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Cells choose their words wisely

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Abstract (50 words)

In their recent Nature paper, Garcia-Martin et al. show that sequences within a microRNA influence how much of that microRNA is sent to another cell through extracellular vesicles. This supports a growing body of data demonstrating that cells use RNA to talk, but we know much less about how they listen.

The field of extracellular vesicles (EVs) has exploded in the last decade, fueled by the hypothesis that these small membrane-enclosed structures transmit information from one biological entity to another as a form of communication. EVs are ubiquitous in diverse living systems and play important functions in maintaining homeostasis and driving different human diseases. Accordingly, there has been intense interest in biology and medicine to understand, disrupt, and harness the information they transmit. RNA is one of the EV cargos whose “information” seems simple: RNA derives from a 4 nucleotide (nt) alphabet and many of the exported RNAs, including microRNAs (miRNAs), are small (~22 nt). Inside of cells, miRNAs function as molecular guides, directing an Argonaute protein (AGO) to specific messenger RNAs (mRNAs) through base pairing interactions, generally requiring only short stretches of complementarity (6-8 nt) at the 5' end of the miRNA (the “seed” region). The interactions between the miRNA, target mRNA and AGO occur within the RNA induced silencing complex (RISC) and typically lead to reduced amounts of the target mRNA and/or protein it encodes (Figure 1). When miRNAs move from one cell to another through EVs, they can regulate gene expression in the recipient, for example under homeostatic conditions (e.g. regulation of metabolism by miRNAs released from adipose tissue), disease (e.g. conditioning of the tumour microenvironment by miRNAs released from tumours) (O'Brien et al., 2020) or infection (both hosts and pathogens can use RNAs to regulate one another, suppressing immunity or virulence factors (Chen and Rechavi, 2021)). Yet a daunting list of mechanistic questions remain unanswered, challenging the field and hindering our ability to learn from and exploit communication mechanisms: how do cells choose which miRNAs to export; how do the miRNAs enter a functional pathway in the recipient cell and how much miRNA must be transferred to be functional?

How do cells choose which miRNAs to export? In the canonical miRNA biogenesis pathway, following transcription, processing and export from the nucleus, the precursor miRNA (pre-miRNA) is cleaved by DICER and the double stranded duplex is directly loaded into AGO. Any miRNA released from AGO is generally assumed to be degraded (Treiber et al., 2019) (Figure. 1). Yet miRNAs are ubiquitously found in EVs whereas AGOs generally are not (Weaver and Patton, 2020). Garcia-Martin et al. shows that miRNAs have evolved motifs that enable them to be recognized by other RBPs and exported in a cell-specific manner. This builds on the discovery in T cells that a short motif (4-6 nt) within specific mature miRNAs is recognized by

hnRNPA2B1 and this RBP directly mediates sorting of these miRNA into EVs (Villarroya-Beltri et al., 2013). Garcia-Martin et al. use a comparative analysis of different cell types to demonstrate the cell-specific nature of this mechanism and identify a range of motifs and RBPs associated with miRNA export “EXOmotifs” or retention “CELLmotif” (Garcia-Martin et al., 2022) (Figure 1). Their findings could help refine strategies for using extracellular miRNAs as biomarkers and for engineering export of miRNAs from different cells. The authors use an *in vitro* transwell system to show that engineering an EXOmotif into miR-34c-5p in brown adipocytes (donor cell) increases the levels of the modified miR-34c-5p transferred to hepatocytes (recipient cell), and results in suppression of miR-34c-5p targets in the hepatocytes (Garcia-Martin et al., 2022). These data underscore the concept that intracellular mechanisms in the donor cell define the level of specific miRNAs found in the recipient cell, and suggest that even low amounts of a transmitted miRNA can suppress targets in certain biological contexts.

How does the miRNA enter a functional pathway in the recipient cell and how much is needed to be functional? EVs can enter the cell through multiple pathways and little is known regarding miRNA trafficking following entry, for example how the miRNA escapes the endosome (Stalder et al., 2013) (Figure 1). There is also little understanding of the mechanism or regulation of mature miRNA loading into AGO, since it is the duplex miRNA that is assumed to be loaded during biogenesis (Treiber et al., 2019). The dialogue in the field has primarily fixated on miRNA abundance (*how much miRNA gets transferred?*). However, one could envision that an imported miRNA could have varying potencies depending on where it localizes, which RISC it occupies and in what biological context it is transferred. For example, inside of cells, many miRNAs bound to AGO are found in low molecular weight (LMW) RISCs that are not engaged in target suppression and specific signaling can dictate the recruitment of proteins (such as GW182) that bind to AGO and form the active RISC (La Rocca et al., 2015). Furthermore, the properties of the target mRNA (e.g., abundance as well as association with other RBPs and regulatory factors controlling its steady-state level) also dictate the extent to which targets will be suppressed by a given quantity of miRNA(s). The functional consequences of miRNA-target interactions will also vary, since miRNAs can act as fine-tuners, buffers or switches, depending on the identity of targets and physiological context. For example, miRNA levels can have much greater consequences in pathogenic stress conditions compared to healthy conditions (Mendell and Olson, 2012). Advancing the technologies and biological models to track transmitted RNA and interrogate its location and binding partners in the recipient cell could help unravel many important questions in RNA communication and miRNA biology, that are also relevant to therapeutic RNA delivery. It is also important to consider that multiple transport mechanisms could evolve to functionally communicate information via RNA. In plants and nematodes, specific AGOs are exported in EVs in association with other types of small RNA (Chen and Rechavi, 2021), and in mammals AGOs are also found in association with miRNAs outside of cells and outside of EVs, in what may represent an alternative secretion pathway (Jeppesen et al., 2019).

In summary, the work by Garcia-Martin illuminates the sophistication of the RNA code and its ability to dictate regulated and cell-specific secretion from cells, which will be increasingly relevant in the era of using RNA as a programmable and scalable biopolymer in vaccines and therapies. Although EV research is young, it provides the opportunity to re-examine our

assumptions and scrutinize gaps in our knowledge about the diverse mechanisms by which miRNAs and other RNAs control cell properties.

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Figure 1. MicroRNA (miRNA)-based communication between cells: linking intra- and inter-cellular RNA biology

Mechanisms of miRNAs in cell-to-cell communication are tied to intracellular miRNA processes including biogenesis, localization, association with Argonaute (AGO) and other RNA binding proteins (RBPs), and integration into different RNA-induced silencing complexes (RISCs). Many aspects of miRNA trafficking inside cells remain poorly understood. (i) The canonical miRNA biogenesis pathway in mammals involves transcription and processing of the primary miRNA in the nucleus, followed by export of the pre-miRNA and processing in the cytoplasm by DICER to yield the double stranded duplex miRNA (~22nt) that is directly passed and loaded into AGO with protein cofactors and chaperones (Treiber et al., 2019). One (guide) strand remains in AGO and the other strand is assumed to be degraded. (ii) The miRNA-AGO binds to target messenger RNAs (mRNAs) through base pairing interactions as part of a larger RISC that leads to inhibited translation and increased mRNA decay. Recent studies have shown that miRNAs also contain short (4-6 nt) motifs in their sequences that are recognized by RBPs, and the identity of the motif and associated RBP define whether the miRNA stays in the cell (iii, “CELL-motif”) or is exported in extracellular vesicles (EVs) (iv, “EXO-motif”). (iii) The fate of miRNA-RBPs that remain in the cell are not known, including whether the miRNA-RBPs can associate with AGO or whether the miRNA re-loads into AGOs. (iv) The RBPs that bind to EXO-motif miRNAs facilitate their sorting into EVs, including those generated within the multivesicular body (MVB) pathway, and possibly those released from the plasma membrane (dotted line). The identity and abundance of the RBPs involved in sorting miRNAs may define cell-specific miRNA secretion in EVs (Garcia-Martin et al., 2022). Whether associated RBPs are exported with the miRNA in an EV remains largely unknown. (v) Various compositions of miRNAs exist in extracellular environments, including those contained within EVs and those bound to lipoparticles or RBPs including AGOs. (vi) AGOs are found in extracellular environments associated with miRNAs yet outside of EVs, and may be exported through unknown pathways (Jeppesen et al., 2019); it is not known if these complexes are internalized by cells. (vii) EVs can be internalized by cells through various mechanisms including endocytosis or direct fusion with the plasma membrane. (viii) The localization and fate of the miRNA following uptake is not well understood, in particular how the miRNA escapes the endosome and how and where loading into AGOs occur. (Stalder et al., 2013). AGOs can exist in different RISCs in the cell, which might have different abilities to regulate targets. These include low molecular weight (LMW) RISC (ix) which is not expected to be active (La Rocca et al. 2015) and high molecular weight (HMW) RISC, which engages and suppresses targets (x) and nuclear RISCs(xi) that could regulate transcription or splicing. The mechanism(s) by which miRNA-AGOs are partitioned into one RISC versus another is not understood.

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