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Evaluating PC12 Cell Differentiation Following Exosome Treatments

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Abstract

Exosomes are extracellular vesicles ranging in size from 40 to 100nm that are used for extracellular communication to control the cell niche environment with regards to differentiation. Initial studies in our lab have shown that exosomes isolated from differentiating neurites can cause differentiation in cells absent typical neuronal hormones. However, these studies only used exosomes isolated after complete differentiation. To further clarify the role of exosomes in cell differentiation, we isolated exosomes from NGF-treated PC12 cells after two, four and six days. The exosomes were taken at the specific times due to a slight majority of cells showing differentiation at day three, with an overwhelming majority showing differentiation at day six. Day two, four and six were thus good markers in the development of cell differentiation. Each set of exosomes was added to a different well of untreated cells, which were then observed for differentiation daily over a six-day period. The results showed a considerably larger amount of percent differentiation in cells treated with the day six exosomes compared to cells treated by the day two and day four exosomes. The change in number of differentiated cells in different exosome treatments indicate that exosomal contents change over time. In an effort to make sure that no NGF contamination occurred in our treatments, an NGF-ELISA was completed testing all the treatment exosomes with results showing no NGF. Additionally, exosomes were isolated from NGF spiked media to show that no NGF was carried over in our exosome isolation process. Both tests further evidence that our exosomes did not contain NGF. Our overall results indicate that the isolated exosomes changed contents over time and that their contents caused greater percent differentiation over time. Funding provided by the Cell Biology Education Consortium (CBEC) and through the AR-EPSCoR Center for Advanced Surface Engineering.

Keywords: Exosomes, differentiation, PC12 cells

1. Background

Exosomes are defined as extracellular vesicles released from cells upon fusion of an endocytic vesicle with the plasma membrane[2]. The importance of exosomes comes from several areas. One important aspect of exosomes is their potential as a drug-delivery system – they are known to be able to cross the blood-brain barrier[6]. With this fact, the potential applications of exosomes are theoretically endless. They could be used as drugs to even affect neurological disorders, like Parkinson's disease[9]. This alone makes exosomes an extremely relevant research topic. Discovering more about them could have many far-reaching medical applications.

While the exact function of exosomes is debated in the literature, the prevailing belief is that cells release exosomes as a means to communicate with other cells[5]. Recently, it has been shown that exosomes contain mRNA and regulatory microRNA,[4] suggesting they could play a role in epigenetic regulation of cells. The same research that looked at the contents of exosomes also looked at if exosomes can spread those contents, with positive results[4]. With this research,

the hypothesis is that if a properly maturating cell's exosomes are isolated, they could be used to help an immature cell grow.
Our research was focused on further elucidating the role of exosomes in cell differentiation. Neurons themselves are not usually self-repairing, which makes humans particularly vulnerable to neurological disorders[7]. However, the potential to deliver exosomes containing differentiation-inducing contents brings hope to those suffering from spinal cord injuries. To test this potential, research needed to done to see if exosomes can cause neuronal differentiation. It has already been shown that exosomes from differentiated Pheochromocytoma (PC12) cells can cause differentiation of mesenchymal stem cells[1]. Without exosomes, nerve growth factor causes the PC12 cells to stop proliferating and project neurite outgrowths similar to a differentiated neuron[10]. In order to determine if the exosomes can induce the same type of differentiation, we wanted to add NGF (nerve growth factor) treated PC12 exosomes taken from different days during differentiation back to PC12 cells and analyze the effects. Our hypothesis is that exosomes taken from later days, and thus from cells with more differentiation, will cause greater differentiation than exosomes taken from cells at an earlier stage in the growth process.

2. Methods

2.1 Cell Culture

PC12 cells were cultured in RPMI Medium with L-glutamine, 10% donor horse serum (DHS), 5% fetal bovine serum (FBS), and 1% Penicillin/Streptococcus antibiotic. They were held at 37°C and 5% CO_2. Cells were added to collagen (Type IV) coated flasks at a density of 5,000 cells/mL. NGF at a concentration of 50ng/mL was added to the +NGF group. On days 2, 4, and 6, the media was removed from the cells and fresh media was added. Pictures were taken every 24 hours to document progress for 6 days.

2.2 Exosome Isolation and Quantification

Cells were centrifuged to remove complete media and re-suspended in differentiation media containing 1% exosome depleted DHS and 1% Penicillin/Streptococcus. The Total Exosome Isolation protocol from Invitrogen (4478359) was used to isolate exosomes from the cell media, re-suspending them in 200µL of PBS for storage. For the media control, the same exosome protocol was followed with differentiation media containing NGF without cells. In order to quantify for treatment, the Thermo Scientific Pierce BCA protein assay kit was used to find protein concentrations of the exosome suspension.

2.3 ELISA Assay

An NGF-ELISA assay was performed on the exosomes used in the differentiation assay. There is always a chance that our filtration protocol in the exosome isolation process was inadequate, allowing NGF to go unfiltered and thus cause differentiation, negating the results of our experiment. In order to show that PC12 differentiation was being caused by exosomes alone and not by NGF, we used this assay to measure the amount of NGF within each exosome sample. The exosome samples came from exosomes isolated from NGF treated cells and no treatment cells over day lengths of 2, 4, and 6 days for each. This was then duplicated into an A and B sample.
The Invitrogen NGF beta Rat ELISA Kit from Thermo Scientific was used. The kit was stored at 4˚ C for three months until usage. Results were calculated by plotting absorbance results of a set of standards provided from the kit, then using the standards to calculate overall concentration of the test samples. (Calculations made using an online point-to-point software, as instructed in the kit directions).

2.4 Differentiation Assay

Cells were plated on rat-tail collagen (Type IV) coated 6-well plates at a density of 5,000 cells/mL in differentiation media. Exosome treated groups were plated in differentiation media and given 5µg of protein from the isolated exosomes. The 5 µg of protein had to be calculated from previously determined concentrations of the exosomes. Media was changed on day 3 with another 5µg of exosome protein added. Pictures were taken every 24 hours for 6 days, and cells were lysed on the last day.

This experiment was replicated with corroborating results during the summer of 2018. The same plates were used, but the cell density was lowered to 3,000 cells/mL in an effort to obtain a larger surface area for the PC 12 cells to differentiate. The previously isolated exosomes were used to add 5µg protein as in the initial assay. Pictures were taken every day, and media was still changed on day 3.

2.5 Determining Differentiation

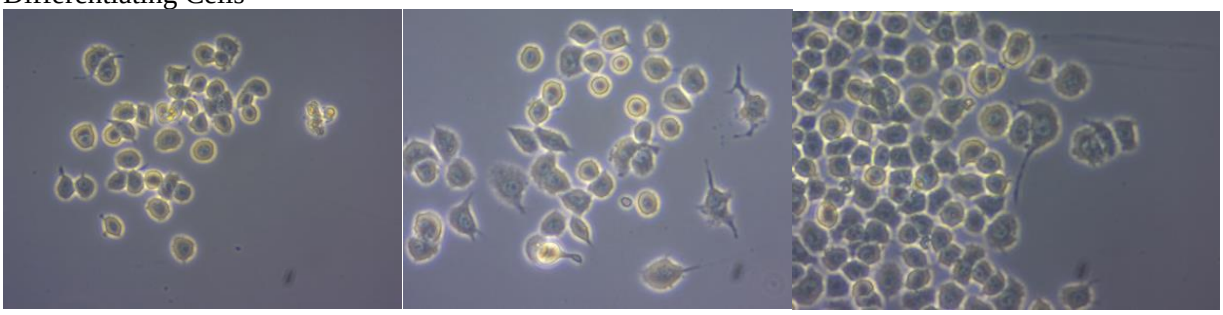
A common debate in exosome-induced differentiation research is the difference between differentiation and attachment of cells to the collagen. Sometimes, it can be hard to tell the difference between the two, creating a possible skew within the results. For our purposes (and what we find to be accepted in the scientific community more frequently), cells are considered differentiated when the neurite outgrowth is equal to or greater than the length of the cell body[8]. Anything less typically indicates attachment, or a developing neurite outgrowth that will become differentiation.

3. Results

3.1 Isolating Exosomes and Determining Differentiation Timing

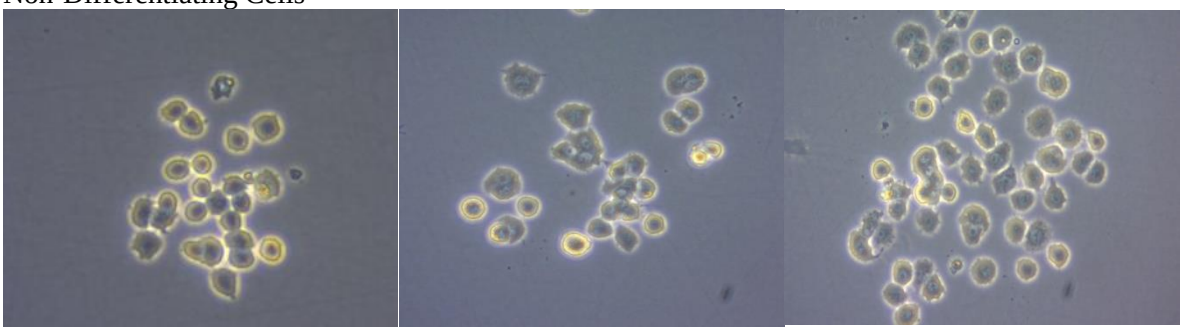
Before a full differentiation assay with exosomes added could be completed, exosomes needed to be obtained. An initial assay was completed to allow for collection of exosomes from both differentiating and non-differentiating cells. Additionally, the pictures from this assay were used to corroborate the finding that PC12 cells begin to show differentiation on the third day[10].

Differentiating Cells



Day 2 Day 4 Day 6

Non-Differentiating Cells

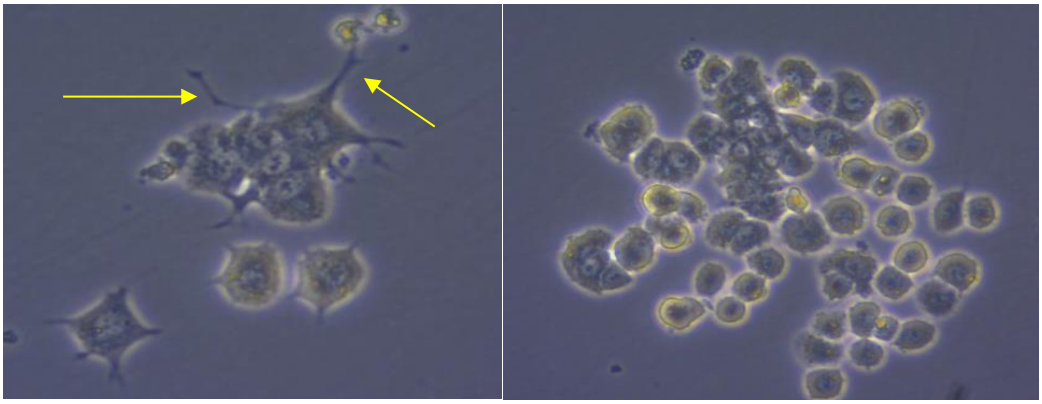


Day 2 Day 4 Day 6

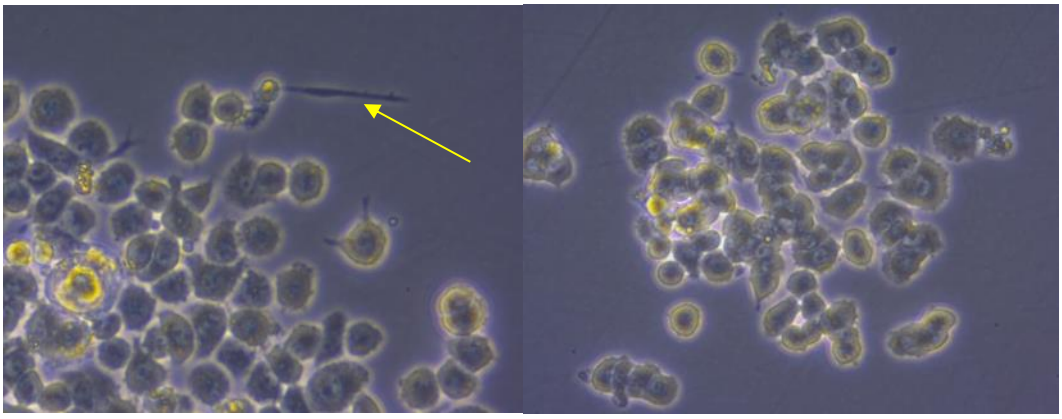
Figure 1 - Pictures show cells on the days when exosomes were isolated. Differentiation became evident between day 3 and day 4 when treated with NGF

3.2 Differentiation Assay – Day 3

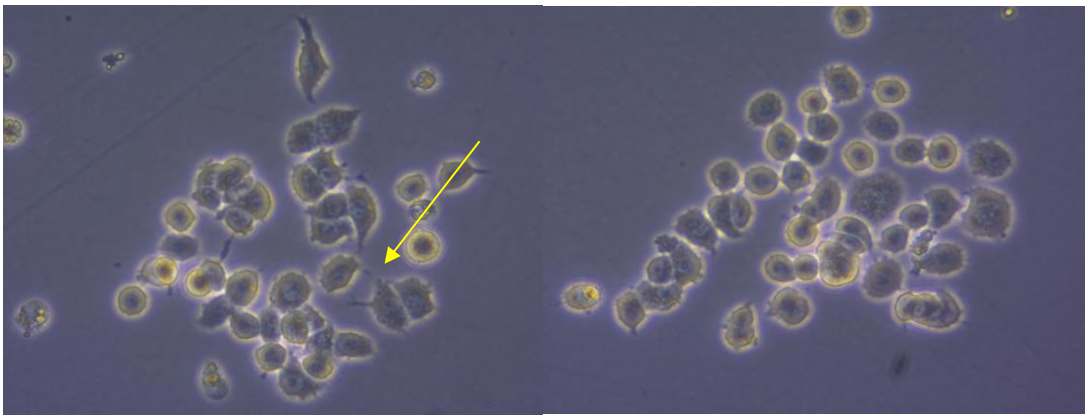
Day 3 of Exosome Treatment



Differentiated control | None differentiated control



Exosomes from day 2 differentiation | Exosomes from day 2 non-differentiation



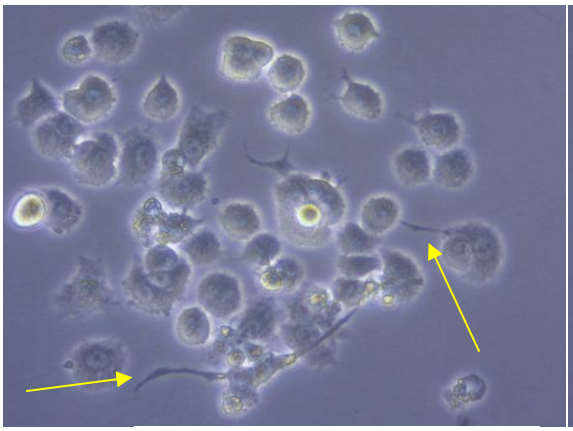
Exosomes from day 6 differentiation | Exosomes from day 6 non-differentiation

Figure 2 – Representation of differentiation (subject of yellow arrows) in the test groups at day 3, when differentiation should start to occur.

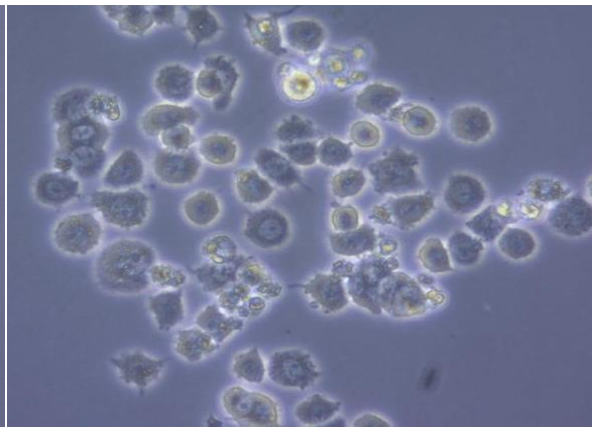
Differentiation is only seen in the control and the differentiation exosomes, not the non-differentiation groups. Again, differentiation is defined in this study as being a neural outgrowth from the cell that is equal to or greater than the width of the cell. This is as expected, showing that the exosomes, at least initially, are influencing the differentiation of PC12 cells.

3.3 Differentiation Assay – Day 6

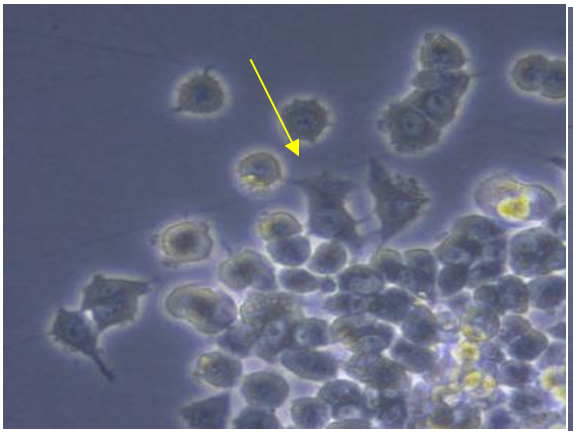
Day 6 of Exosome Treatment



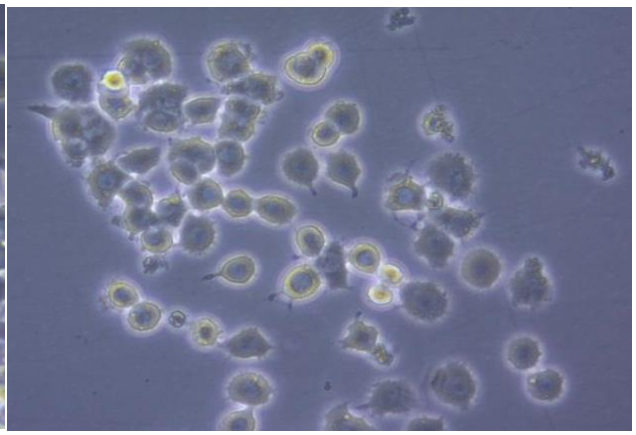
Differentiated control



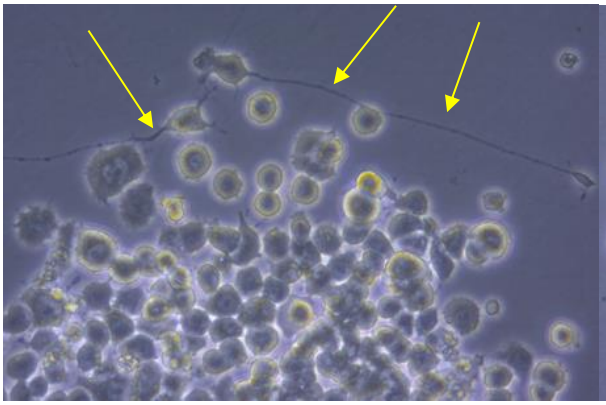
Non-differentiated control



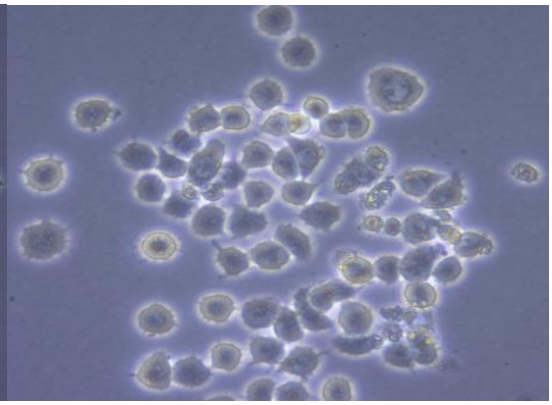
Exosomes from Day 2 differentiation



Exosomes from day 2 non-differentiation



Exosomes from Day 6 differentiation



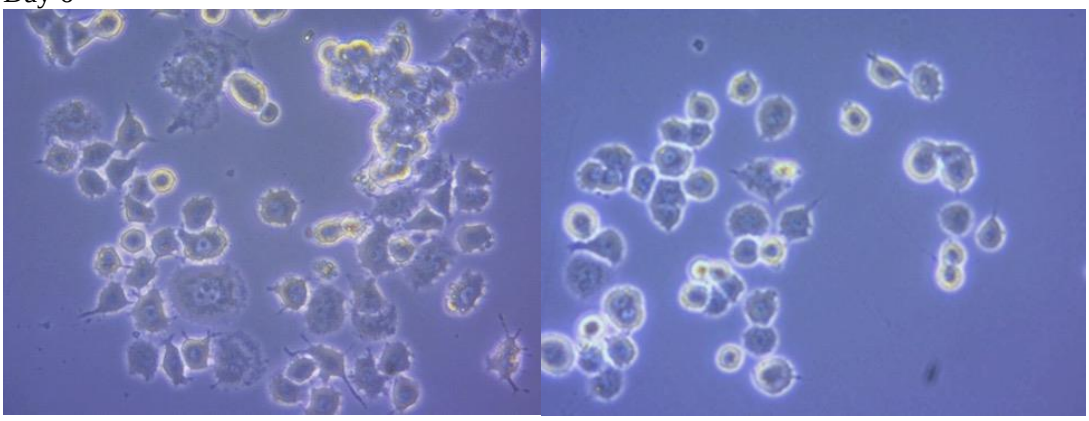
Exosomes from Day 6 non-differentiation

Figure 3 – Representation of differentiation (yellow arrows) in PC12 cells after the full assay, at day 6. This is the final day of the assay, where the most differentiation was seen.

Exosomes taken from non-differentiating cells were shown to have minimal effect on the differentiation of cells they were added to. However, exosomes taken from cells that were differentiating did have an effect, causing differentiation in the test PC12 cells. Cells treated with exosomes from days 2 and 6 of cellular differentiation differentiate in different amounts - indicating that the contents of exosomes change over time.

3.4 Control for NGF Filtration

Day 6



+NGF Exosomes from NGF media

Figure 4 – Test to confirm that no NGF is carried over in exosomes isolation protocol

Treating with exosomes isolated from NGF media alone show that NGF is not being carried over during the isolation protocol contaminating the exosomes – no significant differentiation is seen. If NGF was being carried over, we should see the same results in the sample with exosomes from NGF media added as the results from the +NGF added sample.

3.5 NGF-ELISA Results

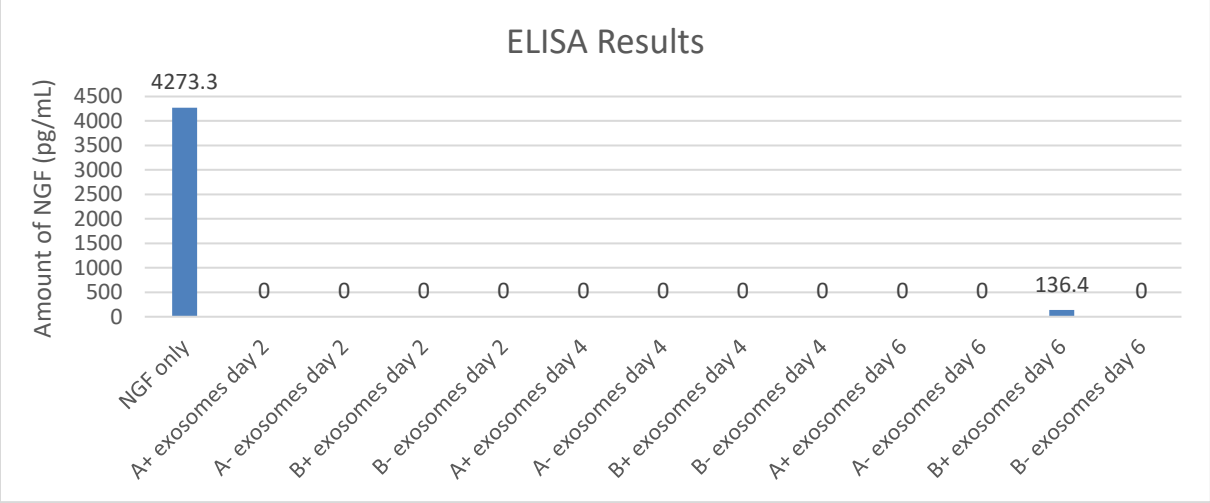


Figure 5 - Results of the NGF ELISA.

Two sets of exosomes were tested (the same sets used in the differentiation assay). The A and B set were two separate isolations of exosomes from PC12 cells. The two sets were taken to ensure accuracy of results. The positive sign means that the exosomes came from cells originally treated with NGF (differentiating cells), the negative signs came from cells without treatment (non-differentiating). There was one sample that was simply an NGF spike, to use as a baseline. An approximate zero NGF was found in all samples except for the B+ Day 6 exosomes, which gave a minimal amount of approximately 136.4 pg/mL. This confirms that no NGF is carried over in the exosome isolation process.

4. Conclusions

Exosomes isolated from different days during NGF treatment produce different results when added to PC12 cells. The differentiation is entirely independent from any caused by the addition NGF to the cells. This result verifies the hypothesis that exosomes taken from different days of differentiation must contain different combinations of RNA and/or proteins. The results can also confirm that the exosome isolation process from Invitrogen is effective at not only isolating exosomes but preventing contaminants from being moved with the exosomes. This conclusion is supported by both observations of the control experiment and through analysis of the NGF-ELISA data. Both of these found no significant quantity of NGF in the sample exosomes, showing that the differentiation of PC12 cells is most likely a consequence of the addition of exosomes taken from differentiated cells.

5. Future Studies

Future studies in our plan to use these exosomes as a basis for further understanding of their contents by isolating and comparing the RNA from each day of exosome isolation. Identifying proteins differentially expressed will help us better understand the mechanism by which exosomes cause differentiation in cells not previously treated with NGF. Other possibilities are to use fluorescent microscopy to measure a percent differentiation for PC12 cells treated with the different exosome groups Another question to address would be the uptake of exosomes. Are the non-differentiating cells still taking in the exosomes, or are they rejecting them all together?

Additionally, the research performed in this article will be used as a basis to develop “cell blocks” which are part of the learning curriculum for the Cell Biology Education Consortium (CBEC), a network of faculty and students incorporating cell culture-based research into the classroom. The cell block will be a model of written and video protocols that other classrooms can use to mimic and expound upon this research. The following link is to the video protocol for the differentiation assay: https://youtu.be/vBDlHFbWSgA.

6. Acknowledgements

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