Farnesol, tyrosol, and oxidative stress modulate the extracellular vesicle proteome and transcriptome in *Candida albicans*

Leitão Jr. NP¹, Amorim MG², Melo A, Mortara RA¹, Sinigaglia-Coimbra R¹, Tashima AK¹, Nunes D², Silva IT², Valieris R², Dias-Neto E² and **Rosana Puccia**

¹Escola Paulista de Medicina-UNIFESP, São Paulo, Brazil; ²A. C. Camargo Cancer Center, São Paulo, Brazil

Background: EVs are closed structures, surrounded by a lipid bilayer membrane, which transport cellular components to the outside environment in all kinds of organisms. EVs are involved in cell signaling and immune modulation. Fungal EVs carry protein, lipid, polysaccharide, melanin, mRNA, s(small)RNA, and probably other molecules not yet described. **Objetive:** To characterize and compare the extracellular vesicles (EVs) produced by *Candida albicans* stimulated by farnesol, tyrosol, and oxidative stress.

Methods: We used concentration and differential centrifugation steps to isolate EVs from culture supernatants of *C. albicans* cultivated for one day under shaking at 37°C in Ham's F12 defined medium (pH 6.3) + 1,5% glucose (controls), in the presence of farnesol (50 µM), tyrosol (20 µM) or 25 mM H₂O₂. The stimuli did not alter viability or cell morphology. EVs were analyzed by nanoparticle-tracking for number and size, by microscopy for interaction with *C. albicans* and macrophages, and by liquid chromatography coupled with in-tandem mass spectrometry for quantitative proteomics. We used Bioanalyzer and RNA-seq (Proton platform, Ion Torrent) for characterization of the mRNA and sRNA content in both EVs and *C. albicans* cells for each condition in biological triplicates.

Results not shown below: *C. albicans* EVs were up to 250 nm in diameter. Controls peaked at 40 and 92 nm. Farnesol evoked about 8-fold increase in EV yields and a decrease in the mean EV size (one peak at 46 nm). Tyrosol and oxidative stress had the opposite effect (lower yields and increased number of EVs around 100 nm). *C. albicans* EVs interacted with and were uptaken by fungal cells, as shown by confocal microscopy. Bioanalyzer profiles of total RNA: EV RNA profiles were similar to control cells samples. EV/cell-farnesol sRNA peaked at 100 nt. EVs tended to be smaller in stressed EVs (100-150 nt) than in controls (100-250 nt). mRNA peaked at 400-700 nt. The stimuli affected EV mRNA at higher rates than cell mRNA. Uptake assays with PKH26-labelled fungal EVs showed that they were either bound to or internalized by 10% of cultured *C. albicans* cells. In our culture conditions about 10% of the cells were in the filamentous form.

General conclusions: Extracellular vesicles from *C. albicans* can signal fungal cells, decreasing their interaction with macrophages. Quorum sense molecules and oxidative stress can modulate the EV size, number, proteome, and transcriptome, thus suggesting that EVs might have a fundamental role in the fungal biology and virulence. Most importantly, comparison between EV and total *C. albicans* transcriptome suggested that the EV population does not reflect the cell mRNA population.

Financial Support: FAPESP, CNPq, CAPES

Candida EV Proteome

C. albicans EV top 10 more abundant proteins; many involved in catalysis

C. abicans EVs (stressed or not) negatively influenced fungal uptake and association index with macrophage Raw 264.7 (5:1 ratio)

mRNA transcriptome (RNA-seq): *Candida* EVs x cells (control, H₂O₂, farnesol, tyrosol)

Distance matrix shows high degree of concordance among samples in cell mRNA (1) and high degree of divergence between EV and cell samples (2, 3); moderate divergence among EVs samples (4). In cells, divergence was mainly between mRNA from oxidative stress versus crt and tyrosol (a). That was partially reflected in EV mRNA (oxidative x crt,b).

Overall results
842 total differentially represented mRNA in EV and cell samples

Number of modulated mRNA from EVs and cells (3x difference, *p*value 10¹³):
EVs x cells = 107
ctr x farnesol = 28
ctr x H₂O₂ = 119
ctr x tyrosol = 19
farnesol x H₂O₂ = 48
farnesol x tyrosol = 20
H₂O₂ x tyrosol = 140

Among 107 differentially represented mRNA in EVs and cells, those in (a) include mRNA for Sec12 and Kip2 (biofilm and polarization); (b) 42 were 7x richer in cells than in EVs, mostly with catalytic functions; (c) 10x more present in EVs tyrosol, H₂O₂, and ctr than in cell RNAs – related to translation.

Highlights from other heat map comparisons:

- ◆ **ctr x H₂O₂:** 66 cell mRNA under oxidative stress were 5x more abundant than other samples. Divergence with stressed EV-H₂O₂ was higher. *STL1* (for a hyphal chaperone) and *TPO3* (for a yeast vacuolar protein) were 10x more abundant in the cells. Nine mRNA were more abundant in EV-H₂O₂ (e.g.: *RTA3*, for a membrane stress protein and *SEC9*, for hyphal t-SNARE related to exocytosis through the plasma membrane).
- ◆ **ctr x farnesol:** the EV content partially reflects the cell mRNA, but EV samples were more distant. mRNA more abundant in EV-farnesol code for Hwp1, C6-02100W-A (stress), Mph1 (DNA replication).
- ◆ **ctr x tyrosol:** the EV content partially reflects the cell mRNA. *CUP9* (transcription), *AOX2* (hyphal growth and biofilm), and *YWP1* (for a cell wall protein; adhesion) were more abundant in cell mRNA.