Scientific and technical report
Project 46PD/2018
Phase 2/2019

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: Investigating the interaction between stem cells and tumor cells (breast cancer) and the miRNA profile in this interaction

Phase 2/2019 objectives:

In the 46PD project, in the 2/2019 phase we aimed to investigate the exosomes released by the breast carcinoma cells, as well as the effect of the interaction between these exosomes and the stem cells destined for breast regeneration. In this regard, the following activities were carried out:
Act 2.1 - Isolation and characterization of exosomes from breast carcinoma cells
Act 2.2 - Realization of the interaction between stem cells and tumor cells
Act 2.3 - Evaluation of gene expression in stem cells after interaction with tumor cells
Act 2.4 - Evaluation of miRNA expression profile in stem cells after interaction with tumor cells
Act 2.5 - Screening of possible epigenetic changes occurring in stem cells after interaction with tumor cells
Act 2.6 - Data analysis and intermediary report

Phase summary:

The second phase of 46PD / MIAMI project included the investigation of exosome-type extracellular vesicles released by breast carcinoma tumor cells (from the MDA-MB-231 line) and the effects of the interaction of these exosomes with adipose-derived stem cells (hASCs). In the first phase, the exosomes were isolated from the tumor cells and were validated by electron microscopy as extracellular vesicles with dimensions between 40 and 100 nm. Isolated exosomes were fluorescently labeled, then added to the stem cell culture system. Stem cell interaction with exosomes in tumor cells was reflected at several levels: (i) at gene expression level, decreased p53

expression and increased telomerase expression were identified; (ii) at the posttranscriptional level - by activating miRNA species - miR-155-5p, miR-21-5p, let-7c, miR-96-5p, miR-210, miR-10b-5p and let-7b-5p; (iii) at epigenetic level - through modifications of the type of methylations and acetylations that appeared in the histones H3 and H4. The data obtained were analyzed and integrated with specialized software and the results obtained were disseminated within 3 specialized conferences and an ISI article.

Scientific and technical description:

Within the activity A2.1. isolation and investigation of exosomes released by the breast cancer line MDA-MB-231 was performed. In this sense, the tumor cell culture was first obtained and cultivated up to passage 15. The culture medium released by the cells was collected and processed in order to isolate the extracellular vesicles of the exosome type, by using a specialized kit and following the instructions in the kit.

Isolated extracellular vesicles were further characterized and validated as exosomes by electron microscopy. By this method, vesicles with a diameter between 51 and 133 nm, dimensions characteristic of exosomes, were highlighted. The method of detection by electron microscopy is currently considered the standard for the characterization of exosomes. Moreover, by western blot technique, the obtained vesicle suspension was analyzed and proved to be positive for the CD9, CD63 and CD81 markers, thus confirming the exosome character of these isolated vesicles.

Within the activity A2.2. the interaction between the adipose tissue stem cells (hASC) and the exosomes isolated from the MDA-MB-231 tumor cells was performed, in order to validate the integration of these tumor exosomes into the stem cells and to monitor the effects of this interaction. In this respect, the exosomes isolated from the tumor cells were labeled to be visualized - green fluorescence, then added to the culture medium of the stem cells for a period of 72 h. Validation of the integration of these labeled exosomes into the cells stem was performed in confocal microscopy.

After a 72h incubation, gene expression was evaluated in the stem cells after interaction with the exosomes isolated from the tumor cells, within the A2.3 activity. For this, RNA was isolated from the hASC monolayer from the obtained culture systems. RNA was isolated with the RNeasy Mini kit (Qiagen), using the manufacturer's instructions. The obtained RNA was verified for concentration, purity and integrity, then re-transcribed to the appropriate cDNA using the HighCapacity cDNA Reverse Transcription kit (ThermoScientific). Gene expression of telomerase and p53 was analyzed by RealTime PCR (qPCR), compared with expression of the same genes in stem cells that were not exposed to tumor exosomes. Following this analysis, a modification of the gene expression was identified, in order to increase the telomerase activity and to decrease the expression of the p53 tumor suppressor in hASCs treated with hASCs not exposed to the tumor exosomes.

Later, in activity 2.4, the miRNA profile in hASC stem cells was investigated after the interaction with exosomes from tumor cells through expression screening with the miRNAkit (Qiagen). This profile was compared with miRNA expression in untreated (control) stem cells. In the first phase, the method and flow of analysis were optimized, which included the following steps: isolation of RNA from the sample and control, enrichment of the isolated fraction in miRNA, reverse-transcription with specific enzymes up to complementary DNA and evaluation of the

expression of miRNA species by RealTime PCR. Following this analysis, the species miR-155-5p, miR-21-5p, let-7c, miR-96-5p, miR-210-3p, miR-10b-5p and let-7b-5p were identified as overexpressed in the sample analyzed compared to control. miR-22-3p, miR-182-5p, miR-100-5p did not show expression differences between sample and control. Among the overexpressed species, let-7c and miR-21-5p showed the most significant fold change and can be considered as markers of the analyzed interaction.

Within A2.5 activity, it was desired to correlate the effects of the interaction of exosomes from breast carcinoma cells with stem cells with epigenetic mechanisms that could be activated after this interaction. For this, we performed a screening of the modifications that can occur at the histones H3 and H4 following the defined interaction, by ELISA analysis methods and multiplex kits. Screening of possible epigenetic changes in stem cells after interaction with tumor cells revealed changes that occur at both histone H3 and H4 levels, in well-defined positions. Thus, the analysis revealed changes in H3K4me1, H3K9me1, H3K27me1, H3K27me2, H3K27me3, H3K36me2, H3K9ac and H3K27ac at the level of histone H3 in cells exposed to exosomes from breast carcinoma cells MDA-MB-231. These changes consist of methylation (me) and acetylation (ac) occurring in the aforementioned positions. In the case of histone H4, changes of acetylation type were observed at the H4K5ac, H4k16ac, H4R3m2a and H4K20m2 levels.

During activity A2.6, data analysis was performed and the data obtained at the screening of the miRNA profile and the screening of epigenetic modifications were attempted. It was concluded that both epigenetic and post-transcriptional mechanisms coordinately regulate pro-tumor signals transmitted through exosomes. Investigation of miRNAs involved in hASC interaction with tumor microenvironment in vitro revealed a number of miRNAs active in this interaction: miR-155-5p, miR-21-5p, let-7c, miR-96-5p, miR-210, miR- 10b-5p and let-7b-5p. The family members let-let-7c, let-7b, let-7i, let-7e modulate this interaction either by controlling adipogenesis, or by controlling the genes involved in tumorigenesis, or by controlling both processes simultaneously. The presence of miRNAs specific exclusively to the tumor medium in breast carcinoma let-7i-5p, miR-10a-5p, miR-7-5p, miR-132-3p, miR-206 in the hASC-MDA interaction environment, however, warns of the possibility gene control of hASC by miRNAs released from exosomes. The molecular mechanisms involved in this interaction are not elucidated and require further studies in the future.

In order to further analyze the data and the correlations that can be made between the miRNA profile and the profile of epigenetic modifications, a specialized software (partially acquired with the project funds) was purchased that allows the elaboration of diagrams and trees for analysis and correlations.

The results obtained during this stage 2/2019 were disseminated as follows:

1) **Dinescu S.,** Ignat SR., Balahura R., Selaru A., Costache M., Human adipose-derived stem cells display altered exosomal miRNAs profile after interaction with breast cancer cells, FSEV Congress, 14-15.10.2019, Nantes, Franta.
2) **Dinescu S**., Zarnescu O., Costache M., Human adipose-derived stem cells produce exosomes that carry pro-regenerative signals, Al 11-lea Congres National de Biologie Celulara cu participare internationala si a 37-a Sesiune Stiintifica Anuala a Societatii Romane de Biologie Celulara, 20-23.06.2019, Constanta, Romania.

3) **Dinescu S**., Chitoiu L., Fertig TE., Zarnescu O., Costache M., Isolation and characterization of the exosomes released by the triple negative breast cancer cell line MDA-MB-231, Conferinta Internationala a SRBBM 2019, 26-27.09.2019, Iasi, Romania.

1 ISI paper:

Dinescu S., Ignat S., Lazar A., Constantin C., Neagu M., Costache M., Epitranscriptomic signatures in lncRNAs and their possible roles in cancer, *Genes* (MDPI ISSN: 2073-4425) 2019, 10(1):52. [ISI 3.331].