Minireview

Tiny RNAs and their voyage via extracellular vesicles: Secretion of bacterial small RNA and eukaryotic microRNA

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Impact statement

The possible endogenous functions of small RNAs such as regulatory small RNAs in bacteria and microRNAs in eukaryotes have been extensively studied since they were first discovered. However, their powerful functions should not be seen as limited to their cells of origin. Recently, several papers have demonstrated that small RNAs function as signaling molecules between cells. This is possible because small RNAs can be shuttled around after being incorporated into environmentally protective extracellular vesicles. It is now clearly plausible that secreted small RNAs can regulate other types of cells through biofluids. Given their ''common molecule'' status, the role of small RNAs in mediating bacteria-human crosstalk is an emerging and competitive area of genetic research. This review provides insight into the function of small RNAs in intercellular and even interkingdom communication.

Abstract

MicroRNAs are small non-coding RNAs that bind to the 3′-untranslated region of target mRNAs and have transcriptional or translational inhibitory function in eukaryotes. Before microRNAs were widely known, bacterial non-coding small RNAs around 50–200 nt in length were discovered whose mechanism of action resembled that of microRNAs. Recently, RNAs that are of similar size to or smaller than microRNAs have been discovered in bacteria and indeed, this class of small RNAs have been found throughout all domains of life. Moreover, recent findings suggest that these tiny RNAs can be released via extracellular vesicles (such as exosomes in eukaryotes and outer membrane vesicles in bacteria), which in turn heralds a new field of research, interkingdom communication. This review discusses two similar classes of small RNAs in evolutionarily distinct eukaryotes and bacteria. In addition to their biogenesis and regulation, we discuss small RNA vehicles and their secretion.

Keywords: Extracellular vesicle, exosomes, outer membrane vesicle, microRNA, small RNA, miRNA-sized small RNA

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Introduction

In recent years, the study of the class of small RNAs including microRNAs (miRNAs) has taken center stage with the recognition that these small molecules function in virtually all cellular pathways. miRNAs are small (approximately 19–23 nucleotides), evolutionarily conserved, single-stranded, and non-coding RNAs. They are endogenously expressed in eukaryotes (also in viruses) mostly work as post-transcriptional regulators.[1,2] miRNAs are involved in various pathological conditions such as cancer, developmental abnormalities, diabetes, and neurological disorders.[3–5]

Long before miRNAs were found in eukaryotic cells, noncoding small RNAs (sRNAs), which are around 50–200 nt in length, were characterized in bacteria.[6,7] sRNAs exist in all kingdoms of life and have become increasingly recognized as a novel class of gene expression regulators. The mechanisms utilized by sRNAs resemble those of miRNAs, which positively or negatively regulate targets by binding to target transcripts posttranscriptionally,[8] suggesting miRNA and sRNA are biologically closely related.

Indeed, small non-coding clustered, regularly interspaced short palindromic repeat (CRISPR) RNAs (crRNAs) have gained great attention as a tool for targeted genome editing.[9] Small non-coding crRNAs are the main means of RNA-mediated silencing for bacterial adaptive immunity against invading viruses.

Currently, miRNAs and sRNAs are known to circulate in nano-sized extracellular vesicles (EVs), referred to as

exosomes in eukaryotes and as outer membrane vesicles (OMVs) in Gram-negative bacteria.[10,11]

Exosomes are microvesicles approximately 30–100 nm in diameter which contain not only miRNAs but also various molecules such as proteins, lipids, and mRNAs.[12]

Although bacterial vesicles have been known for several decades,[13] their significance has only recently become appreciated. OMVs are released from generally Gram-negative bacteria and contain several kinds of molecules just like exosomes. These EVs of bacteria facilitate certain functions, such as secretion of virulence factors and transfer of macromolecules between bacterial cells.[14]

miRNAs and sRNAs secreted via EVs showed differences in abundance in various human pathological conditions and, therefore, have been put forth as novel biomarkers and diagnostic indicators for many diseases.[15–17]

The disease-causing role of pathogens is only one of many host–pathogen interactions. Instead, microbes exist in the host without causing overt disease and constantly communicate each other.[18] Therefore, it is important to elucidate the ''common language'' that both bacteria and host can speak and understand.

In this review, we focus on the biogenesis and regulation of small non-coding RNAs in bacteria and eukaryotes as well as their secretion via EVs and to provide a glimpse into the realm of interkingdom communication.

miRNA in eukaryotes

Primary miRNAs (pri-miRNAs) are first transcribed in the cell nucleus from intragenic or intergenic regions of the genome by RNA polymerase II, just like normal genes.[19] These pri-miRNAs are then processed in the nucleus by the enzymes Drosha and DGCR8 into approximately 60−100 nt-long precursors (pre-miRNAs) that form hairpin structures.[1] The pre-miRNAs are transported with the help of Exportin-5 from the nucleus to the cytoplasm,[20] where the pre-miRNAs are further processed into 18−24 double-stranded RNAs (miRNA:miRNA*) by the RNase III-like enzyme Dicer.[21] After processing by Dicer, one of the two strands of the miRNA duplex becomes a mature miRNA molecule that can be incorporated into the RNA-induced silencing complex (RISC), a multiprotein complex containing RNA inhibitory function such as the Ago family.[22,23] The miRNA-incorporated RISC can completely or incompletely bind to its complementary target transcripts, inducing target mRNA degradation (mostly in plants) or translational inhibition (mostly in animals) or sequestration of mRNA to P-bodies from cellular translational machinery.[24,25]

Most animal miRNAs pair with their 3′-untranslated region (UTR) targets while some miRNAs pair with 5′-UTRs or open reading frames (ORFs).[26,27] This translational inhibition apparently accounts for their involvement in many major cellular events and effects on cellular homeostasis. Additional processing mechanisms involve nonconventional or noncanonical processing of miRNAs. A dicer-independent miRNA biogenesis pathway that requires catalytic activity of Ago2 has been discovered.[27–29] Another review described subsets of miRNAs that are matured by a Dicer-dependent, Drosha-independent mechanism.[30] Moreover, it has also been suggested that certain introns, so called ''mirtrons'', have structural features similar to those of pre-miRNAs and may be involved in Drosha-independent processing of miRNAs.[31] Other small RNA-originated miRNAs such as small nuclear RNA-derived RNAs (sdRNAs) and tRNA-derived RNAs (tdRNAs) also contribute to the miRNA pool. Both sdRNAs and tdRNAs have characteristics far from canonical biogenesis pathways,[32] suggesting this might be a more common process in the cell than previously thought and opening the door to discovery of miRNA sized small sRNA in bacteria.

sRNA in bacteria

Small RNAs that have a regulatory function in bacteria (sRNA) were initially identified from extra-chromosomal genetic materials such as plasmids, transposons, and bacteriophages.[33] Several studies later showed that innate sRNAs were induced by different physiological and stress conditions such as starvation, pH or osmotic shock, and stationary phase.[34] In the bacterial model organism *Escherichia coli*, a majority of the sRNAs are expressed in relation to other pathogen species, such as *Salmonella* and *Yersinia*.[35] Unlike miRNAs, most mRNA-binding sRNAs are incorporated to their target mRNAs in the 5′-UTR or in the ORF.[6] Moreover, in contrast to classical miRNA function, binding of the sRNA and its target mRNA has been found to have both positive and negative effects on the stability and/or translation of the transcript.

sRNAs with a negative regulatory function may inhibit ribosome binding by directly blocking the Shine-Dalgarno (SD) sequence of the mRNA region and accelerate degradation of the mRNA by recruiting RNase E.[36,37] sRNAs may also activate premature transcription termination by binding to a developing (elongating) mRNA.[38,39] On the other hand, sRNAs with a positive regulatory function may affect secondary mRNA structures that block the SD sequence. sRNAs can also inhibit translation of target mRNAs by rearranging the structure of the 5′-UTR, which is critical for ribosomal access to upstream ribosome loading sites.[36,37]

The RNA chaperone protein Hfq is known to bind with and stabilize *E. coli* sRNAs, which have been shown to act by pairing with their target mRNAs.[40] In terms of Hfq in bacteria, it has been reported that AU-rich sequences (which also happen to be RNase E recognition motifs) in sRNAs with hexameric Hfq ring-binding are critical to the enzyme function.[34] In addition, Hfq has been found to facilitate the regulation of target mRNAs by sRNAs, although the mechanism has yet be fully elucidated.[41]

Although the exact roles of most sRNAs need further study, many seem to be expressed only under specific conditions or physiological status.

Recently, miRNA-size sRNAs (msRNA) in bacteria have come to light although their function is unclear or has just begun to be studied.[8,42–44] Their existence in bacteria implies that these kinds of small RNAs are evolutionarily well-conserved from viruses and bacteria to mammals and

that research into msRNA-mediated gene regulation may shed light on bacterial genetics.

EVs and small RNA secretion

Exosomes and secreted miRNA

Exosomes are small EV between 30 and 100 nm in diameter that carry bioactive materials including proteins, lipids, and nucleic acids including miRNAs, thus allowing miRNAs to circulate through the body.[12] The exosomes originate from internal budding of the plasma membrane and are mainly regulated by the machinery of endosomal sorting complexes required for transport or lipid ceramide.[45] The well-known exosomal proteins (or markers for exosomes) are tetraspanins (CD9, CD63, and CD81), heat shock proteins (Hsp60, Hsp70, and Hsp90), membrane transporters and fusion proteins (Annexins, GTPases and flotillin), as well as tumor susceptibility gene 101 (TSG101).[46] The exosome-mediated circulating miRNAs are a novel source of gene transfer between cells[47,48] as well as biomarkers for various diseases.[8,49,50] Exosomal miRNAs are secreted from the endosomal membrane compartment after fusion with multivesicular bodies (MVBs) and can be delivered over long distances within body fluids.[51–53]

Interestingly, it seems miRNA localization in the exosomes is not a random event but occurs through selective mechanisms. Sequencing motifs in exosomal miRNAs are believed to control miRNAs sorting into exosomes, which is mediated by the sumoylated form of ribonuleoprotein hnRNPA2B1.[54] Another RNA-binding protein, SYNCRIP, also functions as a key component of the hepatocyte exosomal miRNA sorting by directly binding to miRNAs sharing a short sequence called the hEXO motif.[55] In addition, there is a report that post-transcriptional modification of 3′ end uridylation in miRNAs can direct exosomal miRNA sorting in B cells.[56] These findings indicate that specific motifs of miRNAs and their interacting proteins make the exosomes carry only certain miRNAs in their cargo, which suggests exosomal miRNAs may have dedicated tasks in the body.

Exosomes have been found under both healthy and disease conditions in a number of human bodily fluids, including blood plasma,[57] urine,[58] breast milk,[59] saliva,[16] bronchoalveolar lavage fluid,[60] and amniotic fluid.[61] Exosomal RNAs have attracted great interest, especially in the context of miRNAs as diagnostic biomarkers.[47] Recent studies have shown that exosomal miRNAs are protected from RNase-dependent degradation and can thus be stably identified in circulating plasma and serum,[62,63] making them model biomarkers for clinical diagnostic applications. Exosomal miRNAs currently demonstrate potential as diagnostic biomarkers for various cancers,[64–66] neurological diseases,[67,68] cardiovascular diseases,[69] and infectious diseases.[49]

OMVs as sRNA vehicles for bacteria

Suspected EVs were first seen in cell-free filtrates of *Vibrio cholera*, which induce an immune response in rabbits.[70] Later, it was shown that lysine-limiting medium *E. coli* cultures were able to secrete outer membrane (lipopolysaccharide) vesicles[71,72] and many other Gram-negative bacteria have also been reported to release similar vesicles, ranging in size from 20 to 250 nm.[73] The composition of the OMVs includes components from other cellular compartments. Therefore, these structures were initially called ''membrane vesicles,'' ''extracellular vesicles,'' ''outer membrane fragments,'' or ''blebs.''[74] Although the traditional term ''OMV'' is widely used for Gram-negative bacteria (and used throughout this review), the term ''extracellular vesicles (EVs)'' is more acceptable because Gram-positive bacteria and Archaea are known to release almost the same vesicles.[75] OMV production depends on growth conditions in regard to their amount and composition but can be found in all growth phases of bacterial culture.[76]

So far, several different mechanisms of OMV biogenesis have been suggested. Most of the biogenesis mechanisms are species-specific but the structure and characteristics of bacterial cell wall components such as the peptidoglycan layer and lipopolysaccharide are important for OMV production.[77] More generally, the phospholipid VacJ/Yrb ATP-binding cassette (ABC) transport system can modulate OMV formation in Gram-negative bacteria through phospholipid accumulation in the outer leaflet of the outer membrane.[78]

Many approaches using OMVs to generate vaccines are being tried,[79] as the virulent character of OMVs and pathogenic bacteria have been shown to stimulate vesicle production during infection.[80,81] Interestingly, it was found that several virulence factors, including PagC, PagK1/K2, and PagJ were translocated via OMVs of *Salmonella* and that *Salmonella* survival inside the host declined in the absence of such factors, suggesting a critical role of OMVs in pathogenesis.[82,83] Many trials to generate vaccines using OMVs for other infectious diseases are still underway,[84–86] and OMVs hold great promise in protecting against diseases.

OMVs also work as cell-to-cell signaling particles, involved in quorum-sensing activity. The signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) of *Pseudomonas aeruginosa* can be delivered to mixed populations of bacteria via OMVs, whose removal from the bacterial population eliminates PQS-mediated group behavior.[87] The hyperthermophilic archaeal genus *Sulfolobus* inhibits nearby growth of other (competing) *Sulfolobus* species by secreting specific proteinaceous toxins via OMVs.[88] *Haemophilus influenza* releases DNA in OMVs (termed transformasomes) that protect DNA during transformation,[89] and archaea of the genus Thermococcus release DNA in virus-like membrane vesicles that are strongly resistant to DNase,[90] hence being likely to facilitate genetic exchange. Thus, microbe-to-microbe (and possibly microbe to other species or host) crosstalk may be provoked by OMV release as OMVs are important for the modulation of pathogenic mechanisms and competing nearby bacteria.

It is noteworthy that OMVs contain various types of nucleic acids such as DNA and RNA fragments, small RNA derived from the chromosome, and plasmids.[91,92]

Recently, we and other colleagues found that msRNAs in OMVs of pathogenic bacteria can be delivered into host cells and thereby modulate host immune responses.[93,94] msRNA in *P. aeruginosa* reduced interleukin-8 (IL-8)

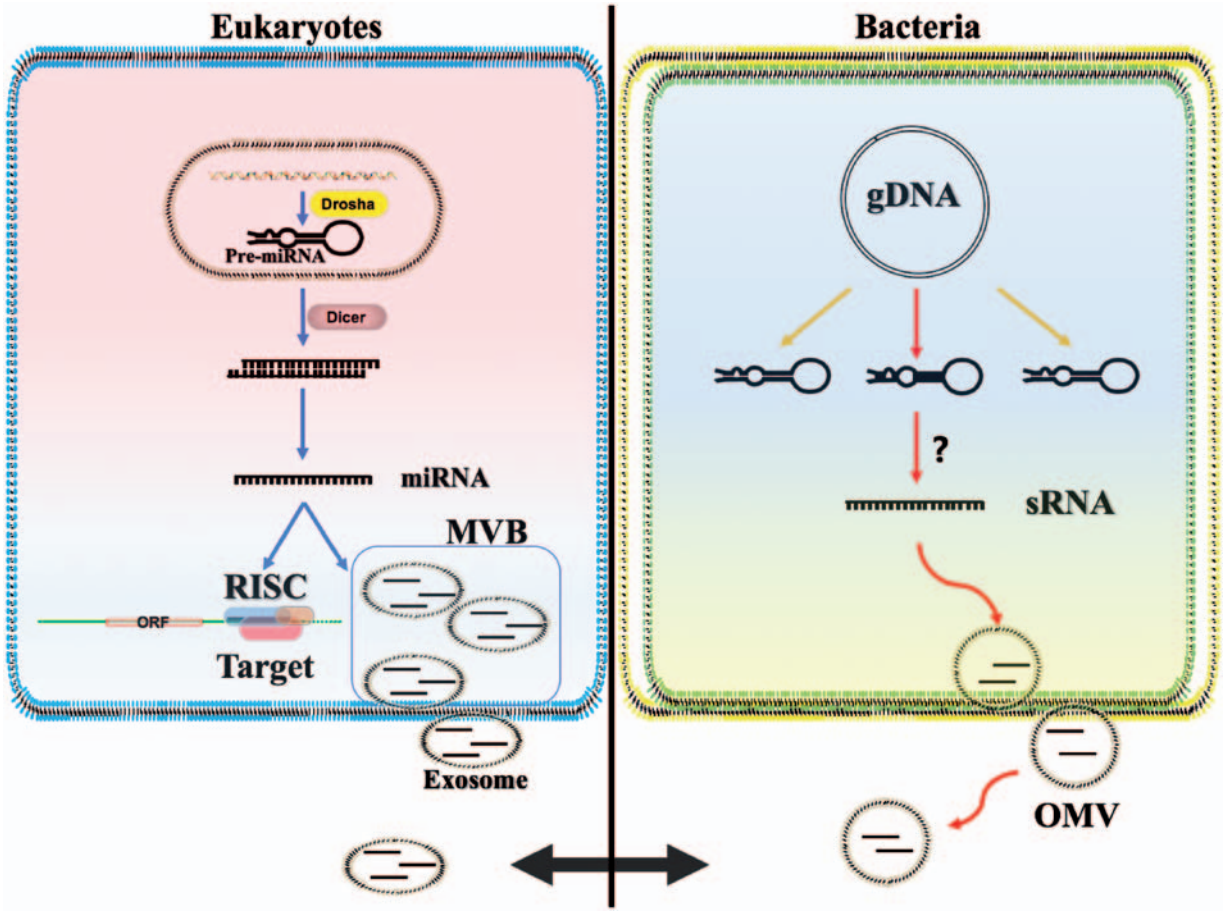

Figure 1 The biogenesis, function, and secretion of miRNA in eukaryotes and sRNA in bacteria. In eukaryotes, miRNA is transcribed and processed into mature form by Drosha and Dicer. miRNA usually has a posttranscriptional inhibitory function on its target transcript. Exosomal miRNAs are released from multivesicular bodies (MVBs) and may get delivered to other types of cells. Similarly, sRNAs in bacteria can be released to extracellular space via outer membrane vesicles (OMVs). Secreted small RNAs may constitute a ''common language'' for interkingdom communication. (A color version of this figure is available in the online journal.)

secretion levels; furthermore, msRNAs in periodontal pathogens deactivated IL-5, IL-13, and IL-15 cytokines via exogenous transfection, suggesting that circulating msRNAs may act as miRNAs in eukaryotic cells by incorporating host RISC. These findings imply a novel mechanism for host–pathogen interaction, wherein these msRNAs act as a miRNA in the host cells and as the vehicles for small RNA. In this way, OMVs may play an important role in interspecies communication.

Although the mechanism of msRNA sorting into OMVs has not been established as bacterial msRNA study has just begun, it has been established that the human oral pathogen *Porphyromonas gingivalis* selectively sorts proteins into OMV,[95] suggesting that bacteria might also have a msRNA sorting mechanism similar to the exosomes in eukaryotes we discussed earlier.

Conclusion and perspectives

The massive data obtained through deep sequencing technologies as well as through advances in bioinformatics make it inevitable that part of the genome previously considered ''useless'' will receive greater attention as it encodes non-coding RNAs and sRNAs important in cellular processes. Previously unknown sRNA mechanisms such as sRNA–protein binding and trans/cis-action, we now realize, can mediate responses to physiological conditions by modulating metabolic pathways or stress responses.

Synthesis of regulating sRNA and miRNA is speedy and requires little energy input, being economic in cellular terms. One merit of sRNA and miRNA is their ability to act on multiple genes in response to exogenous signals distinct from those regulating the target. On the other hand, several different sRNAs (or miRNAs) can also act on a single target at the same time, modulating it efficiently. Studies of sRNA and miRNA regulation in bacteria and human targets hold great promise for unveiling remarkable interspecies cross-talk.

Recently, Liu *et al*. suggested that human gut microbiota can be modulated by human miRNAs.[96] The opposite direction of interaction, from bacteria to human via functional small RNA transfers in EVs, is currently under intense scrutiny.

Collectively, as seen in Figure 1, through the mechanisms of biogenesis and small RNA sorting in both bacteria and eukaryotes, the tiny RNAs are secreted via ''vehicles (exosomes or OMVs)'' which voyage to deliver messages not only in the same species but also between species.

We now see a common channel of communication between bacteria and human, if not among all life forms. Secreted small RNAs in EVs such as exosomes in eukaryotes and OMVs in bacteria represent a new frontier in genetic research: the study of interkingdom communication (Figure 1).

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DECLARATION OF CONFLICTING INTERESTS

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