

**EXOSOME UPTAKE INTO HEY OVARIAN CANCER CELLS AND
ITS POTENTIAL TO SERVE AS A VESSEL FOR GENE THERAPY**

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**EXOSOME UPTAKE INTO HEY OVARIAN CANCER CELLS AND
ITS POTENTIAL TO SERVE AS A VESSEL FOR GENE THERAPY**

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SUMMARY

Exosomes are microvesicles that are released from several different types of cells.¹⁵ Exosomes are thought to play an important role in functions such as immune regulation and coagulation; however their full functionality is not completely understood.⁸ Current research has started to explore their potential utilization in gene therapy and drug delivery. Their derivation from different proteins and RNA make them a versatile transport target in microbiology research.^{3,4,10}

Although exosomes are being increasingly used in current research for gene therapy applications, the actual mechanism is unknown once the exosomes are taken into the cells. Using microfluidic channels, the entire process of exosome uptake can be imaged and monitored. The design of the microfluidic device allows for the manipulation of cellular flow and imitates the real flow of cells during exosome uptake and interaction.¹⁸ The microfluidic device is made from a mold using polydimethylsiloxane (PDMS) and the channels are coated with fibronectin for the cells to adhere to. The device is plasma bonded to a thin sheet of PDMS, incubated, and then left to cure. Because of its ability to grow quickly and efficiently in less-than-ideal conditions, hey ovarian cancer cells are used to seed the device. The hey cells are seeded at a density between five million and 10 million cells in the device, and fresh media is pumped through the device. The cells are left to adhere and proliferate for between 24 hours while fresh media is passed through the device in the 37°C incubator. The hey cells are dyed using a DAPI fluorescent stain which causes the hey cells to illuminate blue fluorescence. Exosomes that are stained with PKH to illuminate green fluorescence are then seeded into the channels, and images are taken using confocal microscopy at several time points. The images showing blue fluorescent hey cells and green fluorescent exosomes are overlaid to show the exosomes uptake into the hey cells. Several images are taken

across approximately a 20-minute time period to show the interaction between the exosomes and the cells in the channels.

Currently, images have been taken of the uptake of exosomes into the key cells without a continuous flow in the channels. The time lapse of how the exosomes are taken into the cells in the microfluidic device channels can be observed, but the point of interest comes in the effect of flow on the exosome uptake. Future studies and plans look to observe a constant flow through the channel while the exosomes are seeded into the device. The effect of flow on the uptake as well as the observation of exosome breakdown after being taken into the cells will help to elucidate the potential of exosomes as a method for gene therapy and drug delivery.

CHAPTER 1

INTRODUCTION

Exosome Background

Exosomes are microvesicles initially thought to be the cell's method of removing unwanted proteins from the cells, which is how the term "exosome" was coined.¹⁴ However, exosomes have recently been found to play an important role in immune regulation as well as coagulation, intercellular signaling, and waste management. They are released by several different types of cells such as reticulocytes⁵, dendritic cells¹², B cells⁸, T cells¹, mast cells⁹, epithelial cells⁶, and tumour cells⁷. Although the exact function of exosomes is not known, evidence has shown that it can be used in specific targeting and signaling pathways in the cell.⁴

Exosomes are generally about 30-120 nm in size and are composed of a lipid bilayer of functional biomolecules such as proteins, mRNA and miRNA. The structure allows for exosomes to be delivered to a target cell through fusion which also indicates its importance in cell-to-cell communication.¹³ Exosomes will contain proteins that are specific to the cell type that they are derived from.²

Literature Review

The research of exosomes and their functionality has only recently become a topic of interest to scientists and researchers. Over 1500 papers about exosomes have been found on PubMed, all within the last 3 years. Basic functionality and biological functions have been explored by researchers, but there is still much unknown about exosomes, including, but not limited to, their mechanistic pathway and uptake into the cells. Because

they are derived from so many different types of cells, exosomes may be an important precursor to early gene therapy and cell-derived targeting.¹⁷

Exosomes show potential in delivering siRNA by using the body's own intercellular "shuttle" for RNAs. Currently, lipids are the preferential vessel for siRNA delivery and efficient and specific delivery is achieved to the liver. However, this approach raises the concern of liver toxicity. Utilizing exosomes for siRNA delivery would alleviate toxicity and the need for additional methods to induce delivery since exosomes naturally embody intercellular communication.¹⁶

In terms of diagnostics, exosomes may help for early detection and diagnosis. As a specific example, research has been conducted exploring the microRNA signatures of exosomes and the microRNA-expression profiles of ovarian cancer tumor cells. By screening the exosomes from the patient, even subtle expressions may indicate early stages of ovarian cancer. In illnesses such as cancer, early detection may mean the difference between the patient's survival and ease of treatment.¹¹

Exosomes are not clearly understood, and as such their potential is currently untapped. As researchers work to understand their mechanism, functionality, and pathways of expression, the possible applications are numerous. This research would contribute to the ongoing understanding of the pathway and interaction with the cell to further gene therapy research.

CHAPTER 2

MATERIALS AND METHODS

Materials

For the PDMS construction, Sylgard® 184 Silicone Elastomer Curing Agent and Sylgard® 184 Silicone Elastomer Base is needed along with a vacuum desiccators and vortex machine to stir the compound thoroughly. Pipettes and bakens are used for accuracy. The device is placed in a 5.365 inch diameter petri dish. In order to seed the cells and pass media through, liquid fibronectin, 8% dextran, and sigmacote is needed to prepare the device for the cells to adhere and proliferate. Both larger and smaller tubing, 0.020" ID x 0.060" OD and 0.012" ID x 0.030" OD respectively as well as a blunt point needle (20 gauge x 1/2") to pump media through the device properly are needed. Standard cell culture procedure is used to passage the hey cells accordingly. A standard 37°C incubator is used to house the cells. When imaging, green PKH dye is used to stain the exosomes and DAPI is used to stain the hey cell nuclei.

PDMS Construction

For this experiment, microfluidic devices were designed in a simple channel, and the mold was constructed using the nanotechnology clean room. The channels are intended to mimic real-condition flow.

When mixing the polydimethylsiloxane (PDMS), a 10:1 (w/w) ratio of base to hardener is used. The air bubbles are removed using a vacuum desiccator, and the mixture is poured onto the mold for an approximate thickness of 5mm. The vacuum is

used once again to further remove any remaining air bubbles that may have resulted from the pour. The mixture is then either cured at 60° C or left to harden overnight.

For this particular petri dish that has a 5.5 inch diameter and a thickness of 1 inch, in order to have the devices with a height of 0.500 cm, calculations for the volume are conducted in order to determine the amount of PDMS mixture necessary. The volume calculation is represented as a cylinder which gives $V = \pi r^2 h$. When plugging in the dimensions of the petri dish and the desired height, it can be determined that a volume of 76.6006 mL of PDMS is needed. With the ratio mixture mentioned previously, it can be determined that 70 mL of base and 7 mL of hardener is needed for the mixture.

The cured PDMS device is cut from the wafer mold using a knife or a scalpel. Inlet and outlet holes are punched in the device using a 1.0 mm hole punch and the surfaces are cleaned thoroughly with scotch tape. A thin sheet of PDMS or a microscope cover slip is used that is slightly larger than the PDMS device for bonding. One side of the PDMS device and the bonding material is exposed to oxygen plasma for 3 minutes, alternating the vacuum and oxygen for periods of 30 seconds each. The plasma knob is turned to High, and the observed color should be a light pink/purple. After the designated time, the vacuum seal is released and the bonded sides are placed together. The device is then placed on a hot plate for approximately 10-15 minutes to solidify the bonding.

Cell Seeding

Smaller tubing is then connected to a small length of larger tubing. A blunt-point needle of a syringe with 50 µg/mL of fibronectin is passed through the channels for cell adherence. The fibronectin is given between 40-60 minutes to coat the device's channels. The large tubing is connected to a syringe with the blunt-point needle and the smaller tubing is inserted in the "inlet" hole in the PDMS device. Pressure is applied to the syringe to seed at a cell density between 5×10^6 and 8×10^6 cells. A fresh syringe with

fresh media is connected to the device and left to pump fresh media through the channels at an approximate rate of 3 $\mu\text{L}/\text{min}$.

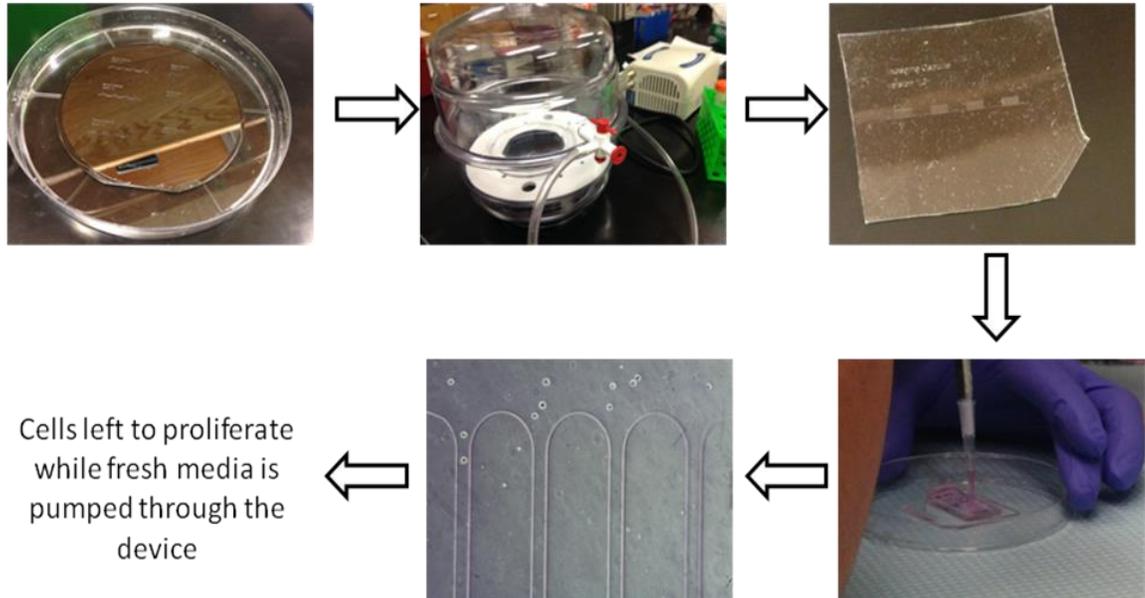


Figure 1: Set-up of the protocol used to run the experiment. The mold is used to pour out the PDMS which is then placed in the vacuum dessicator to remove any air bubbles. The devices are then cut out from the hardened mold, and the devices are seeded with cells and left to proliferate.

The cells are left for 24 hours to adhere and proliferate in the device channels.

CHAPTER 3

RESULTS

In order to image the live uptake of exosomes into the cell, the devices were imaged under a confocal microscope at 20x magnification. The hey cells were stained with a DAPI dye which would cause the nuclei to fluoresce blue. The exosomes that were extracted from the cells and labeled with a green PKH dyed were run through the channels and images were taken to see the live-time uptake of exosomes into the cells. In a matter of minutes (maximum of 20 minutes), the exosomes were completely taken into the cells.

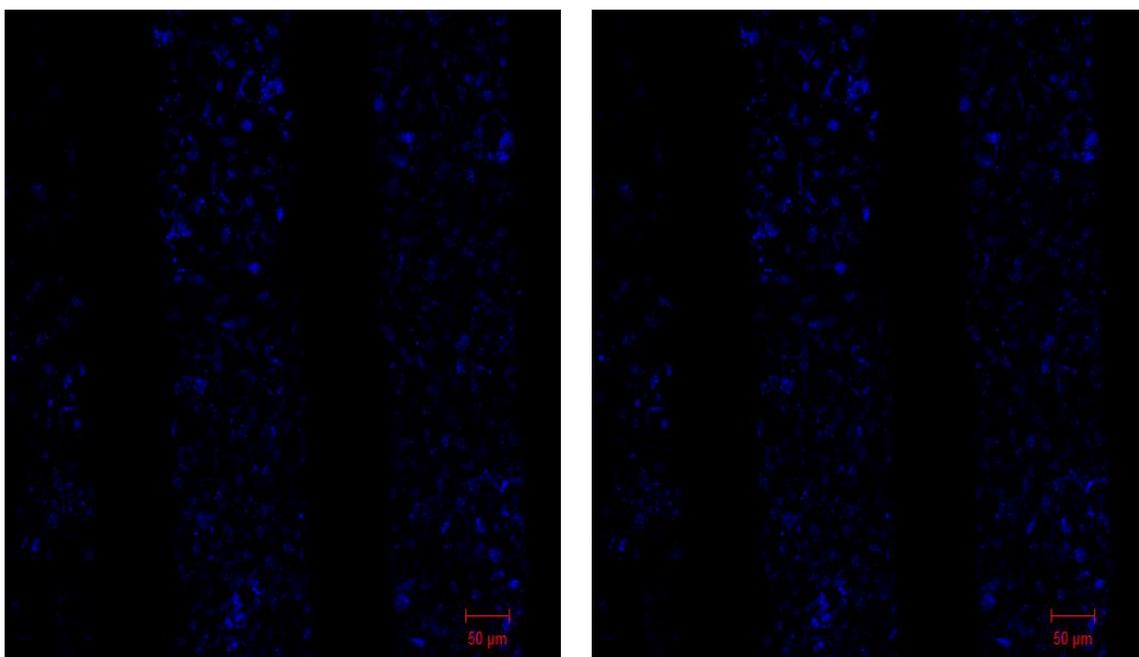


Figure 2: DAPI stained hey cells in microfluidic device channels at 20X magnification.

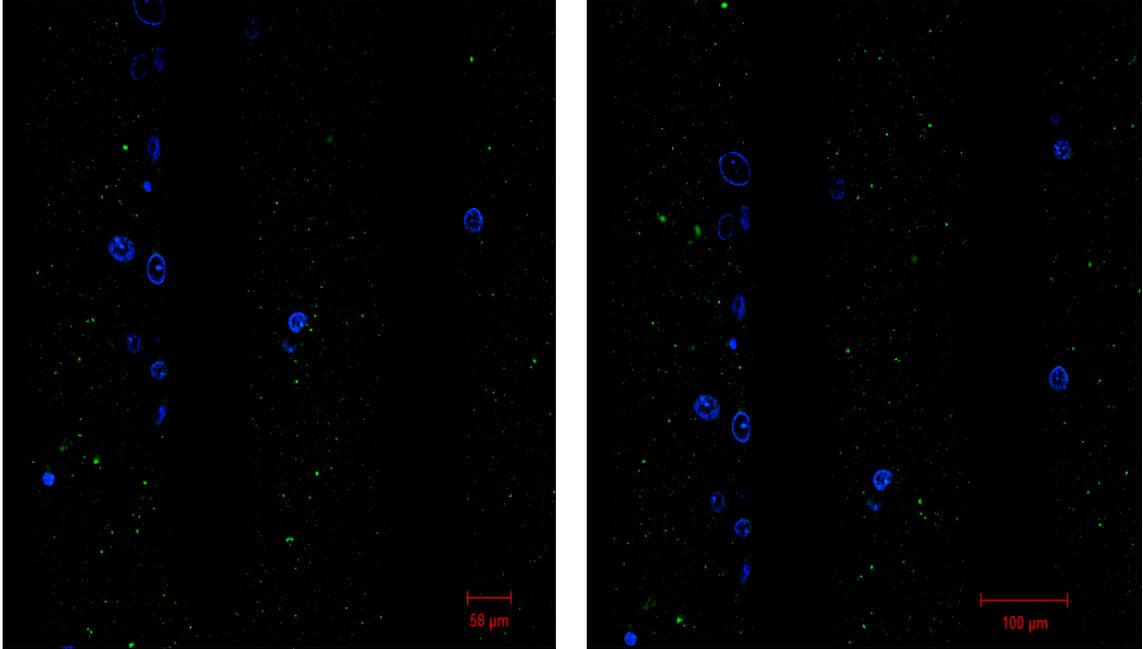


Figure 3: DAPI stained hey cells and PKH stained exosomes in microfluidic device channels at 20X magnification

CHAPTER 4 DISCUSSION

Errors and Modifications

Throughout this experiment, there were several setbacks and needs for modifications to the methods. The first experiment, the cells were seeded at density of approximately 2×10^6 cells/mL. The cells did not adhere or proliferate very well, and so it was determined that a higher density would be necessary, especially to obtain good confocal microscope images and to allow the cells to adhere and proliferate in the channels.

The biggest issue came from false positive readings from the PKH dye. When imaging, the green dye was staining the actual PDMS of the device, causing a high reading of false positives for exosomes. In order to reduce the number of false positives, several alternatives were considered. First, it was decided to leave the dye to stain the exosomes for a longer period of time before adding them to the device. The idea was that this method would allow the dye to stick to the exosomes and not seep out into the PDMS channels of the device. Secondly, a side-by-side positive control would be conducted in order to “subtract out” the false positive readings and be able to tell which were the actual exosome readings. This would also allow for a direct comparison under the same microscope settings, which would also help if other issues arose. A third potential fix for the false positives would be insert a hey cell and exosome stained mixture together into an empty channel. This would cut down the time that the dye is exposed to the device, but the issue would arise in whether or not real time images could be obtained to see the actual uptake in the cells. The most fundamental fix would be to ensure that the threshold settings of the microscope are within the range that minimizes the number of false positive readings caused by the PKH dye (**Appendix A**).

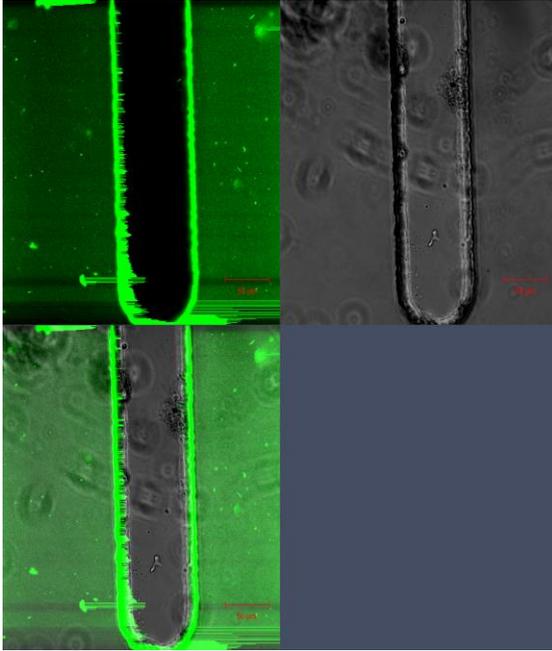


Figure 5: Confocal microscope images of channels with PKH labeled exosome false positives.

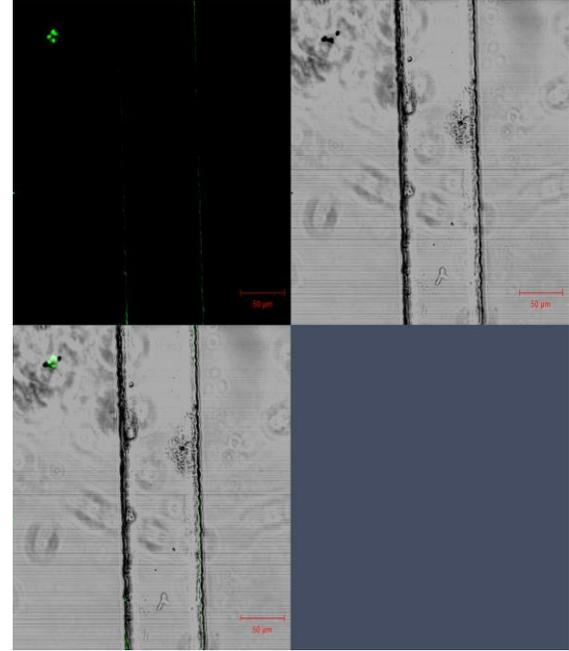


Figure 6: Confocal microscope images of channels with PKH labeled exosomes with adjusted settings to account for false positives.

On the extreme, the dye could be changed possibly to a CDK red dye. Since the CDK dye is released intracellularly, the dye would not seep out into the channels. Also, the RFP cell line which has both the exosomes and cells could also be seeded onto the device to show the real time uptake of the exosomes, which would also solve the problem of false positive readings.

CHAPTER 5

CONCLUSION

This experiment proves the utility of exosomes and the ease with which they are taken into the cells. Because they are derived from the cells, the exosomes are not identified as a foreign body which diminishes the immune response. The exosomes are biocompatible with the cell line they are being inserted back into, making it a potential gateway for gene therapy and possible drug delivery. More research is needed to explore the full uptake process as well as any negative results that may happen after the exosome is delivered. Because so little is known about exosomes and their functionality, there is potential for the exosome to play a major role in drug delivery and gene therapy, as well as diagnostics and other medical applications.

CHAPTER 6

FUTURE PLANS

In continuing this project, different cell lines could be explored in order to see the different uptake time and pathway. Images could be taken at different time points in order to compose a comprehensive timeline of exosome insertion and uptake. The most important next step would be to mimic real body conditions by incorporating a fluid flow of media. Additionally, future plans look to seed the devices with the RFP cell line to get a more real-time time scale of the uptake into the cell.

APPENDIX A

IMAGING CONTROL THRESHOLD SETTINGS

When running the cell imaging with green PKH-labeled exosomes, the settings were taken at a gain of 793, offset of -30, and a digital gain of 0.3. The settings were altered in order to reduce the amount of false positives as mentioned in the discussion, and the threshold settings for the minimum false positive readings are a gain of 600, offset of -60, and a digital gain between 0.3 and 0.5 with a laser strength of 10.0.

REFERENCES

1. Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., & Hivroz, C. (2002). TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ ζ complex. *The Journal of Immunology*, 168(7), 3235-3241.
2. Clayton, A., Court, J., Navabi, H., Adams, M., Mason, M. D., Hobot, J. A., ... & Jasani, B. (2001). Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *Journal of immunological methods*, 247(1), 163-174.
3. Kooijmans, S. A., Vader, P., van Dommelen, S. M., van Solinge, W. W., & Schiffelers, R. M. (2012). Exosome mimetics: a novel class of drug delivery systems. *Int J Nanomedicine*, 7(1525), e41.
4. Lakhali, S., & Wood, M. J. (2011). Exosome nanotechnology: An emerging paradigm shift in drug delivery. *Bioessays*, 33(10), 737-741.
5. Pan BT & Johnstone RM (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 33(3):967-978.
6. Park, J. A., Sharif, A. S., Tschumperlin, D. J., Lau, L., Limbrey, R., Howarth, P., & Drazen, J. M. (2012). Tissue factor-bearing exosome secretion from human mechanically stimulated bronchial epithelial cells in vitro and in vivo. *Journal of Allergy and Clinical Immunology*, 130(6), 1375-1383.
7. Peinado, H., Alečković, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B., Callahan, M., Yuan, J., Martins, V., Skog, J., Kaplan, R., Brady, M., Wolchok, J., Chapman, P., Kang, Y., Bromberg, J., & Lyden, D. (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nature medicine*, 18(6), 883-891.
8. Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., & Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *The Journal of experimental medicine*, 183(3), 1161-1172.
9. Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C., & Desaynard, C. (1997). Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Molecular biology of the cell*, 8(12), 2631-2645.

10. Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., Barnes, S., Grizzle, W., Miller, D., & Zhang, H. G. (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Molecular Therapy*, 18(9), 1606-1614.
11. Taylor, D. D., & Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic oncology*, 110(1), 13-21.
12. Théry, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., & Amigorena, S. (1999). Molecular characterization of dendritic cell-derived exosomes Selective accumulation of the heat shock protein hsc73. *The Journal of cell biology*, 147(3), 599-610.
13. They C, Zitvogel L, & Amigorena S (2002) Exosomes: composition, biogenesis and function. *Nature reviews. Immunology* 2(8):569-579.
14. Trams EG, Lauter CJ, Salem N, Jr., & Heine U (1981) Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. *Biochimica et biophysica acta* 645(1):63-70.
15. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., & Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology*, 9(6), 654-659.
16. Van den Boorn, J. G., Schlee, M., Coch, C., & Hartmann, G. (2011). SiRNA delivery with exosome nanoparticles. *Nature biotechnology*, 29(4), 325.
17. Vlassov, A. V., Magdaleno, S., Setterquist, R., & Conrad, R. (2012). Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1820(7), 940-948.
18. Whitesides, G. M. (2006). The origins and the future of microfluidics. *Nature*, 442(7101), 368-373.