# Extracellular vesicular RNA mediated non-cell autonomous regulation of gene expression.

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**Abstract:**

The transcriptional landscapes of cells are modulated by multi-layered processes, which are endogenous as well as exogenous. Characterization of extracellular vesicular RNA (EV RNA) derived from nine cell types, not only demonstrated the diversity of cell type specific RNA biotypes but also enrichment of specific portions of annotated pre-miRNA, tRNA, YRNA and other RNAs within EVs. Our results also establish the cell state specific dynamicity in EV RNA distributions, qualifying one of the important aspects of communication. In addition, the study highlights both the temporal dynamics and spatial localization of intact EV RNA after transfer into a recipient cell, and their ability to elicit a wide range of transcriptional changes within them. We also demonstrated the cell type specificity in response from two different cell types, when exposed to the same EV stimuli, underscoring the context dependent interpretation of the complex EV message. In doing so, we shed light on a novel and under-appreciated medium of non-cell autonomous regulation of gene expression, through which cells in a complex multicellular organism may shape the transcriptional landscape of other cells and achieve synchronized functioning of involved cells in response to a certain micro-environment at the system level.

# Introduction

Multi-layered processes that are both endogenous and exogenous modulate the complex landscape of transcription of cells in multi-cellular organisms. Endogenous factors include transcription factors, three-dimensional genome organization [[1-4](#_ENREF_1)], chromatin modifications [[5](#_ENREF_5), [6](#_ENREF_6)], DNA methylation [[7-9](#_ENREF_7)], as well as several post-transcriptional molecular processes such as alternative splicing [[10](#_ENREF_10)], microRNA mediated regulation [[11](#_ENREF_11), [12](#_ENREF_12)] and RNA degradation [[13](#_ENREF_13), [14](#_ENREF_14)]. Exogenous factors traditionally refer to extra-cellular conditions (temperature, pH, oxygen, etc.) and secreted factors (proteins, hormones, metabolites, etc.), which stimulate various signaling pathways in the cell. Recently, extracellular vesicles (EVs) have garnered substantial appreciation as a key component of the extracellular milieu. While several studies have implicated EVs in various physiological roles, majority of these studies failed to directly attribute the observed effects to responsible biomolecules encapsulated in them.

Recent studies on EVs have shown the presence of RNA molecules encapsulated within EVs [[15-18](#_ENREF_15)]. However, majority of these studies have limited their attention on two categories of RNA, namely micro-RNA and messenger RNA. Moreover, recent studies have contradicted the earlier observation on the presence of intact messenger RNA [[19](#_ENREF_19)] and has questioned the presence of substantial amounts of microRNA within EVs as well [[20](#_ENREF_20), [21](#_ENREF_21)]. Furthermore, limited studies on EV RNA cargo using next generation sequencing has reported the enrichment of other biotypes of non-coding RNAs within EVs, not microRNA (miRNA) or messenger RNA (mRNA)[[22-25](#_ENREF_22)]. These contradictory reports on EV RNA contents have further convoluted the picture of the true nature and extent of EV RNA repertoire released by cells, and emphasizes the need for a comprehensive characterization of the RNA contents encapsulated within EVs.

Nevertheless, EV mediated transfer of RNA between cells has been suggested to represent a novel mode of communication between cells. Indeed, the ability of RNA to compactly store and transmit information, to produce base pair specific interaction and to act as molecular adaptors for incoming signals (e.g. ribo-switches) makes it an attractive molecule of information transfer [[26](#_ENREF_26)]. Such exchange of genetic information may confer specific advantages to the recipient cell. Uptake of mRNA and its subsequent translation could complement a cell’s own protein repertoire by augmenting its endogenous protein level or by providing it with new protein molecules that may not have been currently expressed by that cell, thereby priming the cell for a new function or state. mi-RNA or other regulatory RNA, upon uptake, may act on its endogenous targets and modulate their expression level, thereby, adding a new layer of regulation of gene expression in-trans [[26](#_ENREF_26)]. Unlike other molecular signaling mechanisms, EVs possess the unique ability to accommodate several RNA and protein molecules within them, which can potentially act *en masse* on the recipient cells to elicit a far more complex response.

However, mere transfer of genetic information between cells may not be sufficient to be termed as a mode of communication, as it must satisfy basic properties that any form of communication must possess[[27](#_ENREF_27)]. Firstly, the message must be meaningful and non-random. This not only requires an understanding of the exact nature and diversity of the messages being exchanged, but more importantly, a specific sorting and packaging of certain messages over others for delivery must be established. Secondly, the message must be dynamic, as a monotonous transfer of a static signal cannot be termed as meaningful communication. Thirdly, the message must be delivered safely to the recipient in a manner such that the message remains available to the recipient’s signal interpretation machinery. Finally, the message must be functional and interpretable, capable of eliciting a meaningful response by the recipient.

In this study, comprehensive characterization of the distribution, enrichment and cell type specificity of annotated RNA cargo within EVs derived from 9 different human cell types was performed by RNA sequencing. While previous studies have reported the presence of RNA fragments derived from longer annotated transcripts, we demonstrated an enrichment of biotype specific RNA fragmentation patterns across cell types, suggesting a conserved processing and sorting machinery of EV RNA. This study further indicates the responsive and dynamic nature of the EV RNA cargoes, with changing physiological conditions of the source cells. While other studies have reported the transfer of EVs between cells, we report the inter-cellular delivery and subsequent sub-cellular localization of EV RNA, resulting in a temporal, dynamic and cell type specific molecular response in recipient cells.

# Results

#### Diversity of EV RNA

Deeply sequenced RNA (RNA-Seq) data from EVs (Figure S1) derived from a diverse group of cell types ( five cancer cell lines, including K562 (chronic myelogenous leukemia), HeLa (cervical adenocarcinoma), MCF7 (breast adenocarcinoma), A549 (lung carcinoma) and U2-Os (Osteosarcoma) and four primary cell types, including BJ (skin fibroblast), HUVEC (human umbilical vein endothelial cells), HFFF (human fetal foreskin fibroblasts) and IMR90 (lung fibroblasts), allowed us to perform comprehensive bio-informatics analyses of global EV RNA diversity both specific to and common between cell types. An essential qualification for EV RNA to be considered as a viable medium of cell-cell communication is to demonstrate reproducibility under similar growth conditions, a lack thereof being indicative of an inability to disseminate meaningful information. A very strong correlation (Pearson’s) was observed between whole cell short RNA replicates, suggesting that the population of cells were in similar transcriptional states when EVs were isolated from them (Figure 1A). The EV RNA profiles derived from them were also very well correlated, suggesting the presence of a relatively consistent RNA appropriation process (Figure 1C, Table S1). This raises the question whether the relatively correlated distribution of EV RNA, is but a reflection of their expression profile within their source cells. However, consistent with previous studies [[16](#_ENREF_16), [28](#_ENREF_28)], we observed a very poor correlation between EVs and their source cells, suggesting only a subset of cellular RNA are being exported into EVs (Figure 1B).

A detailed characterization of EV RNA from all the 9 cells types revealed that EVs are overwhelmingly populated by biotypes of short RNA (sRNA), such as miRNA, miscellaneous RNA, tRNA, rRNA, snRNA and snoRNA. The relative abundance of sRNA biotypes within EVs are demonstrated by their share of reads within the RNAseq library (Figure 2). Though the specific proportions of EV RNA biotypes detected in EVs vary widely from cell to cell, we observed that miscRNA are the most abundant biotype of genes across cell types with an average share of 33% of the reads, followed by rRNA with 30%, tRNA with 11 %, miRNA with 11% , and fragments of protein coding genes with 7% of the reads. This is however in contrast to the distribution of RNA biotypes seen in the whole cells, where rRNA forms the largest biotype with 26%, followed by snoRNA with 22%, miRNA with 12% and snRNA with 10% reads. MiscRNA (0.37 %) and tRNA (1.74%) reads, which are extremely abundant within EVs, are significantly under represented in the cells. On the other hand, the GENCODE annotation biotype “Processed transcript”, described as non-ORF containing RNA, and thus encompassing several possible biotypes, are significantly enriched in the cells (26%) and very rare within EVs (0.6 %). This hints at a strong likelihood of concerted sorting mechanisms responsible for the enrichment of certain EV RNA biotypes relative to others, in a manner independent of their intracellular abundance.

Despite the diversity of sRNA biotypes within EVs, expression levels of genes within each biotype indicate a more homogenous picture (Figure 2). The top five most expressed genes within the miscRNA and rRNA biotypes account on average for 98% of the reads originating from genes within these biotypes. On the other hand, miRNA and tRNA biotypes are relatively more heterogeneous in comparison, where the top five genes accounted for only 68% and 72% of the reads originating from these respective biotypes. Further examination revealed that some of these genes in miscRNA (RNY5, RNY1 and RN7SL2) and rRNA (RNA5-8SP6, RNA5-8SP2 and RNA5-8SP4) biotypes contributed to over 60% of the total reads in EVs in most cell types.

 Since the overwhelming majority of sRNA genes (~99%) within EVs have expression levels less than 100 reads per million (rpm), we defined genes with expression levels above this threshold to be “highly abundant”. Interestingly, we found that there are only 9 genes that are abundantly detected across our cell types, of which two are rRNA (RNA5-8SP2 and RNA5-8SP6), two are miscRNA (RNY5 and RPPH1) and five are tRNA (tRNA23550, tRNA23569, tRNA23643, tRNA23827 and tRNA7867). 23 highly abundant genes were common across all the primary cells, while 15 genes were common to all the cancer cell lines (Figure 3A, B).

#### Cell type Specificity of EV RNA

Next, we evaluated whether the EV RNA landscape of different cell types are stochastic or are truly cell type specific. A global representation of EV RNA expression across nine cell types in a Heatmap with unsupervised hierarchical clustering using Euclidian distance, revealed a cell type specific RNA signature (Figure 3C). This analysis further showed that A549 has the largest number of genes which are uniquely enriched over 4 fold–1082 genes (p-value<3.32e-04), K562–938 genes (p-value<3.46e-02), HUVEC–358 genes (p-value<2.52e-02), U2OS–295 genes (p-value<5.65e-03), HMEC–275 genes (p-values<2.64e-02), MCF7–103 genes (p-value<2.25e-02) and HELA–83 genes (p-value<2.37e-02). In BJ, we found 14 genes (p-value<3.70e-02), which are more than 6 fold, enriched compared to all the other cell lines. With IMR90, even though we found over 116 genes enriched over 4 fold, none of them were statistically significant (p-value<7.86e-02), but there are 613 genes (p-value<1.91e-03) which are over 4 fold enriched in all other cells but IMR90. Furthermore, this cell type specificity was not limited to low abundance transcripts, but many of the cell type specific genes were found to be highly abundant. Among the cancer cell lines, U2-Os had 48 cell type specific highly abundant genes, A549 has 41 genes, K562 26 genes, while MCF7 and HELA 3 genes each (Figure 3A). Among the primary cell types, HUVEC has 34 genes, HMEC 31 genes, IMR90 20 genes and BJ 11 genes (Figure 3B).

## Dynamicity of EV RNA content

Armed with evidence of cell type specificity of EV RNA, we investigated variability of the EV RNA landscape with change in environmental conditions of source cells. Total EV RNA was sequenced from EVs of K562 cells grown under three different conditions – in presence of EV depleted fetal bovine serum, nutritionally constrained media by serum deprivation and chemotherapeutic treatment with DNA cross-linker MitomycinC (Figure 4A, B, C and Figure S3 A, B, C). While 76 highly abundant genes were common between EVs from all three conditions, considerable numbers of genes were uniquely abundant in EVs from each of these conditions (Figure 4E). For example, while 102 genes were uniquely abundant in EVs derived from cells cultured with EV-depleted serum, 12 genes were uniquely abundant in EVs from cells cultured in serum deprived conditioned medium. 18 genes were uniquely abundant in EVs derived from MitomycinC treated K562 cells. EV RNA derived from cells grown in EV depleted serum contained 571 genes enriched (over 4 fold, p-values<1.26e-100) and 51 genes depleted (over 4 fold, p-value<3.27e-11) compared to cells from serum-deprived condition (Figure 4D). Treatment with MitomycinC resulted in enrichment of 135 genes (p-value < 6.36e-16) and depletion of 108 genes (p-value<2.16e-21) in comparison to serum deprived state (Figure 4F). These results suggest that the EV RNA cargo is not static but continuously varies with changing environmental conditions. This variation is not seen in biological replicas of cells grown under the same conditions indicating that these variations are correlated to the change in the growth environments. An additional level of control aside from that responsible for the observed dynamicity can be observed by the unchanged levels of expression of some EV RNAs. These results suggest that not all such selected RNAs respond to the changes in the growth conditions.

#### Enrichment of specific short RNA fragments within EVs

The average read length observed in the EV RNA sequencing libraries from all the 9 cell types is 31.67 nucleotides, corroborating other observations that EV RNA are mainly populated by shorter versions of annotated sRNA genes [[19](#_ENREF_19), [23](#_ENREF_23), [25](#_ENREF_25), [29-31](#_ENREF_29)]. Analysis of the read length distributions by gene biotypes, suggest that fragmentation patterns are non- random and RNA biotype specific (Figure S2). Over half of the reads mapping to pre-miRNA genes are about 22 nucleotides long, both in EVs as well as in cells. Reads mapping to tRNA genes within EVs tend to be larger than miRNA reads, with about half of them being about 35 nucleotides long. In contrast, tRNA read distribution in cells tend to be considerably different, with a sizable proportion of them around ~75 nucleotides) which is not observed in EVs. Similarly, a large population of rRNA reads (40%) are over 101 nucleotides long in cells, while in EVs reads are considerably shorter, with the majority of reads (>90%) being less than 30 nucleotides long. Reads mapping to snRNA also tend to be shorter in EVs than in cells, with about 80% of snRNA reads being less than 60 nucleotides long in EVs, while over 80% are over 100 nucleotides long in cells. However, comparable proportion of reads mapping to snoRNA in both EVs (30%) and cells (35%) tend to be over 70 nucleotides in length. EVs also contain reads that map to protein coding genes, but almost all of these reads are short fragments (<30 nucleotides) of larger mRNA. These findings suggest the enrichment of RNA fragments that have a biotype specific length distribution. As there has been some speculation whether EV RNA are comprised of random fragments or functional parts of their parent RNA molecules, we further examined which portions of the RNA from these genes populate EVs.

Previous study by Chen et.al has suggested that miRNA within EVs are longer pre-miRNA [[32](#_ENREF_32)], which could be processed in the recipient cell after transfer from the source cell, while other studies have suggested that there might be mature miRNA within EVs [[24](#_ENREF_24), [33](#_ENREF_33), [34](#_ENREF_34)]. To obtain a global perspective across all precursor miRNA genes, we analyzed reads mapping to these genes, whose lengths were normalized to 100 nucleotides. Reads mapping to 10% upstream and downstream of the gene were also included in the analysis. The probability of finding a read spanning each normalized position within a gene was used to derive the probability density across all genes within the biotype. This analysis on miRNA demonstrated that though there is a significant enrichment of 3’ mature miRNA reads in EVs, this does not seem to be different from the distribution in source cells (Figure 5A, B, Figure S4 A, D, G). Additionally, in over 97% of the cases, miRNA 22-mers detected in EVs came from the same side as found to be predominant in their source cell, further diminishing the possibility that miRNA within EVs are other than the cellular mature miRNA.

A similar fragmentation analyses on tRNA biotype genes, found within EVs provided a significantly different fragmentation profile compared to cells. Given the difference in read length distribution between tRNA reads from EVs and cells, a look at the probability density distributions in cells and EVs makes it clear that while cells tend to have greater proportions of full length tRNAs, EVs are enriched in 3’ tRNA fragments. Interestingly, there is also a significant under representation of reads upstream and downstream of mature tRNA genes in EVs (previously described as tRF-5 and tRF-1 series [[35](#_ENREF_35)]), suggesting prior processing of tRNA before sorting into EVs (Figure 5C, D & Figure S4 B, E, H).

Reads mapping to snoRNA genes in whole cell were primarily full-length reads. While majority of snoRNA reads within EVs are full length, a small subset of reads map to fragments of snoRNA, which are almost never observed in the cells. However, a gradual 5’- 3’drop in the proportion of the reads mapping to snoRNA were observed in EVs, suggesting at least some of the snoRNA reads detected in EVs may be snoRNA degradation products (Figure S5 A, B).

EVs are also significantly enriched with fragments of snRNA, most of which start at the 5’ end of the genes but vary in length considerably (from halves to full length transcripts), while reads mapping to snRNA in cells are overwhelmingly full length (Figure 5E, F & Figure S4 C, F, I). In contrast to the small-RNA gene biotypes, fragments from protein coding genes in EVs as well as in whole cell did not demonstrate enrichment of any specific fragment, suggesting reads detected in EVs are randomly derived fragments of protein coding genes (Figure S5 E, F).

## Intercellular transfer of EV RNA

The functionality of the diverse repertoire of RNA packaged in EVs relies on its ability to transfer them from one cell to another through the extracellular milieu. Previous studies have demonstrated transfer of EVs from one cell type to another, primarily through labeling lipid and protein components of EVs [[16](#_ENREF_16), [36](#_ENREF_36), [37](#_ENREF_37)]. Here we extended these studies by demonstrating intra and inter-species transfer of EVs and EV RNA cargo, as well as report its subsequent subcellular localization in the recipient cell. To begin with, K562 EVs, labeled with lipid dye PKH67 were added to HeLa cells which confirmed transfer of EVs (Figure S6A). Transfer of K562 EVs, with its RNA cargo labeled with Sytoselect RNA green dye to HeLa cells confirmed the actual transfer of the RNA cargo within the vesicles to another cell (Figure S6B). The subcellular localization of EVs and EV RNA were found to be predominantly cytoplasmic (Figure S6 A, B). Co-labeling of subcellular organelles such as mitochondria, lysosomes and endoplasmic reticulum did not point to any preferential co-localization with any of the labelled structures (Figure S6 C, D, E). The integrity of the transferred RNA and dynamics of EV RNA transfer was studied by tracking the level of human specific gene RNY5 in mouse HB4 cells by direct incubation of GM12878 EVs with HB4 cells for 24 hours and 48 hours (Figure S6F).

## EV mediated transcriptional response in recipient cells

EVs derived from K562 cells grown in serum deprived state were added to primary BJ cells and, their cellular long RNA sequenced 2 and 24 hours post-treatment to capture both acute and delayed changes in the RNA landscape of these cells (Figure S7 A, B, C). A look at the differentially expressed protein coding genes when compared to untreated controls at 2 hours after treatment, demonstrated 285 genes up-regulated and 759 genes down-regulated (over four fold), while 24 hours after treatment, 85 genes were up-regulated and 158 genes were down-regulated (over four fold) (Figure 6A,B). This result suggested a relatively acute onset of transcriptional changes in the recipient cells after treatment with EVs from serum deprived K562 cells, and the persistence of significantly altered, albeit lower degree of transcriptional state changes even 24 hours post treatment. In order to demonstrate that at least some of the transcriptional response in BJ cells after EV treatment is attributable to the EV RNA cargo, the above experiment was replicated with EV RNA isolated from K562 EVs and transfected onto BJ cells (Figure S7D). Cellular long RNA sequenced 24 hours post treatment, revealed a very good correlation (Pearson’s correlation coefficient = 0.934) between the transcriptional landscape of BJ cells treated with both EVs and EV RNA (Figure 6C). Since a minimum of 6 hours is required for adequate transfection using lipofectamine, we were unable to investigate the 2 hours time point with RNA treatment.

Given the lack of specificity with which EVs are transferred *in-vitro*, we studied the transcriptional changes after EV treatment in two different cell types to investigate if a similar response occurred irrespective of recipient cell type. 24 hours after treatment with K562 EVs, HUVEC cells demonstrated up-regulation of 170 genes and down-regulation of 915 genes (over four fold), compared to untreated controls (Figure 6D). Comparing long RNA profiles in BJ and HUVEC cells treated with K562 EVs showed that a significant group of genes related to cell death were commonly differentially expressed as described in our previous study [[36](#_ENREF_36)]. Additionally, we also found that 1088 genes were up-regulated specifically in HUVEC but were either unchanged or down-regulated in BJ, while 203 genes were uniquely up-regulated in BJ cells post treatment (p-adj<0.05, No fold change threshold applied). Similarly, 285 genes were down-regulated in HUVEC but were either unchanged or up-regulated in BJ, while 315 genes were down-regulated uniquely in BJ cells. These unique gene expression changes also suggests a differential molecular response by BJ and HUVEC cells even though they were treated by EVs from the same source cells.

Owing to the dynamicity in the EV RNA cargo (Figure 4 A, B, C), we investigated if this changed cargo is capable of inducing a differential response. Upon treatment of BJ cells with EVs derived from MitomycinC treated K562 cells, long RNA was sequenced at 24 hours post treatment (Figure S7E). It was observed that over 254 genes behaved differently when compared to treatment with EVs derived from serum deprived K562 cells, of which 149 genes were up-regulated and 105 genes were down-regulated over 4 fold (Figure 6E).

**Discussion**

In this study, we performed a comprehensive characterization of the nature and diversity of RNA messages packaged in EVs by at least 9 different cell types, which revealed a reproducible, non-random and cell type specific sorting of the messages into EVs by cells. We observed that the sorting of RNA into EVs is a dynamic process, which varies based on the physiological conditions of the cell. Importantly, we demonstrated that intact RNA molecules are delivered through EVs, capable of eliciting a transcriptional response in recipient cells. We further observed that the molecular response by the recipient cells is dynamic, which varies with change in the RNA composition of EVs. Finally, we revealed the complexity of such a mode of communication by demonstrating that the same EV RNA message may elicit distinct molecular response in different recipient cells, depending on the transcriptional and translational landscape of that cell. Thus, our study demonstrated the involvement of the total EV RNA cargo in EV functionality and prompts us to further investigate the potential involvement of the individual members of the RNA cargo in inducing a response. Taken together, these results support the hypothesis that EV mediated transfer of RNA indeed represents a medium of communication and highlights a novel and underappreciated mode of non-cell autonomous gene regulation in multicellular organisms.

The unique and reproducible fragmentation patterns of each biotype of small non-coding RNA and their enrichment in EVs, points towards specific processing and sorting mechanisms in the cell that are not well understood. Interestingly, tRNA fragments have been previously observed in body fluids [[29](#_ENREF_29), [31](#_ENREF_31)] as well and have been implicated in translation repression and regulation of cell death [[38-40](#_ENREF_38)]. This raises an interesting possibility that these processed fragments from non-coding RNAs may represent a novel class of sRNAs with as-yet-undiscovered functions. The enrichment of such genes makes EVs a valuable source to explore their potential functional role in intercellular communication.

The elegance of EV mediated communication lies in their ability to enclose multiple RNA messages within one packet and deliver them to recipient cells. It is tempting to speculate that during the course of evolution, increased complexity of living organisms necessitated the need to transmit multiple messages (such as replicative, transcriptional or translational status, nutritional or other environmental stress signals, etc.) at the same time to neighboring cells. Instead of evolving distinct regulatory mechanisms for each and every signaling molecule, life may have evolved a common medium/platform of communication in the form of lipid vesicles, which could provide exceptional stability and protection from harsh extracellular environment and robustly and faithfully enclose and deliver multiple messages to intended recipients. Interestingly, the most abundant class of genes detected in EVs is Y-RNAs, a class of short non-coding genes that has been implicated in DNA replication and has been termed as “replication licensing factors” [[41-44](#_ENREF_41)]. It is tempting to speculate that Y RNAs in EVs may play the role of a quorum sensing molecule and play a role in regulating DNA replication in neighboring cells in mammalian systems.

**Material and Methods**

**Validation and quantification of EVs**

The presence of various species of RNA was first demonstrated in extracellular vesicles (EV) in an attempt to define a potential mechanism by which EVs may mediate intercellular transfer of genetic material [[18](#_ENREF_18), [28](#_ENREF_28)]. In this study, isolation of EVs derived from 9 different cell lines was performed using a method adapted from the original ultracentrifugation method described by [[45](#_ENREF_45)]. Empirical validation of the purification was performed using Transmission electron microscopy (Figure S1A), which confirmed the presence of vesicles of size < 200nm and cup-shaped morphology typically described in literature. Immuno-electron microscopy with 5nm gold conjugated antibodies demonstrated the presence of EV surface markers such as CD81 on purified EVs (Figure S1B). Western blot analysis further confirmed the enrichment of EV markers and depletion of other sub-cellular organelle markers [data not shown]. Nanoparticle Tracking Analysis (NTA) further quantified the enrichment of vesicles <200nm (Figure S1C). Consistent with previous studies, Bioanalyzer analysis showed that overwhelming majority of EV RNA are short RNA with size distribution between 20-200 nucleotides (Figure S1D). Intra-vesicular origin of the EV RNA was confirmed by RNase treatment prior to RNA isolation and small RNA sequencing.

**Cell culture and isolation of EV**

Cells were grown in their respective complete medium until they reached 70-80% confluence when the medium was replaced with serum-free conditioned medium and incubated for another 24 hours. The Conditioned medium was centrifuged at 300g for 10 minutes and the cell pellet was discarded and the supernatant was further centrifuged at 2000g for 10 minutes. The Pellet, comprising of mostly cell debris was discarded and the supernatant was again centrifuged at 10000g for 30 minutes. The pellet was discarded and the supernatant was filtered at 3500g for 15 minutes using Centricon Plus70 100KDa NMWL membrane (Millipore). The filtrate was discarded and the residue, enriched with EVs and other proteins was collected. The collected residues were precipitated overnight using ExoQuick-TC (System Biosciences) and the EVs were recovered next day by low speed centrifugation and suspended in 500microliter PBS.

**Electron microscopy of EV**

Aliquots of EVs suspensions were dispensed on parafilm on a petri dish and Butvar coated EM grids were adsorbed on them for 5 minutes at room temperature and kept on ice. The grids were transferred to drops of distilled water thrice for 30 seconds each to wash off excessive salts. The grids were then transferred to a drop of 1% uranyl acetate in 1% methyl cellulose for 30 seconds followed by another transfer to a second drop for 5 minutes. The grids were air dried and excess stain was blotted off. Imaging was performed using Hitachi H7000 electron microscope at 75kV.

**Nanoparticle Tracking Analysis of EVs**

Quantification of the extracellular vesicles were performed by Nanoparticle Tracking Analysis (NTA) using NanoSight LM10 at 25 degrees Celsius. PBS was used as a diluent.

RNA isolation and small RNA sequencing

EVs were treated with Ambion RNase cocktail at 37 degrees Celsius for 15 minutes. RNA isolation was performed immediately using Ambion’s Mirvana miRNA Isolation kit using manufacturer’s protocol. The purified RNA samples were first treated with Tobacco acid pyro phosphatase (TAP) for 1 hour at 37deg and DNase treated with Ambion Turbo-DNase (Life Tech). Ribosomal RNA depletion was performed on Whole cell RNA using Eukaryote Ribominus kit (Life Tech) using manufacturer’s protocol. Libraries were constructed using Illumina TruSeq small RNA kit according to manufacturer’s protocol, except reverse transcription was performed using Superscript III. Amplified libraries were run on 2% agarose gel and 20-200nts region was cut and gel-purified with Qiagen gel extraction kit. Libraries were quantified on Agilent Bio-analyzer HS-DNA chip and sequenced on Illumina HiSeq2000.

Long RNA Sequencing

 Long RNA was isolated with Mirvana miRNA isolation kit and DNase treated with Ambion Turbo-DNase using manufacturer’s protocol. Construction of complementary-DNA libraries was performed using Illumina TruSeq stranded total RNA kit. Libraries were quantified using Agilent Bioanalyzer HS-DNA chip and run on Illumina Hi-Seq 2000 or NextSeq 500 platform.

**Mapping & analysis**

All data from RNA sequencing experiments in the study were mapped to Human Genome version 19 (hg19, GRCh37) obtained from the UCSC genome browser website (<http://hgdownload.cse.ucsc.edu/downloads.html>). RNAseq reads were aligned using the STAR v1.9 [[46](#_ENREF_46)] software, and up to 5 mismatches per alignment were allowed. Only alignments for reads mapping to 10 or fewer loci were reported. Annotations were not utilized for mapping the data. The obtained BAM files were further processed using HTSeq software [[47](#_ENREF_47)] in order to appropriate the number of reads originating from each annotated regions of the genome, utilizing annotations obtained from Gencode v19 [[48](#_ENREF_48)] of the human genome, using the “Union mode” option of the software for all libraries, tRNA annotations were obtained from tRNAscan database [[49](#_ENREF_49)]. Reads per million (rpm) values for each gene was obtained by dividing the number of reads uniquely mapping within the limits of a gene annotation, by the total number of uniquely mapping reads in the library and multiplying by a million. These rpm values were used between replicates to establish correlation between biological replicates of RNA-Seq libraries (Table 3.2, Table 3.3). Relative abundance of RNA biotypes in Figure 3.3 was calculated using the cumulative rpm values of all genes within the Gencode defined RNA biotypes such as miRNA, snoRNA, miscellaneous RNA (miscRNA), protein coding etc. Differential expression analysis of RNAseq libraries was performed using DESeq [[50](#_ENREF_50)] and plots for data visualization were made using R statistical package.

**RNA Fragment Analysis**

To obtain a global perspective of abundance of specific RNA fragments within a biotype, all genes within a biotype are first normalized in length to 100 nt and all reads mapping to the gene or 10 nucleotides up and down-stream of the gene are considered for the analysis. Genes with less than 10 reads mapping to them are excluded from the analysis. The probability of a read spanning a particular normalized position within a gene is calculated as the ratio of the number of reads mapping on a normalized nucleotide position to the total number of reads mapping to that particular gene. This is extended to all genes within a biotype to build a probability distribution for each normalized nucleotide position, which is depicted by the box plots at each position. We then compare these location specific probability distributions between EV and whole cells, using the two sample Kolmogorov–Smirnov test, and the resulting p-value is plotted as a running line.

**Cell Type Specificity of EV RNA**

After adjusting for differences in library sizes using size factor estimations using the DESeq [[51](#_ENREF_51)], we administer the negative binomial test by grouping EV RNA libraries of one cell type as a group and all the other cell types as another, and in turn obtained genes which are enriched in one cell type as compared to all the others in our study. Once we obtained the list of genes which are specifically enriched in each cell type, we obtained p-values to test significance using a one-sample t-test.

**Dynamicity of EV RNA**

K562 cells were grown to 70% confluence in complete medium following which cells were transferred to conditioned medium. Replicates of 1+E8 cells were cultured in serum deprived conditioned medium, conditioned medium supplemented with EV depleted serum (10% final concentration) or in conditioned medium treated with MitomycinC (Sigma) at a final concentration of 20ng/ml for 24 hours. Isolation of EVs, EV RNA and small RNAseq was performed as described above. Bioinformatics analysis of RNASeq libraries were performed using STAR mapping software and HTSeq as described above. Differential expression analysis was performed using DESeq [[50](#_ENREF_50)] and plots for data visualization were made using R statistical package.

**Microscopy**

Transferof EVs wasdemonstrated using lipid labeling of EVs. Briefly, 2 microliter of PKH67 (Sigma) was re-suspended in 500microliter diluent and added to purified K562 EVs for 4 minutes in dark and subsequently EVs were isolated using Exoquick-TC according to manufacturer’s protocol. The labelled EV pellet was re-suspended in complete medium (DMEM +10% FBS+1% Penicillin-Streptomycin) and added to HeLa cells for overnight incubation. Imaging was done on Deltavision OMX microscope and image analysis was performed with Delta-vision SoftWorx software.

Transfer of EV RNA was demonstrated by labeling EV RNA with Syto RNAselect green dye (Life Tech). Briefly, K562 EVs were incubated with Sytoselct RNA green dye in dark and precipitated overnight using ExoQuick-TC. HeLa cells were then incubated overnight with RNA-labeled EVs and next day, imaging was performed using Deltavision OMX microscope and image analysis performed with Deltavision SoftWorx software.

Subcellular localization of EVs was studied by live imaging in HeLa cells by labeling mitochondria with MitoTracker Red (Life Tech), lysosomes with Lysotracker Red (Life Tech) and endoplasmic reticulum with ER-tracker Red (Life Tech) according to manufacturer’s protocols and then incubating labeled HeLa cells with Syto RNAselect green labeled K562 EVs (as described above) for 30 minutes. Imaging was performed on Deltavision OMX microscope and image analysis was performed using SoftWorx software.

**Inter-species transfer of EV RNA**

Interspecies transfer of EV RNA was determined by incubation of mouse HB4 cells with human GM12878 EVs. Approx. 3+E5 mouse HB4 cells were incubated with EVs isolated from human GM12878cells (1+E8 cells, in replicates) for 24 and 48 hours. Mouse HB4 cells were then washed and pelleted with low speed centrifugation. RNA isolation was performed with Mirvana miRNA isolation kit as described above and small RNA sequencing was performed using an A-tailing approach as describedin [[52](#_ENREF_52)]. The data was mapped using STAR [[46](#_ENREF_46)] against combined Human and Mouse genome and reads which mapped uniquely to humans only were considered for further analysis. *RNY5*, a human specific gene enriched in EVs was used to determine inter-species transfer of human GM12878 EV RNA to Mouse HB4 cells.

**Correlated transcriptional response in cells with EV and EV RNA treatment `**

Treatment of cells with EVs was performed by incubating 2+E5 BJ cells with EVs isolated from 1+E8 K562 EVs for 0, 2 and 24hrs. Alternatively, 2E5 BJ cells were transfected with K562 EV RNA (RNA isolated from EVs derived from 1+E8 K562 cells, in replicates) using Lipofectamine for 6 hours in Opti-MEM medium. Lipofectamine medium was replaced with fresh complete medium (DMEM+10% FBS +1% penicillin-Streptomycin) and incubated for another 24hrs. BJ cells were then washed and pelleted and long RNA isolation was performed subsequently using Mirvana miRNA isolation kit. Long RNA libraries were prepared using Illumina total RNA stranded kit, using manufacturer’s protocol and libraries were sequenced on Illumina HiSeq2000. Bioinformatics analysis of RNASeq libraries were performed using STAR mapping software and HTSeq as described above.

**Dynamic response by EVs**

Replicates of K562 cells were grown to 70% confluence and then transferred to serum free conditioned medium with and without MitomycinC (final concentration of 20ng/ml) for 24hrs. EVs were then isolated from both treated and untreated K562 cells. Replicates of 2+E5 BJ cells were incubated with K562 EVs (derived from cells with and without MitomycinC treatment) for 24 hrs. Subsequently, cells were pelleted and long RNA isolation was performed using Mirvana miRNA isolation kit. Long RNA libraries were constructed using Illumina Truseq total RNA stranded kit using manufacturer’s protocol and sequenced on Illumina NextSeq. Bioinformatics analysis of RNASeq libraries were performed using STAR mapping software and HTSeq as described above. Differential expression analysis was performed using DESeq [[50](#_ENREF_50)] and plots for data visualization were made using R statistical package.

**Cell type specificity of EV response**

Replicates of 2+E5 BJ and HUVEC cells were incubated with EVs (derived from 1+E8 K562 cells) for 0hr and 24hrs, following which cells were pelleted and long RNA isolation was performed using Mirvana miRNA isolation kit. Long RNA libraries were constructed using Truseq total RNA stranded kit using manufacturer’s protocol and sequenced on Illumina NextSeq 500. Bioinformatics analysis of RNASeq libraries were performed using STAR mapping software and HTSeq as described above. Differential expression analysis was performed using DESeq [[50](#_ENREF_50)] and plots for data visualization were made using R statistical package. Genes which were not significantly differentially expressed were classified as unchanged in that cell line and a value of 1 was used as default for their fold change and included for this analysis. All genes which were unchanged in both cell lines were excluded from this analysis.

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**Author contributions**

T.R.G managed the project; T.R.G, A.P, and S.K.C designed the experiments; A.P, S.K.C, A.T.S and J.D carried out the experiments. A.P. performed the bioinformatics analyses of the data; T.R.G., A.P., and S.K.C wrote the paper.

**Figure Legends**

**Figure 1: Correlation between EV and cellular RNA.** Scatter plots representing correlation in gene expression levels (log 10 reads per million) between replicates of BJ cellular short RNA **(A)** and BJ EVs **(C)**. **(B)** Volcano plot representing poor correlation in gene expression between BJ EVs and Whole cell. Each purple dot represents an annotated gene. X-axis represents fold change in expression levels between BJ EV and whole cell (log2 scale) and Y-axis represents mean levels of expression of each gene in BJ EVs and Whole cell (reads per million in log10 scale). Genes with fold change less than 2 (log2 scale) between EVs and cell are not represented.

**Figure 2: Relative abundance of gene families in EVs.** Pie-charts representing the distribution of various Gencode annotated gene families in EVs derived from 9 cells: A549 **(A)**, BJ **(B)**, HELA **(C)**, HMEC **(D)**, HUVEC **(E)**, IMR90 **(F)**, K562 **(G)**, MCF7 **(H)** and U2OS **(I)**. The inner pie-chart represents the relative abundance of gene biotypes within EVs (Total RPM of all genes within a biotype combined and represented as a percentage). The outer pie-chart represents the relative abundance (reads per million represented as percentage) of the top five annotated transcripts within the respective gene biotypes that are abundant in EVs.

**Figure 3: Cell type specificity of EV RNA cargo.** **(A, B)** Venn diagrams representing the cell type specificity of highly abundant genes (expression levels greater than 100 reads per million) in EVs derived from **(A)** 5 different cancer cell lines and **(B)** four different primary cell types. **(C)** Heatmap representation of cell type specific of expression profiles of EV RNA derived from these nine cell types.

**Figure 4: Dynamicity of EV RNA cargo. (A, B and C)** Pie-charts representing the distribution of various Gencode annotated gene families in EVs derived from K562 cells grown in three different conditions: serum deprived state **(A)**, Mitomycin-C treatment **(B)**, and in presence of EV depleted serum **(C)**. The inner pie-chart represents the relative abundance of gene biotypes within EVs (Total RPM of all genes within a biotype combined and represented as a percentage). The outer pie-chart represents the relative abundance (reads per million represented as percentage) of the top five annotated transcripts within the respective gene biotypes that are abundant in EVs. **(D)** Differential expression analysis between K562 EV derived from serum deprived conditioned medium and EV derived from K562 cells cultured in EV depleted serum containing medium. X-axis represents levels of gene expression in K562 EV from serum deprived conditioned medium (reads per million in log10 scale), while Y-axis represents gene expression levels of EVs derived from K562 cells cultured in EV depleted serum containing medium (reads per million in log10 scale). Only genes that are differentially expressed over four fold are shown in the figure. **(E)** Venn diagram representing number of unique and shared highly abundant genes (genes with expression level greater than 100 rpm) detected in K562 EVs from different culture conditions. **(F)** Differential expression analysis between K562 EV derived from serum deprived conditioned medium and EVs derived from K562 cells treated with MitoMycinC in serum deprived medium. X-axis represents levels of gene expression in K562 EV from serum deprived conditioned medium (reads per million in log10 scale), while Y-axis represents gene expression levels of EV derived from K562 cells treated with MitomycinC in serum deprived medium (reads per million in log10 scale). Only genes that are differentially expressed over four fold are shown in the figure.

**Figure 5: Biotype specific fragmentation patterns in BJ EV and cellular RNA.** Representation of biotype specific abundance of RNA fragments within BJ EVs and whole cells, with the X-axis as the normalized length of all genes within a particular biotype (from 0 to 100 nt), including 10 nucleotides up and down-stream of the gene. The Y-axis depicts the proportion of reads spanning a particular normalized location compared to all the reads mapping to genes within a biotype. The Cyan line in the EV graphs, represents the p-value of the two sample Kolmogorov–Smirnov test between probility distributions for each position between EV and whole cell, and the red dotted line represents the significance level where p-value is less than 0.01. **(A)** micro-RNA fragmentation patterns in BJ EVand whole cell **(B)**. MicroRNA reads map to mature microRNA region within the microRNA precursor gene in both EVs and whole cell. Reads mapping to the 5’/3’ mature side of microRNA precursor are more abundant in EV as well as in whole cell. tRNA fragmentation patterns in BJ EV **(C)** and whole cell **(D)**. snRNA fragment abundance is depicted in BJ EVs **(E),** while in whole cells mainly full length snRNA are found **(F)**.

**Figure 6: Differential and cell type specific molecular response in recipient cells by K562 EV and EV RNA.** **(A, B)** Differential transcriptional response by BJ cells when treated with K562 EV for **(A)** 2hrs and **(B)** 24hrs compared with untreated control. Only genes that are over four fold differentially expressed are shown in the figure. The axis represents gene expression levels of BJ cells with different treatments (reads per milion in log10 scale).Black dots represents genes downregulated by more than four fold while blue dots represents genes upregulated by more than four fold. Genes that are not differentially expressed by four fold are not shown. (**C)** Correlation in molecular response by BJ cells when treated with K562 EVs and K562 EV RNA for 24hrs. Pearson correlation coefficient between EV and EV RNA treatments was estimated as 0.9456. X and Y-axis represents gene expression levels in BJ cells with 24hr K562 EV and K562 EV RNA treatment (rpm in log10 scale), respectively. **(D)** Differential transcriptional response by HUVEC cells when treated with K562 EVs for 24hrs compared with untreated control. X and Y-axis represents gene expression levels in untreated HUVEC cells and 24hrs K562 EV treatment (rpm in log10 scale), respectively. **(E)** Dynamic molecular response in BJ cells when treated with K562 EVs from serum deprived conditioned medium and K562 EVs derived from MitomycinC treated K562 cells for 24hrs. X-axis represents gene expression levels in BJ cells when treated with serum deprived K562 EVs (rpm in log10 scale) while Y-axis represents gene expression levels in BJ cells when treated with Mitomycin-C treated K562 EVs (rpm in log10 scale). Black dots represents genes downregulated by more than four fold while blue dots represents genes upregulated by more than four fold. Genes that are not differentially expressed by four fold are not shown. **(F)** Cell type specific molecular response by K562 EVs in BJ and HUVEC cells. Scatter plot representation of the fold change of genes that are differentially expressed (adjusted p-value<0.05) in BJ and HUVEC cells when treated with K562 EVs for 24hrs. X-axis represents fold change of genes in BJ cells with 24hrs K562 EV treatment (log2 scale) and Y-axis represents fold change of genes in HUVEC cells with 24hrs K562 EV treatment. 1st and 3rd quadrant represents genes that are similarly differentially expressed between the two cell types, while 2nd and 4th quadrant represents genes that differentially expressed in a cell type specific manner by the same K562 EV treatment. The genes on the axes are those that are up or down-regulated significantly in one cell type but are not differentially expressed in the other cell type. Hence they have been collapsed on the axis using a default fold change of 1 for genes that are not significantly differentially expressed in that cell type.

**Supplementary Figures and Tables:**

**Figure S1: Validation of purification of extracellular vesicles (EVs).** (A) Transmission electron microscopy image of K562 EVs after negative staining shows classic cup-shaped vesicles smaller that are on average smaller than 200nm. (B) Immuno-electron microscopy image of purified EVs labeled with Anti-CD81 (mouse mAb) and detected by Goat anti-mouse IgG secondary conjugated with 5nm gold. (C) Size distribution of K562 EVs by NTA. (D) Bioanalyzer RNA profile (RNA Pico-chip) of K562 EVs. X-axis is nucleotides length and Y-axis is Fluorescent Units.

**Figure S2: Read length distribution by gene family in BJ EVs and whole cell.** Read length distribution by annotated gene families in **(A)** BJ whole cells and **(B)** BJ EV small RNA-Seq libraries. X-axis represents the nucleotide length of reads. Y-axis represents the proportion of all reads mapped to each gene family of a given nucleotide length.

**Figure S3: Replicability of K562 EV short RNA profiles in three different conditions.** Scatter plots representing correlation in gene expression levels (log 10 reads per million) between replicates of K562 EV short RNA grown in serum deprived condition **(A)**, presence of EV depleted serum **(B)** and Mitomycin-C treatment **(C)**.

**Figure S4: Biotype specific fragmentation patterns in K562, U2OS and HUVEC EV RNA.** Representation of biotype specific abundance of RNA fragments within EVs, with the X-axis as the normalized length of all genes within a particular biotype (from 0 to 100 nt), including 10 nucleotides up and down-stream of the gene. The Y-axis depicts the proportion of reads spanning a particular normalized location compared to all the reads mapping to genes within a biotype. The Cyan line in the EV graphs, represents the p-value of the two sample Kolmogorov–Smirnov test between probility distributions for each position between EV and whole cell, and the red dotted line represents the significance level where p-value is less than 0.01. micro-RNA fragmentation patterns in HUVEC **(A)**, K562 **(D)**, and U2OS **(G)** EV. MicroRNA reads map to mature microRNA region within the microRNA precursor gene in EVs. Reads mapping to the 5’/3’ mature side of microRNA precursor are more abundant in EV. tRNA fragmentation patterns in HUVEC **(B)**, K562 **(E)**, and U2OS **(H)** EVs demonstrate over-representation of tRNA halfs. snRNA fragment abundance is depicted in HUVEC **(C)**, K562 **(F)**, and U2OS **(L)** EVs.

**Figure S5: Biotype specific fragmentation patterns in BJ EV and cellular RNA.** Representation of biotype specific abundance of RNA fragments within BJ EVs and whole cells, with the X-axis as the normalized length of all genes within a particular biotype (from 0 to 100 nt), including 10 nucleotides up and down-stream of the gene. The Y-axis depicts the proportion of reads spanning a particular normalized location compared to all the reads mapping to genes within a biotype. The Cyan line in the EV graphs, represents the p-value of the two sample Kolmogorov–Smirnov test between probility distributions for each position between EV and whole cell, and the red dotted line represents the significance level where p-value is less than 0.01. **(A)** snoRNA fragmentation patterns in BJ EVand whole cell **(B)**. rRNA fragmentation patterns in BJ EV **(C)** and whole cell **(D)**. Protein coding gene fragment paucity is depicted in BJ EVs **(E)** andwhole cells **(F)**.

**Figure S6: Intercellular transfer and subcellular localization of EV and EV RNA.** Transfer and subcellular localization of K562 EVs labeled with lipid dye PKH67 (green) **(A)** and Syto RNAselect Green **(B)** in HeLa cells. Subcellular localization of Syto RNAselect Green labeled K562 EV (Green) in recipient HeLa cells with its Endoplasmic reticulum (Red) co-labeled with ER-Tracker Red **(C),** Mitochondria (Red) co-labeled with Mito-Tracker Red **(D)** and Lysosomes (Red) co-labeled with Lyso-Tracker Red **(E)**.Time course RNA sequencing analysis of the level of human specific RNY5 31-mer RNA in mouse HB4 cells when mouse HB4 cells are incubated with human GM12878 EVs for 24 and 48hrs **(F)**. Y-axis indicates the level of RNY5 (in reads per million) in mouse HB4 cells.

**Figure S7: Correlation of cellular long RNA between replicates by RNA sequencing.** Scatter plots representing correlation in gene expression levels (log 10 reads per million) between replicates of BJ cellular long RNA from cells untreated by K562 EVs **(A)**, replicatesof BJ cells 2 hours after treatment with K562 EVs **(B)**, and 24 hours after treatment with K562 EVs **(C)**. Replicates of long RNA from BJ cells treated with EV total RNA derived from K562 EVs **(D)**, and replicates of long RNA from BJ cells 24 hours after treatment for with EVs from Mitomycin-C treated K562 cells **(E)**. The bottom right of each scatter plot reports the Pearson correlation coefficient between the two replicates respectively.

**Table S1: Correlation between replicates of short RNA from EVs of nine cells.** The table reports the Pearson correlation coefficient between the two replicates of short RNA sequencing from EVs of nine different cell types in the study.

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