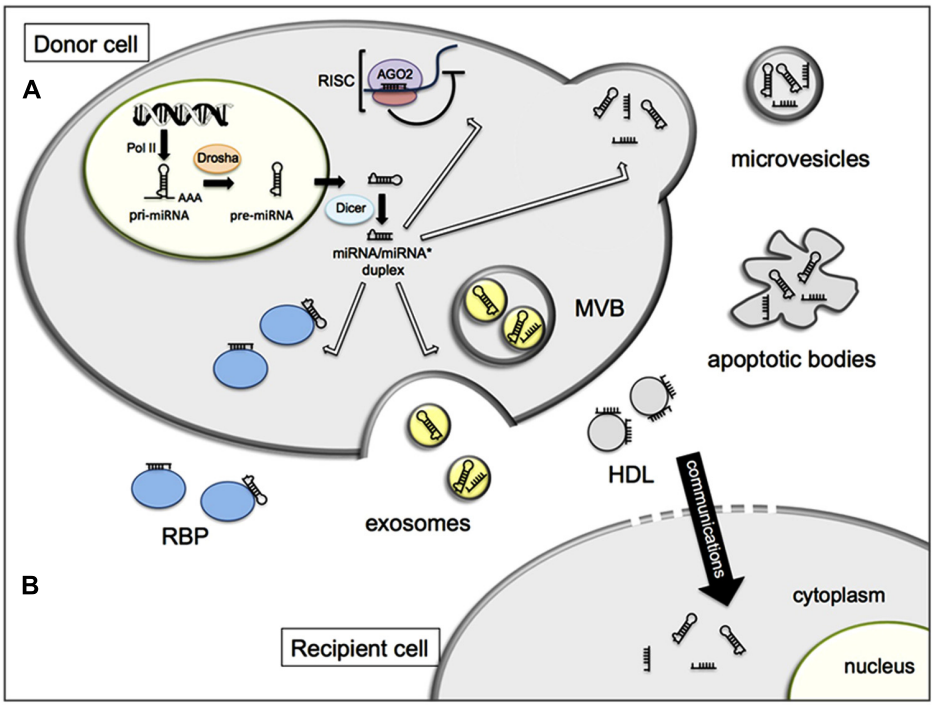

FIGURE 1 | Schematic representation of cellular release (A) and inter-cellular communication (B) of miRNAs. (A) In the nucleus, miRNA genes are mainly transcribed by the RNA polymerase II (Pol II) into primary miRNAs (pri-miRNAs) and processed to precursor miRNAs (pre-miRNAs) by the Drosha complex. Pre-miRNAs are exported to the cytoplasm and cleaved by Dicer to produce a double stranded miRNA duplex. The duplex is separated and a mature miRNA is incorporated into the RNA-induced silencing complex (RISC) while the other strand is likely subject to degradation. Within the RISC complex, miRNAs bind to their target messenger RNAs (mRNAs) to repress their translation or induce their degradation. In addition, miRNAs can be exported out of the cells and transported by various carriers, membrane-derived vesicles (exosomes, microvesicles, apoptotic bodies), miRNA-binding protein complexes (RBP), or high density lipoproteins (HDL). **(B)** Extracellular miRNAs can be transferred to recipient cells where they alter gene expression.

The term exosomes was used for the first time in 1981 to describe exfoliated membrane vesicles (Trams et al., 1981). Exosomes are small (40–120 nm) extracellular microvesicles arising from multivesicular bodies (MVBs) and released by exocytosis of these MVBs (Heijnen et al., 1999). They are produced by a variety of cells including epithelial cells (Zhou et al., 2011), hematopoietic cells (Laulagnier et al., 2004), endothelial cells (Halkein et al., 2013), and tumor cells (Mitchell et al., 2008). Exosomes have also been identified in most circulating body fluids such as plasma, urine, milk, saliva, and sperm (Thery et al., 2006). The interest of exosome biology was increased following the demonstration that exosomes can serve as carriers for miRNAs (Valadi et al., 2007; Gallo et al., 2012). Selection processes must take place of miRNA uploading into exosomes, as some miRNAs can be either more or less expressed in donor cells or in the secreted exosomes (Valadi et al., 2007; Pigati et al., 2010), suggesting the existence of cellular mechanisms that actively concentrate specific miRNA species in exosomes (Valadi et al., 2007; Pigati et al., 2010).

Microvesicles or shedding microvesicles (SMVs) are another form of small, defined vesicles (Pant et al., 2012) that are shed from the plasma membrane by a wide variety of cells (Heijnen et al., 1999). They are larger (0.1–1 μm) than exosomes (Heijnen et al., 1999) and their mechanism of production is also different. While exosomes are produced by exocytic fusion of MVBs, microvesicles are produced by budding of vesicles from the plasma membrane (Mathivanan et al., 2010). The presence of miRNAs in microvesicles were described for the first time in 2008 (Hunter et al., 2008).

A final vesicular form where miRNAs reside are apoptotic bodies or apoptotic blebs, byproducts of apoptotic cells. Apoptotic or dying cells release membrane vesicles into the extracellular environment via bleeding of the plasma membrane (Mathivanan et al., 2010). These are larger particles (1–5 μm) with heterogeneous shape (Gyorgy et al., 2011). In atherosclerotic vascular disease, endothelial cells can produce apoptotic bodies enriched with miR-126. These endothelial cell-derived apoptotic bodies trigger, via miR-126, the production of CXC chemokine CXCL12 in the recipient vascular cells which limits atherosclerosis and confers plaque stability (Zernecke et al., 2009).

Apart from packaging miRNAs in cell-derived vesicles, a significant fraction of extracellular miRNAs is associated with RNA binding proteins, including nucleophosmin (NPM1), that provide protection from degradation (Wang et al., 2010b). It was also demonstrated that many extracellular miRNAs are bound to proteins of the Argonaute family, primarily Ago2, although additional members such as Ago1, Ago3, and Ago4 might be also associated with miRNAs (Arroyo et al., 2011; Turchinovich et al., 2011). These

latter studies are at odds with the findings by Gallo et al. (2012). This discrepancy may arise from the different protocols used for microvesicle- and RNA-isolation and subsequent data normalization, emphasizing the need for further protocol standardization (Turchinovich et al., 2012). Finally, it was recently shown that extracellular miRNAs can be transported by HDL (Vickers et al., 2011; Norata et al., 2013). Whereas vesicle carriers are composed of a bilayer of phospholipids, lipoproteins have a single layer of lipids (Boon and Vickers, 2013).

EXTRACELLULAR miRNAs IN CELL-TO-CELL COMMUNICATION

Interestingly, extracellular miRNAs also present a newly discovered potential of intercellular communication. It is now established that transfer of genetic information in the form of RNA exists (Valadi et al., 2007) and that this form of transfer between cells is of functional relevance by exerting gene silencing in the recipient cells (Kosaka et al., 2010; Mittelbrunn et al., 2011; Halkein et al., 2013; **Figure 1**). While the biological mechanisms driving the secretion of miRNAs are still under debate (Kosaka et al., 2010), this newly discovered manner of genetic exchange between cells opens a new aspect of how adjacent cells within an organ may communicate and how a miRNA can affect a cell type or a tissue where it is not produced. Since the first discovery of the extracellular miRNAs as intercellular communicators, this field of research is still growing. Increasing evidence suggests that this form of communication occurs in various physiological processes such as the regulation of the immunity (Mittelbrunn et al., 2011) or cellular migration (Zhang et al., 2010), but also participates in pathological situations including tumor development (Yang et al., 2011).

For cardiovascular diseases, only three examples of intercellular miRNA communication have been demonstrated. The first study presented evidence that endothelial cell-derived apoptotic bodies are generated during atherosclerosis and lead to the induction of the expression of CXCL12 in recipient endothelial cells. These endothelial cell-derived apoptotic bodies also induce the recruitment of progenitor cells in mice with atherosclerosis and reduce the extent of plaque formation. It was finally demonstrated that the atheroprotective effects of endothelial apoptotic bodies are mediated by miR-126 (Zernecke et al., 2009). Additionally, shear stress as well as the shear-responsive transcription factor Kruppel-like factor 2 (KLF2) induces the expression of the cluster miR-143/145 in endothelial cells and also its enrichment in extracellular vesicles produced by the treated-endothelial cells. It was demonstrated that these endothelial-derived miR-143/145-containing vesicles are transferred to smooth muscle cells and induce an atheroprotective phenotype in recipient cells. MiR-143/145 from endothelial cells repress target genes in recipient smooth muscle cells such as ELK1 and KFL4 implicated in smooth muscle cell fate and plasticity (Hergenreider et al., 2012).

More recently, it was demonstrated that the anti-angiogenic fragment 16K prolactin (PRL) positively regulates the expression of miR-146a in endothelial cells where it affects mainly the cell survival and proliferation by down-regulating NRAS gene expression. Even more, the treatment of endothelial cells with 16K PRL also increases miR-146a level in the exosomes secreted by the donor endothelial cells. There is an uptake of the endothelial cell-derived exosomes by cardiomyocytes and transferred miR-146a reduces the metabolism of the recipient cells. This model was proposed to play a central role in the development of peripartum cardiomyopathy since blocking miR-146a activity attenuated the disease in mice (Halkein et al., 2013).

The use of exosomes as therapeutic vehicles should now also be considered. In the field of cardiovascular diseases, a first study has presented the potential of cardiomyocyte progenitor cells-derived exosomes to stimulate endothelial cell migration in the treatment of myocardial infarction (MI) (Vrijsen et al., 2010). More recently, *in vivo* delivery of cardiac progenitor-derived exosomes has been shown to inhibit cardiomyocyte apoptosis in a mouse acute ischemia/reperfusion model (Chen et al., 2013). In the context of therapeutics, the first report is now also available demonstrating that cells can be engineering to express specific ligands at the surface of the exosomes and load these carriers with therapeutic siRNA species (Alvarez-Erviti et al., 2011). Additional efforts for a better understanding of the mechanisms of extracellular miRNA secretion and the targeting of recipient cells by microvesicles are expected in the future.

CIRCULATING miRNAs AS BIOMARKERS OF CARDIOVASCULAR DISEASES

Circulating B-type natriuretic peptide (BNP) and its amino-terminal fragment, N-terminal pro-brain natriuretic peptide (NT-proBNP) are clinically established as diagnostic biomarkers for heart failure (Januzzi et al., 2006). For patients with acute myocardial infarction (AMI), circulating levels of cardiac troponins (cTns) are considered a gold standard for the early diagnosis of this disease (Jaffe et al., 2000). Unfortunately, elevated levels of cTn concentrations have also been reported in patients with end-stage renal disease (Collinson et al., 1998), which indicates that this marker lacks specificity for AMI. For atherosclerosis, many biomarkers have been proposed, such as C-reactive protein, interleukins IL-1 and IL-6, apolipoproteins apoA-I and apoB, and fibrinogen (Kampoli et al., 2009). It is not clear whether these new biomarkers are useful predictors of future cardiovascular events. Therefore, it remains essential to continue to explore new biomarkers with even greater discriminatory power for the various subtypes of heart disease. In recent years, several studies have reported on the use of miRNAs as circulating biomarkers for diagnosis or prognosis of various human diseases including cardiovascular diseases (Salic and De Windt, 2012; **Table 1**).

CIRCULATING miRNAs IN HEART FAILURE

The first putative miRNA biomarkers in heart failure were discovered in a miRNA array on plasma of 12 healthy controls and 12 heart failure patients (Tijsen et al., 2010). From this array, 16 miRNAs were selected for a second clinical study in 39 healthy controls and in 50 cases with reports of dyspnea, of whom 30 were diagnosed with heart failure and 20 were diagnosed with dyspnea attributable to non-heart failure-related causes. In this study, 6 miRNAs (miR-423-5p, miR-18b-3p, miR-129-5p, miR-1254, miR-675, and miR-622) were elevated in patients with heart failure, with miR-423-5p positively correlated with NT-proBNP levels and most strongly related to the clinical diagnosis of heart failure. The increase of circulating levels of miR-423-5p could

Table 1 | Extracellular miRNAs as biomarkers in cardiovascular diseases.

miRNA biomarkers	Diseases	Source	Regulation	Correlation	Design of study	Reference
miR-423-5p miR-18b-3p miR-129-5p miR-1254 miR-675 miR-622	HF	Plasma	Up	proBNP	30 HF; 20 non-HF with dyspnea; 39 healthy subjects	Tijsen et al. (2010)
miR-126	HF	Plasma	Down	BNP	10 HF; 17 asymptomatic control	Fukushima et al. (2011)
miR-208b miR-499	AMI	Plasma	Up		32 AMI; 36 non-AMI with AP	Corsten et al. (2010)
miR-1	AMI	Plasma	Up	QRS widening	93 AMI; 66 healthy subjects	Ai et al. (2010)
miR-1	AMI	Serum	Up	CK-MB	31 AMI; 20 healthy subjects	Chen et al. (2013)
miR-133a	STEMI	Serum	Up		216 STEMI	Eitel et al. (2012)
miR-499	AMI	Plasma	Up		14 AMI; 15 congestive HF; 10 healthy subjects	Adachi et al. (2010)
miR-1 miR-133a miR-499 miR-208a	AMI	Plasma	Up		33 AMI; 33 non-AMI with other cardiac diseases; 10 healthy subjects	Wang et al. (2010a)
miR-30a miR-195 let-7b	AMI	Plasma	Up		18 AMI; 30 healthy subjects	Long et al. (2012)
miR-126 miR-17/92 cluster miR-155 miR-145	CAD	Plasma	Down		67 CAD; 31 healthy subjects	Fichtlscherer et al. (2010)
miR-135a miR-147	CAD	PBMC	Up Down		25 unstable AP; 25 stable AP; 20 healthy subjects	Hoekstra et al. (2010)

HF, heart Failure; AM, acute myocardial infarction; STEMI, ST-elevation myocardial infarction; proBNP, pro brain natriuretic peptide; BNP, B-type natriuretic peptide; CK-MB, creatine kinase isoenzyme MB; CAD, coronary arterial disease; PBMC, peripheral blood mononuclear cell; AP, angina pectoris.

be confirmed by several other studies including hypertension-induced heart failure patients (Dickinson et al., 2013), systolic heart failure patients (Goren et al., 2012) and patients with dilated cardiomyopathy (Fan et al., 2013). In contrast, in patients with a reduced systemic right ventricular function and decreased ejection fraction, circulating miR-423-5p concentrations were not elevated, suggesting that miR-423-5p discriminates between sub-types of heart failure (Tutarel et al., 2011).

In a different study, the expression of 3 miRNAs in plasma of 10 heart failure patients and 17 asymptomatic control subjects was analyzed, demonstrating that the endothelium-derived miR-126 was negatively correlated with age, NT-proBNP, and New York Heart Association classification. Decreased miRNA-126 was also found in atherosclerotic coronary artery disease (CAD) and in patients with type 2 diabetes mellitus and may reflect the condition of vascular endothelial cells in heart failure patients (Fukushima et al., 2011).

Also plasma levels of several other miRNAs, including the heart-muscle enriched miRNAs miR-1, -133a, -208b, and -499; fibrosis-associated miRNAs miR-21 and miR-29b; and leukocyte-associated miRNAs miR-146, -155, and -223 were tested as candidate biomarkers (Corsten et al., 2010). This study demonstrated that in humans, diverse conditions of myocardial damage are associated with striking perturbations of plasma levels of cardiac specific miR-208b and miR-499 in acute heart failure (minimal), viral myocarditis (marked), and AMI (extensive). An intriguing observation was the correlation of miR-133a plasma levels with NT-proBNP in asymptomatic patients with diastolic dysfunction, which was not observed in acute heart failure patients.

CIRCULATING miRNAs IN MYOCARDIAL INFARCTION

Plasma levels of miR-208b and miR-499 both have been highly associated with AMI. Also, it was demonstrated that measuring

miR-1 in plasma is a good approach for blood-based detection of human AMI (Ai et al., 2010). Circulating miR-1 is significantly increased in the blood of AMI patients compared to non-AMI subjects and were positively correlated with serum CK-MB (creatine kinase-MB; Cheng et al., 2010). In a rat model of AMI induced by coronary ligation, serum miR-1 is increased early after AMI with a peak at 6 h, in which an increase in miR-1 of over 200-fold was demonstrated. Serum miR-1 returned to baseline levels at 3 days after AMI (Cheng et al., 2010). Also, increased miR-1 is well correlated with abnormal QRS complex widening (a reflection of abnormal electrical rhythm) in AMI, and after treatment, plasma miR-1 recovered to normal values (Ai et al., 2010). These data indicate that circulating miR-1 could serve as a biomarker for diagnosis of AMI and associated ischemic arrhythmias.

Next, an array analysis of miRNA production in various human tissues was reported, demonstrating that miR-499 was produced almost exclusively in the heart. To determine whether this miRNA could serve as a biomarker for cardiovascular diseases, the authors assessed the plasma concentrations of miR-499 in 14 individuals with acute coronary syndromes, 15 individuals with congestive heart failure, and 10 individuals without cardiovascular diseases. Plasma miR-499 concentrations were elevated in all AMI patients, but were below the detection limit in the other patient groups (Adachi et al., 2010).

Another miRNA microarray study demonstrated that miR-1, miR-133a, miR-499, and miR-208a were elevated in plasma from 33 AMI patients compared to as well as healthy subjects, patients with non-AMI coronary heart disease, or patients with other cardiovascular diseases. Notably, within 4 h of the onset of symptoms of the disease, miR-208a was easily detectable in AMI patients, but remained undetectable in non-AMI patients (Wang et al., 2010a). Also, circulating miR-133a levels were increased in 216 patients with ST-elevation myocardial infarction (STEMI), and associated with decreased myocardial salvage, larger infarcts, and more pronounced reperfusion injury (Eitel et al., 2012). In contrast, it has been reported that miR-133a levels were not associated with left ventricular remodeling or function after myocardial infarction, nor with BNP, excluding miR-133a as a useful biomarker for left ventricular remodeling after MI (Bauters et al., 2013).

Furthermore, it was reported that miR-30a, miR-195, and let-7b could be used as potential biomarkers for AMI (Long et al., 2012). The authors analyzed plasma samples from 18 patients with AMI and 30 healthy adults, and demonstrated that all 3 miRNAs reached their expression peak 8 h after the onset of AMI and these miRNAs showed significant diagnostic value for AMI using receiver operating characteristic curve analyses.

CIRCULATING miRNAs IN ATHEROSCLEROSIS

Coronary artery disease is characterized by plaque formation along the inner wall of coronary arteries, which narrows the arterial wall and gradually restricts blood flow to the heart (Libby et al., 2011). In one study, circulating miRNA profiles in plasma from eight CAD patients and eight healthy subjects were assessed by a microarray approach. Validation of the obtained results in a larger patient cohort by qPCR revealed that circulating endothelial-associated miR-126, the miR-17/92 cluster, inflammation-associated miR-155 and smooth muscle cell-associated miR-145 were significantly reduced in CAD patients (Fichtlscherer et al., 2010).

Another study using real-time PCR-based profiling showed that among 157 miRNAs expressed in peripheral blood mononuclear cells of CAD patients, miR-135a and miR-147 were fivefold overexpressed and fourfold decreased, respectively. This study also indicated the possibility to discern unstable pectoris angina patients from stable patients due to their relatively high expression of circulating miR-134, miR-198, and miR-370, opening the possibility of a miRNA signature for patients at risk for acute coronary syndromes (Hoekstra et al., 2010).

The potential of circulating miRNAs as biomarkers for cardiovascular diseases is promising. Indeed extracellular miRNAs present many properties of ideal biomarkers, including their detection in many biological fluids, their stability in RNAse-rich body fluids, and their tissue-specific expression patterns. More efforts on much larger cohorts of patients with various cardiovascular diseases are needed to reach sub-stratification of patients. Another appealing outlook of extending available biomarkers is the possibility to perform network analyses and multi-marker biomarker panels for individual patients, allowing increased sensitivity in diagnostics or prognostics than can be expected from the assessment of a single biomarker (Eurlings et al., 2012), as evidenced by the analysis of distinct clusters of miRNAs associated with myocardial infarction in a large study of patients (Zampetaki et al., 2012). Next-generation sequencing is an opportunity for miRNA profiling efforts and for further discovery of new miRNAs in a determined biological or pathological situation (Lee et al., 2010; Lawless et al., 2013). Nevertheless, there are still technical limitations in studying extracellular miRNAs as biomarkers. No consensus has been obtained in terms of normalization methods nor the use of equal amounts of serum or plasma, or the use of spike-in controls or the use of housekeeping miRNAs yield wide-spread consensus (Kroh et al., 2010; Qi et al., 2012).

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Exosomal miRNAs as biomarkers for prostate cancer

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miRNAs are small non-coding RNAs that finely regulate gene expression in cells. Alterations in miRNA expression have been associated with development of cancer, and miRNAs are now being investigated as biomarkers for cancer as well as other diseases. Recently, miRNAs have been found outside cells in body fluids. Extracellular miRNAs exist in different forms – associated with Ago2 proteins, loaded into extracellular vesicles (exosomes, microvesicles, or apoptotic bodies) or into high density lipoprotein particles. These extracellular miRNAs are probably products of distinct cellular processes, and might therefore play different roles. However, their functions *in vivo* are currently unknown. In spite of this, they are considered as promising, non-invasive diagnostic, and prognostic tools. Prostate cancer is the most common cancer in men in the Western world, but the currently used biomarker (prostate specific antigen) has low specificity. Therefore, novel biomarkers are highly needed. In this review we will discuss possible biological functions of extracellular miRNAs, as well as the potential use of miRNAs from extracellular vesicles as biomarkers for prostate cancer.

Keywords: exosomes, microvesicles, extracellular miRNA, prostate cancer, biofluids, biomarkers

INTRODUCTION

miRNAs are 19–23 nucleotides long non-coding RNAs that play important gene-regulatory roles (Carrington and Ambros, 2003; Bartel, 2004). These small RNAs downregulate gene expression through incorporation into the RNA-induced silencing complex (RISC), which then binds to partially complementary sites mainly in the 3′untranslated region (3′UTR) of their mRNA targets (Bartel, 2004). Depending on pairing complementarity, miRNAs operate through translation repression, mRNA cleavage or destabilization, or a combination of these routes (Lee et al., 1993; Wightman et al., 1993; Hutvagner and Zamore, 2002; Song et al., 2004; Lim et al., 2005). Several cellular processes like proliferation, differentiation, and apoptosis are shown to be regulated by miRNAs (Bartel, 2004) and miRNAs are found aberrantly expressed in many types of cancer (Calin et al., 2005; Iorio et al., 2005; Lu et al., 2005; Volinia et al., 2006; Porkka et al., 2007; Croce, 2009). Moreover, studies using knockout mice and transgenic mice overexpressing certain miRNAs indicate that miRNAs do contribute to cancer development (Mu et al., 2009; Hatley et al., 2010; Medina et al., 2010). It has been shown that miRNAs can act both as oncogenes and tumor suppressors, and that they participate in cancer development by regulating cell cycle, cellular senescence, DNA damage response, and apoptosis (Lima et al., 2011; Jansson and Lund, 2012).

Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of death among men with cancer in the Western world (Ferlay et al., 2007; Jemal et al., 2009). A protein mainly secreted by prostate cells, prostate specific antigen (PSA), has been used as a blood-based biomarker for prostate cancer for several decades. Even though PSA is a valuable tool, it lacks specificity and is therefore not considered an optimal biomarker (Nogueira et al., 2010). Thus, new and specific markers for prostate cancer are highly needed. Several miRNA expression profiles have been reported for prostate cancer, showing altered expression levels in prostate cancer tissue compared to control tissue (Lu et al., 2005; Volinia et al., 2006; Ozen et al., 2007; Porkka et al., 2007; Ambs et al., 2008; Szczyrba et al., 2010). Importantly, miRNAs are currently being investigated as prognostic and diagnostic tools for prostate and other types of cancer (Sørensen and Ørntoft, 2009; Kuner et al., 2013).

FORMS OF EXTRACELLULAR miRNAs

Recently, miRNAs have been identified in the medium from cultured cells (Valadi et al., 2007) and in many body fluids like blood (Chim et al., 2008; Lawrie et al., 2008), urine (Hanke et al., 2010), saliva (Park et al., 2009), breast milk (Kosaka et al., 2010b; Weber et al., 2010), and seminal plasma (Weber et al., 2010). Some of these miRNAs appear in extracellular stable forms, which render them interesting as biomarkers for cancer and other diseases. Extracellular miRNA can be found in different forms (**Figure 1**); some miRNAs are loaded into exosomes or microvesicles (Hunter et al., 2008; Skog et al., 2008; Lasser et al., 2011; Gallo et al., 2012; Hessvik et al., 2012), into apoptotic bodies (Zernecke et al., 2009), or into high density lipoprotein particles (Vickers et al., 2011), whereas others are associated with Ago2 proteins – proteins which are part of RISC (Arroyo et al., 2011; Turchinovich et al., 2011). Common to all these forms is that the miRNAs are not degraded by RNase treatment (Valadi et al., 2007; Turchinovich et al., 2011). First, the stability of extracellular miRNAs against RNases was thought to be mainly due to incorporation of miRNAs into small extracellular vesicles (Valadi et al., 2007; Hunter et al., 2008). However, some reports have shown that most of the extracellular miRNAs

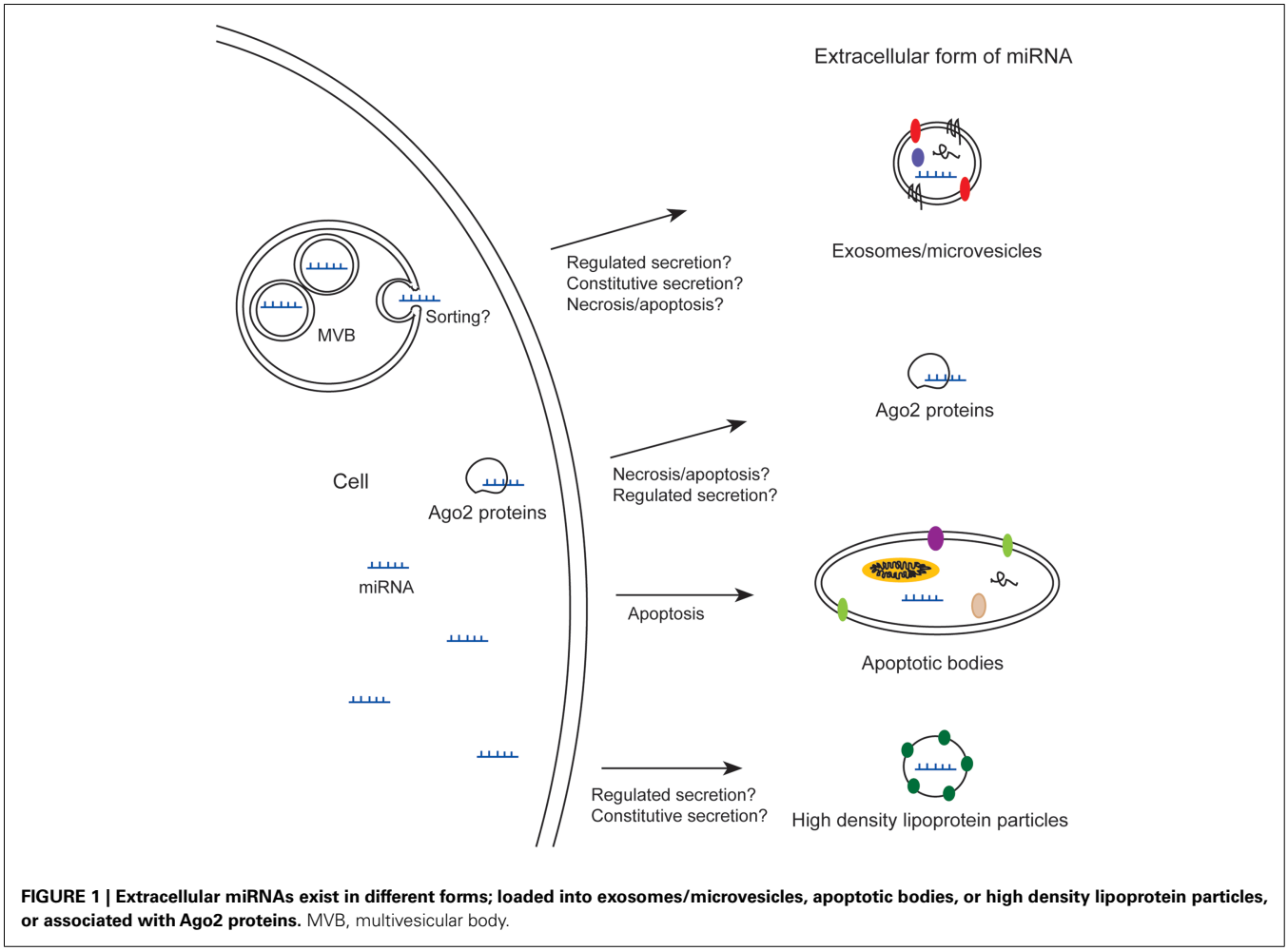

FIGURE 1 | Extracellular miRNAs exist in different forms; loaded into exosomes/microvesicles, apoptotic bodies, or high density lipoprotein particles, or associated with Ago2 proteins. MVB, multivesicular body.

in plasma and cell culture media are found outside vesicles, in stable complexes with Ago2 proteins (Arroyo et al., 2011; Turchinovich et al., 2011), although a recent publication showed that the majority of miRNAs in serum and saliva is enclosed in exosomes (Gallo et al., 2012).

EXTRACELLULAR VESICLES

As mentioned, miRNAs have been found in several types of extracellular vesicles. Extracellular vesicles are mainly classified based on the different mechanism of release, but a consensus in the terminology used to name these small vesicles is still lacking. Normally, exosomes are defined as small membrane vesicles with a diameter of 40–100 nm that are secreted when multivesicular bodies (MVBs) fuse with the plasma membrane (Pan et al., 1985; Johnstone et al., 1987; Bobrie et al., 2011). Shedding vesicles or microparticles are 0.1–1 μm in diameter and formed by budding from the plasma membrane (Cocucci et al., 2009). To collectively describe both exosomes and shedding vesicles the term microvesicles is often used (Cocucci et al., 2009), though this term is sometimes also used to indicate either one of these types of vesicles. A third type of extracellular vesicles is apoptotic bodies, which are 1–4 μm in diameter and formed during apoptosis (Hristov et al., 2004; Zernecke et al., 2009). Recently, the term extracellular vesicles has been used to describe these three classes of vesicles as a group (Kalra et al., 2012).

POTENTIAL ROLES OF EXTRACELLULAR miRNAs

Whether the extracellular forms of miRNAs are simply waste products from cells or have a biological function, such as participating in intercellular communication is not yet clear. There are reports showing increased level of miRNAs in blood upon organ toxicity (Laterza et al., 2009; Zhang et al., 2010; Pritchard et al., 2012), and this could of course represent waste products. Nevertheless, since the various forms of extracellular miRNAs are probably products of distinct cellular processes, they might play different roles, and therefore it is important to distinguish between them. Apoptotic bodies are by definition formed during apoptosis. miRNA bound to Ago2 may be released from cells upon apoptosis or necrosis (Turchinovich et al., 2011), but it is not known if miRNA-Ago2 complexes also can be transported out of viable cells. This means that miRNAs bound to Ago2 proteins and miRNAs incorporated into apoptotic bodies might solely be by-products from dying cells or represent a way for dying cells to communicate with neighboring cells. They could represent a signal warning the organism about cellular dysfunction.

Shedding vesicles and exosomes are thought to be released by viable cells, though it is not ruled out whether these vesicles also are released by dying cells. Therefore, these vesicles have to a greater extent been suggested to play a role in intercellular signaling (Valadi et al., 2007; Hunter et al., 2008). Indeed, it has been shown that miRNAs can be transferred by exosomes from one cell to another *in vitro* and result in downregulation of target genes in the recipient cell (Kosaka et al., 2010a; Kogure et al., 2011; Mittelbrunn et al., 2011; Montecalvo et al., 2012). This finding is intriguing and indicates a role in intercellular communication which could have a huge impact. Yet this remains to be shown *in vivo*. Interestingly, it has been reported that injection of exosomes loaded with siRNA into mice can result in specific gene knockdown in certain cells (Alvarez Erviti et al., 2011). It has been questioned whether the concentration of exosomes in biological fluids is high enough to play a role in intercellular communication, but this does not exclude a role in autocrine or paracrine signaling (Turchinovich et al., 2011; Sverdlov, 2012). Exosomes probably exert their effect on neighboring cells, and thereby participate in creating a specific microenvironment. In this scenario, the exosomes found in body fluids would only be residual amounts, representing a secondary effect.

In addition to their conventional role in post-transcriptional gene regulation, a new role for miRNAs as signaling molecules has recently been described by two independent groups. Interestingly, extracellular let-7 was shown to activate Toll-like receptor 7 in neurons and induce neurodegeneration (Lehmann et al., 2012). By another group, exosomal miR-21 and miR-29a was shown to activate Toll-like receptor 7 and 8 in immune cells, triggering a prometastatic inflammatory response that may lead to tumor growth and metastasis (Fabbri et al., 2012). Thus, extracellular miRNAs could be important regulators of tumor microenvironment as well as exacerbate CNS damage, through agonistic effect on Toll-like receptor 7 and 8.

Another possible role for miRNAs in exosomes and MVBs is that they might function together with the RNAi machinery. RISC proteins have been shown to be associated with MVBs and exosomes (Gibbings et al., 2009; Lee et al., 2009). Moreover, blocking MVB formation by depletion of ESCRT (endosomal sorting complex required for transport) components has been reported to result in impaired miRNA silencing, indicating a role in RNAi dynamics (Gibbings et al., 2009; Lee et al., 2009).

ARE miRNAs SORTED INTO EXOSOMES?

Another debated issue in the field is whether miRNAs are sorted into MVBs and exosomes or not. Several reports have shown that certain miRNAs are selectively identified or expressed at a higher level in exosomes than in parent cells (Valadi et al., 2007; Ohshima et al., 2010; Kogure et al., 2011; Mittelbrunn et al., 2011; Hessvik et al., 2012), indicating a sorting of miRNAs into MVBs. The opposite situation has also been reported; for example one study showed that only 2% of the most abundant miRNA in a breast cancer cell line, miR-720, was found extracellularly, whereas many other miRNAs were presented at comparable levels in the cellular and extracellular populations (Pigati et al., 2010). The findings that certain miRNAs are either enriched in exosomes or retained in cells, indicate that exosomal miRNAs are not simply unsorted waste products from cells. The mechanisms controlling the selection of miRNAs into exosomes or the retention of miRNAs inside cells still remain unknown. Though, it cannot be excluded that the higher expression level of certain miRNAs in exosomes is due to shielding of miRNAs from degradation by exosome membranes, and not due to a sorting mechanism. Moreover, Kim et al. (2012) recently reported that small RNAs with low GC content can be lost during RNA isolation from samples with low RNA content, indicating methodological challenges when working with extracellular miRNAs.

Higher levels of exosomes are found in plasma from cancer patients compared to control individuals (Rabinowits et al., 2009; Tavoosidana et al., 2011), suggesting that cancer cells secrete more exosomes than non-cancerous cells. Therefore, measuring exosomal miRNAs could result in less background from normal cells, and they might then serve as superior biomarkers compared to other extracellular miRNAs. However, the currently used protocols for exosome isolation are extensive and time-consuming. New and faster isolation methods need to be established before exosomal miRNAs can be used in routine diagnostics.

miRNAs AND PROSTATE CANCER

The first profiling of miRNAs in prostate cancer was published in 2007 (Porkka et al., 2007). In this study prostate cancer cell lines and prostate tissue from both benign prostatic hyperplasia and prostate cancer patients were examined. Today, the miRNA expression in prostate cancer has been published in more than 100 reports, showing promising results for miRNAs as tissue-based biomarkers for this disease (Catto et al., 2011). Measurement of extracellular miRNAs obtained from biological fluids constitutes a non-invasive approach for cancer detection and may therefore be preferable. Indeed, several studies (**Table 1**, and discussed below) have investigated the miRNA profile in serum/plasma from prostate cancer patients, pointing toward the use of miRNAs as blood-based biomarkers (Mitchell et al., 2008; Lodes et al., 2009; Brase et al., 2011; Moltzahn et al., 2011).

In the first study, the level of six miRNAs in serum samples from 25 patients with metastatic prostate cancer and 25 healthy controls was analyzed. The authors found that miR-141 was overexpressed in the prostate cancer group compared to the control group, and that this miRNA had the greatest differential expression among the six tested miRNAs (Mitchell et al., 2008). Later, 667 miRNAs in serum samples from patients with metastatic ($n = 7$) or localized prostate cancer ($n = 14$) were screened. In this study 69 miRNAs were found to be higher expressed in the metastatic tumor group compared to the primary cancer group. Five of the upregulated miRNAs (miR-375, miR-9*, miR-141, miR-200b, and miR-516a-3p) were further validated, resulting in the identification of miR-375 and miR-141 as the best markers for high risk prostate cancer (Brase et al., 2011). Another study analyzed the expression of miR-21, miR-141, and miR-221 in plasma from patients with localized/local advanced ($n = 26$) or metastatic ($n = 25$) prostate cancer and healthy controls ($n = 20$). A higher expression level of miR-21 and miR-221, but not statistically significant for miR-141, was observed in plasma from prostate cancer patients compared to controls. Still, all three miRNAs were significantly higher in patients with metastatic prostate cancer than

Table 1 | Studies of extracellular miRNAs in body fluids from prostate cancer patients.

Promising miRNA	Deregulation	Body fluid	Study design	Number of participants	Reference
let-7a	↑	Whole blood	PCa vs. healthy controls	83	Heneghan et al. (2010)
let-7c	↓	Plasma	PCa vs. BPH and healthy controls	Screening: 42 Validation: 178	Chen et al. (2012)
let-7e	↓	Plasma	PCa vs. BPH and healthy controls	Screening: 42 Validation: 178	Chen et al. (2012)
let-7i	↑	Serum	Localized PCa vs. BPH and healthy controls	83	Mahn et al. (2011)
miR-16	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-20a	↑	Plasma	PCa: high risk vs. intermediate risk vs. low risk	82	Shen et al. (2012)
miR-21	↑	Plasma	PCa: high risk vs. intermediate risk vs. low risk	82	Shen et al. (2012)
	↑	Plasma	Metastatic PCa vs. localized/local advanced PCa vs. healthy controls	71	Yaman Agaoglu et al. (2011)
	↑	Serum	Hormone-refractory PCa vs. androgen dependent PCa vs. localized PCa vs. BPH	56	Zhang et al. (2011)
miR-24	↓	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-26a	↑	Serum	Localized PCa vs. BPH and healthy controls	83	Mahn et al. (2011)
miR-26b	↓	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-30c	↓	Plasma	PCa vs. BPH and healthy controls	Screening: 42 Validation: 178	Chen et al. (2012)
	↓	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-34b	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-92a	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-92b	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-93	↑	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-103	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-106a	↑	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-107	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
	↑	Urine (cells)	PCa vs. healthy controls	135	Bryant et al. (2012)
	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-130b	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-141	↑	Serum	Metastatic PCa vs. localized PCa	Screening: 21 Validation: 116	Brase et al. (2011)
	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
	↑	Serum (microvesicles)	Metastatic vs. localized PCa	Validation: 119	Bryant et al. (2012)
	↑	Serum	Metastatic PCa vs. healthy controls	50	Mitchell et al. (2008)
	↑	Serum	Metastatic PCa vs. healthy controls	50	Selth et al. (2012)
	↑	Plasma	Metastatic PCa vs. localized/local advanced PCa vs. healthy controls	71	Yaman Agaoglu et al. (2011)

(Continued)

Table 1 | Continued

Promising miRNA	Deregulation	Body fluid	Study design	Number of participants	Reference
miR-145	↓	Whole blood	PCa vs. healthy controls	83	Heneghan et al. (2010)
	↑	Plasma	PCa: high risk vs. intermediate risk vs. low risk	82	Shen et al. (2012)
miR-155	↓	Whole blood	PCa vs. healthy controls	83	Heneghan et al. (2010)
miR-181a-2*	↓	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-195	↑	Serum	Localized PCa vs. BPH and healthy controls	83	Mahn et al. (2011)
miR-197	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-221	↑	Plasma	PCa: high risk vs. intermediate risk vs. low risk	82	Shen et al. (2012)
	↑	Plasma	Metastatic PCa vs. localized/local advanced PCa vs. healthy controls	71	Yaman Agaoglu et al. (2011)
	↑	Plasma	PCa vs. healthy controls	43	Zheng et al. (2012)
miR-223	↓	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-298	↑	Serum	Metastatic PCa vs. healthy controls	50	Selth et al. (2012)
miR-301a	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-326	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-328	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-331-3p	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-346	↑	Serum	Metastatic PCa vs. healthy controls	50	Selth et al. (2012)
miR-375	↑	Serum	Metastatic PCa vs. localized PCa	Screening: 21 Validation: 116	Brase et al. (2011)
	↑	Serum (microvesicles)	Metastatic vs. localized PCa	Validation: 119	Bryant et al. (2012)
	↑	Serum	Metastatic PCa vs. healthy controls	50	Selth et al. (2012)
miR-432	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-485-3p	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-486-5p	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-574-3p	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
	↑	Urine (cells)	PCa vs. healthy controls	135	Bryant et al. (2012)
	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-622	↑	Plasma	PCa vs. BPH and healthy controls	Screening: 42 Validation: 178	Chen et al. (2012)
miR-625*	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)
miR-636	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-640	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-766	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)

(Continued)

Table 1 | Continued

Promising miRNA	Deregulation	Body fluid	Study design	Number of participants	Reference
miR-874	↑	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-885-5p	↑	Serum	Stage 3 and 4 PCa patients vs. healthy controls	13	Lodes et al. (2009)
miR-1207-5p	↑	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-1274a	↑	Serum	PCa vs. healthy controls	48	Moltzahn et al. (2011)
miR-1285	↑	Plasma	PCa vs. BPH and healthy controls	Screening: 42 Validation: 178	Chen et al. (2012)
miR-2110	↑	Plasma (microvesicles)	PCa vs. healthy controls	Screening: 106	Bryant et al. (2012)

miRNAs ordered by increasing name number. PCa, prostate cancer; BPH, benign prostatic hyperplasia. Asterisks are part of the miRNA names.

in patients with localized/local advanced disease (Yaman Agaoglu et al., 2011). In a study by Lodes et al. 547 miRNAs were screened, 15 miRNAs (miR-16, -92a, -103, -107, -197, -34b, -328, -485-3p, -486-5p, -92b, -574-3p, -636, -640, -766, -885-5p) were found to be overexpressed in serum from stage 3 and 4 prostate cancer patients ($n = 5$) compared to eight healthy controls. They also showed a slightly elevated level of miR-141 in stage 3 and 4 prostate cancer patient serum (Lodes et al., 2009). Selth et al. analyzed the expression of 10 miRNAs in serum from patients with metastatic castration-resistant prostate cancer ($n = 25$) and healthy controls ($n = 25$). They found that miR-141, miR-298, miR-346, and miR-375 were upregulated in serum from prostate patients compared with controls (Selth et al., 2012). Though these independent studies point toward plasma/serum-derived miR-141 and miR-375 as biomarkers, Mahn et al. (2011) did not succeed in detecting miR-141 in serum samples.

In the study by Mahn et al. the expression of four miRNAs in serum from 37 patients with localized prostate cancer, eight with metastatic prostate cancer, 18 with benign prostatic hyperplasia, and 20 healthy controls was analyzed. They found miR-26a, miR-195, and let-7i to be upregulated in patients with prostate cancer compared with patients with benign prostatic hyperplasia. Moreover, they also showed that miR-26a levels could discriminate patients with prostate cancer from patients with benign prostatic hyperplasia (Mahn et al., 2011). Another report showed results from screening of 384 miRNAs in serum from 36 prostate cancer patients and 12 healthy controls. Five miRNAs (miR-874, -1274a, -1207-5p, -93, and -106a) were identified to be upregulated and four (miR-223, -26b, -30c, and -24) downregulated in cancer patients compared to controls (Moltzahn et al., 2011). Heneghan et al. (2010) analyzed the expression of seven miRNAs and found a decreased level of miR-145 and miR-155 and an increased level of let-7a in whole blood from prostate cancer patients ($n = 20$) compared to healthy controls ($n = 63$).

Although a higher level of miR-21 in plasma from prostate cancer patients compared to controls has been observed (Yaman Agaoglu et al., 2011), Zhang et al. did not find a significant difference in the level of serum-derived miR-21 between patients with benign prostatic hyperplasia ($n = 6$), localized ($n = 20$), and androgen dependent prostate cancer ($n = 20$). Still, higher expression of miR-21 was detected in patients with hormone-refractory prostate cancer ($n = 10$), especially in patients resistant to docetaxel-based chemotherapy, suggesting a potential role for miRNAs as markers for disease progression and response to treatment (Zhang et al., 2011). In another study, the level of miR-221 in plasma samples from healthy controls ($n = 20$), androgen dependent ($n = 15$), and androgen independent ($n = 8$) prostate cancer patients was analyzed. In accordance with the findings by Yaman Agaoglu et al. the authors observed increased expression of miR-221 in plasma samples from prostate cancer patients compared with healthy controls, whereas the level of miR-221 was increased in androgen dependent compared to androgen independent prostate cancer patients (Zheng et al., 2012).

Chen et al. screened the miRNA expression in plasma from 25 prostate cancer patients and 17 patients with benign prostatic hyperplasia and validated candidate miRNAs in a larger independent cohort (80 prostate cancer patients, 44 patients with benign prostatic hyperplasia, and 54 healthy controls). These authors showed that five miRNAs (let-7c, let-7e, miR-30c, miR-622, and miR-1285) could differentiate patients with prostate cancer from patients with benign prostatic hyperplasia and healthy controls. They suggested that a panel of the five described miRNAs could distinguish these patient groups from each other with higher sensitivity and specificity compared to one single miRNA (Chen et al., 2012). Another group also suggested a combination of several miRNAs as biomarker after analyzing miRNA levels in plasma samples from 82 prostate cancer patients with varied aggressiveness. In this report the combination of miR-20a, miR-21, miR-145, and miR-221 was shown to distinguish prostate cancer patients with high risk of aggressiveness from those with low risk (Shen et al., 2012).

Neither of the mentioned studies describes in which form the extracellular miRNAs were packaged. In only one study miRNAs from plasma- and serum-derived microvesicles were analyzed, though the vesicles were not well characterized. Eleven miRNAs were found to be differently expressed in prostate cancer patients compared to healthy controls (miR-107, -130b, -141, -181a-2*,

-2110, -301a, -326, -331-3p, -432, -574-3p, and -625*), and 16 miRNAs were upregulated in patients with metastases compared to patients without metastases. The association of miR-141 and miR-375 with metastatic prostate cancer was also confirmed. The authors selected five miRNAs that were analyzed in urine samples, showing that miR-107 and miR-574-3p were found in higher concentrations in the urine of men with prostate cancer compared with healthy controls. However, these miRNAs were not really extracellular, since cell pellets from urine were used in this part of the study (Bryant et al., 2012). Recently, we described the miRNA profile in exosomes from the PC-3 metastatic prostate cancer cell line. Among the aforementioned miRNAs suggested as biomarker candidates in clinical studies, we identified 36 in exosomes from PC-3 cells (miR-141, -9*, -200b, -21, -221, -16, -92a, -103, -107, -197, -92b, -574-3p, -885-5p, -298, -26a, -1274a, -106a, -26b, -30c, -24, let-7i, let-7a, let-7c, let-7e, miR-1285, -20a, -107, -130b, -301a, -331-3p, -625, -485-3p, -874, -155, -181a-2*, and -326) (Hessvik et al., 2012).

As described, an emerging amount of evidence points toward a potential use of blood-based miRNAs as diagnostic and prognostic biomarkers for prostate cancer (**Table 1**). Nevertheless, the data from the clinical studies are to some extent inconsistent. This may not be surprising due to the fact that these studies differ with respect to experimental design and patient cohort. Most of these studies are small-scale, with varying degree of patient description and few are focusing on cancer-related death or progression free survival as endpoints. At least one study included patients under treatment with chemotherapy, therefore it is uncertain whether the altered miRNA levels were due to the chemotherapy or due to the tumor itself (Lodes et al., 2009). In addition, different platforms have been used to quantify miRNA levels, which also could explain some of the conflicting data. High throughput platforms always have biases and confirmation with a secondary analytical approach, such as RT-qPCR, should be performed to validate the results. Isolation of extracellular miRNAs using different methods can contribute to variation due to low recovery of certain miRNA species, and quantification of extracellular miRNAs are challenging due to lack of standards for quality control, normalization, and statistical analysis. Together, these factors might explain most of the conflicting results.

Blood is the body fluid that traditionally has been used as the main source of cancer biomarkers, though the use of urine in cancer diagnosis and prognosis is growing. Choosing urine as a source of biomarkers has several advantages compared to blood; it is non-invasive, easily obtained in large quantities, and the composition of urine is less complex. Particularly important for prostate cancer is the fact that the composition of urine reflects alterations in the urogenital system. However, dilution of the miRNAs could be a possible drawback with the use of urine as a source of biomarkers. To our knowledge there are currently no publications describing the miRNA content of urinary exosomes in prostate cancer.

CONCLUSION

Intensive research is put into the hunt for new diagnostic and prognostic tools for prostate cancer. Representing a non-invasive approach, measurement of extracellular miRNAs in blood, urine, or other biological fluids might turn out as a valuable strategy. Studies on blood-based miRNAs as biomarkers for prostate cancer are emerging, but most of these are small-scale, vary in methodology, and lack a characterization of the form in which the extracellular miRNAs are found. Standards for miRNA quantification need to be established and larger validation studies need to be performed before conclusions can be drawn on the diagnostic potential of specific miRNAs. It might be important to distinguish between different forms of extracellular miRNAs, both for diagnosis and for understanding possible biological functions. More and large-scale studies on exosomal miRNAs in biological fluids from prostate cancer patients and healthy controls are therefore needed. Although there is still very limited knowledge about the biological roles of extracellular miRNAs and extracellular vesicles, they are currently emerging as an important source of biomarkers.

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Extracellular circulating viral microRNAs: current knowledge and perspectives

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MicroRNAs (miRNAs) are small non-coding RNAs responsible of post-transcriptional regulation of gene expression through interaction with messenger RNAs (mRNAs). They are involved in important biological processes and are often dysregulated in a variety of diseases, including cancer and infections. Viruses also encode their own sets of miRNAs, which they use to control the expression of either the host's genes and/or their own. In the past few years evidence of the presence of cellular miRNAs in extracellular human body fluids such as serum, plasma, saliva, and urine has accumulated. They have been found either cofractionate with the Argonaute2 protein or in membrane-bound vesicles such as exosomes. Although little is known about the role of circulating miRNAs, it has been demonstrated that miRNAs secreted by virus-infected cells are transferred to and act in uninfected recipient cells. In this work we summarize the current knowledge on viral circulating miRNAs and provide a few examples of computational prediction of their function.

Keywords: microRNA, viruses, exosomes, circulating microRNA, vesicules, body fluids

INTRODUCTION

MicroRNAs (miRNAs) are the most studied and best characterized molecules in the class of small regulatory non-coding RNAs (Bartel, 2009). They are involved in several important biological processes and functions through post-transcriptional regulation of the expression of messenger RNAs (mRNAs), and their dysregulation is often cause or consequence of a variety of diseases, such as cancer and neurodegenerative disorders (Croce, 2009; Eacker et al., 2009). Cellular miRNAs can be packaged into different carriers and exported to recipient cells or released in small vesicles during apoptosis (Boon and Vickers, 2013; Hilton and Karpe, 2013). The discovery of extracellular miRNAs in biological fluids has started a new exciting field of research. Circulating miRNAs are now considered useful markers of disease conditions and functional mediators of several biological processes in a novel form of cell-to-cell communication. A novel useful resource is the database miRandola, which provides users with a comprehensive manually curated classification of extracellular circulating miRNAs (Russo et al., 2012).

Viruses encode their own sets of miRNAs. Evidence shows that these miRNAs can act as self-regulators of viral gene expression and/or control host cell pathways through silencing of their nodes (Kincaid and Sullivan, 2012). Some viruses can exploit extracellular particles for the initiation and progression of the infection and recent evidence indicates that viruses can export and deliver functional miRNAs through vesicles (Pegtel et al., 2010). This discovery reveals a new layer in the infectious mechanism used by viruses to maintain their latency and control crucial host pathways whose targeting is likely beneficial to the virus.

In this mini review we summarize the current knowledge about circulating miRNAs and their potential regulatory functions, with particular emphasis on extracellular viral miRNAs. We report the promising results of the most recent studies and provide a few examples of computational prediction of viral miRNA function.

CURRENT KNOWLEDGE

CIRCULATING miRNAs ARE FUNCTIONAL IN RECIPIENT CELLS AND CONSTITUTE USEFUL BIOMARKERS FOR VARIOUS CONDITIONS

Extracellular miRNAs have been recently identified stable in most biological fluids, including blood, urine, saliva, semen, cerebrospinal fluid, and breast milk (Mitchell et al., 2008; Hanke et al., 2010; Wang et al., 2011; Alexandrov et al., 2012; Gallo et al., 2012; Zhou et al., 2012). Evidence shows that they may

be selectively packaged into different kinds of carriers, such as membrane-derived vesicles, lipoproteins, and ribonucleoprotein complexes, which protect them from degradation and export them to recipient cells where they exert their regulatory functions. Particularly, exosomes and microparticles (MPs) are two distinct classes of small membrane-enclosed vesicles released from cells, differing in size, biogenesis, and secretory mechanisms (Boon and Vickers, 2013). Exosomes are produced by the inward budding of the limiting membrane of multivesicular bodies (MVBs). They are smaller than MPs, which are instead formed by the outward budding and blebbing of the plasma membrane. Small sealed membrane vesicles that are produced from cells during apoptosis, called apoptotic bodies, can also transport specific sets of miRNAs. Extracellular miRNAs have also been found in high-density lipoproteins (HDL) and low-density lipoproteins (LDL), and bound to Argonaute 2 (AGO2) and other ribonucleoproteins, both in and out of membrane-derived vesicles (Arroyo et al., 2011; Turchinovich et al., 2011; Vickers and Remaley, 2012; Rayner and Hennessy, 2013). Viral surface antigen particles may also carry specific miRNAs, as in the case of hepatitis B surface antigen particles which contain hepatocellular miRNAs bound to AGO2 (Novellino et al., 2012).

MicroRNA profiles of extracellular carriers show distinct sets of miRNAs than their parent cell-type, thus suggesting that some miRNAs might be transcribed only to be exported and not retained in the parent cell (Ohshima et al., 2010; Pigati et al., 2010). Selective packaging of miRNAs into vesicles is probably related to the specific biological functions of the secreted miRNAs.

Circulating miRNAs are highly stable and consistent among individuals of the same species. Specific miRNA expression signatures in extracellular environment have been identified in a variety of human diseases, including cancer and neurological diseases, revealing the diagnostic potential of circulating miRNAs as useful non-invasive biomarkers (Alexandrov et al., 2012; Fayyad-Kazan et al., 2013; Zeng et al., 2013).

Several *in vitro* studies have shown that miRNAs transferred by the different types of carriers are functional and can regulate gene expression in recipient cells.

Apoptotic bodies generated from endothelial cells during atherosclerosis were shown to contain miR-126, which controls endothelial cell signaling *in vitro* and provides atheroprotective effects *in vivo* (Zernecke et al., 2009).

Another study showed that endothelial cells can transfer functional miR-143 and miR-145 to smooth muscle cells where they mediate the reduction of atherosclerotic lesion formation *in vivo* (Hergenreider et al., 2012).

Similarly, circulating miR-150 is released by monocytes and taken up by endothelial cells where it regulates endothelial cell migration (Zhang et al., 2010).

Although the complete mechanism of gene regulation mediated by specifically selected extracellular circulating miRNAs has yet to be clearly demonstrated *in vivo*, these studies suggest a plausible form of cell-to-cell communication in which donor cells send their miRNAs to distant recipient cells where they exert their regulatory functions.

VIRUSES EXPLOIT EXTRACELLULAR PARTICLES TO ESTABLISH AND MAINTAIN THE INFECTION

It has been shown that some viruses exploit extracellular particles, such as microvesicles, for the initiation and progression of the infection (Meckes and Raab-Traub, 2011). According to the trojan exosome hypothesis proposed by Gould et al. (2003), retroviruses may use the pre-existing non-viral exosome biogenesis and uptake pathways for the formation, release, and delivery of viral particles.

This has been later supported by evidence that some viruses utilize endosomal compartments of the host to generate exosome-like vesicles (Hosseini et al., 2013) which can play different roles in the infection, contributing to its spreading (Mack et al., 2000), favoring exosomal biogenesis (daSilva et al., 2009), and providing immune evasion (Temme et al., 2010).

Viral exosomes, for instance, affect the host immune system in different ways according to the type of virus and the stage of its life cycle in which exosome secretion occurs in the infected host. As proving example, during the non-replicative stage, dendritic cells serve as transit location for HIV-1 (human immunodeficiency virus 1) which exploits their intracellular vesicle trafficking pathways to release antigens and viral particles into the extracellular space and *trans*-infect CD4+ T cells (Izquierdo-Useros et al., 2010).

Generally, viruses implement different strategies during infection essentially consisting in escaping the host immune system and facilitating the invasion and proliferation within the host. Observations suggest that the release of microvesicles containing specific cellular and viral components by infected cells contributes greatly to the preservation of the virus even in a hostile antiviral immune environment (de Gassart et al., 2003; Izquierdo-Useros et al., 2009; Klibi et al., 2009; György et al., 2011; Meckes and Raab-Traub, 2011).

Epstein–Barr virus (EBV), cytomegalovirus (CMV), and hepatitis C virus (HCV) have found means to evade immune responses and increase virus-fusing ability and infectivity by exploiting microvesicles, giving rise to a systematic distribution of viral agents from infected cells able to induce genetic and epigenetic modifications in recipient cells (Masciopinto et al., 2004; Klibi et al., 2009; Plazolles et al., 2011; Wurdinger et al., 2012).

Tumor-associated viruses, like EBV, may use exosomal transfer to manipulate the growth characteristics of neighboring cells and enhance tumor progression. In particular, exosomes released from nasopharingeal carcinoma (NPC) cells harboring latent EBV were shown to contain the EBV latent membrane protein 1 (LMP1; Meckes et al., 2010), which is frequently expressed in EBV-associated cancers and has potent effects on cell growth by inducing growth-stimulating signaling pathways (Wang et al., 1985; Kaye et al., 1993) and may modulate the selective sorting of proteins into exosomes, favoring important signaling molecules frequently activated in cancers such as phosphatidylinositol 3-kinase (PI3K) and epidermal growth factor receptor (EGFR; Meckes et al., 2010).

VIRUSES ENCODE miRNAs

RNA interference (RNAi) most probably was originally selected as a primary mechanism of defense against harmful genetic elements

such as viruses. It is of relevant interest that in the evolutionary selection this mechanism was in turn exploited by viruses to their advantage while, as suggested by tenOever (2013), chordate use of small RNAs might exclusively have shifted to the silencing of genome-encoded transcripts and would at least not pose direct threat to RNA viral genome.

The first report of viral-encoded miRNAs was published by Pfeffer et al. (2004) describing the cloning of viral miRNAs from cells infected with EBV. Among DNA viruses, which account for the majority of known virus-encoded miRNAs, 95% of viral miRNAs known today are of herpesvirus origin.

The majority of natural viruses found to encode miRNAs have thus a DNA component to their replication cycle, can exploit the initiating host miRNA biogenesis machinery in the nucleus where they replicate, and cause long-term persistent infections. DNA viruses such as the ones belonging to the Herpesvirus, Polyomavirus, Ascovirus, Baculovirus, Iridovirus, and Adenovirus families clearly match these characteristics (Sullivan et al., 2005; Gottwein et al., 2007; Choy et al., 2008; Hussain et al., 2008; Seo et al., 2009; Seto et al., 2010; Bauman et al., 2011; Marquitz et al., 2011; Suffert et al., 2011; Zhao et al., 2011; Lee et al., 2012) along with at least one member of the retrovirus family, bovine leukemia virus (BLV), which clearly encodes numerous miRNAs (Kincaid et al., 2012).

Despite the established case of BLV, viruses possessing positive or negative sense RNA or double-stranded RNA (dsRNA) genome are not widely accepted to naturally express miRNAs.

Nevertheless, HIV-1 has been proven to encode two miRNAs and potentially a third. In fact, hiv1-mir-H1 was proven to be responsible for inducing apoptosis and repressing host gene expression (Kaul et al., 2009), while hiv-1-miR-N367 has been suggested as functional ortholog of hsa-miR192 (You et al., 2012). Finally, some evidence is present that the HIV-1 TAR element could be a potential viral miRNA (Houzet and Jeang, 2011), also considering its capability to target pro-apoptotic genes (Klase et al., 2009).

All viral miRNAs can essentially be grouped into two classes: host analogs and virus-specific. Generally, though, their functions include prolonging longevity of infected cells, evading the immune response, and regulating host or viral genes to limit the lytic cycle. Interestingly, all these functions are essential for infections to be persistent.

In fact, miRNAs are likely invisible to the adaptive immune system – a valuable trait for viruses that undergo persistent infection (Cullen, 2006). Thus, in viruses that establish a long-lasting latent infection, such as herpesviruses, one important benefit they could gain from employing miRNAs is the ability to regulate host and/or viral gene expression without having to elicit an antigenic immune reaction or directly suppressing components of the host immune system (Sullivan, 2008).

Preventing cell death seems an obvious advantage to viruses that cause persistent or latent infections. Several different viruses including Kaposi's sarcoma-associated herpesvirus (KSHV), EBV, and Marek's Disease Virus type 1 (MDV1) encode miRNAs that can play a subtle role in preventing apoptosis by targeting pro-apoptotic host genes and are also associated with tumorigenesis.

PERSPECTIVES

VIRUSES CAN USE VESICLES TO EXPORT THEIR FUNCTIONAL miRNAs

Pegtel et al. (2010) were the first ones (and, to our knowledge, the only ones together with Meckes et al., 2010) to have demonstrated that virus-infected cells package virus-encoded RNAs, and specifically viral miRNAs, into exosomes which are exported into the extracellular space and eventually delivered to recipient, non-infected cells, favoring the repression of specifically important mRNA targets. EBV is a clear example of a virus that utilizes the exosome pathway for the selective secretion of viral and cellular proteins and miRNAs that likely participate in cell-to-cell communication in the absence of virus production, potentially modulating cell function.

As confirming proof, Pegtel et al. (2010) reported that EBV-infected activated B cells secrete exosomes containing viral miRNAs shown to be delivered and actively internalized by monocyte-derived dendritic cells in co-culture. In particular, the copy number of EBV-miRNA BART1-5p was consistently higher than other EBV-miRNAs and its level increased fourfold after additional 24 h co-culture. This resulted in a dose-dependent, miRNA-mediated repression of confirmed EBV target genes. More specifically, the viral miRNA BHRF1-3 was shown to suppress the expression of the immunostimulatory gene CXCL11 [Chemokine (C-X-C motif) ligand 11] and this repression was proven to be dependent on the amount of exosomes carrying the miRNA and was not recipient cell-type-specific. In addition, expression of EBV-miRNAs in EBV-infected circulating B cells was also investigated. The data collected suggested that in asymptomatic patients BART miRNAs are expressed by latently infected circulating B cells as well as present in non-infected non-B cells, supporting the possibility of miRNA transfer *in vivo*. This further supported the proposal that exosomes could most likely serve as deliverers of small RNA due to their specialized biogenesis and presumed entry route (Zomer et al., 2010).

Later evidence showed that EBV-encoded miRNAs have been detected in exosomes from EBV-infected NPC cells, together with the LMP1 protein and other signal transduction molecules (Meckes et al., 2010), in accordance to other studies proving the presence of cellular miRNAs in tumor-derived exosomes (Taylor and Gercel-Taylor, 2008; Kharaziha et al., 2012; Palma et al., 2012).

Furthermore, differences detected in the levels of intracellular and exosomial miRNAs, in addition to differences even in the amount of enrichment between the individual exosomal miRNAs, suggest that some viral miRNAs might be specifically intended and selected to be packaged into exosomes and exert their functions in cells other than those producing them (Klibi et al., 2009; Meckes et al., 2010; Pegtel et al., 2010). Moreover, exosomes may also deliver cellular components of the RNA-induced silencing complex (RISC) to enhance viral miRNA function (Gibbings et al., 2009).

These results were greatly motivated by the assumption that exosomal exportation of miRNAs in general may have a fundamental role in intercellular communication despite the lack of concrete evidence (Valadi et al., 2007; Skog et al., 2008; Théry et al., 2009).

Although functional significance of all these phenomena requires further investigation, these results suggest that a cellular

miRNA-loading mechanism may exist to direct specific miRNAs into intraluminal vesicles of multivesicular endosomes (MVEs) which could explain why exogenous exosomal miRNAs are capable of repressing targets in recipient cells at new subcellular compartments for RNAi activity such as late endosomes (Morelli et al., 2004; Stern-Ginossar et al., 2007; Gibbings et al., 2009). **Figure 1** depicts all the potential ways in which viruses could exploit extracellular particles to convey their miRNAs to non-infected recipient cells.

FUNCTIONAL ANALYSIS OF CIRCULATING VIRAL miRNAs

The correct identification of targets is fundamental to determine miRNA function. Computational miRNA target prediction is still a big challenge, mostly due to the fact that our knowledge about the mechanisms and the molecular rules of miRNA target recognition is still incomplete (Bartel, 2009). Nevertheless, there are many computational tools available online, which allow to identify the most probable miRNA targets and to uncover non-trivial relationships between miRNAs and other molecular actors (Cascione et al., 2013). These tools collect and integrate heterogeneous miRNA-related data retrieved from different sources, such as target prediction tools and expression profiles of miRNAs and mRNAs, in order to infer miRNA functions and produce general models of miRNA-mediated regulation in the context of complex processes. Few tools are available specifically for the analysis of viral miRNAs and they are limited to the prediction of new miRNAs and targets. RepTar and vHoT are databases of predicted interspecies interactions between viral miRNA and host genomes, while ViTa is a database containing predictions of host miRNA targets on viruses (Hsu et al., 2007; Elefant et al., 2011; Kim et al., 2012). miRiam is a software that has been used to predict potential human targets for viral miRNAs (Laganà et al., 2010). Finally, VMir and Vir-Mir are tools for the prediction of novel virus-encoded miRNAs (Li et al., 2008; Grundhoff, 2011). In regard to functional analysis, despite the lack of specific programs for viral miRNAs, general miRNA

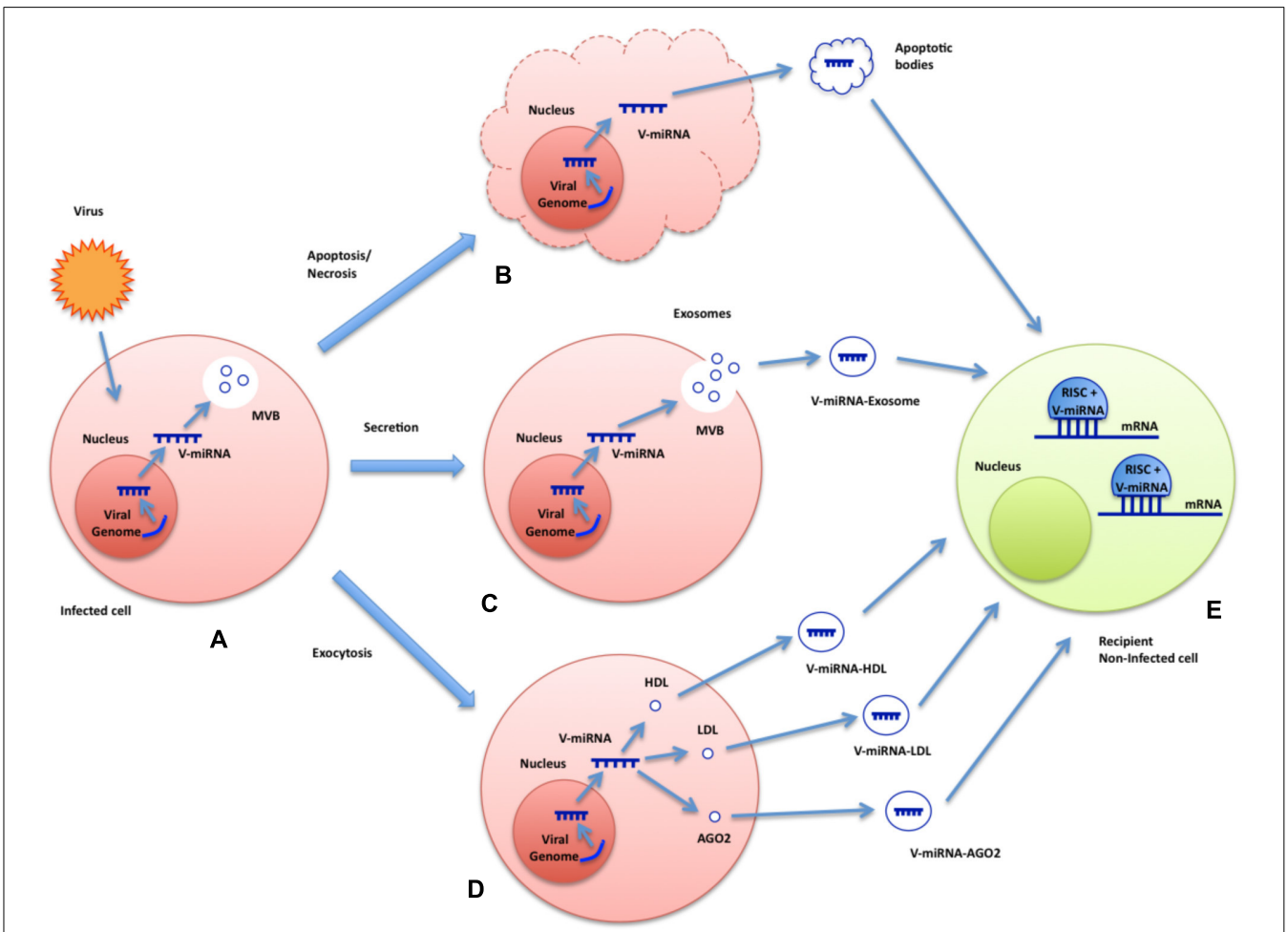

FIGURE 1 | Summary model of plausible mechanisms for export and functional delivery of viral miRNAs. The image depicts the possible means of transcription, packaging, and functional delivery of viral miRNAs during an infection. Virus-encoded miRNAs are transcribed by the infected cell **(A)**. They could exploit various channels to reach extracellular space and, eventually, be delivered to recipient non-infected cells: inside apoptotic bodies after cell death **(B)**, packaged into exosomes **(C)**, or HDL/LDL molecules or even bound to AGO2 **(D)**. Viral miRNAs may be uptaken by non-infected cells where they could exert their regulatory functions **(E)**.

Table 1 | Functional enrichment analysis of circulating EBV miRNAs' predicted targets.

	P-Value
Selected canonical pathways	
Molecular mechanisms of cancer	5.27×10^{-11}
PPARα/RXRα activation	9.39×10^{-6}
Wnt/β-catenin signaling	1.25×10^{-5}
p53 signaling	6.73×10^{-5}
IL-8 signaling	1.56×10^{-4}
Selected molecular and cellular functions	
Cell morphology	$<3.6 \times 10^{-2}$
Cell death and survival	$<3.72 \times 10^{-2}$
Cell cycle	$<4.09 \times 10^{-2}$
Selected diseases and disorders: cancer	
Leiomyomatosis	1.21×10^{-5}
Cell transformation	2.60×10^{-3}
Growth of tumor	5.14×10^{-3}
Mesenchymal tumor	8.31×10^{-3}
Selected tox functions (Clinical Chemistry and Hematology)	
Decreased levels of albumin	1.37×10^{-1}
Increased levels of alkaline phosphatase	2.21×10^{-1}
Increased levels of albumin	3.70×10^{-1}
Increased levels of LDH	3.70×10^{-1}

The table summarizes the most relevant results, particularly associated to EBV infection, of the functional analysis conducted using the software IPA. Results are organized in categories. For each category, the most significant terms, together with their P-Values, are displayed. EBV-encoded miRNAs in which exosomes are particularly enriched were selected (miR-BHRF1-1/1-2-3p and miR-BART1-3p/5p/-2-3p) and their targets predicted using the tool miRiam. The top scoring targets were given as input to IPA.

tools can be successfully applied to the study of viral miRNAs as well. A very recent study shows that the predicted targets of the 135 known viral miRNAs in human viruses and of 6809 putative miRNAs encoded by 23 human viruses, as predicted by Vir-Mir, are enriched for specific host pathways whose targeting is likely beneficial to the virus, such as cancer, axon guidance, ErbB, mitogen-activated protein kinase (MAPK), and wingless-type MMTV integration site family (Wnt) signaling (Carl et al., 2013). The authors performed a functional enrichment analysis by comparing each gene target set with an annotated functional gene set corresponding to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and Gene Ontology biological processes. As further proof of principle, we used miRiam to predict the potential targets of EBV miRNAs in which exosomes are particularly enriched, as reported by Pegtel et al. (2010) (miR-BHRF1-1/1-2-3p and miR-BART1-3p/5p/-2-3p). Then, we used the tool ingenuity pathway analysis (IPA) to perform a functional enrichment analysis of the predicted targets (http://www.ingenuity.com). The results show that subsets of the targets are significantly involved in cancer pathways, in particular leiomyomatosis, and mesenchymal tumors, for which a connection with EBV had already been described (Cheuk et al., 2002; Monforte-Muñoz et al., 2003; Deyrup et al., 2006; Sunde et al., 2010). Other significant pathways include WNT/B-catenin signaling, interleukin 8 (IL-8) signaling, and P53 pathway ($P < 0.0001$), also previously described as related to EBV infections (Morrison et al., 2003; Everly et al., 2004; Ren et al., 2004; Webb et al., 2008; Forte and Luftig, 2009; Husaini et al., 2011; QingLing et al., 2011). The predicted targets are also enriched in GO terms such as cell death and survival and cell cycle ($P < 0.04$). Furthermore, although the significance of the *P*-value is borderline ($P < 0.4$), it is worth to mention that the top tox functions reported by IPA include increased levels of alkaline phosphatase and LDH, tumour-marker characteristics which have been reported to be significant prognostic factors in metastatic NPC, often associated wih EBV infection (Jin et al., 2012). **Table 1** summarizes the most significant associations.

These few examples clearly indicate that miRNA functional analysis tools can be of great help in studying the effects of circulating viral miRNAs, allowing the production of plausible hypotheses about their function and involvement in crucial cellular pathways, encouraging the development of more specific tools for computational investigation of cellular and extracellular viral miRNA.

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Circulating *inflamma-miRs* in aging and age-related diseases

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Evidence on circulating microRNAs (miRNAs) is indisputably opening a new era in systemic and tissue-specific biomarker research, highlighting new inter-cellular and inter-organ communication mechanisms. Circulating miRNAs might be active messengers eliciting a systemic response as well as non-specific "by-products" of cell activity and even of cell death; in either case they have the potential to be clinically relevant biomarkers for a number of physiopathological processes, including inflammatory responses and inflammation-related conditions. A large amount of evidence indicates that miRNAs can exert two opposite roles, activating as well as inhibiting inflammatory pathways. The inhibitory action probably relates to the need for activating anti-inflammatory mechanisms to counter potent proinflammatory signals, like the nuclear factor kappaB (NF-κB) pathway, to prevent cell and tissue destruction. MiRNA-based anti-inflammatory mechanisms may acquire a crucial role during aging, where a chronic, low-level proinflammatory status is likely sustained by the cell senescence secretome and by progressive activation of immune cells over time. This process entails age-related changes, especially in extremely old age, in those circulating miRNAs that are capable of modulating the inflammatory status (*inflamma-miRs*). Interestingly, a number of such circulating miRNAs seem to be promising biomarkers for the major age-related diseases that share a common chronic, low-level proinflammatory status, such as cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), Alzheimer Disease (AD), rheumatoid arthritis (RA), and cancers.

Keywords: inflammation mediators, circulating miRs, age-related diseases, NF-κB signaling, cellular senescence

Inflamma-miRs

The inflammatory response comprises complex biological reactions that require a fine-tuned integration between a range of immune system cell classes and an extensive network of biomolecules, which until recently had been thought to be largely cytokines. However, identification of a vast repertoire of non-coding microRNA (miRNA) in the mammalian genome has completely revolutionized our understanding of most biological processes, including inflammation (Nilsen, 2007). MiRNAs play a significant role in gene regulation acting as repressors as well as activators, mainly at the post-transcriptional level (Breving and Esquela-Kerscher, 2010). Since a single miRNA can target several genes, and multiple miRNAs share common targets, miRNAs are particularly suited for regulating processes and pathways at the "network" level (Inukai and Slack, 2013).

Sensing of dangerous signals by the innate immune system involves a number of germline-encoded pattern recognition receptors (PRRs) that can detect both conserved pathogen-associated molecular profiles (PAMPs) expressed on micro-organisms and altered endogenous ligands, mostly released by necrotic, senescent, and/or damaged cells (DAMPs). Among PRRs, toll-like receptors (TLRs) play a central role, since their engagement activates a potent proinflammatory pathway (Kawai and Akira, 2011). TLR signaling initiates from different adaptor proteins, such as myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter protein-inducing interferon-β, which in turn activate several downstream pathways, leading to activation of transcription factor nuclear factor kappaB (NF-κB), mitogen-activated protein kinases (MAPKs), and members of the interferon regulatory factor family (Dunne and O'Neill, 2005). Fine tuning of TLR signaling prevents generation of harmful and inappropriate inflammatory responses without lowering the surveillance for potentially dangerous signals. Deregulation of the whole network can have destructive effects and lead to tissue damage: this is a hallmark of chronic inflammation, which is often associated with age-related diseases (Olivieri et al., 2013a).

A mounting body of evidence has been documenting a relatively small number of miRNAs that are involved in regulating inflammation: their prototypes are miR-155, miR-21, and miR-146a (Quinn and O'Neill, 2011), hereinafter referred to as *inflamma-miRs*.

In physiological conditions transcription of miR-155, miR-21, and miR-146a is at baseline levels; however, initiation of proinflammatory TLR signaling immediately results in strong co-induction of their expression through a mechanism that is largely NF-κB-dependent (Boldin and Baltimore, 2012).

Although the importance of *inflamma-miRs* in innate immune response regulation is widely accepted, the molecular mechanism

of their action has proved to be highly complex. Early investigations disclosed that miR-146a acts as a negative regulator of TLR signaling by targeting both tumor necrosis factor receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK-1) (Taganov et al., 2006). MiR-21 was found to down-regulate the expression of IRAK and MyD88 (Chen et al., 2013), as well as of programmed cell death protein 4 (PDCD4), switching the cell program from proinflammatory to anti-inflammatory, mainly as reflected by IL-10 production (Sheddy et al., 2010).

Additional findings showing that miR-155 can also negatively modulate TLR signaling by targeting MyD88, Tak1-binding protein 2 (TAB2) (Ceppi et al., 2009), and the I-κ-B kinase ε (IKKε), (Liang et al., 2011) have generated a model where *inflamma-miRs* operate as a negative feedback loop to protect the organism against overwhelming inflammation. However, subsequent research has disclosed that *inflamma-miRs* could play a dual function, inhibiting as well as inducing TLR signaling (Kondo et al., 2012). This is the case of miR-155, which both suppresses and enhances TLR signaling by silencing Src homology 2 domain-containing protein tyrosine phosphatase-1 (SHP-1), a negative regulator of IRAK activity (O'Connell et al., 2009); suppressor of cytokine signaling 1 (SOCS1); and B-cell lymphoma 6 protein (BCL6), a transcription factor that attenuates NF-κB signaling (Nazari-Jahantigh et al., 2012). A similar action is also exerted by miR-21, which can function as an agonist of single-stranded RNA-binding TLRs and can therefore induce NF-κB activation and secretion of inflammatory molecules (Fabbri et al., 2012).

Although *inflamma-miRs* are co-induced during TLR signaling, several observations suggest that they do not act redundantly and simultaneously, but rather cooperate to control TLR signaling through functionally different performances. At least for miR-146a and miR-155 this has recently been confirmed by Schulte and colleagues, who found different induction behaviors and mRNA target profiles in the response to microbial lipopolysaccaride (LPS), (Schulte et al., 2013). Such coordination of *inflamma-miRs* could go beyond suppression of TLR signaling and contribute to regulating the immunity/inflammation balance, as proposed by Akira's group (Kondo et al., 2012).

Knockout mouse models have been instrumental in shedding light on the role of *inflamma-miRs* in inflammation and inflammation-related diseases. Transcriptome analysis of bic/miR-155-deficient CD4+ T cells in mice identified a wide spectrum of miR-155-regulated genes, including cytokines, chemokines, and transcription factors, suggesting that bic/miR-155 plays a key role in immune system homeostasis and function (Rodriguez et al., 2007). Knockout of miR-146a gene in C57BL/6 mice involves increased transcription of NF-κB-regulated genes (Zhao et al., 2011). These animals also develop myeloid sarcomas and lymphomas as well as chronic myeloproliferation in bone marrow. Genetic ablation of NF-κB p50 suppresses the myeloproliferation, demonstrating that NF-κB dysregulation is responsible for the myeloproliferative disease (Zhao et al., 2011).

Details on the role of *inflamma-miRs* in controlling TLR signaling are just beginning to be explored, and further investigations are warranted to gain insights not only into their individual contribution to the homeostasis of the innate immune response, but also into the consequences of their deregulation in conditions characterized by chronic inflammation.

The most recent evidence for the involvement of *inflamma-miRs* in modulating the proinflammatory response at the cell level is summarized in **Table 1**. Few cellular *inflamma-miRs* have been reported to be modulated in plasma/serum samples in different physiopathological conditions, as depicted in **Table 2**.

RELATIONSHIP BETWEEN TISSUE AND CIRCULATING *Inflamma-miRs*

It is becoming increasingly clear that multiple miRNAs can be deregulated in several diseases (Reid et al., 2011). Detection and identification of stable miRNAs in body fluids has opened a new era in systemic biomarker research directed at improving clinical diagnosis/prognosis within translational medicine. The numerous reports of changes in miRNA expression have often failed to clarify whether they are the cause or the effect of malfunction. Identification of multiple miRNA changes in plasma is important, because specific miRNA combinations unique to a normal physiological or pathological state can provide a useful reference. These findings also raise several questions: do circulating miRNA levels match tissue expression levels? If this is the case, does it also apply to pathological conditions? A greater understanding of the relationship between the level of tissue and plasma miRNAs should help uncover the origin and/or function of circulating miRNAs.

So far only a limited number of studies, mostly in oncology, have addressed the issue. Findings have been controversial: some researchers described a similar trend of alteration both in circulating and tissue miRNAs (Brase et al., 2011), whereas others reported that only a subset of circulating miRNAs reflect tissue cellular abundance (e.g., mammary epithelial tumor), and suggested that cells might have developed a mechanism that selects specific miRNAs for release or retention (Pigati et al., 2010). Studies of animal models found distinct roles for circulating and tissue miRNAs (Waters et al., 2012).

The way circulating miRNAs are delivered into the bloodstream is also poorly understood. Evidence from several studies indicates that miRNAs exist freely in the systemic circulation despite their susceptibility to degradation by extracellular RNAses, raising the issue of which mechanisms underpin their unexpected stability. On the one hand, different studies have revealed that miRNAs are secreted into the extracellular space or the bloodstream either in microvesicles or in exosomes (Valadi et al., 2007; Collino et al., 2010; Hosoda et al., 2011). Secretory exosomes are promising candidates for intercellular miRNAs transfer not only because they provide a protected environment, but also because they may directly transfer internal components to target cells by receptor-mediated interactions. This is consistent with the observation that plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes (Wahlgren et al., 2012). On the other hand, a number of reports have shown that a significant fraction of extracellular miRNAs reside outside vesicles and act in an exosome-independent manner, protected by RNA-binding proteins such as nucleophosmin

Table 1 | Cellular *inflamma-miRs*.

MiRs	Cell types	mRNA targets	Signaling	References
MiR-9	Polymorphonuclear neutrophils and monocytes	NFKB1	TLRs	Bazzoni et al., 2009
MiR-21	Monocytes	TLRs	TLRs	Crone et al., 2012
	Myofibroblasts	PDCD4	TLRs	Yao et al., 2011
	HUVEC	PPARα		Zhou et al., 2011
	Immune cells	TLR-8	TLRs	Fabbri et al., 2012
	Hepatocytes	MyD88	TLRs	Chen et al., 2013
	Hepatocytes	IRAK1	TLRs	Chen et al., 2013
MiR-29a	Immune cells	TLR-8	TLRs	Fabbri et al., 2012
MiR-125a MiR-125b	Diffuse large B-cell lymphoma	TNFAIP3	TLRs	Kim et al., 2012
MiR-126	Endothelial cells	VCAM-1	Vascular inflammation	Harris et al., 2008
MiR-146a	Intestinal epithelial cells	IRAK-1	TLRs	Chassin et al., 2012
	Astrocytes	IRAK-1	TLRsTLRs	Chassin et al., 2012
	HUVECs	IRAK-1	TLRs	Iyer et al., 2012
	HUVECs	IRAK-1	TGF-β1	Olivieri et al., 2012a,b
	Myofibroblasts	SMAD4		Liu et al., 2012
MiR-155	MSCs	TAB2	iNOS	Xu et al., 2013
	Macrophages	BCL6	TLRs	Nazari-Jahantigh et al., 2012
	Macrophages	SOCS1	TLRs	Sun et al., 2012
MiR-195	Hepatocellular carcinoma	IKKα, TAB3	TLRs	Ding et al., 2013
MiR-199a	Endometrial stromal cells	IKKβ	TLRs	Dai et al., 2012
MiR-517a/c	Cell lines	TNIP1	TLRs	Olarerin-George et al., 2013
Let-7	Primary cultured T cells	TLR-4	IL-13 secretion,	Kumar et al., 2011
Let-7i	Human biliary epithelial cells		TLRs	Chen et al., 2007

BCL6, B-cell lymphoma 6 protein; HUVECs, human umbilical vein endothelial cells; IKKα and IKKβ, inhibitor of kappa B (IκB) kinase α and β; iNOS, nitric oxide synthase; IRAK-1, interleukin-1 receptor-associated kinase 1; MSCs, Mesenchymal Stem Cell; NFκB1, nuclear factor κB1; PDCD4, programmed cell death 4; MyD88, myeloid differentiation factor 88; PPARα, peroxisome proliferator-activated receptor-α; SMAD4, SMAD family member 4; SOCS1, suppressor of cytokine signaling 1; TAB2, transforming growth factor-β-activated kinase 1 (TAK1)-binding protein 2; TAB3, TAK1-binding protein 3; TLRs, toll-like receptors; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; TNIP1, TNFAIP3 interacting protein 1; VCAM-1, vascular cell adhesion molecule 1.

1 (NPM1) (Wang et al., 2010) or argonaute protein 2 (Ago2), (Turchinovich et al., 2011). Whether circulating miRNAs are found in soluble free form or are predominantly transported *via* secreted microvesicles/exosomes is still unclear, since evidence has been found for both mechanisms. However, it has recently been reported that the form of delivery of circulating miRNAs could depend on the type of tissue injury, suggesting a different role for each mode of systemic transport. Bala and co-workers documented this mechanism for circulating *inflamma-miRs* using different liver disease mouse models: in inflammatory liver injury and alcoholic liver disease (ALD) serum/plasma miR-122 and miR-155 were predominantly associated with the exosome-rich fraction, whereas in drug (acetaminophen, APAP)-induced liver injury (DILI/APAP) the same miRNAs were found mainly in protein-rich, soluble free form (Bala et al., 2012).

Evidence has also been found that blood cells are the major contributors to circulating miRNAs (Pritchard et al., 2012), However, analysis of *inflamma-miRs* from patients with acute coronary syndrome (ACS) documented that their levels in plasma and peripheral blood mononuclear cells (PBMCs) did not correlate in all subjects (Yao et al., 2011). This suggests that other cells may contribute to circulating *inflamma-miRs*. Endothelial cells could exert such a role, especially during normal aging and in age related-diseases. Indeed human endothelial cells and circulating progenitor endothelial cells can acquire the senescence-associated secretory phenotype (SASP) during replicative senescence *in vitro* (Olivieri et al., 2012b, 2013c). The accumulation of endothelial senescent cells *in vivo*, and stimulation of immune cells over time, could strongly contribute to promoting and maintaining low-level chronic systemic inflammation (Orjalo et al., 2009; Davalos et al., 2010; Campisi et al., 2011).

Table 2 | Circulating *inflamma-miRs* in age-related diseases.

Circulating miRs	Disease	Sample(s)	References
MiR-9	AD	Cerebrospinal fluid	Alexandrov et al., 2012
MiR-21	AMI, CVD	Plasma	Olivieri et al., 2012a,b
	AD	Cerebrospinal fluid	Alexandrov et al., 2012
	Breast cancer	Serum	Mar-Aguilar et al., 2013
MiR-29a	Colorectal liver metastasis	Serum	Wang et al., 2012
MiR-126	T2DM	Plasma	Zampetaki et al., 2010
	Malignant mesothelioma	Plasma	Tomasetti et al., 2012
MiR-146	T2DM	Plasma	Balasubramanyam et al., 2011
	AMI, CVD	Plasma	Olivieri et al., 2012a,b
	AD	Cerebrospinal fluid	Alexandrov et al., 2012
MiR-155	AMI	Plasma	Matsumoto et al., 2012
	Atherosclerosis	Plasma	Wei et al., 2013
	Breast cancer	Serum	Mar-Aguilar et al., 2013
			Liu et al., 2013

AD, Alzheimer Disease; AMI, acute myocardial infarction; CVD, cardiovascular disease; T2DM, type 2 diabetes mellitus.

CIRCULATING *Inflamma-miRs* IN AGING

A progressive increase in circulating acute-phase proteins and proinflammatory mediators, i.e., proteases, cytokines, chemokines, and growth factors, has been described as a general feature of the aging process and has been denominated *inflamm-aging* (Franceschi et al., 2000). Age-associated chronic inflammation has mainly been attributed to progressive activation of immune cells over time and to accumulation of senescent cells with a proinflammatory secretory phenotype (Olivieri et al., 2013b). The complex *inflamma-ging* phenotype is the result of age-related cell/tissue adaptation and remodeling interacting with genetic/epigenetic factors.

Even though TLR family members do not show consistent age-dependent changes across model systems, there is evidence for impaired downstream signaling events during aging, including inhibition of positive effectors and activation of negative modulators of TLR signaling (Olivieri et al., 2013a). Therefore, during aging *inflamma-miR* levels progressively increase in order to stem the cell and tissue damage induced by the low-level chronic inflammation, also likely sustained by the cell senescence secretome (Murray and Smale, 2012).

We have recently described an increased expression of miR-146a in human umbilical vein endothelial cells (HUVECs) and in aortic and coronary endothelial cells (respectively HAECs and HCAECs) during replicative senescence, thus demonstrating that TLR/NF-κB activation and cell senescence can be modulated by the same miRNAs (Olivieri et al., 2012b). MiR-146a is highly expressed also in aged mice (Jiang et al., 2012). In addition, lack of response by aged mouse macrophages to stimulation with LPS and proinflammatory cytokines indicates interruption of the negative feedback loop of miR-146a (Jiang et al., 2012). Altogether, these data lend support to the hypothesis that cellular senescence and TLR signaling activation may be closely interconnected and share common regulators.

Given the involvement of miRNAs in gene expression regulation, a peculiar modulation of their expression might contribute to efficient homeostasis in human aging. It is worth stressing that exceptionally long survival requires dynamic preservation of optimal levels of physiological variables, and that the mean levels of many biomarkers of aging are not stable, but change in the course of life (Spazzafumo et al., 2013). Only four studies have compared the miRNA expression profile of centenarians and younger subjects. They have shown a significant overlap between the miRNA profiles of centenarians and young individuals and a different profile in octogenarians, supporting the hypothesis that achievement of extreme longevity probably requires a special gene expression regulation (ElSharawy et al., 2012; Gombar et al., 2012; Olivieri et al., 2012a; Serna et al., 2012). Interestingly, all these studies showed miR-21 deregulation in centenarians compared with younger subjects. Age-related changes in the expression of miR-21 and miR-21* have recently been reported also in mouse heart, the major changes occurring from middle to old age (Zhang et al., 2012).

Modulation of miR-21 expression in plasma, circulating cells, and tissues of very old subjects and animals is not surprising to those who believe that miR-21 lies at the intersection of senescence, inflammation, and age-related diseases. Our group showed a positive correlations between circulating miR-21 and two important biomarkers of inflammation: C-reactive protein (CRP) and fibrinogen (Olivieri et al., 2012a,b). These data suggest that centenarians may have a better balance of their systemic inflammatory status compared with elderly subjects. Notably, we also found age-related changes in circulating miR-146a levels that were quite similar to those described for miR-21 (Olivieri et al., 2012a,b, our unpublished data). Interestingly, analysis of global miRNA expression in the peripheral blood of adult women has shown that miR-155 is one of the most up-regulated miRNAs among older women (Sredni et al., 2011).

CIRCULATING *Inflamma-miRs* IN AGE-RELATED DISEASES

Abundant data continue to support the hypothesis that progressive up-regulation of inflammatory gene expression and high levels of inflammatory signaling facilitate the development and progression of the major age-related diseases, such as cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), Alzheimer Disease (AD), rheumatoid arthritis (RA), and cancers. Patients suffering from such diseases show subclinical/clinical inflammation and, interestingly, deregulation of most circulating *inflamma-miRs* (Wang et al., 2012). Since multiple co-expressed miRNAs can cooperatively regulate a given biological process by targeting common components of that process, the development and progression of human diseases could

be associated with abnormal regulation of multiple miRNAs functioning cooperatively. Some of the most recent data showing that cellular and circulating *inflamma-miR* deregulation is shared by the major human age-related diseases are summarized below.

Inflamma-miRs AND T2DM

Recently, some of the miRNAs that we have designated *inflamma-miR*s, including miR-21 and miR-146a, were identified as novel players in β-cell failure elicited by proinflammatory cytokines in both *in vitro* and *in vivo* animal models (Roggli et al., 2010). Reduced miR-146a levels were also reported in PBMCs from Asian Indian patients with T2DM, in association with insulin resistance, poor glycaemia control, specific proinflammatory cytokine gene variants, and high levels of plasma TNFα and IL-6 (Balasubramanyam et al., 2011). Importantly, glucose concentrations were also reported to correlate negatively with circulating miR-126 in endothelial apoptotic bodies (Zampetaki et al., 2010); similarly, in patients with diabetes the reduction in miR-126 was seen to be confined to circulating vesicles in plasma (Zampetaki et al., 2010). Interestingly, miR-126 can modulate endothelial cell activation in response to systemic inflammatory stimuli, and can down-regulate the expression of IKBα, an important inhibitor of NF-κB signaling (Asgeirsdóttir et al., 2012; Feng et al., 2012).

Inflamma-miRs AND CVD

In patients with CVD activation of innate immunity leads to an acute inflammatory reaction. There is evidence that *inflamma-miR* may contribute to the development/restraint of this inflammatory response: circulating *inflamma-miRs* could thus be clinically relevant diagnostic/prognostic biomarkers in CVD patients. MiR-21 plays important roles in cardiovascular and pulmonary disorders, including cardiac and pulmonary fibrosis and myocardial infarction, and also regulates various immunological and developmental processes (Kumarswamy et al., 2011).

We recently reported significantly higher circulating miR-21 and miR-146a levels in elderly patients with acute myocardial infarction (AMI) and/or heart failure compared with healthy subjects (Olivieri et al., 2012c). Circulating miR-155 were higher in those post-AMI patients who experienced cardiac death within 1 year (Matsumoto et al., 2012). Elevated miR-155 levels are also found in proinflammatory macrophages and atherosclerotic lesions, even though the effects of miR-155 seem to be different in early vs. advanced atherosclerosis (Wei et al., 2013). Recent studies suggest that miRNA deregulation may limit cardiovascular repair responses and result in an altered function and differentiation of cardiovascular progenitor cells and endothelial progenitor cells (EPCs), modulating endothelial regeneration and cardiomyocyte homeostasis and playing a crucial role in CVD (Jakob and Landmesser, 2012). Aging-associated senescence results in reduced EPC number and function, contributing to enhanced cardiac risk, reduced angiogenic capacity, and impaired cardiac repair effectiveness. Mounting evidence supports a role for miRNAs in vascular homeostasis, and miR-21 was found to regulate EPC senescence (Zhu et al., 2013).

Overall, circulating miR-146a, miR-155, and miR-21 are up-regulated in plasma of CVD patients.

Inflamma-miRs AND AD

Increasing evidence supports a major role for TLRs in brain injury and their involvement in neurodegenerative disorders including AD. Components of this inflammatory pathway are known to contribute to AD, in part through overexpression of IL-1α and promotion of 42-amino acid amyloid β 42 (Aβ42) peptide generation; in turn IL-1α and amyloid β induce transcription of the proinflammatory prostaglandin synthase cyclooxygenase-2 (COX-2) gene and stimulate apoptotic brain cell death and neural tissue degeneration (Mrak and Griffin, 2001). Consistent with these findings many *inflamma-miRs*, including miR-9, miR-146a, and miR-155, are strongly expressed in human cerebrospinal fluid (CSF)- and brain tissue-derived extracellular fluid (ECF) from AD patients and are significantly up-regulated compared with age-matched controls, suggesting that they may be involved in modulation or promotion of miRNA-triggered pathogenic signaling throughout the brain and the CNS (Alexandrov et al., 2012). In AD patients miR-125b, miR-146a, and miR-155 have been shown to down-regulate complement Factor H (CFH), an important repressor of innate immunity acting on the cerebral inflammation response (Lukiw and Alexandrov, 2012; Lukiw et al., 2012a).

MiR-146a is among the more extensively investigated miRNAs in AD: it is up-regulated in response to IL-1, Aβ42 and oxidative stress in cultured human neuronal glial cells (Lukiw et al., 2008; Cui et al., 2010; Holohan et al., 2012; Lukiw and Alexandrov, 2012). In line with these data it is over-expressed in temporal cortices and in Aβ42-stressed human astroglial cells, where it down-regulates IRAK1 while inducing compensatory up-regulation of IRAK2 (Cui et al., 2010).

Interestingly, miR-146a and miR-155 were detected in the secretion of stressed human primary neural cells, and the conditioned medium containing miR-146a and miR-155 was found to induce Alzheimer-type gene expression changes in control brain cells (Lukiw et al., 2012b). These data suggest that paracrine transfer of genetic information between cells—either within the local brain environment or in the cerebrospinal or systemic circulation—may be the source of both beneficial and detrimental signals that further modulate the amyloidogenic, inflammatory, or neurotrophic aspects of the AD process (Lukiw, 2012).

Further confirmation that inflammatory pathways can contribute to AD development comes from the recent identification of a role for triggering receptor expressed on myeloid cells 2 (*TREM2)* in modulating the risk of AD onset (Guerreiro et al., 2013; Jonsson et al., 2013). TREM2 is an innate immune receptor expressed on the cell surface of microglia, macrophages, immature dendritic cells, and white matter in the hippocampus and neocortex, two areas that partially overlap with those affected by AD (Jiang et al., 2013). It controls two signaling pathways, one enhancing phagocytosis and another suppressing inflammatory reactivity. Reduced *TREM2* expression is associated with an increase in microgliosis and neurodegeneration, suggesting that it modulates AD by enhancing inflammation (Jiang et al., 2013).

Although the analysis of miRNAs in AD is a relatively new research area, the data reported so far strongly indicate that *inflamma-miRs* could play a key role also in this condition.

Inflamma-miRs AND RA

Altered miRNA expression has been demonstrated in the inflamed joints of RA patients (Blüml et al., 2011). Interestingly, miR-155 up-regulation has been documented in synovial membrane and synovial fluid (SF) macrophages from RA patients in association with reduced expression of its target, SHIP-1, an inhibitor of inflammation (Kurowska-Stolarska et al., 2011). Moreover miR-155(−/−) mice show significantly reduced local bone destruction, attributed to reduced osteoclast generation, although the severity of the joint inflammation is similar to the one seen in wild-type mice. These data demonstrate that miR-155 is critically involved in the adaptive and innate immune reactions leading to autoimmune arthritis (Blüml et al., 2011). In addition up-regulation of miR-146a, detected in PBMCs from RA patients, suggests that this miRNA could be a useful marker of disease activity (Abou-Zeid et al., 2011; Xie et al., 2013). Interestingly, circulating miR-21 is increased in the blood of patients with systemic lupus erythematosus (SLE) or RA, whereas circulating miR-146a and miR-155 show a trend toward significantly reduced levels only in SLE (Carlsen et al., 2013).

Inflamma-miRs AND CANCERS

MiRNAs are increasingly being recognized as oncogenes or oncosuppressors, since they contribute to cell transformation. Solid tumors are infiltrated by different stromal cells, e.g., fibroblasts, endothelial cells, lymphocytes and macrophages, that strongly influence neoplastic processes through a continuous heterotypic cross-talk (Squadrito et al., 2013). It is therefore not surprising that *inflamma-miRs* have been recognized as modulators of cancer development and progression (Williams et al., 2008). The contribution of miR-146a deregulation has been widely documented (Hurst et al., 2009; Li et al., 2010; Labbaye and Testa, 2012). However, its mechanism of action remains elusive, since both raised and decreased levels have been described depending on the type of cancer (Williams et al., 2008). Since miR-146a acts as a negative feedback loop of inflammation, dynamic changes in its expression can be expected in cancer tissues depending on the context of the heterotypic cross-talk.

Interestingly, mice with miR-146a deletion spontaneously develop subcutaneous flank tumors (Zhao et al., 2011). MiR-146a has been reported to suppress metastatic activity (Hou et al., 2012; Hwang et al., 2012), in particular its up-regulation inhibits cancer cell invasion and metastasis *in vitro* and *in vivo* (Hou et al., 2012). Altogether these findings show that by counteracting the inflammatory state associated with cell senescence, miR-146a can exert a general tumor suppressing action by inhibiting cancer development and cancer cell invasion and metastasis.

MiR-21 is frequently up-regulated in cancer and is implicated in practically every stage of the cancer process: promotion of cell proliferation, invasion, and metastasis; genome instability and mutation; inflammation; replicative immortalization;abnormal metabolism; angiogenesis; evasion of apoptosis; immune destruction and growth suppression. This suggests that miR-21 is an oncogene with a key role in resisting programmed cell death in cancer cells (Buscaglia and Li, 2011). MiR-155 is also an established "*oncomiR*" in breast cancer and regulates several pro-oncogenic pathways including angiogenesis (Czyzyk-Krzeska and Zhang, 2013).

CONCLUSION

Recent data on inflammation-related miRNAs in normal and pathological aging outline a complex scenario characterized by an altered expression of specific miRNAs that we have named *inflamma-miRs*, which mainly target the TLRs/NF-κB pathway. Up-regulation of inflammation markers is a general feature of the aging process and has been named *inflamm-aging*. The *inflamm-aging* phenotype results from age-related cell and tissue adaptation/remodeling interacting with the genetic/epigenetic background. It is a complex phenotype involving not only innate but also adaptive immunity and affecting a range of tissues and organs such as gut, fat, liver, muscle and brain. Importantly, *inflamm-aging* appears to be accelerated in a variety of age-associated diseases. Tissue and circulating *inflamma-miRs* could contribute to restrain the activity of the senescent cell secretome and to check the destruction induced by activation of the inflammatory response (Murray and Smale, 2012; Olivieri et al., 2013a). *Inflamma-miRs* have been implicated in regulation of the immune and inflammatory response, and their abnormal expression may contribute to the low-level chronic inflammation that has been documented both in normal aging and in the major age-related diseases. Circulating *inflamma-miRs* could thus have diagnostic/prognostic relevance in human diseases, e.g., CVD, T2DM, AD, RA, and cancer, which share a common inflammatory background. Recent data show up-regulation of *inflamma-miRs* in the circulation of healthy elderly and old individuals: the increase is less pronounced in centenarians and greater in patients with CVD, AD, or cancer. Notably, T2DM patients show decreased levels of circulating *inflamma-miRs*, but these data need to be confirmed in patients with diabetic complications. It is conceivable that the main sources of circulating *inflamma-miRs* in aging and age-related diseases are immunity circulating/tissue cells and endothelial circulating/resident cells. Cell senescence and inflammatory stimulation can contribute to induce and perpetuate systemic inflammation over time, inducing up-regulation of *inflamma-miRs* to stem the excessive activation of inflammatory pathways.

Since this field of research is new and growing, it would not be surprising if in the near future novel miRNAs were recognized as fine tuners of inflammation and thus added to the list of *inflamma-miRs*.

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