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Using information theory to assess the communicative capacity of circulating microRNA

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ABSTRACT

The discovery of extracellular microRNAs (miRNAs) and their transport modalities (i.e., microparticles, exosomes, proteins and lipoproteins) has sparked theories regarding their role in intercellular communication. Here, we assessed the information transfer capacity of different miRNA transport modalities in human serum by utilizing basic principles of information theory. Zipf Statistics were calculated for each of the miRNA transport modalities identified in human serum. Our analyses revealed that miRNA-mediated information transfer is redundant, as evidenced by negative Zipf's Statistics with magnitudes greater than one. In healthy subjects, the potential communicative capacity of miRNA in complex with circulating proteins was significantly lower than that of miRNA encapsulated in circulating microparticles and exosomes. Moreover, the presence of coronary heart disease significantly lowered the communicative capacity of all circulating miRNA transport modalities. To assess the internal organization of circulating miRNA signals, Shannon's zero- and first-order entropies were calculated. Microparticles (MPs) exhibited the lowest Shannon entropic slope, indicating a relatively high capacity for information transfer. Furthermore, compared to the other miRNA transport modalities, MPs appeared to be the most efficient at transferring miRNA to cultured endothelial cells. Taken together, these findings suggest that although all transport modalities have the capacity for miRNA-based information transfer, MPs may be the simplest and most robust way to achieve miRNA-based signal transduction in sera. This study presents a novel method for analyzing the quantitative capacity of miRNA-mediated information transfer while providing insight into the communicative characteristics of distinct circulating miRNA transport modalities.

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1. Introduction

Communication – the systematic transfer of information from a source to its intended recipient – is thought to be a conserved feature of biological systems. Communication can be characterized by information transfer between whole organisms, such as spoken or written language, or within whole organisms, e.g., intercellular communication. Several different methods of intercellular communication have been identified, including cell junctions [1,2] and the transfer of soluble factors [3]. Recently, the discovery of extracellular miRNA and the potential horizontal transfer of miRNA from one cell to another have led to speculation that circulating miRNAs are intercellular signaling molecules [4–6].

Evidence suggests that the selective packaging of miRNAs into distinct transport modalities can affect the specificity and biological function of secreted miRNAs [6]. For example, Vickers et al. demonstrated the importance of high density lipoprotein (HDL)-associated miRNAs in modulating gene expression in hepatocytes [4]. Other reports have indicated that vesicle-encapsulated miRNAs are biologically active and capable of modulating gene expression in a variety of cells [5,7–9]. Taken together, these findings suggest the existence of a complex miRNA-mediated intercellular communication network, one that may depend on the modes of extracellular miRNA transport.

In this study, we used basic principles of information theory to begin to decipher the underlying structural organization of the miRNA-mediated intercellular communication network. From this analysis, we were able to quantify the information transfer capacity and internal organization of distinct miRNA transport modalities while revealing previously unknown communicative characteristics of miRNA-mediated intercellular signaling. By offering statistical support for a miRNA-based communication network, this study further substantiates a role for circulating miRNAs as signaling molecules and potential biomarkers for disease.

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2. Materials and methods

2.1. Sample populations

Frozen human serum samples from healthy subjects ($n = 8$) and patients with significant CHD ($n = 8$) were obtained from the Emory Cardiovascular Biobank. CHD donors were identified as individuals with $\geqslant 1$ epicardial coronary artery with >50% stenosis on coronary angiogram, as previously described [10,11]. The clinical characteristics of the two study groups are shown in Table 1. The study was approved by the Institutional Review Board at Emory University, Atlanta, GA, USA. All study subjects provided written informed consent at the time of enrollment into the Emory Cardiovascular Biobank.

2.2. Serum fractionation

Serum samples were thawed in a water bath at 37 °C. After thawing, serum was diluted 1:3 with phosphate buffered saline (PBS). Using a previously described ultracentrifugation protocol [12], diluted serum was fractionated. The four fractions obtained were: (i) a microparticle fraction containing microvesicles and apoptotic bodies; (ii) an exosome fraction; (ii) an aggregated protein fraction [13]; and (iv) a lipoprotein fraction.

2.3. MiRNA abundance measurement

MiRNA was isolated from serum fractions using the commercially available miRNeasy isolation kit according to manufacturer's protocol (Qiagen Inc., Valencia, CA). MiRNA reverse transcription was conducted using the TaqMan microRNA Reverse transcription Kit (Life Technologies, Foster City, CA) at 16 °C for 30 min, 42 °C for 30 min, and denaturation of the enzyme at 85 °C for 5 min. TaqMan microRNA assays (Life Technologies) were performed using the 7500 Fast Real-Time PCR System at the 9600 emulation run mode. Cycle threshold (Ct) values of the analyzed miRNAs were converted into relative miRNA expression (Relative Expression = RE = $2^{(-\mathrm{Ct})}$). Normalized relative expression levels (nRE) for miRNAs were calculated by dividing the RE values of each miRNA by the RE values obtained for the non-coding, small nuclear RNA molecule, U6, as previously described [14]. U6 levels were similar in both study groups (data not shown). The following 8 miRNAs were assessed: miR-17, -19a, -21, -92a, -126, -146a, -222, -223. These miRNAs were chosen for this analysis because they are thought to be involved in the signaling processes that contribute to coronary heart disease (CHD) initiation and progression [15–18].

Table 1
Characteristics of sample cohort.

	Healthy volunteers ($n = 8$)	Patients with CHD ($n = 8$)
Gender male	4 (50%)	5 (62.5%)
Race white	4 (50%)	6 (75%)
Age (years)	39.13 ± 14.09	53.88 ± 8.34[#]
Stable CHD	0	100%[#]
Hypertension	0	8 (100%)[#]
Active smoker	0	1 (12.5%)
Adipositas (BMI >25)[a]	2 (25%)	8 (100%)[#]
Diabetes mellitus	0	4 (50%)[#]
History of (AMI, PCI, stroke)[b]	0	2 (25%)[#]
Concurrent medication		
Aspirin/Plavix	0	8 (100%)[#]
ACE-Inhibitor/ARB	0	7 (87.5%)[#]
Statin therapy	0	7 (87.5%)[#]

[#] $P < 0.05$ (significance).
[a] BMI = body mass index.
[b] PCI = percutaneous coronary intervention.

2.4. Zipf's Statistic calculation

The Zipf Statistic for a given repertoire is calculated by ranking the signals contained within the repertoire and then plotting the log of the rank against the log of the frequency [19,20] – the most frequently used signal is given a rank of one, the second most frequently used signal is given a rank of two, etc. Once plotted, the slope of the curve can be compared to a Zipf slope of −1.00. Communication repertoires with a Zipf's Statistic (i.e., slope) of −1.00 are considered optimal because they have the perfect balance between diversity and redundancy – such repertoires contain the maximum capacity for information transfer [19,20].

To adapt the Zipf analysis to circulating miRNAs in human serum, the concept of "signal" was defined as the mature miRNA sequence. The concept of "communication system" or "repertoire" was defined as the transport modality in which the circulating miRNA was identified and measured. Here, four transport modalities were assessed: microparticle (MP), exosome (Exo), aggregated protein (AP), and lipoprotein (LP). The metric that was used to describe signal "frequency" was adapted from the U6-normalized, relative miRNA expression, as measured by qRT-PCR analysis (described above). Relative miRNA frequency was calculated by dividing the U6-normalized miRNA copy number for a specific miRNA by the sum of all the U6-normalized relative expression levels obtained for all the miRNAs assessed (relative miRNA frequency = nRE $\mathrm{miRNA}_x / \sum \mathrm{nRE\ miRNA}_{\text{all miRNAs}}$). To obtain Zipf's Statistics for each of the four repertoires under investigation, the log of the relative miRNA signal frequencies was plotted against the log of the ranks of the signal frequencies. The slopes of the generated curves were subsequently analyzed.

2.5. Shannon's entropy calculation

Shannon's entropies [19,21] were calculated to assess the internal organization of the communication repertoire and provide insight into how signals within the repertoire interact to convey information. Shannon's zero-order entropy (H_0) measures repertoire diversity [19]. Shannon's first-order entropy (H_1) measures repertoire internal organization; higher-order entropies (second, third, fourth, etc.) measure repertoire complexity. In brief, Shannon's theory states that an effective communication system will have entropies (H_0, H_1, H_2,..., H_n) that are similar in value [21]. A communication repertoire exhibiting similar entropic values at each entropy level is likely to have an increased capacity for information transfer.

To analyze the entropic characteristics of the known miRNA transport modalities, we calculated Shannon's zero- and first-order entropies for the different transport modalities under consideration. Calculations were conducted using the following formulas [19]:

$$H_0 = \log_2(N) \tag{1}$$

$$H_1 = \sum_{j=1}^{N} -p(A_j)\log_2 p(A_j) \tag{2}$$

where N is the number of miRNAs (i.e., signals) analyzed in the repertoire and $p(A_j)$ is the frequency of occurrence of signal A. The frequency of occurrence of a particular miRNA was taken as the relative miRNA frequency calculated from the U6-normalized relative miRNA expression of a specific miRNA (nRE $\mathrm{miRNA}_x / \sum \mathrm{nRE\ miRNA}_{\text{all miRNAs}}$). H_0 and H_1 were independently calculated for each of the miRNA transport modalities under investigation. After

calculating H_0 and H_1, Shannon slopes were generated by plotting the values of H_0 and H_1 and calculating the slope of the best-fit line.

2.6. Assessment of miRNA signal transduction

Human umbilical vein endothelial cells (HUVECs), passages 2–5, were grown on 75 cm^2 cell culture flasks (Corning Inc., Corning, NY). Cells were split onto 6-well cell culture plates (Corning) 1 day prior to miRNA experiments. On the day of experiments, cell confluence was confirmed and media in each well was replaced with 1 mL of fresh medium in addition to 100 μL of the MP, Exo, AP or LP fractions obtained by serum ultracentrifugation. Concurrently, 100 μL of PBS was added to separate cell-containing wells to serve as a control. MiRNA experiments were independently carried out using serum fractions from healthy subjects and patients with CHD. After a 2 h incubation at 37 °C, media was removed and HUVECs were washed with PBS then harvested. MiRNA was isolated from HUVECs using the mirVana miRNA isolation kit (Life Technologies) according to manufacturer's protocol and miR-223 was quantified using qRT-PCR analysis. Intracellular miR-223 abundance in treated HUVECs was represented as a normalized fold change over the miR-223 abundance measured in HUVECs that were incubated with PBS only according to the formula: nRE_{miRNA} in treatment well/ nRE_{miRNA} in PBS control well.

2.7. Statistical analysis

Statistical analyses were performed using the GraphPad Prism software package (GraphPad Software Inc., LaJolla, CA). Values are expressed as means ± SEM. Regression analysis, Student's *t* test and the parametric analysis of variance (ANOVA) test were used to analyze and compare data. $P \leqslant 0.05$ was considered statistically significant.

3. Results

3.1. MiRNA-based information transfer is redundant

Zipf's Statistics were obtained for the different miRNA transport modalities (Fig. 1A). All miRNA transport modalities analyzed exhibited Zipf's Statistics with magnitudes that were significantly greater than one (Fig. 1B). Redundant information transfer mechanisms are characterized by a high rate of signal repetition. The Zipf's slopes obtained for extracellular miRNA transport modalities indicate that extracellular miRNA-mediated information transfer is redundant: MP (-2.45 ± 0.12), Exo (-2.56 ± 0.13), LP (-2.50 ± 0.11) and AP (-2.66 ± 0.11). The AP repertoire, having exhibited the steepest slope, is the most redundant in terms of its capacity to transfer miRNA signals.

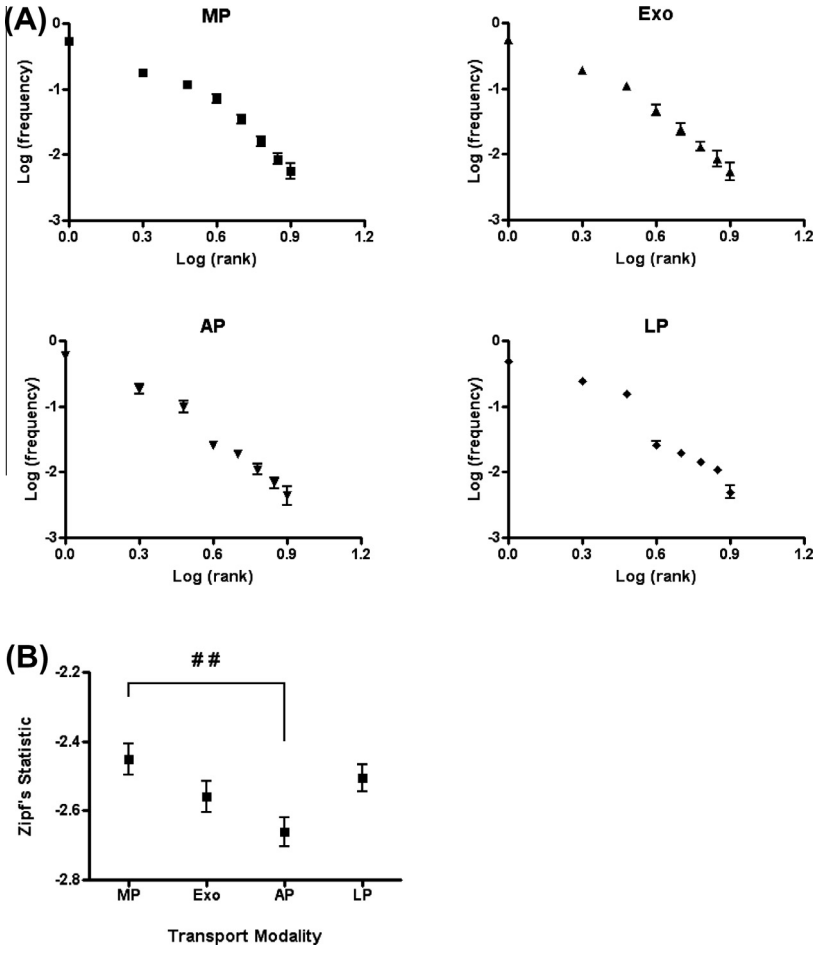

Fig. 1. MiRNA transport in serum is redundant. (A) Log miRNA frequency versus log miRNA rank for distinct miRNA transport modalities isolated from sera of healthy subjects: microparticle (MP), exosome (Exo), aggregated protein (AP) and lipoprotein (LP). (B) Zipf Statistics calculated for the miRNA transport modalities isolated from the sera of healthy subjects. ($N = 8$; $^{\#\#}P < 0.01$, ANOVA, Bonferroni Posthoc).

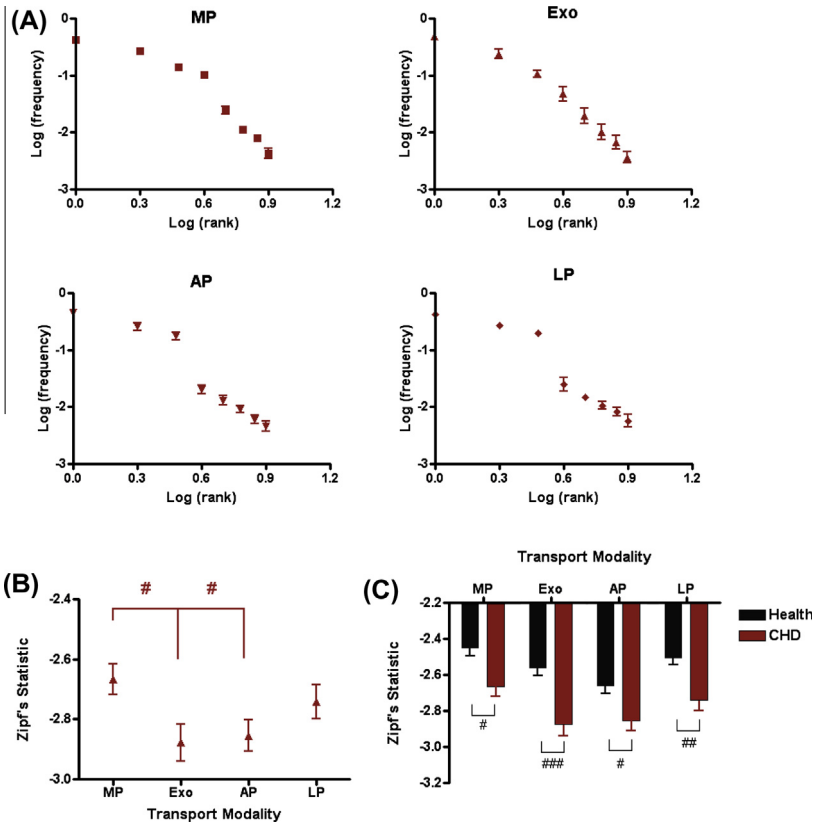

Fig. 2. CHD increases redundancy of miRNA transport in serum. (A) Log miRNA frequency versus log miRNA rank for distinct miRNA transport modalities isolated from sera of patients with significant coronary heart disease (CHD): microparticle (MP), exosome (Exo), aggregated protein (AP) and lipoprotein (LP). (B) Zipf Statistics calculated for the miRNA transport modalities isolated from the sera of patients with CHD. (C) Calculated Zipf Statistics for distinct miRNA transport modalities compared across healthy subjects and patients with CHD. (Panel A, B: $N = 8$, $^{\#}P < 0.05$, ANOVA, Bonferroni Posthoc; Panel C: $N = 8$ for healthy and $N = 8$ for CHD; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, Student's t test).

3.2. CHD increases redundancy of miRNA-based information transfer

Coronary heart disease (CHD) is associated with abnormal intercellular signaling [22] and recent work suggests that circulating miRNAs may play a role in this phenomenon [23,24]. To evaluate whether CHD alters the communicative capacity of distinct miRNA transport modalities, we calculated the Zipf's Statistics for miRNA transport modalities obtained from the sera of patients with statistically significant CHD (Fig. 2A). All four miRNA transport modalities exhibited non-optimal Zipf's Statistics with magnitudes greater than one (Fig. 2B). Comparing the Zipf's Statistics for all four miRNA transport modalities, across healthy subjects and CHD patients, we observed that the Zipf's slopes obtained for CHD subjects were significantly higher (more steep) than those obtained for healthy subjects (Fig. 2C). This finding suggests that miRNA transport in CHD is more redundant than miRNA transport in healthy subjects.

Table 2
Slopes of higher-entropic orders for distinct miRNA transport modalities.

Transport modality	Slope	SEM	Entropy	
			H_0	H_1
Microparticle (MP)	−1.003	0.03	3.0	2.00
Exosome (Exo)	−1.180	0.06	3.0	1.81
Aggregated protein (AP)	−1.314	0.05	3.0	1.68
Lipoprotein (LP)	−1.136	0.03	3.0	1.86

3.3. Microparticles exhibit the lowest Shannon entropic slopes

We calculated the zero- and first-order (H_0 and H_1, respectively) Shannon entropic values for each of the four miRNA transport modalities under investigation (Table 2). Once calculated, H_0 and H_1 were used to generate entropic slopes (Fig. 3A). Shallow Shannon entropic slopes indicate that a majority of the information being transmitted is captured in the miRNA signals themselves, as opposed to the way in which the miRNA signals are organized. The entropic slope of the MP fraction (Slope = −1.0 ± 0.03) was significantly lower than both the Exo ($P < 0.05$; Slope = −1.18 ± 0.06) and AP ($P < 0.01$; Slope = −1.31 ± 0.05). This suggests that the internal organization of miRNAs encapsulated in MPs may not significantly impact information content. Based on this interpretation, microparticles are likely to be the simplest and most straight-forward means of transporting miRNA-based information from one cell to the next.

3.4. MPs are efficient at transferring miRNA to cultured HUVECs

Microparticles were found to be least redundant in terms of their capacity for miRNA-based information transfer and they exhibited the lowest Shannon entropic slope, compared to other miRNA transport modalities. Given these observations, we hypothesized that microparticles would be the most efficient transport modality for miRNAs. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were incubated with the different serum fractions, respectively. After incubation, intracellular levels of miR-223 were assessed in the HUVECs and used as an indirect

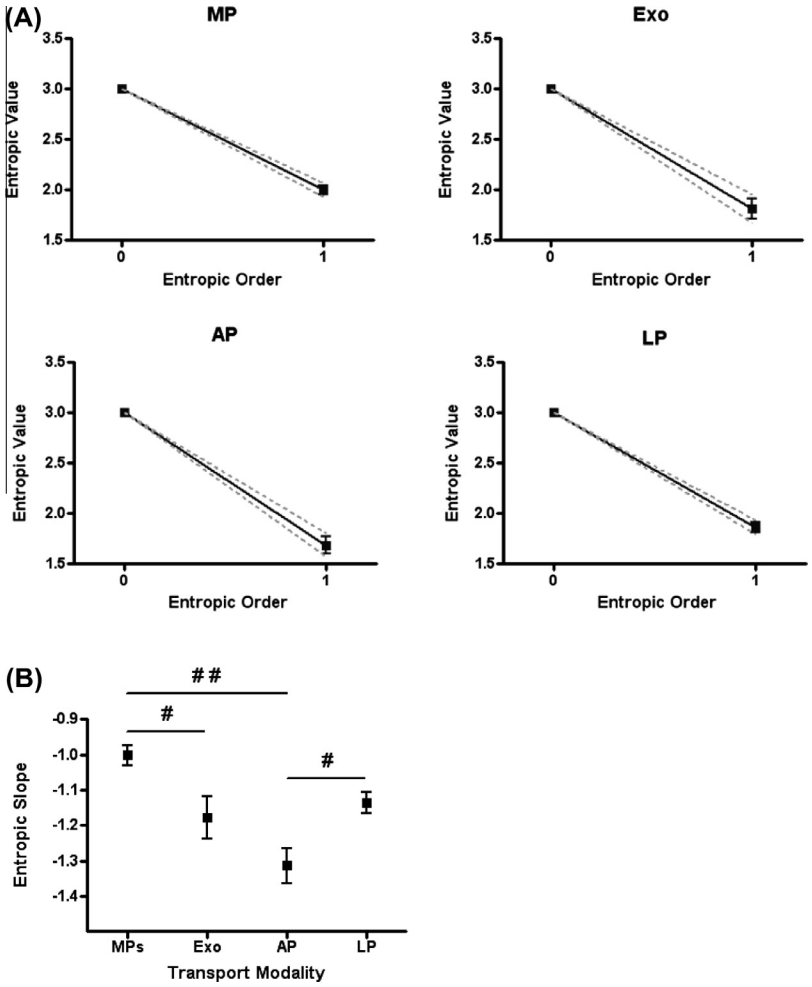

Fig. 3. Microparticles exhibit shallow Shannon entropic slopes. (A) Shannon's entropic order (H_0 and H_1) versus entropic value for distinct miRNA transport modalities. (B) Shannon entropic slopes calculated for distinct miRNA transport modalities. ($N = 16$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, ANOVA, Bonferroni Posthoc).

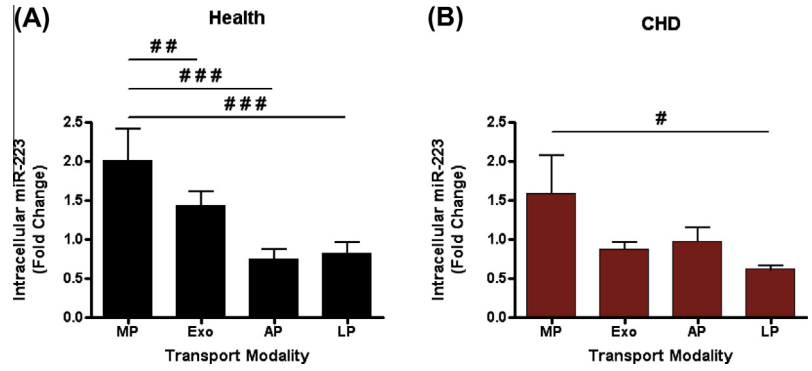

Fig. 4. Microparticles are most efficient at transferring miRNA to cultured HUVECs. Intracellular miR-223 levels (fold change) measured in HUVECs exposed to serum fractions isolated from (A) healthy subjects and (B) patients with CHD, respectively. ($N = 3$ for each cohort; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, ANOVA, Bonferroni Posthoc).

measure of miRNA-based information transfer. MicroRNA 223 was a good candidate because it is primarily expressed in myeloid lineage cells and is not otherwise highly expressed in endothelial cells [16,17,25]. Additionally, miR-223 levels did not differ significantly across the four transport modalities assessed in healthy subjects or patients with CHD, so the amount of extracellular miR-223 in each aliquot of serum fraction added to the endothelial cells was similar (data not shown).

The MP serum fraction obtained from healthy subjects induced a 2-fold increase in intracellular miR-223 expression (Fig. 4A). The MP fraction obtained from healthy subjects was significantly more efficient than the Exo, AP and LP fractions at transferring miR-223 to cultured HUVECs. Incubation of HUVECs with microparticles from CHD patients induced a 1.5-fold increase in intracellular miR-223 expression, which was significantly higher than that induced by the incubation of HUVECs with lipoproteins ($P < 0.5$;

0.62 ± 0.05; Fig. 4B). Taken together, these results suggest that MPs are most efficient at inducing changes in miRNA levels in recipient cells.

4. Discussion

Extracellular miRNAs have emerged as potential mediators of intercellular signaling in disease [6,17,18,26,27], but the details of this phenomenon remain uncertain [28]. This study is the first to use information theory, specifically, Zipf and Shannon analyses, to provide statistical observations on the characteristics of miRNA-mediated information transfer. We show that different the miRNA transport modalities present in human sera possess varying capacities for information transfer. We highlight statistical and experimental observations that suggest circulating microparticles are the simplest and least redundant method for transporting miRNA-based signals.

Zipf's analysis of circulating miRNA suggests that miRNA-based intercellular communication is redundant and highly repetitive. This finding is not at all surprising considering that human blood can be a fairly noisy communication channel. Human blood contains a variety of biological components, including red blood cells, white blood cells, platelets, and circulating proteins. Given the noisy nature of the human blood, redundancy may be necessary for efficient communication. McCowan et al. describe how noisy communication channels require redundancy and repetition in their means of information transfer to ensure that messages are accurately received [29].

The observed redundancy in miRNA-based communication, therefore, is likely an adaptation to the noisy environment that is present in blood. Correspondingly, the observation that CHD increased the redundancy of all four transport modalities is in line with previous reports that indicate CHD is associated with an increased number of circulating microparticles, platelets, proteins and lipoproteins [30,31]. In fact, changes in the abundance and content of circulating miRNA transport vehicles, namely, microparticles, exosomes and lipoproteins, is thought to impact CHD initiation and progression [4,5,32–35]. We speculate that the Zipf's Statistics calculated for miRNA transport modalities contained in biological fluids that are less noisy than blood will reveal decreased redundancy in miRNA-mediated information transfer.

Shannon's higher order entropies reveal information related to the way in which signals are organized and how this organization affects information content. The information content of written English, for instance, is strongly dependent on the way in which the signals, i.e., letters, are organized. Repertoires with shallow Shannon entropic slopes contain signals that are rich in information content independent of signal organization [19]. The Shannon entropic slope calculated for miRNAs in the MP fraction was significantly lower than that calculated for miRNAs in aggregated protein complexes, or miRNAs in exosomes. This observation suggests that the information content of miRNAs encapsulated in microparticles is contained primarily in the miRNA signals themselves, as opposed to the way in which the miRNAs are organized.

Because organizational complexity is a necessary feature of all communication systems, we speculate that most of the organizational complexity associated with MP-encapsulated miRNA is contained in the molecules and proteins found on the microparticle membrane, as opposed to the way in which the miRNAs are organized within the microparticle. MP uptake from the circulation can be targeted to specific cells based on the presence or absence of particular membrane proteins and receptors [34,36]. For example, the glycoproteins Del-1 and lactedherin mediate the clearance of circulating MPs by endothelial cells and splenic macrophages, respectively [34]. Given the organizational complexity present on MP membranes, the organization of miRNA sequences within MPs themselves may be less important.

We have proposed a novel method of analyzing miRNA-based signals as it relates to intercellular communication in normal physiology and disease. In doing so, certain assumptions regarding the concept of "signal" and "communication repertoire" were made. Future analyses with greater sample sizes will be necessary to determine the accuracy of these assumptions. Moreover, as is the case with all statistical models, conclusions drawn from information theory must be vetted by experimental studies that aim to extract physiological meaning from statistical data. Although information theory can provide statistical validation that information exists, it cannot provide insight into the actual meaning of the information [29]. It is likely that specific miRNAs have distinct communicative properties once they get taken up by recipient cells, so future studies will have to focus on elucidating the meaning of the miRNA signals that are contained in distinct miRNA transport modalities.

Using information theory, we have begun to decipher the organizational structure of miRNA-mediated intercellular communication. By highlighting the communicative capacity of distinct miRNA transport modalities in human serum, we have shown that certain transport modalities are likely more efficient than others at relaying miRNA-based signals. While microparticles exhibited the highest capacity for information transfer, the other miRNA transport modalities should not be assumed to be completely irrelevant. Given the dynamic nature of biological systems, we speculate that different biological fluids, different disease conditions and even different stimulatory environments will likely impact the information transfer capacity of distinct miRNA transport modalities. Future work in this area can lead to a greater understanding of the organizational structure of miRNA-based intercellular signaling and the development of novel therapeutics based on engineered miRNA-delivery systems that more closely resemble physiological miRNA transport modalities.

Acknowledgments

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