Scientific and technical report
Project 46PD/2018
Phase 3/2020

Project Title: Molecular investigation of the exosomes-mediated interaction between stem and tumor cells (MIAMI)

General aim:

Stem cells derived from adipose tissue (hASCs) are mesenchymal stem cells with beneficial properties for tissue engineering (TE) and wound healing, and are now considered a safe source for regenerative implants. However, there are a number of studies that have shown that hASCs (1) can respond to tumor signals by migrating from adipose tissue to a tumor site and (2) are susceptible to genomic instability and neoplastic transformation. Exosomes are nanoparticles containing RNA and proteins and mediate intercellular communication. In this context, the hypothesis of the study is that communication between hASCs and breast cancer cells (BCCs) through exosomes could lead to a different response of hASCs during a healing / regenerative process by altering gene expression and signaling pathways in hASCs. The overall purpose of the project is to investigate the exosomal mediated intercommunication between hASCs and BCCs, aiming at testing the safety of hASCs for stem cell therapies.

Phase title: Investigation of the profile of exosomes released by stem cells after interaction with tumor cells

Phase 3/2020 objectives:

Within the 46PD project, in stage 3/2020, the aim was to investigate the exosomes released in the culture medium by the stem cells after the interaction with the tumor cells from the same culture system. In this sense, the following activities were carried out:

Act 3.1 - Isolation of exosomes released into the culture medium by the stem cells after interaction with tumor cells

Act 3.2 - Characterization and validation of exosomes isolated from stem cells after interaction with tumor cells

Act 3.3 - Isolation of total RNA from exosomes

Act 3.4 - Evaluation of the miRNA expression profile in exosomes released by stem cells after interaction with tumor cells

Act 3.5 - Evaluation of the profile of non-coding long RNA expression (lncRNA) in exosomes released by stem cells after interaction with tumor cells

Act 3.6 - Data analysis and correlation of the obtained expression profiles

Phase summary:

The third stage of the 46PD / MIAMI project aimed at investigating extracellular vesicles of the type exosomes secreted by adipose tissue stem cells (hASCs), after an interval of interaction

with exosomes secreted by breast carcinoma tumor cells from the line MDA-MB-231. After isolating the vesicles from the hASC culture medium, they were tested by electron microscopy and Nanoparticle Tracking Analysis (NTA) to validate their size (nm) and exosomal nature. The isolated vesicle fraction showed on average 150 nm, as well as specific exosomal markers. Subsequently, RNA was isolated from these exosomes and the miRNA and lncRNA fractions were analyzed to identify possible changes in the expression of non-coding RNA circulating in the exosomes, following the interaction with breast carcinoma cells. The interaction of stem cells with exosomes from tumor cells was reflected in the exosomal content by overexpression of miRNA species: miR-10a-5p, miR-93-5p, let-7i-5p, miR-206, miR-132-3p and miR -7-5p, as well as some lncRNA species: Malat1, Xist and Hotair. The obtained data were analyzed and interpreted with specialized software, and the obtained results were disseminated by publishing two ISI articles and a book chapter in the international publishing house.

Scientific and technical description:

In the previous stage of the project, the interaction between isolated stem cells from adipose tissue (hASC) and exosomes isolated from MDA-MB-231 tumor cells was performed, in order to integrate these tumor exosomes into stem cells and monitor the effects of this integration at the genetic and post-transcriptional.

In ***activity A3.1***, this interaction was reconstituted for 72 hours, then the culture medium of the stem cells in which the tumor exosomes were integrated was replaced with a new one and after another 24 hours of culture, the new exosomes released by the stem cells were isolated according to the protocol developed in steps 1 and 2. The culture medium obtained after 24 hours was taken and processed using the specialized kit (ExoQuick for Tissue Culture media, SBI) and following the instructions in the kit. Thus, extracellular vesicles released by hASC were isolated after interaction with tumor exosomes, vesicles that may contain altered molecular signals as a result of this interaction. The purpose of the subsequent activities in this stage was to characterize the content of these vesicles and the changes that occurred at the molecular level.

Characterization and validation of exosomes isolated from stem cells after interaction with tumor cells took place in ***activity A3.2***. At this stage, the analysis of the exosomal nature of the isolated vesicles was performed both by electron microscopy and by Nanoparticle Tracking Analysis (NTA), both of which are techniques recognized as exosome validation methods. By means of electron microscopy, vesicles with a diameter between 30 and 150 nm were highlighted, which proves their exosomal nature.

By using NTA technique, several populations of particles were highlighted, classified according to size. Of these, the majority was the fraction of particles (vesicles) with an average size of 157 nm, proving by this technique that in the isolated sample at activity 3.1 exosomes are present. Also, the NTA technique showed a high concentration of exosomes in the isolated sample, respectively 6.63x108 vesicles / ml. The three-dimensional profile of the majority exosomal fraction is shown in fig. 2b, showing a dependence between the size and concentration of these

particles in the isolated sample. In conclusion, by both techniques used, the fraction of extracellular vesicles isolated from the stem cell culture medium was characterized and validated as exosomes.

Once validated as exosomal, the vesicle fraction was subjected to analysis of its contents. In this regard, in ***activity A3.3***, total RNA was isolated from these exosomes using the RNeasy Mini kit (Qiagen), using the manufacturer's instructions and the isolation protocol developed in steps 1 and 2. The total RNA obtained was checked for concentration, purity and integrity and a concentration of 300 ng / µL, good purity ratios and a RIN value of 9.8 were obtained. These data suggest a good quality of isolated RNA.

In ***activity A3.4***, the miRNA fraction was selected from totally isolated RNA in order to evaluate the profile of miRNA expression in exosomes released by stem cells after interaction with tumor cells. Thus, the miRNA profile in hASC stem cells was investigated after interaction with exosomes in tumor cells through miScript miRNA (Qiagen) expression screening and the PCR array technique. The screening consisted in evaluating the expression of 90 species of miRNA (of which the most important are let-7b-5p, let-7c, let-7i-5p, miR-7-5p, miR-93-5p, miR -101-3p, miR-10a-5p, miR-132-3p miR-146b-5p, miR-155-5p, miR-182-5p, miR-192-5p, miR-194-5p, miR-196a- 5p, miR-21-5p, miR-206, miR-214-3p, miR-22-3p, miR-24-3p, miR-498), and the aim was to evaluate the expression level of these miRNAs comparatively between stem cells exposed to tumor exosomes and untreated stem cells.

The miRNA profile obtained revealed changes in certain miRNA species after exposure to the content of tumor exosomes released by breast cancer cells, compared to the miRNA profile obtained for stem cells not exposed to these conditions. Of these, the most relevant changes were highlighted in miR-10a-5p, miR-93-5p, let-7i-5p, miR-206, miR-132-3p and miR-7-5p, as shown. shown in Figure 3. Of the overexpressed species, let-7i-5p, recognized for its contribution to tumor evolution, was expressed 11-fold more in stem cells exposed to tumor exosomes than in control stem cells, indicating the transmission of Non-coding RNA (which may be tumor stimuli) by trafficking in exosomal vesicles between tumor cells and normal cells.

Activity A3.5 evaluated the non-coding long RNA (lncRNA) expression profile of exosomes released by stem cells after interaction with tumor cells. This activity involved amplifying the lncRNA fraction from total RNA isolated from exosomes isolated in activity 3.1., Followed by evaluation of expression by RealTime PCR, based on a dedicated kit (LncRNA profiler qPCR array kit, SBI).

The results of the analysis indicated an overexpression of three important species of lncRNA- Xist, Malat1 and Hotair, all with important roles in tumor evolution. Of these, the most significant expression was obtained for Malat1, suggesting that following the interaction between stem cells and tumor exosomes, this lncRNA was transferred through exosomes to stem cells.

During ***activity A3.6***, data analysis and correlation of data obtained from miRNA profile screening and screening of long non-coding RNA expression (lncRNA) were performed. In order to further analyze the data and the correlations that can be made between the miRNA and lncRNA profile, in the previous stage/ 2019 a specialized software was purchased that allows the elaboration of schemes and trees of analysis and correlations.

These correlations indicated the presence of tumor-specific miRNAs and lncRNAs in exosomes secreted by stem cells after interaction with tumor cells. The presence of miRNA specific exclusively to the tumor environment in breast carcinoma let-7i-5p, miR-10a-5p, miR-7-5p, miR-132-3p, miR-206, as well as Malat1, Xist and Hotair in hASC exosomes suggests efficacy intercellular communication through exosomes, as well as a strong influence of exosome content in non-coding RNA on gene-level control of hASC by exosome-released miRNAs. It is desired to deepen and understand these effects in the future, through further studies of the signaling pathways directly targeted by these changes.

During stage 3/2020, the dissemination consisted in the publication of 2 ISI articles and a book chapter in the international publishing house, in the thematic area of the project:

2 ISI papers:

Dobre, E.G.; Dinescu, S.*; Costache, M. Connecting the Missing Dots: ncRNAs as Critical Regulators of Therapeutic Susceptibility in Breast Cancer. *Cancers* 2020, 12(9), 2698. DOI: 10.3390/cancers12092698 [ISI 6,126]

Chitoiu, L.; Dobranici, A.; Gherghiceanu, M.; Dinescu, S.*; Costache, M. Multi-Omics Data Integration in Extracellular Vesicle Biology- Utopia or Future Reality? *International Journal of Molecular Sciences* 2020, 21, 8550. DOI: 10.3390/ijms21228550 [ISI 4,556].

1 book chapter in an international publishing house:

Dinescu S., Dobranici A., Tecucianu R., Selaru A., Balahura R., Ignat S., Costache M. (2020) Exosomes as Part of the Human Adipose-Derived Stem Cells Secretome- Opening New Perspectives for Cell-Free Regenerative Applications. In: Advances in Experimental Medicine and Biology. Springer, New York, NY; DOI: 10.1007/5584_2020_588