

Extracellular small RNAs: what, where, why?

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Abstract

miRNAs (microRNAs) are a class of small RNA that regulate gene expression by binding to mRNAs and modulating the precise amount of proteins that get expressed in a cell at a given time. This form of gene regulation plays an important role in developmental systems and is critical for the proper function of numerous biological pathways. Although miRNAs exert their functions inside the cell, these and other classes of RNA are found in body fluids in a cell-free form that is resistant to degradation by RNases. A broad range of cell types have also been shown to secrete miRNAs in association with components of the RISC (RNA-induced silencing complex) and/or encapsulation within vesicles, which can be taken up by other cells. In the present paper, we provide an overview of the properties of extracellular miRNAs in relation to their capacity as biomarkers, stability against degradation and mediators of cell-cell communication.

Introduction

Mandel and Métais first described the presence of extracellular nucleic acids in human plasma in 1948 [1]. The concept of extracellular RNA emerged in a different context in the 1970s, when Kolodny et al. [2,3] showed that RNA is transferred between fibroblast cells *in vitro* and Stroun et al. [4] demonstrated that highly methylated RNA is secreted by diverse cell types through a process not associated with cell death. In parallel, a range of reports in the 1960s suggested that RNA from one tissue (e.g. liver) could induce tissue-specific expression in other cell types [5], although the mechanisms surrounding this phenomenon were never described. The recent discovery of RNA encapsulation within extracellular vesicles [6] is consistent with some of these earlier studies and provides a framework for conceptualizing RNA transport in mammals. At present, however, there is little connection between secretion and uptake of RNA observed *in vitro* and the meaning of RNA in systemic circulation. In the present paper, we highlight some of the key issues surrounding the biological and medical meaning of extracellular miRNA (microRNA).

miRNA classification

Several classes of small RNA have been identified in animals, fungi and plants which play diverse roles in gene regulation and genome defence (reviewed in [7]). The defining features of a small RNA include its origin and interaction partners. In the present paper, we focus on miRNAs, which are derived from stem-loop structures located within the introns or exons of coding genes or transcribed from 'intergenic' regions of the

genome. In animals, the stem-loop structures are processed by Drosha in the nucleus, followed by Dicer in the cytoplasm, resulting in a ~22 nt duplex RNA (reviewed in [8]). One strand of this duplex is incorporated into RISC (RNA-induced silencing complex), which binds to mRNAs at specific sites with base-pair complementarity to the miRNA; generally these sites are located in the 3'-UTR (untranslated region) of the mRNA [9]. The interactions of the miRNA and mRNA within RISC leads to destabilization of the mRNAs and/or inhibition of translation [9]. Currently, 1921 mature human miRNAs have been annotated in miRBase version 18 [10], each of which is predicted to target hundreds of mRNAs [9]. Given the vast scope for combinatorial regulation of targets, it is difficult to find a cellular pathway not regulated at some level by a miRNA. Indeed, the majority of protein-coding genes contain miRNA-binding sites under selective pressure [11] and misexpression of miRNAs is associated with many disease processes, encompassing all cancers, as well as metabolic, cardiovascular, neuronal and immune-related diseases [12].

miRNAs as extracellular biomarkers

The first evidence that miRNAs exist outside cells was reported by Valadi et al. [6] in 2007, who showed that exosomes secreted by mast cell lines contain both mRNA and miRNA. In parallel, several reports in 2008 demonstrated that miRNAs are present in a cell-free form in human and mouse serum [13–16]. Given the numerous associations between miRNAs and disease, their presence in blood has sparked enormous interest in using them as non-invasive biomarkers [17]. However, the actual composition of extracellular miRNAs in blood is likely to derive from a variety of cell types and factors dictating secretion of RNA are not yet known (discussed further below). Tissue injury appears to be one pathological state that leads to differential expression of specific miRNAs in blood. Wang

Key words: extracellular small RNA, microRNA (miRNA), RNA, RNA-induced silencing complex (RISC).

Abbreviations used: Ago2, Argonaute 2; AMI, acute myocardial infarction; HDL, high-density lipoprotein; HMEC-1, human microvascular endothelial cell 1; miRNA, microRNA; MVB, multivesicular body; NPM1, nucleophosmin; nSMase2, neutral sphingomyelinase 2; RISC, RNA-induced silencing complex; snRNA, small nuclear RNA; UTR, untranslated region.

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et al. [18] demonstrated that the liver-specific miRNA *miR-122* is elevated ~500-fold in mouse plasma following liver injury by acetaminophen overdose. Others have reported increases in *miR-122* levels in human serum following liver damage induced by acetaminophen [19] or hepatitis B infection [20]. Differential expression of extracellular miRNAs is also associated with AMI (acute myocardial infarction). Cheng et al. [21] reported a transient 200-fold increase in the level of *miR-1* in rat serum at 6 h after AMI and a similar increase in human serum taken within 24 h of AMI. Whether *miR-122* and *miR-1* are released during cell death and/or there is specificity in the secretion of these miRNAs is unknown. Both of these miRNAs are highly abundant and tissue-specific [22], which might be essential criteria for any good miRNA biomarker, since many cell types can secrete miRNAs into circulation [23].

Beyond tissue injury, miRNA changes in serum or plasma are also associated with different cancers (reviewed in [24]). Mitchell et al. [14] used a mouse xenograph model with human prostate cancer cells expressing human-specific miRNAs (*miR-629** and *miR-660*) to demonstrate that tumour-derived miRNAs enter the circulation and are detectable in plasma [14]. They also observed a 46-fold elevation in *miR-141* in the serum of patients with prostate cancer and reported 60% sensitivity and 100% specificity in detecting individuals with cancer. Ironically, *miR-141* in human plasma was first reported in association with pregnancy; this miRNA is enriched in the placenta, increases in maternal plasma with gestational age and falls off significantly 24 h after delivery [16]. Clearly, prostate cancer and pregnancy would not be examined in the same individual, but these examples highlight the complexity of using circulating miRNAs as biomarkers. Further understanding of when, where and how miRNAs are released or secreted by different cell types will guide investigations into their capacity as biomarkers.

Who else is out there?

Analyses of small RNA content in bodily fluids has been largely directed toward miRNAs, given their tissue-specific expression, the precedence for their differential expression in disease, and existing commercial interest [25]. However, other classes of small RNA have also been detected in the extracellular environment: the 3'-end fragments of rRNAs and tRNAs are detectable in human milk [26] and U6 snRNA (small nuclear RNA) is up-regulated in the sera of cancer patients [27]. It is therefore possible that various small RNAs exist in an extracellular form that may be of diagnostic or biological interest. Interestingly, studies in the 1970s suggested that much of the RNAs secreted by cells were small (sedimenting between 2.5 and 4 S) and highly methylated [4]. It is possible that miRNAs are not the only, or even the most interesting, player in the extracellular space. Existing sequence datasets from human serum and plasma suggest that miRNAs dominate the small RNA fraction (40–96% of reads), but in some cases, a significant proportion of

reads map to rRNA (3–56%) or tRNA (11–51%), with <1% mapping to snoRNA (small nucleolar RNA) and snRNA [14,15,28,29]. However, in most publications, only those reads annotated as miRNAs are reported, and it should be noted that rigorous controls/standards for contamination of cellular RNA have not yet been defined. On the other hand, the extracellular environment is known to be full of RNases, and a key question in interpreting the meaning of any extracellular RNA is how and why it is stabilized.

The extracellular environment

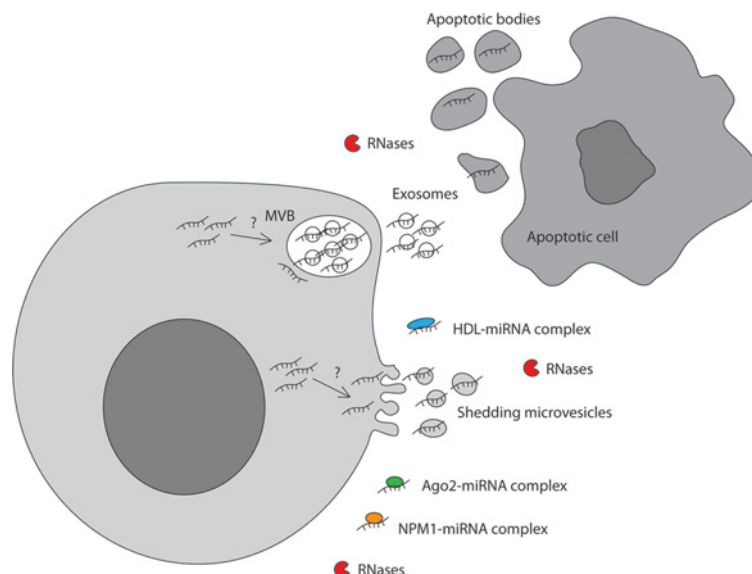
miRNAs have been found in various body fluids, including serum, plasma, saliva, tears, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid and seminal fluid [30]. Yet, highly stable RNases are an abundant component of these fluids; the concentrations of RNases in human serum or plasma are estimated at several hundred nanograms/ml, and can be elevated in cancer patients [31,32]. Consistent with this, Tsui et al. [33] showed that synthetic RNA is degraded in less than 5 s when incubated with human plasma, yet they found that mRNAs present in serum and plasma are stable for many hours. Further studies (before analysis of miRNAs) have confirmed this apparent RNA stability: mRNA in plasma is not degraded by RNase-A/T, RNase H or DNase [34], arguing against the possibility that it is stabilized by association with DNA [4]. A range of reports have shown similar results with miRNAs, and researchers have gone on to test the extremes of miRNA stability in serum and plasma using long periods at room temperature, freeze–thawing, exposure to boiling and different pHs [14,15,35]. These and other investigations suggest that a large component of the RNA found in fluids is extremely stable. On the other hand, pre-treatment of serum or plasma with detergents (e.g. Triton X or SDS) makes mRNAs susceptible to degradation by RNases [33,34], and miRNAs secreted from THP-1 cells similarly lose protection following treatment with detergents [36]. As postulated by many researchers since the 1970s, it appears that at least one mechanism for the extracellular stability of RNAs is the natural encapsulation of these molecules in vesicles [37].

Stability and function of extracellular RNA

Vesicles are secreted by many (if not all) cell types and have been detected in body fluids through density sedimentation, electron microscopy and analysis of specific markers on their surfaces. Owing to a lack of precise nomenclature, many terms are used to describe extracellular vesicles, including microparticles, microvesicles, exosomes and membrane particles; in the present paper, we refer to exosomes, apoptotic bodies and shedding microvesicles as defined in a recent review [38]. The term exosome defines vesicles ~40–100 nm in diameter, of endocytic origin, derived from MVBs (multivesicular bodies) that fuse with the plasma membrane [39]. Shedding microvesicles are ~100–1000 nm

Figure 1 | Diverse origins of stabilized extracellular miRNA

Extracellular miRNAs are protected from degradation by RNases through encapsulation within exosomes, shedding microvesicles and apoptotic cells. They have also been identified in lower-molecular-mass complexes bound to Ago2, NPM1 or HDL.



in diameter and derive directly from cell membranes by budding. Apoptotic bodies are ~50–5000 nm in diameter and are membranous vesicles shed from the plasma membranes of dying/apoptotic cells via blebbing. A description of the biogenesis and distinguishing features of these vesicles is summarized in [38]. Of interest is the fact that RNA has been identified in vesicles (cartoon in Figure 1) and specific small RNAs are enriched in vesicles in relation to the pool of intracellular small RNA [6,40]. Furthermore, various reports suggest that vesicular miRNAs are a medium for cell–cell communication. For example, *miR-150* is selectively packaged into exosomes secreted by THP-1 cells (a human acute monocytic leukaemia cell line), which are taken up by HMEC-1 cells (a human microvascular endothelial cell line). Increased concentrations of *miR-150* in HMEC-1 cells leads to down-regulation of *miR-150* targets, including the transcription factor c-Myb, resulting in enhanced cell migration [36]. Functional transfer of RNA between cells also occurs through apoptotic bodies: *miR-126* is the most abundant miRNA in endothelium-derived apoptotic bodies generated during atherosclerosis and can be transferred and taken up by vascular cells [41]. Using a variety of controls, including apoptotic bodies from cells derived from *miR-126*^{-/-} mice, Zernecke et al. [41] demonstrated that the uptake of *miR-126* is responsible for the down-regulation of its target, RGS16, an inhibitor of G-protein-coupled receptors. Regulation of RGS16 by *miR-126* leads to an increase in CXCL12 (CXC chemokine ligand 12), which causes mobilization of progenitor cells and incorporation into plaques, conferring protective effects in diet-induced atherosclerosis [41].

Before it was known that RNA was contained within secreted vesicles, numerous studies have demonstrated important functions of exosomes in cell–cell communication, in particular in neuronal and immune signalling (reviewed in [42]). On the basis of the work cited in the present paper, it seems likely that RNAs within these vesicles could be involved directly in altering the functional properties of recipient cells. However, the actual mechanisms governing specificity, in terms of which RNAs are selectively packaged into vesicles, remain unknown. Given the short length of a mature miRNA, there are limited *cis*-acting elements within the sequence to dictate specificity. However, it is possible that miRNA–interaction partners could somehow be involved in localization of miRNAs for export. Gibbins et al. [43] demonstrated that exosomes secreted from monocytes contain components of the RISC machinery, including Ago2 (Argonaute 2) and GW182, which co-migrate with endosomal–MVB fractions in density gradients [43]. miRNA–RISC silencing activity is also enhanced when MVB turnover is impaired and MVBs positively regulate RISC loading [44]. However, to date, there is no experimental support for a mechanism whereby specific miRNAs are loaded into MVBs for secretion; neither is it known how specificity is achieved in apoptotic bodies and shedding microvesicles.

Non-vesicular extracellular miRNA

The studies cited above support the existence of miRNA-containing vesicles, but this may not be the most prevalent form of extracellular miRNA. Several reports suggest that

a substantial fraction of extracellular miRNAs are not encapsulated within vesicles, but are protected against RNase digestion by association with proteins. Wang et al. [45] showed that miRNAs secreted by A549 and HepG2 cells associate with RNA-binding protein nucleophosmin (NPM1), a multifunctional histone-binding protein. On the basis of size-exclusion chromatography, Arroyo et al. [46] reported that the majority of plasma miRNAs (~90%) are present as Ago2-miRNA complexes not contained within vesicles. They showed further that these miRNAs become susceptible to RNase digestion following proteinase K treatment of plasma [46]. Similarly, Turchinovich et al. [47] showed that the majority of miRNAs found in human plasma, as well as those secreted from the MCF7 breast cancer cell line, are associated with Ago2, but exist in a form that is <300 kDa. The authors suggested that the high proportion of Ago-miRNA may represent by-products of dead cells, since Ago-miRNA complexes are known to be extremely stable within cells [47]. However, Ago2 is also present within exosomes [36,43], and it may be that some of the Ago2-RNA complexes identified derive from vesicles, potentially damaged during purification. Another study has shown that specific miRNAs are complexed to HDL (high-density lipoprotein) in serum, and this also protects miRNAs from degradation and mediates transport into recipient cells [48]. The mechanisms controlling which export/import pathways are active in a cell are unknown. However, exosome release is blocked by inhibiting nSMase2 (neutral sphingomyelinase 2), an enzyme involved in ceramide biosynthesis [49]. Interestingly, inhibition of nSMase2 actually increases the export of miRNAs by HDL [48], suggesting distinct mechanisms and/or competition in the export pathways. Further research is required to understand the mechanisms dictating specificity in the secretion and uptake pathways. Nonetheless, the capacity to mimic and exploit these natural RNA-transport vehicles, whether they be vesicles or protein co-factors, has exciting implications for therapeutic RNA delivery [50].

The extracellular communicator hypothesis revisited

In a hypothesis paper in 1988, entitled "Extracellular 'communicator RNA'", Steven Benner proposed that RNA could be involved in cell-cell communication as a short distance messenger [51]. He based this on the fact that extracellular fluid is full of proteins with RNase activity, as well as RNase inhibitors, and the balance between these molecules is associated with disease conditions including cancer and angiogenesis. We are now aware of even more secretory RNase-like proteins with functions in innate and acquired immunity [52]. One might expect, as Benner proposed [51], that it is the substrates of extracellular RNases, i.e. extracellular RNA, that are important. However, all of the examples cited in the present paper suggest that extracellular RNA is protected from RNases through association with

proteins and/or encapsulation within vesicles. It is therefore possible that we have just scratched the surface in understanding the functions of short- or long-lived extracellular RNAs, and the meaning of the numerous RNase-like proteins that have evolved in different species [53].

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