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Profiling of small RNA cargo of extracellular vesicles shed by *Trypanosoma cruzi* reveals a specific extracellular signature

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ABSTRACT

Over the last years, an expanding family of small regulatory RNAs (e.g. microRNAs, siRNAs and piRNAs) was recognized as key players in novel forms of post-transcriptional gene regulation in most eukaryotes. However, the machinery associated with Ago/Dicer-dependent small RNA biogenesis was thought to be either entirely lost or extensively simplified in some unicellular organisms including *Trypanosoma cruzi*, *Saccharomyces cerevisiae*, *Leishmania major* and *Plasmodium falciparum*. Although the biogenesis of small RNAs from non-coding RNAs represent a minor fraction of the normal small RNA transcriptome in eukaryotic cells, they represent the unique small RNA pathways in *Trypanosoma cruzi* which produce different populations of small RNAs derived from tRNAs, rRNAs, sn/snoRNAs and mRNAs. These small RNAs are secreted included in extracellular vesicles and transferred to other parasites and susceptible mammalian cells. This process represents a novel form of cross-kingdom transfer of genetic material suggesting that secreted vesicles could represent new relevant pieces in life cycle transitions, infectivity and cell-to-cell communication. Here, we provide for the first time a detailed analysis of the small RNA cargo of extracellular vesicles from *T. cruzi* epimastigotes under nutritional stress conditions compared to the respective intracellular compartment using deep sequencing. Compared with the intracellular compartment, shed extracellular vesicles showed a specific extracellular signature conformed by distinctive patterns of small RNAs derived from rRNA, tRNA, sno/snRNAs and protein coding sequences which evidenced specific secretory small RNA processing pathways.

1. Introduction

Over the last years, an expanding family of small regulators RNAs (e.g. microRNAs, siRNAs and piRNAs) were recognized as key players in novel forms of post-transcriptional gene regulation in most eukaryotes [17]. Soon after its initial description the presence and relevance of small RNA pathways in the control of post-transcriptional gene regulation was reported for bacteria and most eukaryotes. However, the machinery associated with small RNA biogenesis was thought to be either entirely lost or extensively simplified in some unicellular organisms including *Trypanosoma cruzi*, *Saccharomyces cerevisiae*, *Leishmania major* and *Plasmodium falciparum* [5].

In a previous work aimed to identify the presence of alternate small RNA pathways in *T. cruzi* [15], we reported the specific production of small RNAs derived from mature rRNAs and tRNAs representing ~60% and ~25% respectively of the small RNA population. The tRNA-derived fragments of ~32 nt in length mainly derived from the 5′arm of a restricted group of precursors tRNAs, favoring the idea of a controlled process. Shortly after, the specific production by *T. cruzi* of different populations of small RNAs derived not only from tRNAs and rRNAs but from sn/snoRNAs and coding genes (CDS) was also reported [12,31].

Cleavage of tRNAs, snoRNAs, rRNAs and snRNAs to generate a novel class of small regulators associated with the AGO/Piwi

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family of proteins represent evolutionary conserved small RNA pathways (from prokaryotes to higher eukaryotes), frequently initiated after nutritional, biological or physicochemical stress [19,21,22,24,25,40]. Although the biological significance remains to be completely elucidated, recent experimental evidence suggested that small RNAs derived from rRNA (srRNAs) and tRNA (tsRNAs) are able to modulate gene expression in response to certain survival pressures [9,10,20,26,27,29,38].

In a recent work we reported that small RNAs derived form rRNA, tRNA and sno/snRNAs associated with *T. cruzi* specific Piwi proteins in ribonucleoprotein complexes which are secreted to the extracellular medium through extracellular vesicles (EVs) [14,16]. Surprisingly, EVs cargo could be delivered to other parasites and to mammalian susceptible cells promoting metacyclogenesis and conferring susceptibility to infection, respectively [14,35].

Studies over the past few years reported that cell-free mRNA, miRNAs and other non characterized small RNAs are normally secreted from a variety of normal and diseased cells to the extracellular media either through membranous vesicles [4] or included in ribonucleoprotein complexes [5,34]. It is now accepted that secreted exosomes and shed microvesicles (ectosomes) [6] serve as a mean of delivering genetic information and proteins between cells playing pivotal roles in cell-to-cell communication [4]. In an elegant study, Valadi et al. [37] reported that exosomes from different mammalian cells carry at least 1300 mRNAs and more than 120 known microRNAs and other non characterized small RNAs. Interestingly, these exosomal mRNAs and microRNAs were completely functional in recipient cells.

In this respect, we recently reported that EVs secreted by *T. cruzi* were able to induce epigenetic changes in susceptible mammalian cells suggesting that, *T. cruzi* secreted vesicles could have a high impact in host cells responses against pathogens and that tRNA-derived halves could be one of the molecules inducing these changes [13]. Taken together, these results highlight the relevance of EVs and their cargo as novel forms of intercellular communication in protozoan parasites revealing a cross-kingdom transfer of genetic material from parasites to mammals.

Given the information raised so far, the characterization of all small RNA species that could be playing critical roles in each stage of protozoan parasites life cycle is extremely important for fully understanding life cycle transitions, infectivity and their interactions with hosts. In this respect, Bayer-Santos and col. have recently sequenced *T. cruzi* small RNA from epimastigote extracellular vesicles, metacyclic extracellular vesicles and the intracellular compartment of metacyclic forms [1]. They categorized sequenced RNAs in seven categories (tRNAs, snRNAs, snoRNAs, CDS, pseudogenes and unspecified reads) and analyzed only the small RNAs derived from transfer RNAs describing the issoaceptor precursors and their quantitative contribution to the total population of tRNA halves sequenced. In their work they specifically collected epimastigote extracellular vesicles from cultures with parasites at exponential growth. To gain insights on the role of extracellular vesicles from *T. cruzi* as vehicles of genetic exchange and their potential significance in host-pathogen biology we provide for the first time a comparative and detailed analysis of small RNAs patterns of the intracellular and the extracellular compartments of *T. cruzi* epimastigotes under nutritional stress. Our data revealed a specific extracellular signature conformed mainly by specific small RNAs from rRNA and tRNA which evidenced specific secretory small RNA processing pathways. In particular, our data suggests that in the previous steps of metacyclogenesis a specific population of small RNAs is packaged into vesicles which may be intended to interact with hosts. Also, this extracellular signature differs with the one described by Bayer-Santos and col. in epimastigote extracellular vesicles from cultures with parasites at exponential growth, suggesting that specific tsRNAs signatures could be associated to different growth conditions or parasite stages.

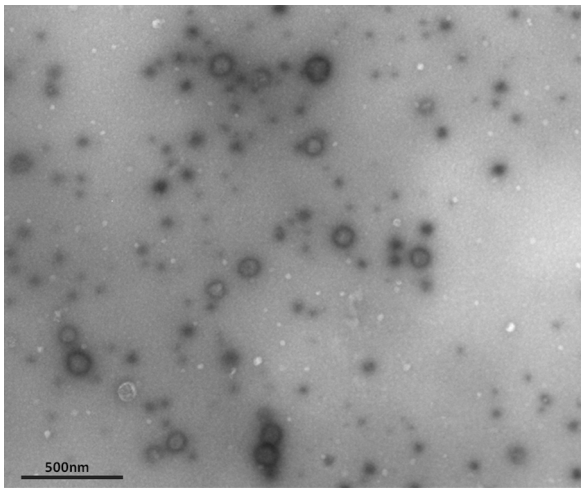

Fig. 1. Extracellular vesicles from *T. cruzi*. Representative transmission electron micrograph of purified extracellular vesicles.

2. Materials and methods

2.1. Trypanosoma cruzi *cultures and extracellular vesicle purification*

T. cruzi epimastigotes from the Dm28c clone [7] epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% of heat-inactivated fetal bovine serum (FBS) at 28 °C. Parasite cultures were started at 1×10^6/ml with passages every 4 days. For total RNA isolation parasites were harvested in late logarithmic growth phase at a cell density of 50×10^6/ml and washed twice in phosphate-buffered saline supplemented with D-glucose at 2 g/l.

Parasites cultured for 48 hours in FBS free RPMI medium were used for total RNA isolation for each fraction. When submitted to this medium parasite viability was monitored by light microscopy through the analysis of motility and by flow cytometry with propidium iodide which showed >98% viable parasites within over a period of 72 h. For purification of EVs the supernatants of 1×10^{10} were collected and centrifuged at $2000 \times g$ for 15 min to eliminate remnant cells. The $2000 \times g$ supernatants were collected and centrifuged at $15{,}000 \times g$ at 4 °C for 30 min to remove cell debris and eventual apoptotic blebs. The $15{,}000 \times g$ supernatant was ultra centrifuged at $110{,}000 \times g$ at 4 °C for 70 min to pellet small extracellular vesicles. The pellet was washed twice in PBS and further ultracentrifuged at $110{,}000 \times g$ for 1 h. Isolation procedures were evaluated by transmission electron microscopy (Fig. 1) and quantification of EVs was done by determining the total protein concentration by the Bradford protein quantification assay (Pierce). By these procedures the total protein yield of the small vesicular fraction was about 1.2 μg per 1×10^{10} parasites.

2.2. *Total RNA isolation and small RNA libraries*

Total RNA was extracted by using Tri Reagent (Sigma) following manufacturer's recommendations. Minor modifications were performed in order to improve small RNA recuperation including the addition of glycogen as carrier after isopropanol precipitation and the use of ethanol 80% for washing.

Total RNA of EVs vesicular fractions was performed in parallel from two different preparations of 1×10^{10} epimastigotes cultured for 48 h in FBS free RPMI medium. Isolation of intracellular RNA was