Published in final edited form as:

Annu Rev Nutr. 2016 July 17; 36: 301–336. doi:10.1146/annurev-nutr-071715-050711.

Sources and functions of extracellular small RNAs in human circulation

Joëlle V. Fritz^{1,†}, Anna Heintz-Buschart¹, Anubrata Ghosal², Linda Wampach¹, Alton Etheridge³, David Galas^{1,3}, and Paul Wilmes^{1,†}

¹Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Campus Belval, 7, avenue des Hauts-Fourneaux, Esch-sur-Alzette, L-4362 Luxembourg

²Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

³Pacific Northwest Diabetes Research Institute, Seattle, Washington, 98122, USA

Abstract

Various biotypes of endogenous small RNAs (sRNAs) have been detected in human circulation including microRNAs, tRNA, rRNA and yRNA fragments. These extracellular sRNAs (ex-sRNAs) are packaged and secreted by many different cell types. Ex-sRNAs exhibit differences in abundance in several disease contexts and have therefore been proposed as well-suited biomarkers. Furthermore, exosome-borne ex-sRNAs have been reported to elicit physiological responses in receiving cells. Albeit controversial, exogenous ex-sRNAs derived from plants and microorganisms have also been described in human blood. Essential questions which remain to be conclusively addressed in the field concern the (i) presence and mechanistic sources of exogenous ex-sRNA in human bodily fluids, (ii) detection and measurement of ex-sRNA in human circulation, (iii) selectivity of ex-sRNA export and import, (iv) sensitivity and specificity of ex-sRNA delivery to cellular targets, and (v) cell-, tissue-, organ- and organism-wide impacts of ex-sRNAs. We will survey the present state of knowledge of most of these questions in this review.

Keywords

blood; vesicle; gene regulation; microRNA; microorganisms; plant

1. Introduction

In addition to the well-established roles that RNA plays in the coding, decoding, regulation and expression of genes intracellularly, it has recently become apparent that mammalian cells also release RNA into their extracellular milieu, a process which suggests that RNA moieties expressed by cells might carry functions which transcend the confines of single cells (195).

[†]Corresponding authors: Joëlle V. Fritz and Paul Wilmes, Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Campus Belval, 7 avenue des Hauts-Fourneaux, Esch-sur-Alzette L-4362 Luxembourg, joelle.fritz@uni.lu and paul.wilmes@uni.lu.

Distinct biotypes of extracellular RNA have been described in many human bodily fluids (147, 205). In blood for instance non-coding RNAs (ncRNAs), including small regulatory RNAs (sRNAs) (12, 21, 50, 52, 63, 102, 111, 125, 139, 188, 196), non-coding RNAs (86, 199) as well as long coding RNAs (155, 179, 195) (Table 1), have been identified. sRNA are typically in the size range of between 15–200 nucleotides and comprise multiple different RNA biotypes including microRNAs (miRNAs) and others (Table 1). Initially regarded as “junk” RNA (144), ncRNAs have more recently been found to represent a very large fraction of the transcribed eukaryotic genome, and these RNAs play central roles in regulating gene expression at multiple levels, including a wide range of known mechanisms, and certainly many more yet unknown (63, 226). While long ncRNAs (lncRNA) are known for their intracellular function, we have only limited knowledge of their extracellular function (170, 226). Although this review focuses on the wide spectrum of extracellular small RNAs (ex-sRNAs), it is nevertheless noteworthy that extracellular mRNAs (ex-mRNAs) were the first circulating RNAs found to be functionally active following exosomal transfer from a distant donor cell to a target cell (195).

The most widely studied ex-sRNAs in mammals are miRNAs. They are known to play key functions in regulating gene expression intracellularly and specific miRNAs are known to be dysregulated in certain diseases. Because these dysregulated sRNAs can be reflected in the overall ex-sRNA complement (7, 152), they have been proposed as potentially important biomarkers for a panoply of different diseases (102, 111, 126, 152, 210). Apart from miRNAs, it has only recently become clear that other ex-sRNAs, such as tRNA halves (tRHs), tRNA fragments (tRFs), rRNA fragments (rRFs), yRNA fragments (yRFs), piwi-interacting RNAs (piRNAs) and circular RNAs (circRNA) may also be suitable biomarkers for human diseases (16, 51, 52, 175, 196) (Table 1). Moreover, these ex-sRNA types, distinct from ex-miRNAs, are highly abundant in human circulation (3, 40, 50–52, 111, 126) (Table 1 and D. Yusuf, A. Heintz-Buschart, B.B. Upadhyay, J.V. Fritz, A. Ghosal, M. Desai, J.M. Dhahbi, P. May, D. Huang, E. Muller, P. Shah, H. Roume, C. De Beaufort, J. Schneider, A. Hogan, K. Wang, D. Galas and P. Wilmes, unpublished observation) where they appear to be significantly enriched relative to their intracellular counterparts (139, 188). Although the field of ex-sRNA is still in its infancy, it has already been demonstrated that different sRNA families exhibit distinct characteristics in the extracellular space including specific processed sequences and abundance patterns (139, 188, 205). Taken together, these characteristics raise important questions about the function of ex-sRNAs in relation to their potential cellular targets and their role in intercellular communication.

RNA export appears to be a generally well-conserved process in biology as it has been described in nematodes (91), plants (28), bacteria (23, 68), fungi (149) as well as mammals (195). Intriguingly, a number of recent studies suggest the presence of exogenous ex-sRNAs derived from the diet and/or microbiome in human plasma and serum (21, 117, 205). These findings, albeit controversial (53, 180, 187, 216, 220), suggest the possibility of ex-sRNA-mediated trans-kingdom regulation of gene expression (223, 231). Analogous to endogenous ex-sRNAs, the dominant exogenous ex-sRNA biotypes within human circulation are families of rRFs and tRFs, whereas exogenous yRFs are found at lower levels (D. Yusuf, A. Heintz-Buschart, B.B. Upadhyay, J.V. Fritz, A. Ghosal, M. Desai, J.M. Dhahbi, P. May, D.

Huang, E. Muller, P. Shah, H. Roume, C. De Beaufort, J. Schneider, A. Hogan, K. Wang, D. Galas and P. Wilmes, unpublished observation).

Independent of their origin, endogenous or exogenous ex-sRNA within human circulation are relatively stable and are protected from the action of extracellular RNases. It is generally accepted that ex-sRNA are either present in extracellular vesicles (EVs) (47, 139, 188, 195, 230), and/or in protein-complexed forms (15, 44, 194, 199, 204, 209). Even though there are different possible mechanisms for protection, how and which sRNA are selected for export remains somewhat unknown. Similarly, the potential import of ex-sRNA into target cells remains poorly understood.

It is now generally accepted that ex-sRNAs are specifically processed and that they are present in stable forms within their extracellular milieus. However, the main question that remains to be addressed is whether these ex-sRNAs are present at physiologically relevant concentrations (which is a poorly defined concept) and if specific delivery mechanisms exist for them to reach and affect cellular targets. Even though numerous studies suggest functional roles for ex-sRNAs in human health and disease, only a few studies show that ex-sRNAs are functionally active in distant target cells in vivo. Direct intercellular sRNA-mediated communication has been demonstrated for specific miRNAs (74). However, a major question in relation to ex-sRNA now focused on whether such exchanges also occur over longer distances and whether human circulation is involved? The ranges of known functions of different ex-sRNAs families identified in human circulation are described in this review, as well as our current understanding of endogenous and exogenous ex-sRNA compositions, means of transfer and their potentials as biomarkers for disease diagnosis and prognosis. Finally, the review concludes with two sections on current challenges and perspectives.

2. Origins and compositions of ex-sRNAs complements in human circulation

2.1 Cellular origins of endogenous ex-sRNAs

The identification of ex-sRNA in many body fluids has been the subject of many investigations over the past few years (33, 147, 205). Several techniques have been used for the characterization of extracellular RNA repertoires including RNA-seq for broader coverage of extant RNA and RT-qPCR for known or suspected sequences. There are a number of challenges associated with the identification of circulating RNAs which render the comprehensive description of sRNA complements challenging (see also Section 5 of this review). While there is a wide range of RNA biotypes that have been identified in human circulation (Table 1), many of these types have incomplete or mistaken annotations compounding the challenges of identifying the sources (200). While it is apparent that most of the ex-sRNAs in human circulation are derived from human cells, it remains largely unclear which specific cells, tissues and organs contribute to these RNAs. Knowledge of the origins of ex-sRNAs, is however particularly pertinent in the context of disease states where knowledge about sources of specific ex-sRNAs would suggest mechanistic studies. Such

knowledge would also inform how relevant particular sRNAs might be for the development of disease biomarkers.

So far, three overall processes have been described that result in sRNA being shed into the extracellular milieu by their cells of origin. These include (i) cell lysis and apoptosis, (ii) export into exosomes and microvesicles, and (iii) a pathway that has been inferred but not yet mechanistically described that involves cellular export of RNA bound to specific ribonucleoproteins (15, 194, 199, 204, 209) (see also Section 3 of this review and Figure 1). Although the propensity of different cell types to use distinct export mechanisms is highly likely, such differences remain yet to be described. Furthermore, only a few cases have been reported so far for which the origins of endogenous ex-sRNA appear reasonably clear. For example, the release of miR-122 during acetaminophen toxicity, is almost certainly driven by liver cells, and likely due, in part at least, to the lysis of liver cells as concurrent cell necrosis is observed in the liver (208). There are, however, only a few such examples of tissue-specific RNAs where the source cells can be traced to specific tissues (210). One additional relatively large class of circulating RNAs which are released in the context of disease, involves those secreted by cancer cells. However, sRNA export has been extensively studied in the context of the development of potential cancer diagnostics and, thus, will not be reviewed here (169) (see also section 4.3. of this review).

2.2 Composition of endogenous ex-sRNAs

The diversity of intra- and extra-cellular RNA reported in the literature is large, and has grown dramatically in recent years as more investigations are performed. A wide range of distinct sRNAs as well as sRNA fragments derived from long RNA species have been observed (Table 1). From RNA-seq data, fragment sRNAs exhibit consistent alignment patterns supported by high numbers of reads which suggest sRNA-specific cleavage events. One major class, the tRNA-derived sRNAs, consists of at least four subtypes: tRNA-derived sRNA fragments (tRFs) and tRNA-derived halves (tRHs), which represent two distinct subclasses that probably have distinct biological functions (Table 1) (67). While the molecular functions of these tRFs and tRHs are only emerging, they are clearly generated in response to cell stress and the enzymes responsible for their cleavage have been elucidated (67). More specifically, tRHs are generated by angiogenin-mediated cleavage near the anticodon loop in response to starvation, oxidative stress, or other forms of cellular stress (77, 83). One subclass, the 3'CCA tRFs, has been reported to function like miRNAs in silencing specific targets (77, 83). tRHs have also been shown to bind to eukaryotic translation initiation factor 4 γ (eIF4G) through a distinct sequence motif on the tRH 5'-terminal end and titrate eIF4G away from the pre-initiation complex (181). Most importantly, tRHs are highly abundant in blood plasma, where they are found associated with protein complexes, but are not found in exosomes (52). There are many other longer RNAs, such as lncRNAs and others that can be cleaved to produce smaller RNA fragments that can appear in circulation (116, 207). Some of these are listed in Table 1, but the list is growing rapidly and requires regular updating. The functions of the fragments from most of these are unknown.

It is perhaps surprising that all body fluids examined to date contain some levels of ex-sRNA (212), but their inclusion into vesicles (including exosomes) appears distinct in different fluids (see also section 5.4 of this review) (6, 16, 31, 172). Since the pathways of export from cells play an integral part in shaping these differences, this suggests that exporting cells in different tissues/organs (e.g. renal cells for urine, salivary cells for saliva etc.) most likely use these pathways to different extents or that cell type-specific sorting and packaging of sRNA exists. Intricate intracellular sorting and packaging mechanisms are the most likely alternative and the body fluid-specific sRNA proportions are probably due to different cell populations using sRNA export mechanisms to different extents. Since the blood is exposed to a wide variety of cell-types, it is difficult to identify the contribution of one specific cell type, tissue or organ. Another caution in inferring the cellular origins of ex-sRNA come from the fact that cell stress, and responses to a variety of stimuli are known to alter cell export (49, 182). Thus, stress, exercise, external stimuli, and nutrition can affect not only the specific plasma ex-sRNA species, but also the proportions of plasma ex-sRNA in vesicles (161). The situation in relation to ex-sRNA is even more complex, as evidence has accumulated that human cells are not the only source of ex-sRNA in the blood.

2.3 Exogenous origins of ex-sRNAs

exRNA from a panoply of different organisms including other animals (18), plants (21, 117, 129, 172, 205, 232), microorganisms (21, 117, 172, 205) and nematodes (32) has been reported in mammalian circulation (Figure 1). The first published report of plant miRNAs in human blood came from Chen-Yu Zhang's group in 2012 (231). While the report was met with skepticism (53, 180, 216, 219), subsequent studies also reported small amounts of plant RNA in blood (21, 205, 236). A careful study by Hirschi et al. (223) recently confirmed the presence of plant RNA in mouse serum and urine. They identified at least two different environmental factors, including diet and the cancer drug cisplatin, which can lead to the clear detection of diet-derived miRNAs in mice—specifically plant miRNAs. It is clear that there are discrepancies among the identities and levels of exogenous ex-sRNAs reported in the literature (234) and a wide range of incommensurate experimental designs (219). Nonetheless, it now seems clear that diet-derived RNA can indeed be detected in mammalian internal body fluids like plasma, and that more carefully designed, standardized and executed studies are needed to elucidate the processes of transfer and factors influencing them. Less controversial, but still novel and complex to analyze, are the reports of microbial and parasitic RNA in mammalian blood and tissues (21, 32, 124, 135, 148, 205) (Figure 1).

In our work of a few years ago we reported the presence of RNA molecules that matched exogenous species, particularly those of microorganisms constituting the gastrointestinal microbiome, in human circulation (205). Subsequent studies in mice confirmed these prior observations in human plasma (207). The most abundant exogenous sRNAs could be mapped to the bacterial phyla Proteobacteria and Firmicutes, and the Ascomycota was the predominant fungal phylum in the plasma samples analyzed. Because many of the sRNA sequences are conserved among closely related species, it is difficult to determine precisely the source of these sRNAs, at the level of bacterial and fungal species. Since mice used in experiments were kept in a carefully controlled, pathogen-free environment, these exogenous miRNAs were most likely introduced through their microbiomes and through

food intake (207). These and other observations indicate a wide range of potential sources of ex-sRNAs, but so far to our knowledge no microbial ex-sRNA has been implicated in a cellular function in mammalian host cells. There are other cases, however, where the functional impact of non-host ex-sRNA is clearly indicated—specifically among several parasitic species (32).

Several parasites can apparently secrete sRNAs into their hosts during infection (97) and RNA from protozoa, *Trypanosomas cruzi* (66), *Schistosoma japonicum* (43) and the nematode *Litomosoides sigmodontis* (153) have been found in the body fluids of infected individuals, indicating that circulating sRNAs from parasites in host systems may be common. The helminth nematode *Heligmosomoides polygyrus* utilizes exosomal vesicles to increase virulence in a fashion similar to that of the mammalian miRNA transport mechanism, since its miRNA-loaded vesicles are accompanied by a nematode AGO protein, mimicking the mammalian system (32). In another striking parallel, *H. polygyrus* vesicles are internalized by mice cells, resulting in a suppression of host immunity (32). Some of these *H. polygyrus* miRNAs can be shown in vitro to target host mRNAs that are related to host immunity (32). These parasite vesicles seem to resemble their mammalian exosomal miRNA transport counterparts in function. Apart from ex-sRNA secreted from parasites, the discovery of ex-sRNA from various commensal and mutualistic species constituting the human microbiome, including bacteria, archaea and fungi (21, 205) within human blood circulation (Figure 1), along with the immunostimulatory effects of exogenous extracellular sRNA (1, 141), suggest that microbial ex-sRNAs may play a much wider role in immune system regulation. In this context, it is noteworthy that the model enteric bacterium *Escherichia coli* has been recently found to secrete specific sRNAs into its extracellular milieu both through secretion of outer membrane vesicles (OMVs) and other so far unknown secretory mechanisms (68).

It is thus clear that there are both wide ranges of RNA species as well as sources of the RNA found in human circulation. We are clearly at the beginning of a new set of discoveries related to these RNAs which will result in understanding much more about their appearance, their impact, and their overall molecular, cellular and systemic functions.

3. Transfer of ex-sRNAs into human circulation

3.1. Transfer of endogenous ex-sRNA via extracellular vesicles

Extracellular vesicle (EV) is a generic term for all cell-secreted vesicles found in bodily fluids, including exosomes, microvesicles and apoptotic bodies. All human extracellular vesicles have so far been found to contain sRNA and/or to have sRNA associated with them, e.g. through their binding of ribonucleoprotein complexes. However, the varieties of extracellular vesicles are distinct in their origin, composition and possible functional roles.

Exosomes originate from endosomes and are released from cells when multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) fuse with the plasma membrane (Figure 1). They have a reported diameter from 30 to 100nm (86, 197). Microvesicles, also named shedding vesicles or ectosomes, are larger in diameter (0.1–1µm) than exosomes and are released from cells through blebbing (budding out) and fission of the plasma membrane (86,

186) (Figure 1). Cells undergoing apoptosis, release apoptotic bodies which are phosphatidylserine-exposing vesicles with a diameter of 0.5–2µm (86, 197) (Figure 1).

3.1.1. Sorting of sRNA into EVs—Among the different biotypes of endogenous ex-sRNA, miRNA has gained most attention and, given a general lack of knowledge about most other sRNAs, this section describes mainly the sorting and packaging of miRNAs. miRNA profiles in exosomes can differ from their parent cell (195) and the proportion of some specific miRNAs is higher in exosomes compared to their parent cells. Therefore, it has been hypothesized that a selective sorting mechanism for miRNA packaging into EVs must exist. Indeed, a specific exosome-sorting RNA motif (GGAG), named EXOmotif, has been described (202). This motif is located in the 3' half of some miRNAs and it controls their loading into exosomes. The heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1) appears to recognize this EXOmotif and this interaction is essential for the loading of EXOmotif-containing miRNAs into exosomes (201, 202). However, not all miRNA which are sorted into exosomes present this specific motif and, thus, additional mechanisms for the targeted loading of miRNAs into exosomes must exist. Another possibility which may explain some of the specific sorting of miRNAs into EVs is based on the fact that miRNAs can be posttranscriptionally modified. Indeed, 3'-uridylated miRNA isoforms are enriched within exosomes compared to their intracellular levels whereas 3'-adenylated miRNAs are relatively depleted (100).

It seems that not only the sequence characteristics or possible posttranslation modifications of the RNA are important for miRNA-sorting into exosomes, but also their subcellular localisation. Gibbings et al. proposed that miRNAs-loaded argonaute-2 (AGO2) are sorted into exosomes via the GW182 protein when these ribonucleoprotein complexes are located in so called GW-bodies (69). These bodies are located in areas of the cytoplasm where endosomes, MVBs, miRNAs and miRNA-repressible mRNA accumulate (85). GW-bodies also contain GW182 and AGO2, proteins of the RISC complex which have also been identified in exosomes (69). Interestingly Yao et al. reported that a knock-down of GW182 decreases the miRNA release via secretory exosomes (225). Furthermore, Guduric-Fuchs et al. showed that a knock-out of AGO2 also reduces the incorporation of miRNAs into exosomes (72, 225). Whether this phenomenon is due to instability of the miRNAs, when unbound to AGO2 and/or GW182, or to the possibility that the miRNAs do not reach GW-bodies anymore, still needs to be elucidated.

Specific lipids appear to be another important component governing the sorting of miRNA into exosomes, as it has been shown that exosomal miRNAs are released through ceramide-dependent secretory pathway and that the whole process is partially controlled by the ceramide biosynthesis enzyme nSMase2 (104, 127, 193). It is interesting to note, that lipid raft-like regions contain ceramide (45) and that differential affinities of RNAs to such structures have been identified (87). Moreover, posttranslational changes, such as hydrophobic modifications (e.g. Methylation by NSun2 of miR-125b) also increase the lipophilicity of sRNAs (86, 229). Therefore, the aforementioned findings have recently been summarized in a model whereby lipophilic sRNA molecules, carrying an EXOmotif are transferred via an RNA binding protein (e.g. hnRNPA2B1) towards raft-like regions in the

MVB limiting membrane (could be inside GW-bodies) where the sRNAs are then internalized via ILVs (86).

Apart from the specific packaging and export of sRNAs, at least a fraction of the circulating miRNA complement in bodily fluids has been found to represent mere exported byproducts of cellular activity and cell death (182, 188, 192, 193) (Figure 1). Indeed, it has been shown that IL-4 activation of macrophages does influence the miRNA biotypes, as well as their quantity, loaded into exosomes (182).

Intracellular concentrations of miRNAs do also influence their sorting into exosomes, as an overexpression of a given miRNA inside cells does also increase its incorporation into exosomes (182). Furthermore, while a DICER deficient cell does exhibit a reduced intracellular miRNA profile, it shows an even more pronounced decrease in exosome-associated miRNAs (182).

Interestingly, RNA-seq analyses have revealed that miRNAs are not necessarily the most abundant RNA species in extracellular vesicles. Rather small rRNA, vaultRNA, yRNA, signal recognition particle (SRP)-RNA and tRFs (139) are present at higher levels in many cases. Moreover, it has been recently shown that 5'-tRFs and 5'-yRFs are significantly enriched in the extracellular space compared to their breast epithelial parental cell line in contrast to most miRNAs (188). How these sRNAs are sorted into EVs remains unclear, but it is interesting to note that SRP-RNA binding to the SRP protein is mediated by the exact the same motif, GGAG, that has been recently described as the EXOmotif (201, 213). Eventually the specific sorting mechanism described so far for the loading of miRNA into exosomes could thus also hold true for the specific introduction of other sRNAs into EVs.

Other loading mechanisms of ex-sRNAs into EVs likely exist as ex-sRNAs have also been described in MVs (193) and apoptotic bodies (230).

In summary, most recent reports are consistent with a non-selective model for most ex-miRNAs, although some miRNAs do exhibit patterns consistent with preferential sorting into exosomes. Consequently, both models are likely valid, do not mutually exclude each other and may serve different functions.

3.1.2. Release of sRNA from extracellular vesicles into circulation, targeting and entering recipient cells—Once incorporated into EVs, sRNAs reach the extracellular milieu upon exit from their parental cells. sRNAs packages into exosomes, microvesicles or apoptotic bodies are released from the cells' plasma membranes by distinct mechanisms which are summarized in detail in other recent reviews (5, 170, 201). Whereas exosomes and microvesicles are secreted during normal cellular processes, apoptotic bodies are only formed during programmed cell death (5) (Figure 1).

How EVs target a specific cell population still remains unclear at present, although many EV proteins have been found to interact with membrane receptors on recipient cells (summarized in a recent review (132)) (Figure 2). As no common pathway has been identified so far, it seems likely that the targeting of EVs to particular cell types depends on the physiological state of the producer and recipient cells (86, 132, 201).

Once attached to the target cell, EVs can be taken up from circulation by target cells by at least three different mechanisms: 1) the vesicular membrane can directly fuse with the plasma membrane of the recipient cell and thereby release its cargo into the cytoplasm of the recipient cell (86); 2) vesicles can be transported into the recipient cell using distinct endocytotic pathways (86); 3) once bound to the surface of recipient cells, vesicles may remain stably attached to the plasma membrane leading to activation of signaling pathways via protein-protein interactions or possibly also via sRNA-mediated activation of Toll-like receptors (TLRs) (58) (Figure 2).

3.2. Transfer of endogenous ex-sRNA via ribonucleoproteins

Although the association of miRNA with EVs has received most research attention, over 90% of circulating miRNAs have been found to be associated with proteins outside of EVs, highlighting the importance of ribonucleoprotein complexes in stabilizing, protecting and transferring sRNA (44, 194, 209) (Figure 1).

Different proteins have been found to be secreted together with miRNAs. These include AGO2 (15, 194), the key effector protein of miRNA-mediated gene silencing, nucleophosmin 1 (NPM1) (209) and high-density lipoprotein (HDL) particles (199, 204). How these proteins interact and package miRNAs remains unclear, but it seems that the miRNA exporting process is an active and energy-dependent process (209) (Figure 1). Furthermore, it has been suggested that AGO2/miRNA complexes are released non-specifically into circulation following cell death, as it has been observed that cell damage from toxicity in certain organs increases the level of specific miRNAs in blood (192, 194, 207) (Figure 1). For HDL particles it has been shown that the cellular export of miRNAs enriched in HDLs is increased when neutral sphingomyelinase 2 (nSMase2), a key regulator of exosomal-miRNA secretion (101) is inhibited (199), suggesting a clear requirement for sRNA export which is balanced between the different packaging and secretion mechanisms. Moreover, the scavenger receptor class B member 1 (SR-BI) surface receptor seems to be involved in the uptake of functional miRNAs associated with HDLs as demonstrated in mouse macrophages (199) (Figure 2). However, no significant uptake of HDL-bound miRNAs has so far been observed in human endothelial cells, smooth muscle cells or peripheral blood mononuclear cells (204). This suggests that there are differences in the propensity of different cell types to take up HDL-bound sRNAs while the targeted uptake of these by immune cells suggests that they may play important roles in immune system regulation.

To decipher the exact mechanism involved in the release of ribonucleoprotein complexes into circulation and their uptake into recipient cells, further investigations clearly are required, but a recent report showed that protein-bound and exosome-free extracellular miRNAs could functionally target cells by transfecting antibody-coated exosomes released by cells distinct from their parental cells (30). Conversely, Almanza et al. showed in the context of ex-sRNA transfer from B to T cell that only vesicle-associated sRNA could be taken up by CD8 T cells, whereas vesicle-free ex-sRNA could not be detected inside the target cells (8). Interestingly, it has also been described that miR-122 is able to shift between extracellular carriers under specific pathological conditions i.e. present within exosomal

fraction in the context of alcoholic liver disease and inflammation, and in the protein-bound form in the context of acetaminophen-induced liver necrosis (19, 210). The latter study indicates that even if no specific uptake and delivery mechanisms exist for ribonucleoprotein complexes, a specific delivery of ex-sRNAs is still not restricted to EV-associated sRNA. Finally it should be pointed out that SID-1 (identified in a screen for systemic RNAi defective mutants), a receptor transferring double stranded RNA, present in *Caenorhabditis elegans* (215), mammals (54), flies (59) and plant (227) has also been reported to transport miRNA precursors and larger hairpin RNAs (178), and thus it will be interesting to learn what role such receptors may play in the uptake of circulating sRNAs.

3.3. Transfer of sRNA by direct cell-to-cell contact

Although this review focuses on ex-sRNA in human circulation, it is important to note that transfers of sRNA by direct cell-to-cell contact has also been described (27). More specifically, sRNA has been shown to shuttle between mammalian cells through organized structures such as gap junctions (118, 128), intercellular bridges and synapses (127, 128). Furthermore, the transcytosis of sRNA through biological barriers has been suggested (219) (Figure 1).

3.4. Transfer of exogenous ex-sRNA from microorganisms and parasites

Much of the controversy in the field of exogenous ex-sRNAs has revolved about the presence and possible effects of plant miRNA in mammalian circulation (42, 53, 180, 219, 220, 223, 231, 232). However, in more general terms exogenous sRNA from different species have been identified in different human body fluids in a number of independent studies (18, 21, 32, 93, 148, 205) and several mechanisms have either been proposed or recently demonstrated which can explain the presence of bacterial, fungal, archaeal, viral and diet-derived RNA in human blood (Figure 1).

For most of the identified exogenous ex-sRNA, the means of entry into circulation remains enigmatic. However, mechanisms for exogenous sRNA transfer and stability in human circulation have been described for a number of viral- and nematode-derived sRNA (32, 124, 148, 221). For example, the Epstein-Barr-virus (EBV) hijacks the human exosomal machinery in order to transfer EBV-encoded miRNAs from infected B lymphocytes to non-infected recipient cells where these miRNAs downregulate immunoregulatory genes and, thus, help EBV to evade detection by the human immune system (148). Furthermore, the gastrointestinal nematode, *Heligmosomoides polygyrus*, which infects mice, has been demonstrated to secrete vesicles containing miRNAs and Y-RNAs (32). These vesicles are of intestinal origin, are taken up by mouse small intestinal epithelial cells and suppress type 2 innate response probably via the repressive function of parasite-derived miRNAs (32). Moreover, *Cryptococcus neoformans*, an encapsulated yeast, which causes systemic mycosis in immunosuppressed individuals, secretes RNA-containing vesicles into its extracellular space both in vitro and in vivo (137). These vesicles are also taken up by mammalian cells (142). Thus, some exogenous sRNA are protected from degradation by exosomes and human pathogens appear to hijack EVs in order to evade immune detection (Figure 1).

Bacterial sRNAs, with a probable gastrointestinal origin, have been detected in human circulation (21, 172, 205). Interestingly, both Gram-positive and Gram-negative bacteria are known to secrete extracellular vesicles (29, 171), which have recently been found to contain sRNAs (62, 68) (Figure 1). Such vesicles have been found to be taken up by human gastrointestinal epithelial cells (146), and they may trigger responses in distant target cells of the host. In mammals, vesicles released by gastrointestinal pathogens can be harmful or even lethal as vesicles derived from pathogenic bacteria transport diverse virulence factors to host cells enabling them to modulate host defense and response in order to assure their survival and replication (26, 107, 145). Furthermore, it has only recently become apparent that vesicle-induced signaling by commensal bacteria is of utmost importance to the host. More specifically, a recent study has demonstrated that polysaccharide A enriched vesicles derived from the human commensal bacterium *Bacteroides fragilis* mediate host immune regulation and prevent colitis in a mouse model (13, 177). At present, the precise means by which these vesicles affect host responses are unknown. Analogous to observations in nematodes, fungi and viruses it is tempting to speculate that vesicles protect bacterial sRNAs found in circulation and that these exogenous ex-sRNAs may be functionally active in recipient human cells. Whether these vesicles are however able to cross the epithelial barrier in healthy individuals or only deliver their cargo to epithelial cells that are in direct contact with the microbiome, or if these vesicles are used to “transcytose” sRNA–vesicular uptake of RNA on one side and release on the other side of a biological barrier, e.g. the gastrointestinal epithelium - still needs to be investigated (Figure 1).

3.5. Transfer of diet-derived exogenous sRNAs

Most work as well as most controversy surrounds the transfer of diet-derived RNA into the human blood stream. miRNAs present in chicken eggs (93) or in cow’s milk exosomes (18) have been found to be bioavailable upon consumption by healthy adults and it has been proposed that they do affect human gene expression (18, 93). However, how these exogenous miRNAs pass the epithelial barrier is yet not clear and more recent work challenges the notion of miRNA transfer from milk (187) (Figure 1). Similarly, plant miRNAs have been suggested to cross the epithelial barrier and to influence gene expression of the host when administered along with honeysuckle decoction (231, 236) (Figure 1). However, the mechanism explaining the crossing of the epithelial barrier by these specific exogenous miRNAs remains enigmatic and these reports have been rather vehemently questioned (53, 180, 216, 220). However, recent work by the group of Kendal Hirschi provides first evidence of how specific exogenous sRNAs may pass the epithelial barrier (223). Most importantly, this work also provides an explanation for the contradictory previous findings by different group and attributes this to likely differences in barrier function (223). More specifically, administration of a chow diet containing honeysuckle to mice lead to the detection of plant-based small RNAs in the sera and urine of the mice, whereas no plant miRNAs could be observed in the described bodily fluids in absence of the honeysuckle decoction (223). Honeysuckle-fed animals appeared to exhibit hallmarks of kidney failure indicated by clinical blood analysis although they did not show any histopathological abnormalities in kidney tissues. Moreover, a treatment of mice with cisplatin, an important chemotherapeutic agent known to induce kidney damage, prior to their gavage with synthetic plant miRNA, did result in measurable levels of dietary miRNAs

in the murine sera and urine, whereas untreated mice, gavage-fed with the same cocktail, did not display measurable levels of exogenous miRNAs in any measured body fluid (223). This work thus suggests that consumers of particular diets and/or alterations in intestinal permeability have an improved capacity to absorb dietary sRNAs (Figure 1). A recent study supports this hypothesis by demonstrating that dietary intake of a specific compound (plant cysteine protease) does favor the transmission of plant-derived RNA from plant to midgut insect cells, where this RNA triggers target gene suppression (121). Moreover, Mlotshwa et al. demonstrated oral delivery of a cocktail of tumor suppressor miRNAs produced in plants into the bloodstream and these subsequently reduced tumor burden in a mouse model of colorectal cancer (129). In this context of colorectal cancer, intestinal permeability is known to also be increased (151) (Figure 1). Finally, bacterial factors which impact epithelial barrier function may actively contribute to the transfer of ex-sRNA from the gut to the bloodstream (222). In addition to direct translocation of RNA across the gastrointestinal barrier, a possible alternative route for RNA molecules to pass into blood could be via immune cells, particularly dendritic cells, which form part of the epithelium and which continuously sample mucus-penetrating bacteria or exogenous products present in the gut lumen (81, 219) (Figure 1).

The uptake pathways reported for dietary originating ex-sRNA described within this section does point towards a panoply of uptake mechanisms rather than specific transfer (Figure 1). Nevertheless, this does not exclude the possibility that exogenous sRNA cannot trigger host responses. Indeed, exogenous sRNA encapsulated in EVs could target a specific cell population depending on the origin and composition of the EVs using similar mechanisms described for endogenous sRNA present in EVs (Figure 1 and Table 2).

3.6. Concentration ranges of ex-sRNA in circulation

While it has been shown that endogenous as well as exogenous ex-sRNA can be functionally active (see also sections 4.1. and 4.2. of this review), it seems unlikely that all ex-sRNAs are present in concentrations that allow functional activity. Indeed, RNA-seq has revealed that the concentration of total miRNA in plasma is within the 100fM range and the concentration of any individual miRNA is only a fraction of this number (214). Moreover, quantitative and stoichiometric analyses of the miRNA present in exosomes have revealed that even for the most abundant miRNAs in exosome preparations, the ratio is one specific miRNA molecule per ten exosomes (44). Furthermore, not all miRNAs present in a cell, abundant or not, do suppress their cognate sensors (133, 219). Thus, the transfer of physiological significant miRNA via circulating EVs over long distances may be a rare event. However, localized high concentrations of ex-miRNA from donor cells are likely and their impact on neighboring recipient cell may therefore be physiologically relevant.

Apart from miRNA, other sRNA species, such as yRFs and tRHs make up a large fraction of all small RNAs present in circulation. More specifically, RNA-seq data from blood plasma from healthy individuals revealed that yRFs comprise up to 10% of the total circulating miRNA pool. Moreover, RT-qPCR analyses on the same samples revealed that specific species out of the different RNA families can be similarly abundant or even more abundant than specific circulating miRNAs (Y4-5p: 5×10^9 molecules/ml plasma; tRF-val: 2×10^7

molecules/ml plasma versus miR-486-5p: 8×10^7 molecules/ml plasma) (D. Yusuf, A. Heintz-Buschart, B.B. Upadhyay, J.V. Fritz, A. Ghosal, M. Desai, J.M. Dhahbi, P. May, D. Huang, E. Muller, P. Shah, H. Roume, C. De Beaufort, J. Schneider, A. Hogan, K. Wang, D. Galas and P. Wilmes, unpublished observation). These sRNAs are complexed with proteins within the serum which protect them from nuclease-mediated degradation (50–52, 188). These RNA species were also detected in immune cells-derived EVs (139) and tRHs concentrate within blood cells (52). Although, biological functions have been reported for intracellular tRHs (reviewed in (11, 67)), further studies are required to evaluate whether their extracellular counterparts are biological active and are present in concentrations allowing them to trigger physiological responses.

4. Ex-sRNAs' functional and biomarker potential

4.1. Functional potential of endogenous ex-sRNAs

Extracellular vesicles have been shown to be able to transfer functional RNA from a donor to a target cell or to shuttle RNA between neighboring identical cell types; analogous to hormones which can signal in a paracrine and autocrine mode (55, 130, 155, 195) (Table 2). To date, most studies have focused on miRNA and/or mRNA as a cargo within exosomes. In these studies, the patterns of translation and its repression upon exposure of a given cell population to specifically packaged RNAs have been analyzed. As summarized in table 2, a number of studies have shown that exosomes can indeed transfer miRNAs and that specific target genes in recipient cells are affected by specific miRNAs. However, it is noteworthy that these experiments mostly involve exposing recipient cells to concentrations of exosomes which are much higher than those that cells, tissues or organs would be exposed to in vivo (Table 2). Therefore, it seems at present absolutely essential to investigate whether exosomal-delivered miRNA are able to trigger functional responses in a given target cell in vivo. First studies, seem to underscore the functional impact of ex-miRNA transported via exosomes in vivo (103, 131, 182, 233, 235). Squadrito et al. have demonstrated that exosomal-miRNA are transferred from macrophages to endothelial cells in vivo where they have been shown to detectably repress targeted reporter gene sequences (182). Zhou et al. have demonstrated in vivo that only exosomes with a high content of miR-105 derived from a metastatic breast cancer cell line significantly reduce the expression of the tight junction protein ZO-1 (miR-105 target gene) in endothelial cells which in turn enhances vascular permeability (182, 235) (Table 2). The work of Zhou et al. illustrates that exosomal miRNA may be implicated in metastatic progression, whereas a study by Kosaka et al. shows that miR-143, a tumor-suppressive miRNA, derived from normal epithelial cells inhibit the proliferation of cancer cells in vitro and in vivo, indicating that the functional impact of exosomal miRNAs is driven by the producer cells (103, 235) (Table 2). Apart from cells with relevance to the human circulatory system, the transfer of exosomal miRNAs from a donor cell to an acceptor cell has been described in the murine brain, whereby neuronal miR-124a indirectly increased the glutamate production in astrocytes (131). Finally, ex-mRNA-based signaling between the hematopoietic system and various organs including the brain has been observed in response to inflammation (158) (Table 2). From these recent studies, there is mounting evidence that exosomal miRNA and mRNA are functional in vivo and are indeed involved in intercellular communication. However, as the field of ex-sRNAs

is still in its infancy, further independent experiments are required to validate these pioneering findings, especially since most of the aforementioned studies have involved injecting mice with exosomes derived from cultured cells which were overexpressing the miRNA of interest (103, 131, 182, 235). Moreover, in one of these studies it has been shown that when such exosomes are injected systemically, no RNA-based signal could be observed in the brain which might be due to an overall low amount of exosomes in the preparation or that exosomes are not able to cross the blood–brain barrier (158). Thus, more studies are required to investigate the functional potential of circulating ex-sRNA *in vivo*, especially to determine if their concentration within circulation is high enough to really reach their specific targets and to understand their mechanism of release, targeting as well as their impact on gene expression and resulting changes in cell physiology.

Even though most of the studies showing a functional transfer of circulating miRNAs concentrated on exosomes as a carrier (although in most studies it cannot be excluded that a mixed population of different vesicles had been analyzed), this does not necessarily mean that ex-miRNAs transported by other vesicles are not functional (Figure 2). In particular, Zernecke et al. have shown that, in atherosclerosis, miR-126-enriched apoptotic bodies derived from endothelial cells convey paracrine alarm signals to recipient vascular cells which in turn triggers the production of CXCL12 thereby culminating in a decrease in atherosclerosis in mouse models (230) (Table 2). Moreover, Vickers et al. have provided evidence that high-density lipoprotein (HDL) can transport endogenous miRNAs and deliver them to recipient cells with functional targeting capabilities (199) (Table 2 and Figure 2). More specifically, they have demonstrated that HDL particles can suppress the expression of intercellular adhesion molecule 1 (ICAM-1) through the transfer of miR-223 to endothelial cells (184) (Table 2).

Although the majority of miRNA, or sRNA in general have been found to be associated with protein complexes (44, 194, 209), it remains enigmatic if ex-sRNAs present in blood ribonucleoprotein complexes are able to trigger functional responses in target cells. Currently, studies aiming at understanding the composition and the potential functional impact on human physiology of these ribonucleoprotein complexes are on-going and first indications whether or not ex-sRNA complexed to proteins are functional should be revealed soon (147).

Independent of the different ex-sRNA carriers (exosomes, apoptotic bodies, HDL particles or ribonucleoproteins), these highlighted studies demonstrate that ex-sRNA do appear to have repercussions on functional gene expression *in vivo*. However, in order to comprehensively evaluate which other ex-sRNAs (including exogenous moieties) might have specific effects *in vivo*, large-scale screening efforts will be essential.

Besides the fact that ex-miRNA have been shown to regulate the expression of target genes at the post-transcriptional level, it has recently also become apparent that ex-miRNAs can be transported from donor cells to acceptor cells, in which they may function as ligands for TLRs and induce downstream signaling (41, 58, 113) (Figure 2). Indeed, Fabbri et al. found that lung tumor cells can secrete miR-21 and miR-29a via exosomes, and that these exosomes can be transferred from cell-to-cell (58). Furthermore, they found that these can

be taken up by macrophages localized at the tumor-normal tissue interface and eventually reach TLR-containing endosomes (41, 58). Similarly, extracellular let-7b has been found to induce neurodegeneration through the activation of TLR7 in vitro and in vivo, and it has been suggested that ex-let7b is transferred via apoptotic bodies from dying neurons to neighboring healthy neurons where neurodegeneration is then triggered (41, 113). Finally, Boelens et al. showed that the RNA within cancer cell-derived exosomes, which is largely composed of non-coding transcripts and transposable elements, is able to stimulate the pattern recognition receptor retinoic acid-inducible gene 1 (RIG-I) to activate signal transducer and activator of transcription 1 (STAT1)-dependent signaling which seems to be involved in chemotherapy and radiation resistance (25) (Figure 2). Similarly, as for ex-miRNA, additional functions have also been demonstrated for ex-mRNA. Batagov et al. demonstrated that exosomal-mRNA, which seems to be present in vesicles mostly as fragments, may act as competing RNA to regulate stability, localization and translation activity of mRNAs in recipient cells (20). Thus translation or translation repression induced by ex-mRNA or ex-miRNA, respectively, are not the only functions that may be affected by ex-sRNA (Figure 2).

It is important to recognize that apart from ex-miRNA and ex-mRNA, other ex-RNA species may also act as extracellular signaling molecules, as repressors of gene expression or both. This notion is primarily based on the observations that (i) other sRNA families have been identified in large amounts in circulation (Table 1), (ii) intracellular tRFs and tRHs possess distinct biological roles when compared to their parental tRNA (67), (iii) in a tissue damage situation full-length tRNAs and rRNAs can trigger vasculogenesis and leukopoiesis of embryonic stem cells (175) and (iv) yRNAs seems to play a central role in endothelial cell-to-cell communication as this RNA family seems to be important in the regulation of the degradation of specific RNA molecules in endosomes prior to their export as exosomes (196). Taken together, these observations raise the question whether, apart from ex-miRNA and ex-mRNA, other ex-RNA species have major impacts on human cell physiology? An important caveat in this discussion about the roles of RNA biotypes is that there are several extensive classes of RNAs that are rather poorly annotated, like the piRNA, and others, like lncRNA that have been reported to generate processed fragments that function like miRNA (Table 1). This makes it difficult to be definitive about attributing or restricting functions to class or biotypes of RNA. Clearly, extensive future experiments and further annotations are required to answer these questions.

4.2. Functional potential of exogenous ex-sRNAs

As described previously in the section 2, ex-sRNA molecules also appear to include a number of stable exogenous sRNA moieties (18, 21, 32, 93, 148, 231). Given the relatively large diversity of sRNAs derived from organisms other than human, there likely is tremendous functional potential reflected in this exogenous ex-sRNA pool. The fact that exogenous ex-sRNAs may have functional impacts is well established since the discovery of RNAi in the nematode *Caenorhabditis elegans* (61). More broadly speaking, other nematodes, planaria and many insects assimilate double-stranded RNA from their environment and induce RNAi. In *C. elegans*, once absorbed, it has been proposed that exogenous ex-sRNA is distributed systemically, regulates gene expression and physiological

conditions of *C. elegans* (119). More specifically, the stress-inducible OxyS non-coding RNA in *E. coli* was found to cause the down-regulation of the *che-2* mRNA in *C. elegans* when expressed in the *E. coli* food (119) but this finding has yet to be replicated (4). In this context, it is also noteworthy that *C. elegans* can be fed *E. coli* engineered to overexpress a certain sRNA and subsequently elicits RNAi (92).

In addition to taking up ex-sRNA, nematodes have also been found to secrete their own ex-sRNA. More specifically, the gastrointestinal nematode *Heligmosomoides polygyrus* secretes sRNA containing exosomes which are transferred from the parasite to mammalian cells where they have been found to modulate the innate immunity (32) (Table 2 and Figure 1&2). In humans, plant miRNA and more generally diet-derived miRNAs have been suggested to have a functional impact on human gene expression (18, 93, 232) (Table 2). This assertion is primarily based on the pioneering work of Zhang et al. which demonstrated that a specific rice-derived miRNA, namely miR-168a, detectable in relatively high concentrations in the blood of Chinese individuals may have systemic effects on metabolism. More specifically, Zhang et al. found through extensive in vitro and in vivo work that miR-168a binds human and murine low-density lipoprotein receptor 1 (LDLRAP1) mRNA and downregulates LDLRAP1 expression in the liver of mice (232). Plant derived miR-168a thereby reduces low density lipoprotein removal from blood which in turn may greatly impact overall lipid metabolism (232). As discussed previously (section 3.5.), the notion of the transfer of plant-derived sRNA into circulation and its consequential organ-specific and systemic effects have proven controversial (53, 180, 216, 220). Much of this controversy has focused on the effects of plant-derived miRNAs, whereas other areas of exogenous RNA-mediated cross-kingdom regulation of gene expression have attracted less contention. Human breast (105), and cow's milk (18) contain miRNAs which are encapsulated in exosomes. Recent work demonstrates that bovine miRNAs at nutritionally relevant doses affect gene expression in human and murine cells which in turn suggests that milk-derived miRNAs may likely have organism-wide effects (18), although these results have also been questioned recently (187). Apart from the apparent potential of honeysuckle to facilitate transfer of exogenous sRNAs across the gut epithelium (section 3.5.), a highly stable and transferrable miRNA, namely miR-2911, derived from honeysuckle has been found to significantly inhibit replication of influenza A viruses and viral infection-induced phenotypes (236). This study along with another recent study (129), underline the exciting therapeutic avenues which exogenous ex-RNA may offer.

Apart from therapeutic possibilities, exogenous RNA has been found to also be implicated in disease pathogenesis. It is well accepted that viral RNA is sorted into exosomes which allows remote action (38). More specifically, human tumor viruses can exploit exosomes as delivery vectors to transfer functional exogenous miRNAs from the originally infected cell to other non-infected cells where gene expression is then affected (124, 148) (Table 2 and Figure 1&2). Moreover, exosomes carrying HIV-1-derived miRNA from an acceptor to an adaptor cell induce a reduction in apoptosis by lowering Bim and Cdk9 proteins in the target cell (135) (Table 2).

Fungal RNA has also been detected in circulation (21, 205) and fungi endemic to humans, including *Cryptococcus neoformans*, *Paracoccidiales brasiliensis*, *Candida albicans* and

Saccharomyces cerevisiae have been shown to secrete extracellular vesicles carrying sRNAs (149). Therefore, it is tempting to speculate that fungal RNA-containing vesicles may also be determinant for various biological processes, including inter-kingdom communication and pathogenesis (149) (Figure 1&2). Although the functional transfer of fungal RNA towards human host cells remains hypothetical, it is noteworthy to mention that in recent years many examples of RNA-signal exchange have been described to occur between organisms of different kingdoms (97).

Similarly no direct evidence of a functional transfer of bacterial-derived ex-sRNA from a donor to a mammalian recipient cell has been established so far. The functional implications of the transfer of ex-sRNA from a bacterium to a mammalian recipient cell are, however, underlined by studies which have demonstrated that extracellular RNA secreted by *Listeria* spp. are key components in the development of immunity against the bacterial infection (1), and small extracellular RNA fragments of *Mycobacterium tuberculosis* (comprised primarily of tRFs and rRFs), induce early apoptosis in human monocytes (140). Another example illustrating the interplay between bacterial ex-RNA and an infected host is that the highly conserved 23S rRNA motif 'CGGAAAGACC', is recognized by the TLR13 receptor in mice, and modifications in the motif allow bacteria, e.g. erythromycin-resistant *Staphylococcus aureus*, to bypass host immune recognition (141) (Table 2). Although TLR13 is not found in humans, this may suggest that exogenous ex-sRNAs may carry similar functions in humans by binding to other Pattern Recognition Receptors (PRRs).

So far no extracellular bacterial RNA moieties have been identified which would function identically to mammalian miRNAs. However, it is becoming clear that the expression of particular genes, e.g. those involved in quorum sensing (bacterial cell-to-cell communication), are regulated by noncoding small RNAs which themselves act directly or indirectly to control gene expression by post-transcriptional mechanisms (162, 174). It is also important to highlight that recent evidence suggests that bacterial DNA is able to integrate into the human genome through an RNA intermediate (10). Finally, bacteria possess a mechanism of defense against foreign nucleic acid molecules, primarily comprised of bacteriophages and plasmids, the CRISPR-Cas system, which functions in an analogous fashion to eukaryotic RNA interference (RNAi) (for a recent review: (168)). When responding to an invading phage, bacteria transcribe spacers and palindromic DNA encoded in the CRISPR array into a single long RNA molecule. This long RNA molecule is then cut into pieces called crRNAs through the action of a specific endonuclease. The mature crRNAs then associate with Cas proteins to form complexes, which interfere with foreign DNA ultimately leading to its degradation. Whether crRNAs may also play other function and whether they might be secreted extracellularly is presently unknown. Thus, bacterial sRNA do show an enormous functional potential but much remains to be clarified in particular how bacterial RNA derived from gastrointestinal microbiota might pass the epithelial barrier and how they might impact human physiology once they are present in circulation (Figure 1&2). Based on the rather compelling number of studies published over the last decade, it seems that both endogenous and exogenous ex-sRNAs do have a direct impact on human gene expression and trigger immune system responses. Whether this is the case for all or most ex-sRNA or only for specific ones still needs to be determined.

4.3. Ex-sRNAs' biomarker potential

Ideally, a good clinical biomarker should allow discrimination between a healthy and diseased state, monitor early indications of a given disease. Most importantly a good biomarker should suggest early appropriate treatment strategies: it may be diagnostic or predictive, or both. Furthermore, a biomarker should be easy and quick to measure using established and robust analytical assays which can be applied to easy accessible biological samples, such as body fluids (whole blood, blood plasma, blood serum, urine, etc.) (183, 210).

The interest in ex-sRNAs, especially in extracellular miRNAs, as clinical biomarkers for distinct pathologies has grown tremendously over the past decade, since they present numerous essential attributes which make them qualify as potentially ideal candidates for biomarkers including (i) their key regulatory functions (7, 152) and dysregulation in disease (7, 120, 152, 157); (ii) their cell type- and tissue-specific expression patterns (108, 163, 210); (iii) their relative ease of sampling and measurement (21, 40, 50, 52, 126, 205) and (iv) their stability (40, 52, 126, 176, 190, 237).

In addition to being potentially good biomarkers for disease, the use of circulating miRNAs has also been assessed to provide information on the overall physiological status, dietary habits and other lifestyle choices of individuals. For instance, blood-borne ex-sRNAs have been used to evaluate healthy individuals according to their peak oxygen consumption (VO₂max levels: an indicator of the level of fitness) and it has been shown that miR-222 significantly correlates with self-reported habitual exercise intensity (35, 161). Moreover, miR-92a has been associated with low body mass index and it has been found to be elevated in plasma samples of vegans and vegetarians when compared to omnivores, indicating that circulating miRNA level may be modulated by diet and might thus be used to ascertain how nutrition and lifestyle is influencing metabolic pathways and consequently human health and disease (161, 185). Following the same lines it was also demonstrated that dietary zinc intake influences the level of at least 20 circulating miRNAs (166) and let-7 expression has been negatively correlated with vitamin D intake (22). Interestingly, the correlation between vitamin D intake and let-7 levels was however only significant in cohort participants who displayed one out of two common vitamin D receptor polymorphisms highlighting the importance of considering underlying genotypic variance in miRNA expression studies (22). Besides ex-miRNAs, a few studies have concentrated on other circulating sRNA families to determine their potential as biomarkers. So far changes in levels of specific subtypes of tRHs and yRFs, as well as of RNU6-1 (a specific snRNA) have been associated with a breast cancer diagnosis (12, 51).

Taken together, endogenous circulating sRNAs hold great promise for identifying biomarkers with clinical relevance for the diagnosis of a number of human diseases and gathering information on the overall health status of individuals.

The recent discovery of gastrointestinal microbiome-derived sRNAs in human blood plasma (21, 172, 205) in addition to the identification of disease-associated microbiome characteristics (36), as well as the findings that some of these microbial sRNAs are detectable in the plasma of colorectal cancer patients (205), suggest that not only

endogenous ex-sRNA, but also exogenous ex-sRNA might be potential biomarkers for specific diseases. Although the presence of exogenous (plant) sRNA in mammalian circulation (231) has been the subject of much recent controversy (53, 216), recent findings demonstrate that alterations in intestinal permeability lead to an increased capacity to absorb exogenous sRNAs from the gastrointestinal tract (224). Since several intestinal inflammation and gastrointestinal diseases including celiac disease, colorectal cancer, food allergy, inflammatory bowel disease, type I diabetes and others have been associated by localized breakdown of the epithelial barrier at early disease stages, and are often accompanied by bacterial infections (71, 80), disease-associated microbiome-derived ex-sRNAs might be detectable at significant levels in human blood plasma to inform early diagnosis. Furthermore, given that certain medical therapies, e.g. intense systemic chemotherapy regimes, can have dramatic effects on gastrointestinal barrier function, which might culminate in severe treatment side-effects, e.g. sepsis (191).

The ex-sRNAs detected in blood might also have a range of turnover rates affected by their sequences and their carriers, which of course will also impact their potency as biomarkers. So far it has been shown for miR-122 that this specific miRNA is able to shift between extracellular compartments under specific pathological conditions: it is found at elevated levels within exosome-rich fractions in alcoholic liver disease and inflammation and as a protein-rich fraction in acetaminophen-induced liver necrosis (19). Thus, by analyzing not only the expression pattern of ex-sRNAs under distinct conditions but also their carriers, with which they are associated, may provide further specificity to ex-sRNAs biomarkers.

5. Challenges

The study of ex-sRNAs in circulation is hampered by several challenges, impacting the comparability between studies employing different analytical techniques. These challenges affect most studies in the field, and most importantly biomarker studies, where a lack of standardization and the range of method-specific biases have created a sense of irreproducibility and lack of consistency.

5.1 Sample preparation

Several steps during sample preparation can significantly affect the outcome of sRNA studies, especially those focusing on circulating RNAs. Plasma and serum are the two most frequently used sample types in circulating RNA studies; however, given their distinct composition, i.e. blood plasma still contains clotting factors with potentially associated sRNAs, the results from serum and plasma are not directly comparable to each other (206). In addition, different methods for preparing serum or plasma can also greatly affect the spectrum of RNAs detected in the sample. For example, removal of platelets by high-speed centrifugation, can influence the resulting sRNA profile compared to samples in which platelets have not been comprehensively removed (143). Sample processing times can also greatly affect the observed RNA profile. For example, Page et al., (143) reported that the levels of several miRNAs are significantly reduced in plasma isolated from blood that has been stored for 6 hours compared to 2 hours of storage before the processing. Serum RNA levels are more drastically influenced by coagulation duration (190). In comparison to

sample preparation, the actual isolation protocol of sRNA from prepared cell-free blood fractions seems to have little impact on the results (143).

Hemolysis, the rupturing of erythrocytes during phlebotomy or sample storage, can also severely affect the sRNA composition of serum or plasma, as erythrocytes contain a relatively large amount of RNA (95). Exclusion of samples based on hemoglobin content or visual inspection is therefore absolutely critical. In addition, documentation and reporting of the haemoglobin levels in samples may help eliminate a potential confounding factor in circulating RNA studies.

The use of anticoagulants in plasma preparations also affects the circulating RNA profiles. More specifically, heparin, an anti-clotting agent which is commonly used for blood draws, interferes with subsequent reverse transcription reactions (17, 65), which are a necessary step for most RNA measurement technologies including RT-qPCR, microarray analysis as well as RNAseq. In addition, heparin has been found to affect different sRNA species to different extents (24). While treating heparinized samples with heparinase or lithium chloride precipitation can resolve some of these problems but it may introduce other, additional variations into RNA measurements (78, 150). Therefore, plasma samples prepared using EDTA-based anticoagulants are preferred for circulating RNA analysis.

sRNA from blood is remarkably stable after phlebotomy and the total levels of plasma RNA have been found to not be affected by storage of plasma at room temperature for 24 h (190). However, it is likely that different sRNA species display different stabilities, depending on their secondary structure as well as their carrier.

Especially in relation to exogenous ex-sRNAs, contamination during sample preparation needs to be avoided and all preparations need to be accompanied by suitable controls to trace potential introduction of sRNAs by reagents or extraction equipment (167, 220) (A. Heintz-Buschart, D. Yusuf, A. Kaysen, Alton Etheridge, J.V. Fritz, D.J. Galas, P. Wilmes, unpublished observation).

RNAs in circulation are packaged in different ways (see also section 3 of this review). Studies have shown that the distribution of RNA among protein complexes and vesicles is different and may have unique biological activities. Therefore, there is great interest in detailed characterizations of RNA in the different compartments, especially in exosomes. However, there is currently a lack of standardized procedures and quality controls to ensure the consistent the isolation of exosomes, ribonucleoprotein-complexes or high-density lipoprotein particles (218). While early studies on exosomes relied on differential ultracentrifugation and sucrose gradient centrifugation (56, 154), the needs to use less laborious methods and work with smaller input volumes has generated several alternative protocols. These include size-based filtration (70) and polyethylene glycol precipitation. Unfortunately, these protocols usually lead to co-purification of a very different assortment of cellular fragments and protein-complexes in addition to canonical exosomes (156, 198). Immunoprecipitation based on exosome surface proteins such as CD9 or CD63 is another commonly used method for exosome purification. However, this method does not allow, of course, the capture of exosomes lacking these surface markers. An easy and reliable method

for purifying exosomes is therefore urgently required which will allow the investigation of the different ex-sRNA fractions.

5.2. Measurement methods

Several methods, including microarrays, RT-qPCR and RNAseq, are commonly used to profile RNA in circulation. Each method has distinct advantages, limitations and biases. Because the overall levels of RNA in circulation are low (217), the detection limits (both the absolute detection levels and the ability to detect distinct concentration differences) of analytical methods are important. The detection limit of microarrays is relative high compared to RT-qPCR. The detection limits of RNA-seq mainly depend on sequencing depth. While the detection limit of RT-qPCR is theoretically three templates per reaction (34), small differences in template abundance may be better resolved using droplet digital PCR (79).

Besides the low detection limit, another advantage of RT-qPCR-based methods for circulating RNA profiling is that they are arguable less sensitive to sequence-specific biases than microarrays or next generation sequencing platforms (238). The ligation of 3' and 5' adaptors to small RNAs, which comprise the first two steps of most currently used sRNA sequencing protocols, are strongly affected by sRNA sequence (88) and secondary structures (64, 75, 239). Besides structure- and a range of sequence-dependent biases, 5'- and 3'-end modifications also greatly influence the representation of sequences in sRNA libraries, and the conditions in the ligation reactions have strong effects on the detection of specific RNAs (57). Currently available commercial kits are designed to capture RNAs with 5' monophosphate and 3' hydroxyl groups. Consequently, RNA fragments with other end modifications (5' hydroxyl or 3' phosphate, for example) are not efficiently ligated to adaptors and are therefore poorly represented in sequenced libraries. In addition, 2'-O-methylation of the 3'-terminal nucleotide of sRNAs commonly observed in plants (228) reduces their representation in RNA sequencing libraries (134). While reverse transcriptase and PCR steps seem to introduce negligible bias into sRNA libraries (75), length and GC-content can impact amplification efficiency of cDNA species (2).

Both microarray- and RT-qPCR-based studies share the limitations of requiring prior knowledge, i.e. sequences of interest need to have been previously defined. Furthermore, frequently occurring variants in sRNA sequences (isomiRs), and particularly those affecting the ends of the sRNA, cannot be readily distinguished from unedited sequences by RT-qPCR or microarray (112). In addition, both microarray and RT-qPCR also have difficulties in distinguishing RNA family members with closely related sequences, such as miR-199a and miR-199b. These closely related sequences can only be reliably distinguished by sequencing-based approaches (73, 112, 122, 203).

5.3. Data analysis and validation

Unlike microarray or RT-qPCR analyses, data generated from next generation sequencing-based approaches requires significant efforts in data processing prior to analysis. More specifically, the resultant reads have to be filtered to remove adapter sequences, low quality reads and homopolymers before they are mapped to various databases. Depending on the

alignment tools used and the levels of error tolerance allowed, mapping results can vary significantly. Therefore, for sequencing-based datasets, a detailed description of the analysis conditions is essential in order for others to reproduce workflows.

One of the most frequently encountered problems in comparative extracellular sRNA analyses or sRNA validations by RT-qPCR is the lack of a generally accepted stable reference RNAs (12). There are two levels of “normalization” processes commonly used in gene expression analyses – one is to use the same amount of input RNA from each sample and the other is to adjust the signal between samples using “housekeeping” transcripts, which are assumed to be unchanged between samples. As the concentration of RNA isolated from plasma or serum samples is usually too limited to allow accurate quantification, the use of the same mass of input is typically not possible; equal volumes of extract are commonly used instead. Moreover, spike-in RNAs are commonly used for signal adjustment rather than housekeeping transcripts. However, spike-ins, which are usually added after isolation of sRNAs (106), cannot account for the varying total concentrations of sRNA associated to the different carriers and, thus, sRNAs in the original body fluid. Absolute quantification of sRNAs using calibration to synthetic RNAs with the same sequence as the sRNA of interest is another option. However, this method is limited by potential *in vivo* RNA modifications, which may affect the efficiency of cDNA synthesis or PCR and, therefore, equivalent efficiencies in the amplification of actual samples and synthetic standards needs to be ensured. Microarray and sequencing-based studies usually rely on across-sample normalization or standardization (9, 160, 189), which usually assumes similar distributions over all detected sRNA species or use spike-in sequences (159, 173).

5.4. Biomarker discovery

Despite the fact that many circulating sRNAs have in recent years been identified as potential biomarkers, issues of diagnostic sensitivity, specificity and reproducibility of blood-borne sRNAs still needs to be addressed (recently reviewed in (217)). Indeed, very low levels of consistency are apparent in terms of identified potential ex-sRNA biomarkers between very similar studies, for example those carried out in the context of the same disease. These discrepancies have most recently been highlighted in a meta-analysis by Leidner and coworkers in which they compared data from 15 previous reports on circulating miRNA as potential biomarkers for breast cancer diagnosis with their own data and found widespread inconsistencies across the different studies (114, 217). This poor overlap probably arises from differences in study design as well as the applied methodologies.

One of the reasons for limited reproducibility of biomarker studies is that in the absence of standardized protocols, each study is affected by its individual set of challenges and biases, as discussed above, in addition to common challenges in biomarker studies, relating to cohort sizes, identification and treatment of confounding factors, and choice of control groups,. Sources of variation of ex-sRNA levels in healthy individuals (152) include age (52), gender (211), circadian rhythm (D. Yusuf, A. Heintz-Buschart, B.B. Upadhyay, J.V. Fritz, A. Ghosal, M. Desai, J.M. Dhahbi, P. May, D. Huang, E. Muller, P. Shah, H. Roume, C. De Beaufort, J. Schneider, A. Hogan, K. Wang, D. Galas and P. Wilmes, unpublished observation), and nutrition (52, 161). Several medications have also been shown to leave

their mark on circulating sRNA profiles, some of which are commonly used by a large proportion of the population, such as aspirin (48) or statins (60). Therefore, in addition to commonly recorded data such as age and gender, the times of sampling in relation to the probands' circadian rhythm as well as intake of food and medication also need to be recorded in ex-sRNA biomarker studies and considered as potential confounding factors.

To avoid technique-derived biases, protocols should be kept constant within one study, including clinical routines such as storage times of samples and blood fractionation. Standardized reporting of methodological detail (34, 96) can highlight potential differences incurred by methodological differences and should therefore be a minimum requirement for any biomarker study. Cross-validation of potential biomarker RNAs using independent techniques would decrease reporting on false-positive results. Standardization of protocols for sampling, sRNA isolation, and analytics would furthermore facilitate the comparability of studies and help pinpoint the origin of different findings to cohort characteristics rather than methodology.

The lack of specificity of ex-sRNAs to discriminate between specific diseases is also considered a major limitation explaining in part why clinical use has not yet been established. For example, miR-141 has been shown to exhibit elevated levels in the blood of pregnant women (46). However, it has also been proposed as a biomarker for prostate cancer, breast cancer, lung cancer and colorectal cancer (126, 217).

To address the issue of specificity, several databases summarizing the so far identified associations between human ex-miRNAs and diseases have been created (89, 90, 115) and these represent essential efforts which can help to assess the specificity of potential biomarkers. In order to improve specificity, it might be useful to concentrate the search for biomarkers on ex-sRNAs that are not implicated in general pathology-related mechanisms, such as inflammation, but rather on ex-sRNAs specific for a given disease and which are normally not present or only rarely present in circulation. To establish such rare ex-sRNAs as suitable biomarkers, their cellular origin needs to be investigated and their functional and mechanistic associations to a specific disease need to be verified.

Finally, it is clear that the complexity of potential information in the spectrum of circulating ex-sRNA can become an advantage as methods for developing multi-RNA panels, whose collective patterns indicate important medical information, are developed and perfected.

6. Future directions and unanswered questions

The prevalence and characteristics of ex-sRNA in human circulation offer particularly exciting prospects for the development of novel diagnostic and therapeutic approaches as the biological information inherent in their levels is potentially large. However, as might be expected for a field barely 10 years old, several controversies remain to be resolved and numerous questions remain unanswered (Sidebar).

For endogenous ex-sRNA-mediated endocrine and paracrine signaling, major points of contention revolve around questions of (I) selectivity, (II) specificity, and (III) sensitivity.

- (I)** Endogenous ex-sRNAs have been suggested to be majorly comprised of byproducts of dead cells (194, 195). Therefore, the overall selectivity of sRNA export and import may therefore be questioned. However, the apparent distinctive packaging and enrichment of certain ex-sRNA into extracellular vesicles, exosomes and high-density lipoprotein complexes does suggest selection of distinct moieties prior to export. However, the exact processes underlying ex-sRNA export into the extracellular space, their selective transfer to recipient cells and their subsequent import into recipient cells require detailed elucidation.
- (II)** In order for specific processes in distinct cell types, tissues or organs to be targeted, a precise ex-sRNA delivery mechanism must exist. At present, ex-sRNAs have been found to be associated with microvesicles, exosomes, high-density lipoproteins and apoptotic bodies in circulation. However, the vast majority of extracellular miRNA has been found to be complexed with ribonucleoproteins, e.g. argonaute, nucleophosmin, etc. which may suggest that specificity of delivery is largely absent (192). The relative importance of each of these potential delivery vehicles in relation to the delivery of sRNAs to specific cells/tissues requires further in-depth in vitro, ex vivo and in vivo studies in particular against the apparent background of non-specific sRNAs derived from cell lysis.
- (III)** Given that total miRNA concentrations in plasma are in the 100 fM range and that single miRNAs are typically present at a fraction of that concentration, doubts have been raised whether the amounts of sRNA in circulation would be sufficient to induce a response in recipient cells, tissues and organs analogous to those of hormones which are typically present in the pM range (214). Consequently, the precise threshold concentrations for ex-sRNAs to trigger a physiological response in recipient cells/tissues need to be elucidated in relation to the distinct delivery mechanisms which have so far described in addition to their endocytotic pathways, their intracellular RNA receptors and likely signal amplification mechanisms. To increase the degree of the challenge, concentration thresholds are likely to be specific for a given affected molecular pathway.

In addition to the above questions, the study of the characteristics and impact of exogenous ex-sRNA in human circulation are also faced with several controversies. Major points of contention revolve around their (IV) presence, (V) transfer, and (VI) impact.

- (IV)** The presence of exogenous ex-sRNA in mammalian circulation has been the subject of much recent debate and controversy. Early studies claimed that exogenous ex-sRNAs are present in mammalian blood (172, 205, 232), these results were subsequently questioned (53, 180, 216, 220) but recent studies support the earlier findings (21, 117, 129, 223, 236). Given the number of studies on both sides of the argument, one may cautiously conclude that the jury is still out regarding the presence of exogenous ex-sRNA in mammalian circulation. However, given the weight of evidence and likely importance of

exogenous ex-sRNAs in human circulation, we suggest that multi-center studies involving partners from different geographical locales should be organized and conducted to describe the exogenous ex-sRNA complement in human circulation. Furthermore, experiments involving animal models need to be carefully standardized and sRNA detection methods harmonized. In particular, a consensus should be reached on methods for treating animals with respect to factors that may affect barrier function, detecting exogenous ex-sRNAs in human circulation in particular allowing the detection of plant miRNAs which carry 2'-O-methyl modifications on the ribose of the 3' terminal nucleotide. A reference compendium of commonly encountered exogenous ex-sRNAs in human circulation should also be compiled. A joint, international effort driven by a quest for consensus would likely be most successful in this context.

- (V) Although recent work has linked the transfer of exogenous ex-sRNA to epithelial barrier function (223), the precise mechanisms involved in the uptake of exogenous ex-RNA from the gastrointestinal tract and its transfer into blood remain unclear especially in view of the apparent differences between studies which have detected exogenous ex-RNA in circulation (21, 68, 94, 117, 192, 205, 214, 220), compared to those that have failed to do so (53, 180, 220). The exact mechanisms involved in the transfer of exogenous ex-sRNAs from the gastrointestinal tract into human cells, tissues, organs and circulation therefore require detailed elucidation. More specifically, the processes of packaging, absorption, endocytosis and transcytosis need to be further described. Furthermore, given the importance of the human gastrointestinal microbiome in modulating barrier function (94) as well exogenous ex-RNAs *sui generis* (68, 165, 205), the precise role of the microbiome in facilitating the transfer of and contributing to the exogenous ex-sRNA complement calls for more advanced study.
- (VI) Early studies (205, 232) as well as more recent studies (129) suggest that exogenous ex-sRNAs impact cellular, tissue and organismal physiology. However, negative results about the presence of exogenous ex-sRNA in circulation as well as apparent low levels have cast doubts over such suggestions. Similar to the situation regarding endogenous ex-sRNAs (see point III above), assessment of the biological impact of exogenous ex-sRNAs requires further detailed study. Both localized (129) as well as systemic (232) effects have been reported, but these need to be validated in independent experiments. Standardized cell-based screening assays as well as appropriate animal models for assessing the impacts of exogenous ex-sRNAs should be established and shared within the community. In relation to exogenous ex-sRNAs, its role in immune system function should be specifically addressed as well as the question whether there is continuous rather than sporadic trans-kingdom regulation of gene expression via exogenous ex-sRNAs *in vivo*.

The potential of ex-sRNAs for the development of novel diagnostic and therapeutic approaches are indeed tremendous. However, given the currently missing knowledge, numerous questions and controversies, we suggest that organized, concerted efforts, like the

US NIH's Extracellular RNA Communication Consortium (138) by the international research community are required in coming years to find answers to these in order to move the field forward.

Acknowledgments

The present work was supported by a CORE programme grant (CORE14/BM/8066232) to JVF as well as an ATTRACT programme grant (A09/03), CORE programme grant (CORE11/BM/1186762), European Union Joint Programming in Neurodegenerative Diseases grant (INTER/JPND/12/01), and Proof-of-Concept grant (PoC/13/02) to PW, all funded by the Luxembourg National Research Fund (FNR).

References

1. Abdullah Z, Schlee M, Roth S, Mraheil MA, Barchet W, et al. RIG-I detects infection with live *Listeria* by sensing secreted bacterial nucleic acids. *EMBO J*. 2012; 31(21):4153–64. [PubMed: 23064150]
2. Aird D, Ross MG, Chen W-S, Danielsson M, Fennell T, et al. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome Biol*. 2011; 12(2):R18. [PubMed: 21338519]
3. Akat KM, Moore-McGriff D, Morozov P, Brown M, Gogakos T, et al. Comparative RNA-sequencing analysis of myocardial and circulating small RNA s in human heart failure and their utility as biomarkers. *Proc Natl Acad Sci U S A*. 2014; 111(30):11151–56. [PubMed: 25012294]
4. Akay A, Sarkies P, Miska EA. *E. coli* oxyS non-coding RNA does not trigger RNA i in *c. elegans*. *Sci Rep*. 2015; 5:9597. [PubMed: 25873159]
5. Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*. 2013; 113(1): 1–11. [PubMed: 23456661]
6. Akers JC, Ramakrishnan V, Kim R, Skog J, Nakano I, et al. Mir-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. *PLoS One*. 2013; 8(10):e78115. [PubMed: 24205116]
7. Allegra A, Alonci A, Campo S, Penna G, Petrungaro A, Gerace DMC. Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer (review). *Int J Oncol*. 2012; 41(6):1897–1912. [PubMed: 23026890]
8. Almanza G, Anufreichik V, Rodvold JJ, Chiu KT, DeLaney A, et al. Synthesis and delivery of short, noncoding RNA by B lymphocytes. *Proc Natl Acad Sci U S A*. 2013; 110(50):20182–87. [PubMed: 24277816]
9. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol*. 2010; 11(10):R106. [PubMed: 20979621]
10. Anderson MT, Seifert HS. Opportunity and means: horizontal gene transfer from the human host to a bacterial pathogen. *MBio*. 2011; 2(1):e00005–00011. [PubMed: 21325040]
11. Anderson P, Ivanov P. tRNA fragments in human health and disease. *FEBS Lett*. 2014; 588(23): 4297–4304. [PubMed: 25220675]
12. Appaiah HN, Goswami CP, Mina L, Badve S, Sledge GW, et al. Persistent upregulation of U6:SNORD44 small RNA ratio in the serum of breast cancer patients. *Breast Cancer Res*. 2011; 13(5):R86. [PubMed: 21914171]
13. Archambaud C, Sismeiro O, Toedling J, Soubigou G, Bécavin C, et al. The intestinal microbiota interferes with the microRNA response upon oral *Listeria* infection. *MBio*. 2013; 4(6):e00707–13. [PubMed: 24327339]
14. Arkov AL, Mankin A, Murgola EJ. An rRNA fragment and its antisense can alter decoding of genetic information. *J Bacteriol*. 1998; 180(10):2744–48. [PubMed: 9573162]
15. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011; 108(12):5003–8. [PubMed: 21383194]

16. Bahn JH, Zhang Q, Li F, Chan T-M, Lin X, et al. The landscape of microRNA, PIWI-interacting RNA, and circular RNA in human saliva. *Clin Chem*. 2015; 61(1):221–30. [PubMed: 25376581]
17. Bai X, Fischer S, Keshavjee S, Liu M. Heparin interference with reverse transcriptase polymerase chain reaction of RNA extracted from lungs after ischemia-reperfusion. *Transpl Int*. 2000; 13(2): 146–50. [PubMed: 10836652]
18. Baier SR, Nguyen C, Xie F, Wood JR, Zemleni J. MicroRNA s are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. *J Nutr*. 2014; 144(10):1495–1500. [PubMed: 25122645]
19. Bala S, Petrasko J, Mundkur S, Catalano D, Levin I, et al. Circulating micrornas in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology*. 2012; 56(5):1946–57. [PubMed: 22684891]
20. Batagov AO, Kurochkin IV. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. *Biol Direct*. 2013; 8:12. [PubMed: 23758897]
21. Beatty M, Guduric-Fuchs J, Brown E, Bridgett S, Chakravarthy U, et al. Small RNAs from plants, bacteria and fungi within the order Hypocreales are ubiquitous in human plasma. *BMC Genomics*. 2014; 15:933. [PubMed: 25344700]
22. Beckett EL, Martin C, Duesing K, Jones P, Furst J, et al. Vitamin D receptor genotype modulates the correlation between vitamin D and circulating levels of let-7a/b and vitamin D intake in an elderly cohort. *J Nutrigenet Nutrigenomics*. 2014; 7(4–6):264–73. [PubMed: 25969372]
23. Biller SJ, Schubotz F, Roggensack SE, Thompson AW, Summons RE, Chisholm SW. Bacterial vesicles in marine ecosystems. *Science*. 2014; 343(6167):183–86. [PubMed: 24408433]
24. Boeckel J-N, Thomé CE, Leistner D, Zeiher AM, Fichtlscherer S, Dimmeler S. Heparin selectively affects the quantification of microRNAs in human blood samples. *Clin Chem*. 2013; 59(7):1125–27. [PubMed: 23613337]
25. Boelens MC, Wu TJ, Nabet BY, Xu B, Qiu Y, et al. Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. *Cell*. 2014; 159(3):499–513. [PubMed: 25417103]
26. Bomberger JM, Maceachran DP, Coutermarsh B, Ye S, O'Toole G, Stanton B. Long-distance delivery of bacterial virulence factors by *pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog*. 2009; 5(4):e1000382. [PubMed: 19360133]
27. Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD. Genetically haploid spermatids are phenotypically diploid. *Nature*. 1989; 337(6205):373–6. [PubMed: 2911388]
28. Brosnan CA, Voinnet O. Cell-to-cell and long-distance siRNA movement in plants: mechanisms and biological implications. *Curr Opin Plant Biol*. 2011; 14(5):580–87. [PubMed: 21862389]
29. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol*. 2015; 13(10):620–30. [PubMed: 26324094]
30. Bryniarski K, Ptak W, Martin E, Nazimek K, Szczepanik M, et al. Free extracellular mirna functionally targets cells by transfecting exosomes from their companion cells. *PLoS One*. 2015; 10(4):e0122991. [PubMed: 25923429]
31. Bryzgunova OE, Laktionov PP. Extracellular nucleic acids in urine: sources, structure, diagnostic potential. *Acta Naturae*. 7(3):48–54.
32. Buck AH, Coakley G, Simbari F, McSorley HJ, Quintana JF, et al. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nat Commun*. 2014; 5:5488. [PubMed: 25421927]
33. Burroughs AM, Ando Y, de Hoon MJL, Tomaru Y, Suzuki H, et al. Deep-sequencing of human Argonaute-associated small RNAs provides insight into mirna sorting and reveals Argonaute association with RNA fragments of diverse origin. *RNA Biol*. 8(1):158–77.
34. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009; 55(4): 611–22. [PubMed: 19246619]
35. Bye A, Røsjø H, Aspenes ST, Condorelli G, Omland T, Wisløff U. Circulating microRNAs and aerobic fitness--the HUNT-study. *PLoS One*. 2013; 8(2):e57496. [PubMed: 23469005]

36. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis*. 2015; 26:26191. [PubMed: 25651997]
37. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell*. 2009; 136(4):642–55. [PubMed: 19239886]
38. Chahar HS, Bao X, Casola A. Exosomes and their role in the life cycle and pathogenesis of RNA viruses. *Viruses*. 2015; 7(6):3204–25. [PubMed: 26102580]
39. Chakraborty SK, Prakash A, Nechooshtan GaL, Hearn S, Gingeras TR. Extracellular vesicle-mediated transfer of processed and functional RNY5RNA. *RNA*. 2015; 21(11):1966–79. [PubMed: 26392588]
40. Chen X, Ba Y, Ma L, Cai X, Yin Y, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008; 18(10):997–1006. [PubMed: 18766170]
41. Chen X, Liang H, Zhang J, Zen K, Zhang C-Y. MicroRNAs are ligands of Toll-like receptors. *RNA*. 2013; 19(6):737–9. [PubMed: 23554231]
42. Chen X, Zen K, Zhang C-Y. Reply to lack of detectable oral bioavailability of plant microRNAs after feeding in mice. *Nat Biotechnol*. 2013; 31(11):967–9. [PubMed: 24213764]
43. Cheng G, Luo R, Hu C, Cao J, Jin Y. Deep sequencing-based identification of pathogen-specific microRNAs in the plasma of rabbits infected with *Schistosoma japonicum*. *Parasitology*. 2013; 140(14):1751–61. [PubMed: 23942009]
44. Chevillet JR, Kang Q, Ruf IK, Briggs Ha, Vojtech LN, et al. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc Natl Acad Sci*. 2014; 111(41):14888–93. [PubMed: 25267620]
45. Chiantia S, Kahya N, Ries J, Schwille P. Effects of ceramide on liquid-ordered domains investigated by simultaneous AFM and FCS. *Biophys J*. 2006; 90(12):4500–8. [PubMed: 16565041]
46. Chim SS, Shing TK, Hung EC, Leung T-Y, Lau T-K, et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem*. 2008; 54(3):482–90. [PubMed: 18218722]
47. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol*. 2009; 19(2):43–51. [PubMed: 19144520]
48. De Boer HC, van Solingen C, Prins J, Duijs JM, Huisman MV, et al. Aspirin treatment hampers the use of plasma microRNA-126 as a biomarker for the progression of vascular disease. *Eur Heart J*. 2013; 34(44):3451–57. [PubMed: 23386708]
49. De Maio A. Extracellular heat shock proteins, cellular export vesicles, and the stress observation system: a form of communication during injury, infection, and cell damage. it is never known how far a controversial finding will go! dedicated to ferruccio ritossa. *Cell Stress Chaperones*. 2011; 16(3):235–49. [PubMed: 20963644]
50. Dhahbi J, Spindler S. 5'-YRNA fragments derived by processing of transcripts from specific yRNA genes and pseudogenes are abundant in human serum and plasma. *Physiol Genomics*. 2013; 45(21):990–8. [PubMed: 24022222]
51. Dhahbi JM, Spindler SR, Atamna H, Boffelli D, Martin DI. Deep sequencing of serum small RNAs identifies patterns of 5' tRNA half and YRNA fragment expression associated with breast cancer. *Biomark Cancer*. 2014; 6:37–47. [PubMed: 25520563]
52. Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Boffelli D, et al. 5' tRNA halves are present as abundant complexes in serum, concentrated in blood cells, and modulated by aging and calorie restriction. *BMC Genomics*. 2013; 14:298. [PubMed: 23638709]
53. Dickinson B, Zhang Y, Petrick JS, Heck G, Ivashuta S, Marshall WS. Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. *Nat Biotechnol*. 2013; 31(11):965–67. [PubMed: 24213763]
54. Duxbury MS, Ashley SW, Whang EE. RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem Biophys Res Commun*. 2005; 331(2):459–63. [PubMed: 15850781]
55. Ekström K, Valadi H, Sjöstrand M, Malmhäll C, Bossios A, et al. Characterization of mRNA and microRNA in human mast cell-derived exosomes and their transfer to other mast cells and blood CD34 progenitor cells. *J Extracell Vesicles*. 2012; 1

56. Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem*. 1998; 273(32):20121–7. [PubMed: 9685355]
57. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. *Mutat Res*. 2011; 717(1–2):85–90. [PubMed: 21402084]
58. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A*. 2012; 109(31):E2110–16. [PubMed: 22753494]
59. Feinberg EH, Hunter CP. Transport of dsRNA into cells by the transmembrane protein SID-1. *Science*. 2003; 301(5639):1545–7. [PubMed: 12970568]
60. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, et al. Circulating microRNAs in patients with coronary artery disease. *Circ Res*. 2010; 107(5):677–84. [PubMed: 20595655]
61. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391(6669):806–11. [PubMed: 9486653]
62. Fritz, JV. Extracellular RNA in Bacteria - exRNA. exRNA research portal. 2015. <http://exrna.org/extracellular-rna-bacteria/>
63. Fu X-D. Non-coding RNA: a new frontier in regulatory biology. *Natl Sci Rev*. 2014; 1(2):190–204. [PubMed: 25821635]
64. Fuchs RT, Sun Z, Zhuang F, Robb GB. Bias in ligation-based small RNA sequencing library construction is determined by adaptor and RNA structure. *PLoS One*. 2015; 10(5):e0126049. [PubMed: 25942392]
65. García ME, Blanco JL, Caballero J, Gargallo-Viola D. Anticoagulants interfere with PCR used to diagnose invasive *Aspergillosis*. *J Clin Microbiol*. 2002; 40(4):1567–68. [PubMed: 11923400]
66. Garcia-Silva MR, das Neves R, Cabrera-Cabrera F, Sanguinetti J, Medeiros LC, et al. Extracellular vesicles shed by *Trypanosoma cruzi* are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. *Parasitol Res*. 2014; 113(1):285–304. [PubMed: 24241124]
67. Gebetsberger J, Polacek N. Slicing tRNA to boost functional ncRNA diversity. *RNA Biol*. 2013; 10(12):1798–1806. [PubMed: 24351723]
68. Ghosal A, Upadhyaya BB, Fritz JV, Heintz-Buschart A, Desai MS, et al. The extracellular RNA complement of *Escherichia coli*. *Microbiologyopen*. 2015; 4(2):252–66.
69. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol*. 2009; 11(9):1143–9. [PubMed: 19684575]
70. Grant R, Ansa-Addo E, Stratton D, Antwi-Baffour S, Jorfi S, et al. A filtration-based protocol to isolate human plasma membrane-derived vesicles and exosomes from blood plasma. *J Immunol Methods*. 2011; 371(1–2):143–51. [PubMed: 21741384]
71. Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol*. 2009; 124(1):3–20. [PubMed: 19560575]
72. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC Genomics*. 2012; 13:357. [PubMed: 22849433]
73. Guduric-Fuchs J, O'Connor A, Cullen A, Harwood L, Medina RJ, et al. Deep sequencing reveals predominant expression of miR-21 amongst the small non-coding RNAs in retinal microvascular endothelial cells. *J Cell Biochem*. 2012; 113(6):2098–2111. [PubMed: 22298343]
74. Gutiérrez-Vázquez C, Villarroya-Beltri C, Mittelbrunn M, Sánchez-Madrid F. Transfer of extracellular vesicles during immune cell-cell interactions. *Immunol Rev*. 2013; 251(1):125–42. [PubMed: 23278745]
75. Hafner M, Renwick N, Brown M, Mihailovi A, Holoch D, et al. RNA-ligase-dependent biases in miRNA representation in deep-sequenced small RNA cDNA libraries. *RNA*. 2011; 17(9):1697–1712. [PubMed: 21775473]

76. Halkein J, Tabruyn SP, Ricke-Hoch M, Haghikia A, Nguyen N-Q-N, et al. MicroRNA-146a is a therapeutic target and biomarker for peripartum cardiomyopathy. *J Clin Invest.* 2013; 123(5): 2143–54. [PubMed: 23619365]
77. Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ, Kay Ma. Human tRNA-derived small rnas in the global regulation of RNA silencing. *RNA.* 2010; 16(4):673–95. [PubMed: 20181738]
78. Hebels DG, van Herwijnen MH, Brauers KJ, de Kok TM, Chalkiadaki G, et al. Elimination of heparin interference during microarray processing of fresh and biobank-archived blood samples. *Environ Mol Mutagen.* 2014; 55(6):482–91. [PubMed: 24740823]
79. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods.* 2013; 10(10):1003–5. [PubMed: 23995387]
80. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer.* 2004; 4(1):45–60. [PubMed: 14681689]
81. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science.* 2012; 336(6086):1268–73. [PubMed: 22674334]
82. Ismail N, Wang Y, Dakhallah D, Moldovan L, Agarwal K, et al. Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. *Blood.* 2013; 121(6):984–95. [PubMed: 23144169]
83. Ivanov P, Emara MM, Villen J, Gygi SP, Anderson P. Angiogenin-induced tRNA fragments inhibit translation initiation. *Mol Cell.* 2011; 43(4):613–23. [PubMed: 21855800]
84. Iwasaki YW, Siomi MC, Siomi H. PIWI-interacting RNA: its biogenesis and functions. *Annu Rev Biochem.* 2015; 84:405–33. [PubMed: 25747396]
85. Jakymiw A, Pauley KM, Li S, Ikeda K, Lian S, et al. The role of GW/P-bodies in RNA processing and silencing. *J Cell Sci.* 2007; 120(Pt 8):1317–23. [PubMed: 17401112]
86. Janas T, Janas MM, Sapota K, Janas T. Mechanisms of RNA loading into exosomes. *FEBS Lett.* 2015; 589:1391–98. [PubMed: 25937124]
87. Janas T, Janas T, Yarus M. Specific RNA binding to ordered phospholipid bilayers. *Nucleic Acids Res.* 2006; 34(7):2128–36. [PubMed: 16641318]
88. Jayaprakash AD, Jabado O, Brown BD, Sachidanandam R. Identification and remediation of biases in the activity of RNA ligases in small-RNA deep sequencing. *Nucleic Acids Res.* 2011; 39(21):e141. [PubMed: 21890899]
89. Ji Diana Lee Y, Kim V, Muth DC, Witwer KW. Validated microRNA target databases: an evaluation. *Drug Dev Res.* 2015; 76(7):389–96. [PubMed: 26286669]
90. Jiang Q, Wang Y, Hao Y, Juan L, Teng M, et al. miR2Disease: a manually curated database for microRNA deregulation in human disease. *Nucleic Acids Res.* 2009; 37(Database):D98–104. [PubMed: 18927107]
91. Jose AM, Garcia GA, Hunter CP. Two classes of silencing RNAs move between *Caenorhabditis elegans* tissues. *Nat Struct Mol Biol.* 2011; 18(11):1184–88. [PubMed: 21984186]
92. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, et al. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature.* 2003; 421(6920):231–37. [PubMed: 12529635]
93. Scott, Baier. MicroRNA are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow's milk and chicken egg affect gene expression in peripheral blood mononuclear cells, cell cultures, and mouse liver. *Nutrition and Health Sciences Dissertation & Theses.* 2015. Paper 50 <http://digitalcommons.unl.edu/nutritiondiss/50>
94. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature.* 2011; 474(7351):327–36. [PubMed: 21677749]
95. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, et al. Haemolysis during sample preparation alters microRNA content of plasma. *PLoS One.* 2011; 6(9):e24145. [PubMed: 21909417]
96. Kirschner MB, van Zandwijk N, Reid G. Cell-free microRNAs: potential biomarkers in need of standardized reporting. *Front Genet.* 2013; 4:56. [PubMed: 23626598]
97. Knip M, Constantin ME, Thordal-Christensen H. Trans-kingdom crosstalk: small RNAs on the move. *PLoS Genet.* 2014; 10(9):e1004602. [PubMed: 25188222]

98. Kogure T, Lin W-L, Yan IK, Braconi C, Patel T. Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. *Hepatology*. 2011; 54(4):1237–48. [PubMed: 21721029]
99. Kogure T, Yan IK, Lin W-L, Patel T. Extracellular vesicle-mediated transfer of a novel long noncoding RNA TUC339: a mechanism of intercellular signaling in human hepatocellular cancer. *Genes Cancer*. 2013; 4(7–8):261–72. [PubMed: 24167654]
100. Koppers-Lalic D, Hackenberg M, Bijnsdorp I, van Eijndhoven M, Sadek P, et al. Non-templated nucleotide additions distinguish the small RNA composition in cells from exosomes. *Cell Rep*. 2014; 8(6):1649–58. [PubMed: 25242326]
101. Kosaka N, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, Ochiya T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *J Biol Chem*. 2013; 288(15):10849–59. [PubMed: 23439645]
102. Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci*. 2010; 101(10):2087–92. [PubMed: 20624164]
103. Kosaka N, Iguchi H, Yoshioka Y, Hagiwara K, Takeshita F, Ochiya T. Competitive interactions of cancer cells and normal cells via secretory microRNAs. *J Biol Chem*. 2012; 287(2):1397–1405. [PubMed: 22123823]
104. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem*. 2010; 285(23):17442–52. [PubMed: 20353945]
105. Kosaka N, Izumi H, Sekine K, Ochiya T. MicroRNA as a new immune-regulatory agent in breast milk. *Silence*. 2010; 1(1):7. [PubMed: 20226005]
106. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-pcr (qRT-PCR). *Methods*. 2010; 50(4):298–301. [PubMed: 20146939]
107. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev*. 2005; 19(22):2645–55. [PubMed: 16291643]
108. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2002; 12(9):735–39. [PubMed: 12007417]
109. Lara PC, Pruschy M, Zimmermann M, Henríquez-Hernández LA. MVP and Vaults: a role in the radiation response. *Radiat Oncol*. 2011; 6:148. [PubMed: 22040803]
110. Lasda E, Parker R. Circular RNAs: diversity of form and function. *RNA*. 2014; 20(12):1829–42. [PubMed: 25404635]
111. Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol*. 2008; 141(5):672–75. [PubMed: 18318758]
112. Lee LW, Zhang S, Etheridge A, Ma L, Martin D, et al. Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA*. 2010; 16(11):2170–80. [PubMed: 20876832]
113. Lehmann SM, Krüger C, Park B, Derkow K, Rosenberger K, et al. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat Neurosci*. 2012; 15(6):827–35. [PubMed: 22610069]
114. Leidner RS, Li L, Thompson CL. Dampening enthusiasm for circulating microRNA in breast cancer. *PLoS One*. 2013; 8(3):e57841. [PubMed: 23472110]
115. Li Y, Qiu C, Tu J, Geng B, Yang J, Jiang T, Cui Q. HMDD v2.0: a database for experimentally supported human microRNA and disease associations. *Nucleic Acids Res*. 2014; 42(Database Issue):D1070-4. [PubMed: 24194601]
116. Li Z, Ender C, Meister G, Moore PS, Chang Y, John B. Extensive terminal and asymmetric processing of small RNAs from rRNAs, snoRNAs, snRNAs, and tRNAs. *Nucleic Acids Res*. 2012; 40(14):6787–99. [PubMed: 22492706]
117. Liang H, Zhang S, Fu Z, Wang Y, Wang N, et al. Effective detection and quantification of dietetically absorbed plant microRNAs in human plasma. *J Nutr Biochem*. 2015; 26(5):505–12. [PubMed: 25704478]

118. Lim PK, Bliss Sa, Patel Sa, Taborga M, Dave Ma, et al. Gap junction-mediated import of microRNA from bone marrow stromal cells can elicit cell cycle quiescence in breast cancer cells. *Cancer Res.* 2011; 71(5):1550–60. [PubMed: 21343399]
119. Liu H, Wang X, Wang H-D, Wu J, Ren J, et al. *Escherichia coli* noncoding RNAs can affect gene expression and physiology of *Caenorhabditis elegans*. *Nat Commun.* 2012; 3:1073. [PubMed: 23011127]
120. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature.* 2006; 441(7090):231–34. [PubMed: 16648837]
121. Mao Y-B, Xue X-Y, Tao X-Y, Yang C-Q, Wang L-J, Chen X-Y. Cysteine protease enhances plant-mediated bollworm RNA interference. *Plant Mol Biol.* 2013; 83(1–2):119–29. [PubMed: 23460027]
122. Martí E, Pantano L, Bañez-Coronel M, Llorens F, Miñones-Moyano E, et al. A myriad of miRNA variants in control and huntington's disease brain regions detected by massively parallel sequencing. *Nucleic Acids Res.* 2010; 38(20):7219–35. [PubMed: 20591823]
123. Marz M, Gruber AR, Höner zu Siederdissen C, Amman F, Badelt S, et al. Animal snoRNAs and scaRNAs with exceptional structures. *RNA Biol.* 2011; 8(6):938–46. [PubMed: 21955586]
124. Meckes DG, Shair KHY, Marquitz AR, Kung C-P, Edwards RH, Raab-Traub N. Human tumor virus utilizes exosomes for intercellular communication. *Proc Natl Acad Sci U S A.* 2010; 107(47):20370–75. [PubMed: 21059916]
125. Mishima E, Inoue C, Saigusa D, Inoue R, Ito K, et al. Conformational change in transfer RNAs is an early indicator of acute cellular damage. *J Am Soc Nephrol.* 2014; 25(10):2316–26. [PubMed: 24833129]
126. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A.* 2008; 105(30):10513–18. [PubMed: 18663219]
127. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltri C, González S, Sánchez-Cabo F, et al. Unidirectional transfer of microRNA-loaded exosomes from t cells to antigen-presenting cells. *Nat Commun.* 2011; 2:282. [PubMed: 21505438]
128. Mittelbrunn M, Sánchez-Madrid F. Intercellular communication: diverse structures for exchange of genetic information. *Nat Rev Mol Cell Biol.* 2012; 13(5):328–35. [PubMed: 22510790]
129. Mlotshwa S, Pruss GJ, MacArthur JL, Endres MW, Davis C, et al. A novel chemopreventive strategy based on therapeutic micromas produced in plants. *Cell Res.* 2015; 25(4):521–24. [PubMed: 25721325]
130. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan MLG, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood.* 2012; 119(3):756–66. [PubMed: 22031862]
131. Morel L, Regan M, Higashimori H, Ng SK, Esau C, et al. Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. *J Biol Chem.* 2013; 288(10):7105–16. [PubMed: 23364798]
132. Mulcahy LA, Pink RC, Carter DRF. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles.* 2014; 3:1–14.
133. Mullokandov G, Baccarini A, Ruzo A, Jayaprakash AD, Tung N, et al. High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries. *Nat Methods.* 2012; 9(8):840–46. [PubMed: 22751203]
134. Munafó DB, Robb GB. Optimization of enzymatic reaction conditions for generating representative pools of cDNA from small RNA. *RNA.* 2010; 16(12):2537–52. [PubMed: 20921270]
135. Narayanan A, Iordanskiy S, Das R, Van Duyne R, Santos S, et al. Exosomes derived from HIV-1-infected cells contain trans-activation response element RNA. *J Biol Chem.* 2013; 288(27):20014–33. [PubMed: 23661700]
136. Nguyen VT, Kiss T, Michels AA, Bensaude O. 7SK small nuclear RNA binds to and inhibits the activity of CDK9/cyclin T complexes. *Nature.* 2001; 414(6861):322–25. [PubMed: 11713533]

137. Nicola AM, Frases S, Casadevall A. Lipophilic dye staining of *Cryptococcus neoformans* extracellular vesicles and capsule. *Eukaryot Cell*. 2009; 8(9):1373–80. [PubMed: 19465562]
138. NIH. Extracellular RNA Communication Consortium. <http://exrna.org/>
139. Nolte-'t Hoen EN, Buermans HP, Waasdorp M, Stoorvogel W, Wauben MH, 't Hoen PA. Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions. *Nucleic Acids Res*. 2012; 40(18):9272–85. [PubMed: 22821563]
140. Obregón-Henao A, Duque-Correa M, Rojas M, García LF, Brennan PJ, et al. Stable extracellular RNA fragments of *Mycobacterium tuberculosis* induce early apoptosis in human monocytes via a caspase-8 dependent mechanism. *PLoS One*. 2012; 7(1):e29970. [PubMed: 22253841]
141. Oldenburg M, Krüger A, Ferstl R, Kaufmann A, Nees G, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science*. 2012; 337(6098):1111–15. [PubMed: 22821982]
142. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect Immun*. 2010; 78(4):1601–9. [PubMed: 20145096]
143. Page K, Guttery DS, Zahra N, Primrose L, Elshaw SR, et al. Influence of plasma processing on recovery and analysis of circulating nucleic acids. *PLoS One*. 2013; 8(10):e77963. [PubMed: 24205045]
144. Palazzo AF, Lee ES. Non-coding RNA: what is functional and what is junk? *Front Genet*. 2015; 6:2. [PubMed: 25674102]
145. Park K-S, Choi K-H, Kim Y-S, Hong BS, Kim OY, et al. Outer membrane vesicles derived from *Escherichia coli* induce systemic inflammatory response syndrome. *PLoS One*. 2010; 5(6):e11334. [PubMed: 20596524]
146. Parker H, Chitcholtan K, Hampton MB, Keenan JJ. Uptake of *Helicobacter pylori* outer membrane vesicles by gastric epithelial cells. *Infect Immun*. 2010; 78(12):5054–61. [PubMed: 20876296]
147. Patton JG, Franklin JL, Weaver AM, Vickers K, Zhang B, et al. Biogenesis, delivery, and function of extracellular rna. *J Extracell Vesicles*. 2015; 1:1–8.
148. Pegtel DM, Cosmopoulos K, Thorley-Lawson Da, van Eijndhoven MA, Hopmans ES, et al. Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A*. 2010; 107(14):6328–33. [PubMed: 20304794]
149. Peres da Silva R, Puccia R, Rodrigues ML, Oliveira DL, Joffe LS, et al. Extracellular vesicle-mediated export of fungal RNA. *Sci Rep*. 2015; 5:7763. [PubMed: 25586039]
150. Plieskatt JL, Feng Y, Rinaldi G, Mulvenna JP, Bethony JM, Brindley PJ. Circumventing qPCR inhibition to amplify miRNAs in plasma. *Biomark Res*. 2014; 2:13. [PubMed: 25075309]
151. Puppa MJ, White JP, Sato S, Cairns M, Baynes JW, Carson JA. Gut barrier dysfunction in the APC(Min/+) mouse model of colon cancer cachexia. *Biochim Biophys Acta*. 2011; 1812(12):1601–6. [PubMed: 21914473]
152. Quinn JF, Patel T, Wong D, Das S, Freedman JE, et al. Extracellular RNAs: development as biomarkers of human disease. *J Extracell vesicles*. 2015; 4(27495)
153. Quintana JF, Makepeace BL, Babayan SA, Ivens A, Pfarr KM, et al. Extracellular onchocerca-derived small RNAs in host nodules and blood. *Parasit Vectors*. 2015; 8:58. [PubMed: 25623184]
154. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. 2013; 200(4):373–83. [PubMed: 23420871]
155. Ratajczak J, Miekus K, Kucia M, Zhang J, Reca R, et al. Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia*. 2006; 20(5):847–56. [PubMed: 16453000]
156. Rekker K, Saare M, Roost AM, Kubo A-L, Zarovni N, et al. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem*. 2014; 47(1–2):135–38. [PubMed: 24183884]
157. Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE. The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform. *Gynecol Oncol*. 2009; 112(1):55–59. [PubMed: 18954897]

158. Ridder K, Keller S, Dams M, Rupp A-K, Schlaudraff J, et al. Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. *PLoS Biol.* 2014; 12(6):e1001874. [PubMed: 24893313]
159. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol.* 2014; 32(9):896–902. [PubMed: 25150836]
160. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015; 43(7):e47. [PubMed: 25605792]
161. Rome S. Use of miRNAs in biofluids as biomarkers in dietary and lifestyle intervention studies. *Genes Nutr.* 2015; 10(5):33.
162. Romilly C, Lays C, Tomasini A, Caldelari I, Benito Y, et al. A non-coding rna promotes bacterial persistence and decreases virulence by regulating a regulator in *Staphylococcus aureus*. *PLoS Pathog.* 2014; 10(3):e1003979. [PubMed: 24651379]
163. Rosa A, Brivanlou AH. MicroRNAs in early vertebrate development. *Cell Cycle.* 2009; 8(21):3513–20. [PubMed: 19875943]
164. Rosenblad MA, Larsen N, Samuelsson T, Zwieb C. Kinship in the srp RNA family. *RNA Biol.* 2014; 6(5):508–16.
165. Roume H, Muller EEL, Cordes T, Renaut J, Hiller K, Wilmes P. A biomolecular isolation framework for eco-systems biology. *ISME J.* 2013; 7(1):110–21. [PubMed: 22763648]
166. Ryu M-S, Langkamp-Henken B, Chang S-M, Shankar MN, Cousins RJ. Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. *Proc Natl Acad Sci U S A.* 2011; 108(52):20970–75. [PubMed: 22171008]
167. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 2014; 12(1):87. [PubMed: 25387460]
168. Samson JE, Magadán AH, Sabri M, Moineau S. Revenge of the phages: defeating bacterial defences. *Nat Rev Microbiol.* 2013; 11(10):675–87. [PubMed: 23979432]
169. Sana J, Faltejiskova P, Svoboda M, Slaby O. Novel classes of non-coding RNAs and cancer. *J Transl Med.* 2012; 10(1):103. [PubMed: 22613733]
170. Sato-Kuwabara Y, Melo SA, Soares FA, Calin GA. The fusion of two worlds: non-coding RNAs and extracellular vesicles - diagnostic and therapeutic implications (Review). *Int J Oncol.* 2014; 46(1):17–27. [PubMed: 25338714]
171. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nat Rev Microbiol.* 2015; 13(10):605–19. [PubMed: 26373371]
172. Semenov DV, Baryakin DN, Brenner EV, Kurilshikov AM, Vasiliev GV, et al. Unbiased approach to profile the variety of small non-coding RNA of human blood plasma with massively parallel sequencing technology. *Expert Opin Biol Ther.* 2012; 12(Suppl 1):S43–51. [PubMed: 22509727]
173. Sewer A, Gubian S, Kogel U, Veljkovic E, Han W, et al. Assessment of a novel multi-array normalization method based on spike-in control probes suitable for microRNA datasets with global decreases in expression. *BMC Res Notes.* 2014; 7:302. [PubMed: 24886675]
174. Shao Y, Feng L, Rutherford ST, Papenfort K, Bassler BL. Functional determinants of the quorum-sensing non-coding RNAs and their roles in target regulation. *EMBO J.* 2013; 32(15):2158–71. [PubMed: 23838640]
175. Sharifpanah F, Jayarathne SWG, Bekhite MM, Hurtado-Oliveros J, Preissner KT, et al. Stimulation of vasculogenesis and leukopoiesis of embryonic stem cells by extracellular transfer RNA and ribosomal RNA. *Free Radic Biol Med.* 2015; 89:1203–17. [PubMed: 26524400]
176. Shen J, Stass SA, Jiang F. MicroRNAs as potential biomarkers in human solid tumors. *Cancer Lett.* 2013; 329(2):125–36. [PubMed: 23196059]
177. Shen Y, Giardino Torchia ML, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. *Cell Host Microbe.* 2012; 12(4):509–20. [PubMed: 22999859]
178. Shih JD, Hunter CP. SID-1 is a dsRNA-selective dsRNA-gated channel. *RNA.* 2011; 17(6):1057–65. [PubMed: 21474576]

179. Skog J, Wurdinger T, Van Rijn S, Meijer D, Gainche L, et al. Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2012; 10(12):1470–76.
180. Snow JW, Hale AE, Isaacs SK, Baggish AL, Chan SY. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. *RNA Biol.* 2013; 10(7):1107–16. [PubMed: 23669076]
181. Sobala A, Hutvagner G. Small RNAs derived from the 5' end of tRNA can inhibit protein translation in human cells. *RNA Biol.* 2013; 10(4):553–63. [PubMed: 23563448]
182. Squadrito ML, Baer C, Burdet F, Maderna C, Gilfillan GD, et al. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. *Cell Rep.* 2014; 8:1432–46. [PubMed: 25159140]
183. Strimbu K, Tavel JA. What are biomarkers? *Curr Opin HIV AIDS.* 2010; 5(6):463–66. [PubMed: 20978388]
184. Tabet F, Vickers KC, Cuesta-Torres LF, Wiese Carrie B, Shoucri BM, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2012; 29(6):997–1003.
185. Tarallo S, Pardini B, Mancuso G, Rosa F, Di Gaetano C, et al. MicroRNA expression in relation to different dietary habits: a comparison in stool and plasma samples. *Mutagenesis.* 2014; 29(5):385–91. [PubMed: 25150024]
186. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol.* 2009; 9(8):581–93. [PubMed: 19498381]
187. Title AC, Denzler R, Stoffel M. Uptake and function studies of maternal milk-derived microRNAs. *J Biol Chem.* 2015; 290(39):23680–91. [PubMed: 26240150]
188. Tosar JP, Gambaro F, Sanguinetti J, Bonilla B, Witwer KW, Cayota. Assessment of small RNA sorting into different extracellular fractions revealed by high-throughput sequencing of breast cell lines. *Nucleic Acids Res.* 2015; 43(11):5601–16. [PubMed: 25940616]
189. Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol.* 2013; 31(1):46–53. [PubMed: 23222703]
190. Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem.* 2002; 48(10):1647–53. [PubMed: 12324479]
191. Tsuji E, Hiki N, Nomura S, Fukushima R, Kojima J, et al. Simultaneous onset of acute inflammatory response, sepsis-like symptoms and intestinal mucosal injury after cancer chemotherapy. *Int J Cancer.* 2003; 107(2):303–8. [PubMed: 12949812]
192. Turchinovich, a, Samatov, TR., Tonevitsky, aG, Burwinkel, B. Circulating miRNAs: cell-cell communication function? *Front Genet.* 2013; 4(JUN):1–10.
193. Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin and function. *Trends Biochem Sci.* 2012; 37(11):460–65. [PubMed: 22944280]
194. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 2011; 39(16):7223–33. [PubMed: 21609964]
195. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007; 9(6):654–59. [PubMed: 17486113]
196. Van Balkom BWM, Eisele AS, Pegtel DM, Bervoets S, Verhaar MC. Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting. *J Extracell Vesicles.* 2015; 4:26760. [PubMed: 26027894]
197. Van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev.* 2012; 64(3):676–705. [PubMed: 22722893]
198. Van Deun J, Mestdagh P, Sormunen R, Cocquyt V, Vermaelen K, et al. The impact of disparate isolation methods for extracellular vesicles on downstream RNA profiling. *J Extracell Vesicles.* 2014; 3

199. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011; 13(4):423–33. [PubMed: 21423178]
200. Vickers KC, Roteta LA, Hucheson-Dilks H, Han L, Guo Y. Mining diverse small rna species in the deep transcriptome. *Trends Biochem Sci.* 2015; 40(1):4–7. [PubMed: 25435401]
201. Villarroya-Beltri C, Baixauli F, Gutiérrez-Vázquez C, Sánchez-Madrid F, Mittelbrunn M. Sorting it out: regulation of exosome loading. *Semin Cancer Biol.* 2014; 28:1–11. [PubMed: 24993580]
202. Villarroya-Beltri C, Gutiérrez-Vázquez C, Sánchez-Cabo F, Pérez-Hernández D, Vázquez J, et al. Sumoylated hnRNP2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat Commun.* 2013; 4:2980. [PubMed: 24356509]
203. Voellenkle C, van Rooij J, Guffanti A, Brini E, Fasanaro P, et al. Deep-sequencing of endothelial cells exposed to hypoxia reveals the complexity of known and novel microRNAs. *RNA.* 2012; 18(3):472–84. [PubMed: 22282338]
204. Wagner J, Riwanto M, Besler C, Knau A, Fichtlscherer S, et al. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. *Arterioscler Thromb Vasc Biol.* 2013; 33(6):1392–1400. [PubMed: 23559634]
205. Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, et al. The complex exogenous RNA spectra in human plasma: an interface with human gut biota? *PLoS One.* 2012; 7(12):e51009. [PubMed: 23251414]
206. Wang K, Yuan Y, Cho J-H, McClarty S, Baxter D, Galas DJ. Comparing the microRNA spectrum between serum and plasma. *PLoS One.* 2012; 7(7):e41561. [PubMed: 22859996]
207. Wang K, Yuan Y, Li H, Cho J-H, Huang D, et al. The spectrum of circulating RNA: a window into systems toxicology. *Toxicol Sci.* 2013; 132(2):478–92. [PubMed: 23358195]
208. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci U S A.* 2009; 106(11):4402–7. [PubMed: 19246379]
209. Wang K, Zhang S, Weber J, Baxter D, Galas DJ. Export of microRNAs and microRNA-protective protein by mammalian cells. *Nucleic Acids Res.* 2010; 38(20):7248–59. [PubMed: 20615901]
210. Wang W, Shi Q, Mattes WB, Mendrick DL, Yang X. Translating extracellular microRNA into clinical biomarkers for drug-induced toxicity: from high-throughput profiling to validation. *Biomark Med.* 2015
211. Wang Y-T, Tsai P-C, Liao Y-C, Hsu C-Y, Juo S-HH. Circulating microRNAs have a sex-specific association with metabolic syndrome. *J Biomed Sci.* 2013; 20:72. [PubMed: 24093444]
212. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, et al. The microRNA spectrum in 12 body fluids. *Clin Chem.* 2010; 56(11):1733–41. [PubMed: 20847327]
213. Wild K, Sinning I, Cusack S. Crystal structure of an early protein-rna assembly complex of the signal recognition particle. *Science.* 2001; 294(5542):598–601. [PubMed: 11641499]
214. Williams Z, Ben-Dov IZ, Elias R, Mihailovic A, Brown M, et al. Comprehensive profiling of circulating microRNA via small RNAsequencing of cDNA libraries reveals biomarker potential and limitations. *Proc Natl Acad Sci U S A.* 2013; 110(11):4255–60. [PubMed: 23440203]
215. Winston WM, Molodowitch C, Hunter CP. SystemicRNAi in *c. elegans* requires the putative transmembrane protein SID-1. *Science.* 2002; 295(5564):2456–59. [PubMed: 11834782]
216. Witwer KW. Contamination or artifacts may explain reports of plant miRNAs in humans. *J Nutr Biochem.* 2015
217. Witwer KW. Circulating microRNA biomarker studies: pitfalls and potential solutions. *Clin Chem.* 2015; 61(1):56–63. [PubMed: 25391989]
218. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles.* 2013; 2
219. Witwer KW, Hirschi KD. Transfer and functional consequences of dietary microRNAs in vertebrates: concepts in search of corroboration. *BioEssays.* 2014; 36(4):394–406. [PubMed: 24436255]
220. Witwer KW, McAlexander MA, Queen SE, Adams RJ. Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of

- dietary miRNAs: limited evidence for general uptake of dietary plant xenomiRs. *RNA Biol.* 2013; 10(7):1080–86. [PubMed: 23770773]
221. Wurdinger T, Gatson NN, Balaj L, Kaur B, Breakefield XO, Pegtel DM. Extracellular vesicles and their convergence with viral pathways. *Adv Virol.* 2012; 2012(1):767694. [PubMed: 22888349]
  222. Xiang S, Fruehauf J, Li CJ. Short hairpin RNA -expressing bacteria elicit RNA interference in mammals. *Nat Biotechnol.* 2006; 24(6):697–702. [PubMed: 16699500]
  223. Yang J, Farmer LM, Agyekum AA, Hirschi KD. Detection of dietary plant-based small RNAs in animals. *Cell Res.* 2015; 25:517–20. [PubMed: 25721324]
  224. Yang J, Farmer LM, Agyekum AAA, Hirschi KD. Detection of dietary plant-based small RNAs in animals. *Cell Res.* 2015; 25(4):517–20. [PubMed: 25721324]
  225. Yao B, La LB, Chen Y-C, Chang L-J, Chan EKL. Defining a new role of GW182 in maintaining miRNA stability. *EMBO Rep.* 2012; 13(12):1102–8. [PubMed: 23090477]
  226. Yarmishyn AA, Kurochkin IV. Long noncoding RNAs: a potential novel class of cancer biomarkers. *Front Genet.* 2015; 6:145. [PubMed: 25954300]
  227. Yoo B-C, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, et al. A systemic small RNA signaling system in plants. *Plant Cell.* 2004; 16(8):1979–2000. [PubMed: 15258266]
  228. Yu B, Yang Z, Li J, Minakhina S, Yang M, et al. Methylation as a crucial step in plant microRNA biogenesis. *Science.* 2005; 307(5711):932–35. [PubMed: 15705854]
  229. Yuan S, Tang H, Xing J, Fan X, Cai X, et al. Methylation by NSun2 represses the levels and function of microRNA 125b. *Mol Cell Biol.* 2014; 34(19):3630–41. [PubMed: 25047833]
  230. Zerneck A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, et al. Delivery of microRNA -126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal.* 2009; 2(100):ra81. [PubMed: 19996457]
  231. Zhang L, Hou D, Chen X, Li D, Zhu L, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res.* 2012; 22(1):107–26. [PubMed: 21931358]
  232. Zhang L, Hou D, Chen X, Li D, Zhu L, et al. Exogenous plant mir168a specifically targets mammalian ldlrap1: evidence of cross-kingdom regulation by microRNA. *Cell Res.* 2012; 22(1): 273–74.
  233. Zhang Y, Liu D, Chen X, Li J, Li L, et al. Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol Cell.* 2010; 39(1):133–44. [PubMed: 20603081]
  234. Zhang Y, Wiggins B, Lawrence C, Petrick J, Ivashuta S, Heck G. Analysis of plant-derived miRNAs in animal small RNA datasets. *BMC Genomics.* 2012; 13(1):381. [PubMed: 22873950]
  235. Zhou W, Fong MY, Min Y, Somlo G, Liu L, et al. Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis. *Cancer Cell.* 2014; 25(4):501–15. [PubMed: 24735924]
  236. Zhou Z, Li X, Liu J, Dong L, Chen Q, et al. Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses. *Cell Res.* 2014; 25(1):39–49. [PubMed: 25287280]
  237. Zhu W, Qin W, Atasoy U, Sauter ER. Circulating microRNAs in breast cancer and healthy subjects. *BMC Res Notes.* 2009; 2:89. [PubMed: 19454029]
  238. Zhuang F, Fuchs RT, Robb GB. Small RNA expression profiling by high-throughput sequencing: implications of enzymatic manipulation. *J Nucleic Acids.* 2012; 2012:360358. [PubMed: 22778911]
  239. Zhuang F, Fuchs RT, Sun Z, Zheng Y, Robb GB. Structural bias in T4 RNA ligase-mediated 3'-adapter ligation. *Nucleic Acids Res.* 2012; 40(7):e54. [PubMed: 22241775]

Perspectives

Key questions for future research

- Are ex-sRNAs selectively secreted or are they mere relicts of dead cells?
  - Distinct packaging and enrichment point to a selective process, which need to be conclusively elucidated.
- Are there tissue- or cell-specific delivery mechanisms for ex-sRNAs?
  - Specificity of delivery has not yet been observed in mammalian systems and needs to be investigated.
- How many molecules of ex-sRNAs does it take to trigger a cellular response?
  - As miRNA concentrations in circulation are in the fM range, their function may depend on selective enrichment and amplification pathways, which have yet to be discovered.
- Are exogenous ex-sRNAs present in mammalian circulation? And how might they be taken up?
  - In view of the current debate, standardization of animal models and sequencing techniques to detect and trace exogenous ex-sRNAs is absolutely essential.
- Can exogenous ex-RNAs influence human physiology?
  - Localized and systemic effects of exogenous ex-sRNAs have been described, but clear mechanistic explanations are lacking.

Figure 1. Origins and modes of transfer of ex-sRNAs into extracellular spaces and circulation
 During cell death, apoptotic bodies, which contain sRNA moieties, are released into the extracellular space (Process 1). Ribonucleoprotein complexes are probably also released during cell death (Process 1) but also from healthy cells (Process 2), although no specific mechanisms have yet been described. Healthy cells also secrete vesicular structures associated with sRNA including exosomes (Process 3), HDL (high density lipoproteins) (Process 4) and microvesicles (Process 5) shed from the plasma membrane. Bacteria, viruses and fungi are able in some cases to hijack the mammalian export machinery, whereby infected cell types could release exogenous sRNA associated with carriers (exosome, microvesicle, ribonucleoproteins) into circulation (Process 6). In a “leaky gut” scenario, occurring in different pathologies, exogenous sRNAs (derived from diet, microorganisms or nematodes) associated with carriers (vesicles or ribonucleoproteins) may reach the mammalian circulation by breaching of the diseased epithelium (Process 7). Dendritic cells, underlying the gut epithelium, continuously sample antigens and, thus, exogenous sRNA may be transferred into circulation via these immune cells (Process 8). Transcytosis of exogenous sRNA - vesicular uptake of RNA on one side and release on the other side of the epithelial barrier – may represent an alternative route for exogenous sRNA to reach circulation (Process 9).

Note: All graphical components are for illustration purposes and are not representative of any real size dimensions. Although represented here with double-layered membranes, single-layered outermembrane vesicles (OMVs) also exist. Single RNA moieties have been placed into the different carriers for representation purposes only, but more or fewer molecules may associate with the highlighted carriers.

Figure 2. Uptake and functional potential of ex-sRNAs in recipient cells

The scavenger receptor class B member 1 (SR-BI) has been suggested to be involved in the uptake of functional miRNA associated with HDLs (Process 1). The uptake mechanism of ribonucleoproteins of either endogenous or exogenous origin remains largely unknown (Process 2). Endogenous RNA-transporting vesicles are able to enter a target cell by different types of endocytosis (Process 3) or by fusing with the plasma membrane of the target cell (Process 4). This scenario possibly also occurs when target cells encounter exogenous sRNA (derived from diet, microorganisms or nematodes) containing vesicles (Process 3 & 4). The binding of the different originated vesicles to the target cell is probably mediated via recognition of a specific surface receptor present on the target cell. Viruses are known to enter target cells by endocytosis (Process 3), fusion with the plasma membrane of the target cell (Process 4) or by binding through a specific surface receptor present on target cell (Process 5). Thus, sRNA packaged within a virus may reach the intracellular compartment via different routes. In the uptake of apoptotic bodies TIM-4, a surface receptor recognizing phosphatidylserine, may be involved (Process 6). Once present inside the cell, some ex-sRNAs have been described to repress translation (Process 7) or to induce signaling via Toll-like receptors (TLRs) or retinoic acid-inducible gene 1 (RIG-I; Process 8).

Some of the classes of extracellular RNAs identified in human blood circulation or extracellular fluids ^a

Table 1

Name	Abbreviation(s)	Size range (nt)	Definition /Function ^b
Micro RNA	miRNA; miR	18–25	• epigenetic control of gene expression (37)
			• mRNA degradation (37)
			• suppression of mRNA translation (37)
			• activation of innate immune receptors (58, 113)
Small interfering or silencing RNA	siRNA	20–25	• mRNA degradation, overlapping with miRNAs
Small nucleolar RNAs	snoRNA	70–240	• chemical modification of RNA (123)
			• telomere maintenance (123)
			• pre-rRNA processing (123)
			• regulatory role in alternative splicing (123)
Piwi-interacting RNA	piRNA	24–31	• repression of transposons (84)
			• maintenance of germline genome integrity (84)
			• gene repression (84)
			• chromatin modification (84)
Small nuclear RNA	snRNA	~150	• modulation of the activity of RNA polymerase II (136)
			• involvement in splicing (136)
tRNA fragments	tRFs	10–45	• translation control (67)
			• RNA silencing (67)
5' & 3' tRNA halves; tRNA-derived stress-induced RNAs	tRH; tRNA	20–35	• translation control (67)
			• RNA silencing (67)
Vault RNA	vRNA	80–150	• constitutive component of the Vaults, which are ribonucleoprotein complexes involved in transport, signal transmission and immune responses (109)
rRNA fragments	rRFs	20–1600	• translation control (14)
			• RNA silencing (236)

Name	Abbreviation(s)	Size range (nt)	Definition /Function ^b
yRNA fragments	yRFS	27, 30–33	• apoptosis (39)
signal recognition particle RNA	SRP-RNA	~300	• RNA component of the signal recognition particle which is involved in the protein trafficking (164)
Long non-coding RNA	Long ncRNA	> 200	• chromatin remodelling (226) • transcription stability (226) • posttranscriptional modifications (226) • translation (226)
Circular RNA	circRNA	80–90000	• templates for viroid and viral replication (110) • intermediate in RNA processing reactions (110) • regulator of transcription in cis (110) • function as snoRNAs and as miRNA sponges (110)

^aThe labels and annotations should be viewed as tentative, as the information in this field is rapidly expanding and many corrections of annotations are likely in the not too distant future.

^bFunctions are related to intracellular and extracellular moieties.

Table 2

Examples of functionally-described ex-sRNAs

RNA species*	Origin	Recipient	Transfer	Mechanism	Function	Reference
miR-210	Metastatic breast cancer cells	Endothelial cells	Exosome	–	Induction of angiogenesis	(101)
miR-143	Epithelial prostate cells	Prostate cancer cells	Exosome	–	Tumor suppression	(103)
miR-126	Endothelial cell	Vascular cells	Apoptotic bodies	Production of the chemokine CXCL12	Counteraction of apoptosis	(230)
miR-451	Hepatocellular carcinoma cells	Hepatocellular carcinoma cells	Exosome	Targeting of transforming growth factor β activated kinase-1	Cell growth	(98)
miR-223	Macrophage	Monocyte	EV ^a	–	Induction of differentiation	(82)
miR-150	Monocyte	Endothelial cell	EV ^a	Repression of c-Myb	Enhancement of cell migration	(233)
miR-105	Plasma of hypercholesterolemia patients	Hepatocytes	HDL	Alteration of gene expression	Lipid metabolism, inflammation, and atherosclerosis	(199)
miR-223	Blood from healthy donors	Endothelial cells	HDL	ICAM-1 repression	Anti-inflammatory properties	(184)
miR-105	Metastatic breast cancer cells	Endothelial cells	Exosome	Targeting of the tight junction protein ZO-1	Regulation of migration and promoting of metastasis	(235)
miR-124a	Neurons	Astrocytes	Exosome	–	Indirect regulation of glutamate transporter	(131)
miR-146a	Endothelial cells	Cardiomyocytes	Exosome	Downregulation of NRAS	Attenuation of angiogenesis	(76)
miR-451	Bone marrow-derived dendritic cells	Specific cell line of dendritic cells (DC2.4)	Exosome	Repression of luciferase activity in a luciferase reporter assay for miRNA activity	–	(130)
miR-148a	Bone marrow-derived dendritic cells	Specific cell line of dendritic cells (DC2.4)	Exosome	Repression of luciferase activity in a luciferase reporter assay for miRNA activity	–	(130)
miR-142-3p	Macrophage	Endothelial cells	Exosome	Repression of lentiviral (LV) activity in a LV reporter assay for miRNA activity	–	(182)
EBV miRNAs	EBV-transformed lymphoblastic B cells	Primary immature monocyte-derived dendritic cells	Exosome	Repression of CXCL11/ITAC	Repression of immune response	(148)
TAR miRNA (HIV-1)	HIV-1 infected cells or patients sera	293 T recipient cells	Exosome	Repression of Bim and Cdk9	Repression of apoptosis	(135)
rRF (23S rRNA)	<i>Staphylococcus aureus</i>	TLR13-expressing mice cell	–	Motif modification	Evasion of immune recognition	(141)
Nematode miRNAs	Gastrointestinal nematode: <i>Heligmosomoides polygyrus</i>	Mouse intestinal epithelial cells	Exosome	Repression of Dusp1 in a reporter assay	Modulation of innate immunity	(32)

RNA species*	Origin	Recipient	Transfer	Mechanism	Function	Reference
miR-168a	Food-borne: rice	Liver cell	2'-O-methyl modified on their terminal nucleotide	Repression of LDLRAP1	Involvement in lipid metabolism	(231)
miR-29b	Food-borne: cow's milk	Peripheral blood mononuclear cells	-	Repression of RUNX2	-	(18)
miR-181a/b	Food-borne: chicken egg	Lymphocytes	-	Repression of BCL2 and BCL2A1	-	(93)

^aEV stands for extracellular vesicle which are composed of exosomes, microvesicles and apoptotic bodies.