# SMALL EXTRACELLULAR VESICLES PRODUCTION BY BRAF MELANOMA CELLS

**Key words: MELANOMA, ISOLATION PROTOCOL, EXTRACELLULAR VESICULES**

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## INTRODUCTION:

Melanomas are extremely aggressive tumors that originate from mutations in melanocytes (SHELTON et al., 2020). In 2020, melanomas were responsible for more than 324.000 new cases and 57.000 deaths worldwide (SUNG et al., 2021). The high metastatic potential of this type of tumor and an innate resistance of tumor cells are the main challenges for the treatment of this disease (DOMINGUES et al., 2018; FALCONE et al., 2020).

Currently it's a well stablished fact that the development and progression of tumors is strongly influenced by the tumor microenvironment (ARNETH, 2020; ANDERSON e SIMON, 2020; HINSHAW e SHEVDE, 2019). Small extracellular vesicles (sEVs), as exosomes, are constituents of the tumor microenvironment. These vesicles are smaller than 200nm and have a lipid bilayer, as established by the International Society for Extracellular Vesicles (THÉRY et al., 2018). The involvement of sEVs in oncogenic events has been associated with their ability to reprogram target cells, in a process known as tumor education (PARAYATH et al., 2020; QUESENBERRY et al., 2015). This process involves the release of oncogenic signaling mediators by tumor cells which can result in functional and/or phenotypic changes in targeted cells (LAZAR et al., 2018; LUCCHETTI et al., 2020).

Several studies have shown that melanoma-derived cells are associated with the regulation and reprogramming of diverse cell types through the release of sEVs, resulting in changes that promote tumor growth and progression as well as metastasis (CHENG et al., 2021; PIETROWSKA et al. 2021). Then, the biological understanding of extracellular vesicles in the tumoral process might contribute to the development of more effective treatments against this tumor. Therefore, the aim of this study is to establish a protocol for the isolation of melanoma-derived sEVs in order to allow the investigation of the role played by these particles in the skin cancer progression process.

## METHODS:

Melanoma cells (Skmel-28) were cultured in a humidified atmosphere with 5% $CO_2$ at 37°C. DMEM medium was supplemented with 10% of fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin sulfate). For the sEVs isolation, the cells had to be temporarily cultured in serum-free medium. Therefore, to assess cell viability and morphology after the serum deprivation period, MTT reduction assay and morphological aspect (crystal violet staining) were performed. In both cases, skmel-28 cells were cultured in 24-well plates at a density of 70,000 cells/well for 24h. Subsequently, the culture medium was removed and replaced with a new medium without the addition of FBS, in which the cells were maintained for up to 24h. For the MTT reduction assay, after serum deprivation, cells were incubated with MTT (0.6 mg/mL) for 3h at 37°C. The medium was then removed and the formazan, solubilized with ethanol. The absorbance was measured at OD = 570 nm (Epoch Microplate Spectrophotometer - Biotek).

For the crystal violet staining, the medium was removed and the wells washed with 1mL of PBS. Then, 500uL of crystal violet solution (0.5%) was added and the cells were incubated for 45 minutes at 37°C. After incubation, the solution was removed and the plates were inverted for overnight drying. Cell images were recorded with the aid of a microscope (Nikon Phase Contrast).

Following these steps, the sEVs isolation was performed. Skmel-28 cells were cultured in 75 $cm^2$ bottles with 25 mL of DMEM medium containing FBS for 24h. The medium was, then, removed and the cells were washed 3 times with 5 mL of PBS filtered with a syringe filter (pore size: 0.22 µm). Subsequently, 15mL of medium without FBS, also filtered, was added to each bottle. After another 24h, the culture medium was collected and centrifuged for 30 minutes at 4500 g. The supernatant was collected and filtered through a syringe filter (pore size: 0.22 µm). The sample was enriched by allocating the filtered supernatant in a concentrator tube (Vivaspin Turbo 15 with 10000 MWCO filter membrane - Sartorius) which was centrifuged for 15 minutes at 4500 g. To the recovered concentrate, Total Exosome Isolation Reagent (Life Tech) was added at the ratio of 1 volume of reagent to 2 volumes of concentrate. The solution was mixed gently by inversion, and incubated overnight at 4°C with gentle agitation. Then, the samples were centrifuged for 60 minutes at 10,000g, 4°C. The supernatant was discarded, and the pellet was ressuspended in PBS filtered through a 0.22 µm filter pore. The number of cells in each bottle was calculated by counting in Neubauer chambers, for yield calculations of vesicle production.

To determine the size and concentration of the EVs was used the NanoSight NS300 equipment (Malvern Instruments, UK). The EV samples obtained were diluted 1:10 (volume of 1 mL injected into the equipment) in filtered PBS (pore size: 0.22 µm). Each experiment was carried out at 25°C with the acquisition of 3 videos of 30 seconds each. The capture parameters, slider gain 15, camera level 8 and detection threshold 4, were maintained in all captures. Analyzes were performed by NanoSight 2.3 software.

## RESULTS AND DISCUSSION:

To isolate extracellular vesicles it is necessary to culture the cells in serum-free medium once the serum naturally contain vesicles that might contaminate the medium with extracellular vesicles originated from melanoma cells. Therefore, viability assays were performed by crystal violet and MTT to assess whether the depletion of serum for 24h interferes in the viability of melanoma cells (Skmel-28).

The data obtained demonstrate that serum deprivation for 24h didn't affect the viability of cells. Cell morphology was preserved when compared to the control group (no serum deprivation) (figures 1 and 2). Analysis of MTT reduction showed that the cells in serum-free medium, although present variations in the ability to reduce MTT, maintained a viability of approximately 70% in relation to the control group. This percentage is within the expected range since serum deprivation can lead to a certain metabolic decrease, without this necessarily indicating cell impairment (figure 3).

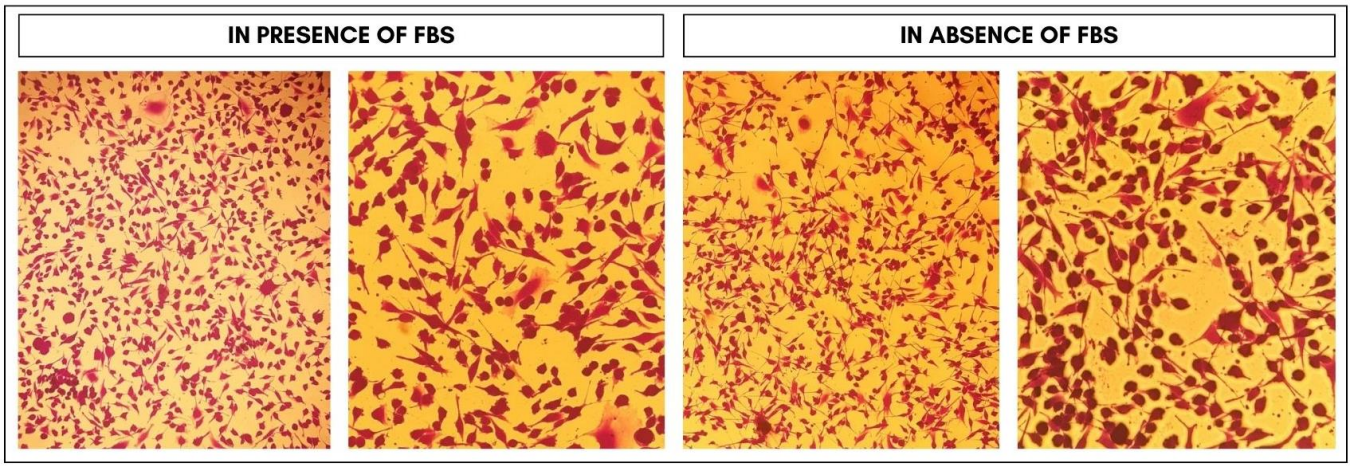


**Figure 1:** Skmel-28 cells stained with crystal violet for morphological evaluation after 24 hours of treatment. Images were captured at different magnifications. Control group cells were maintained in medium with FBS. The treated group was maintained in serum-free medium for 24 hours.

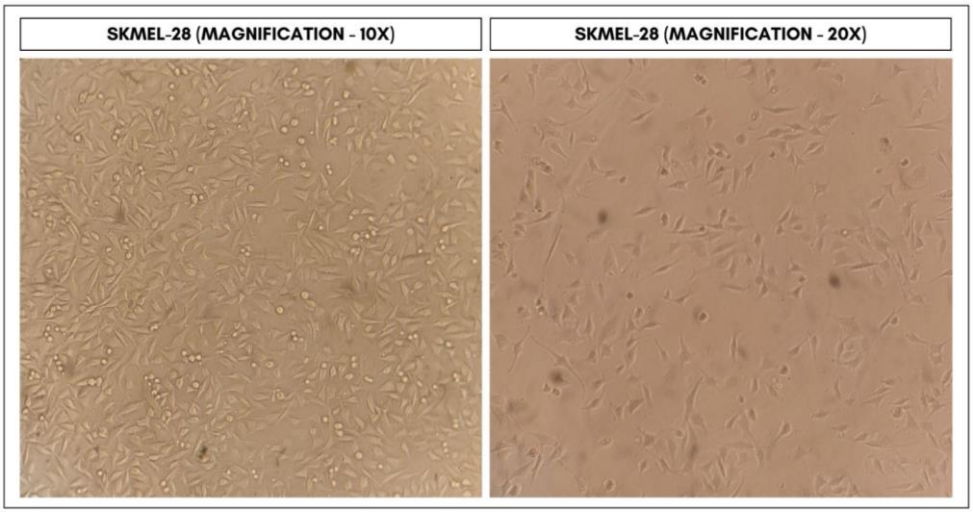


**Figure 2:** Skmel-28 cells in bright field, 24 hours after addition of serum-free medium. Images were captured at different magnifications from T75 bottles prepared for vesicle extraction.

After isolation of vesicles, the NTA analysis were performed. NTA data showed a vesicle concentration of $7{,}21 \times 10^8$ particles/mL and average size of 163.9 nm (figure 4). Notably, this size is compatible with exosomes characteristics according to literature data. The small variation in particle size,

as can be seen in figure 4, is also indicative of a homogeneous population of extracellular vesicles, highlighting the adequacy of the protocol. Interestingly, the high yield of isolation of extracellular vesicles ($7{,}21x10^8$ particles/mL) may be associated with the greater aggressiveness observed in melanoma-type tumors once these vesicles are associated with the reprogramming of target cells, leading to the establishment of a pro-tumor phenotype. Furthermore, studies have demonstrated that more aggressive cellular lineages release more extracellular vesicles.

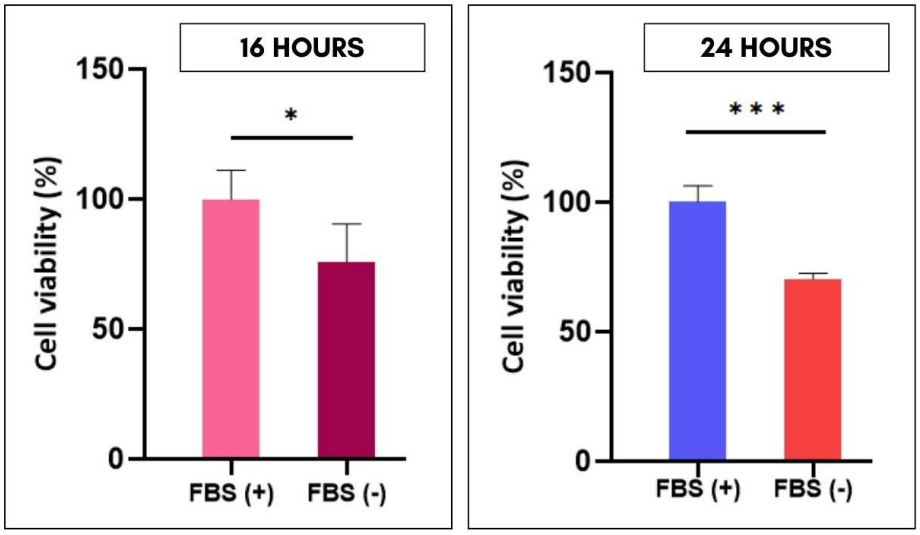


**Figure 3**: Cell viability graphs (Skmel-28), 16/24 hours after addition of serum-free medium. For data analysis, t test (unpaired) was performed. *** P-value < 0.0001. *P-value = 0.0186. Absorbance was measured in a spectrophotometer (570nm).

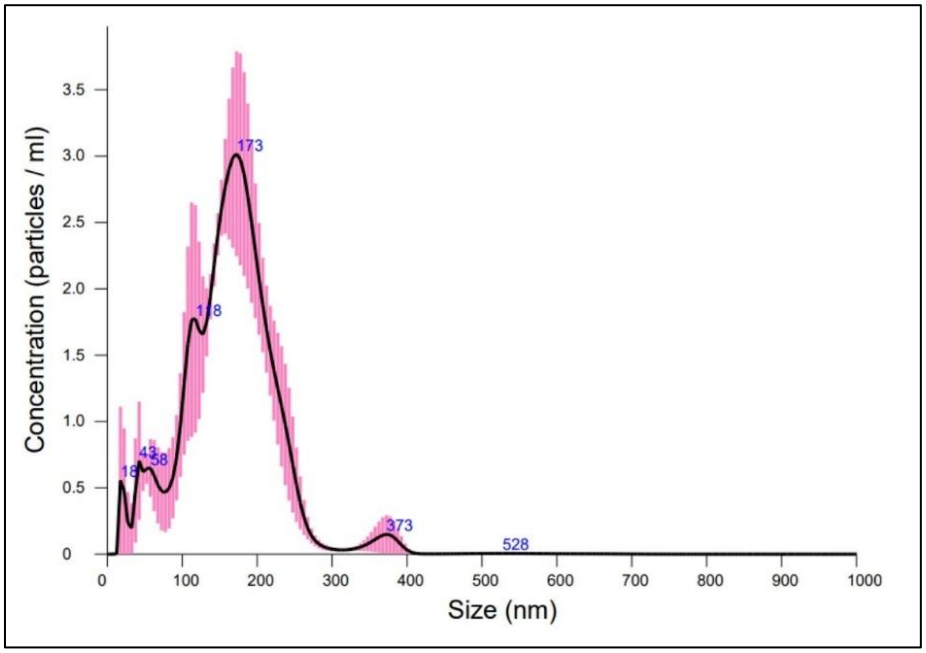


**Figure 4**: Size distribution/ concentration of extracellular vesicles extracted from Skmel-28 cells. Data were obtained from 3 videos of 30 seconds. The parameters used were: laser type (green), camera level (8), slider gain (15), detect threshold (4). Error bars represent +/- 1 standard error of the mean.

## CONCLUSIONS:

The results indicate that cell viability was maintained after 24h of serum deprivation, condition required to isolate the extracellular vesicles. The size of the vesicles is in agreement with the expected for sEVs and the yield was significant. Importantly, the established protocol appears to be useful as it provided a high yield of isolated vesicles, which is crucial for the biofunctional analysis of sEVs.

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