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OPTIMIZING PROCEDURAL TOV-112 OVARIAN CARCINOMA CELL TRANSFECTION VIA INITIAL SEEDING AND pBABE/PEI JET RATIO VARIATIONS

Cassondra Walker

A thesis submitted to the University Honors Program in partial fulfillment of the requirements for the Honors Extended Certificate

Southern Illinois University
6 December 2017
I. Abstract

Cell transfection is an exemplary tool in protein analysis and examining transcription factor activity. While general procedures are commonly followed, each cell line requires slight differentiation in pBABE/PEI JET ratios and initial cell count for optimal transfection results. This research focusses on determining a successful procedure for TOV-112 carcinoma cells for future work with 2-methoxyestradiol and NFkB. 2-methoxyestradiol is an angiogenesis inhibitor believed to decrease inflammatory processes. NFkB promoter controls the luciferase gene on the MetLuc plasmid and is pro-inflammatory. The objective of this experiment is to modify the variables until successful results at 24 and 48 hours are observed. Four experimental transfections were conducted during the three months of research, which analyzed the effectiveness of high and low pBABE/PEI JET ratios and increased vs. decreased initial plate seeding. Results show that optimal TOV-112 transfection occurs with a 1:2 pBABE/PEI JET ratio and an initial cell count of around 3x10^5 cells/mg. Future work will hope to emphasize this conclusion and continue with the overarching NFkB ovarian cancer research, which aims to reduce the cases of lethal occurrences.

II. Introduction

Cell transfection, the introduction of foreign nucleic acids on eukaryotic cells for the purpose of genetic uptake and experimental observance, is a useful experimental tool to measure activity of various proteins and transcription factors, [6]. While similar in technique, diversified kinds of cells require slight manipulations in the overall procedure. TOV-112 are human endometrial histotype carcinoma cells known to cause ovarian cancer, [8]. This form of cancer has an excessively high mortality rate due to late-stage diagnostic findings. With no modern mechanisms for early diagnosis, ovarian carcinoma female lethality is fifth in cancer deaths, [2, 4-5]. In successful ovarian cancer transfections, there are great potentials in advancement for ovarian cancer studies and with deeper information, earlier detection may be possible. In this form of cell transfection, TOV-112 cells are grown in 90% Dulbecco's Modified Eagle Medium, 10% Fetal Bovine Serum, and 0.5% Penicillin streptomycin. DMEM is rich in glucose and other growth nutrients, which allow for optimal and healthy cell growth, [3]. TOV-112 cells double in about a period of 22 hours, which requires greater upkeep and nutrient availability. FBS and Penstrep also ensure proper growth where FBS has increased growth serum and Penstrep is antibacterial. Penstrep is later removed from the growth medium to establish limited barriers for genetic uptake. The ovarian cancer cells are transfected with pBABE plasmid. This carries the gene for the Green Fluorescent Protein (GFP). When the plasmid is translated, transfected cells can be visualized by fluorescent microscopy, [7]. Image 1.0 below shows the pBABE plasmid map that contains the genetic material for GFP translation. Ampicillin-resistant genetic material with the pBABE plasmid was amplified via Polymerase Chain Reaction (PCR) and the pBABE plasmid was extracted and isolated. Since transfection, in most cases, would be lethal to the delicate cells, Opti-MEM media
buffers the solution with HEPES and Sodium Bicarbonate, [3]. PEI-JET is used for efficient gene delivery. The purpose of this research is to find an optimal transfection procedure for TOV-112 cells by varying the pBABE and PEI JET ratios and manipulating the original cell count at the beginning of the transfection. This determination leads into future research with Metridia Luciferase (MetLuc) transfection and 2-methoxyestradiol introduction to see if there is a decrease in luciferase protein activation proportional to Nuclear Factor kappa Beta (NFkB) activity. MetLuc is a plasmid that contains the genetic coding for luciferase. The MetLuc gene is controlled by the NFkB promoter. NFkB is a transcription factor that promotes angiogenesis in the presence of ovarian cancer. It participates in inflammatory processes and since ovulation is pro-inflammatory, it is believed to be involved with ovarian cancer cases. Determining if 2-methoxyestradiol attracts anti-inflammatory processes in-vitro could later be conducted in-vivo as a means to reduce the virulence of the carcinoma. Previous studies show that 2-methoxyestradiol induces tumor apoptosis and has anti-inflammatory properties, [1].

Image 1.0: pBABE Plasmid Map (Snapgene)
III. Materials and Methods

Transfection 1: pBABE-PEI JET Variations with Low Ratios
I thawed a vial of TOV-112 ovarian cancer cells frozen from 04/2017 with a passage number of 36 on a 100-ml cell culture plate and grew/transferred the cells until five passages have passed. Each cell culture plate had 12-ml of 90%DMEM, 10%FBS, and 0.5% Penstrep media. In each passage, cells grew for 48-72 hours (until 100% confluence). Passage 41 TOV-112 cells were transferred to a 6-well plate with a cell count of 7.0x10⁵ cells/mg and 2-ml of 90% DMEM, 10%FBS, and 0.5% Penstrep media in each well. In five-1ml conical tubes, I mixed 200-µL OptiMEM Media; 1.0-µg, 1.25-µg, 1.5-µg, 1.75-µg, and 2-µg pBABE (0.47µg/µL concentration) respectively, and 6.0µg of vortexed PEI-JET (1.0µg/µL concentration). Each 1-ml conical tube was vortexed every five minutes for fifteen minutes at T 0min, 5min, 10min, and 15min. The 90% DMEM, 10% FBS, and 0.5% Penstrep media was replaced with 90%DMEM and 10% FBS media in each well (2-ml). The pBABE mixtures were added to wells 1-5 respectively, leaving well 6 as the control. The cells grew for 48 total hours with observations under a compound microscope to see the fluorescence at T-24hours and T-48hours.

Transfection 2: Initial Cell Count Variations
I transferred the 100-ml cell culture plate of P43 TOV-112 cells from my maintained cell line to a 6-well cell culture plate. Initial cell counts were 6.0x10⁵, 6.5x10⁵, 7.0x10⁵, 7.5x10⁵, and 8.0x10⁵ cells/mg respectively in wells 1-5. The control in well 6 was plated with 7.0x10⁵-the average of all experimental values. Each well had 2-ml of 90%DMEM, 10%FBS, and 0.5% Penstrep media. In five 1-ml conical tubes, I mixed 200-µL OptiMEM Media, 2-µg pBABE (concentration 0.47 µg/µL), and 6-µg of vortexed PEI-JET (1.0 µg/µL concentration). Each 1-ml conical tube was vortexed every five minutes for fifteen minutes at T 0min, 5min, 10min, and 15min. The 90% DMEM, 10% FBS, and 0.5% Penstrep media was replaced with 90%DMEM and 10% FBS media in each well (2-ml). The pBABE mixtures were added to wells 1-5 respectively, leaving well 6 as the control. The cells grew for 48 total hours with observations under a compound microscope to see the fluorescence at T-24hours and T-48hours.

Transfection 3: Initial Cell Count Variation
I transferred the 100-ml cell culture plate with P47 TOV-112 cells from my maintained cell line to a 6-well cell culture plate. Initial cell counts were 6.5x10⁴ (in wells 1 and 4), 6.5x10⁴ (wells 2 and 5), and 6.5x10⁵ (wells 3 and 6) cells/mg. Wells 4, 5 and 6 were used as controls. Each well had 2-ml of 90%DMEM, 10%FBS, and 0.5% Penstrep media. In three 1-ml conical tubes, I mixed 200-µL OptiMEM Media, 2-µg pBABE (concentration 0.811 µg/µL), and 6-µg of vortexed PEI-JET (1.0 µg/µL concentration). Each 1-ml conical tube was vortexed every five minutes for fifteen minutes at T 0min, 5min, 10min, and 15min. The 90% DMEM, 10% FBS, and
0.5% Penstrep media was replaced with 90% DMEM and 10% FBS media in each well (2-mL). The pBABE mixtures were added to wells 1-3, respectively, leaving well 4, 5, and 6 as the controls. The cells grew for 48 total hours with observations under a compound microscope to see the fluorescence at T-24 hours and T-48 hours.

**Transfection 4: pBABE-PEI JET Variations**

The passage 51 TOV-112 cells were transferred to a 6-well plate with a cell count of 3.0x10^5 cells/mg and 2-mL of 90% DMEM, 10% FBS, and 0.5% Penstrep media in each well. In three 1-mL conical tubes, I mixed 200-μL OptiMEM Media; 3.0-μg, 2.0-μg, 1.0-μg pBABE (0.811 μg/μL concentration), respectively, and 6.0μg of vortexed PEI-JET (1.0μg/μL concentration). Each 1-mL conical tube was vortexed every five minutes for fifteen minutes at T 0 min, 5 min, 10 min, and 15 min. The 90% DMEM, 10% FBS, and 0.5% Penstrep media was replaced with 90% DMEM and 10% FBS media in each well (2-mL). The pBABE mixtures were added to wells 1-3 respectively, leaving wells 4, 5, and 6 as the controls. The cells grew for 48 total hours with observations under a compound microscope to see the fluorescence at T-24 hours and T-48 hours.

IV. Results

**Transfection 1: pBABE/PEI JET Ratio Variations**

Results show an increase number of pBABE transfected TOV-112 cells with lower pBABE/PEI JET ratios. There is also increased expansion with the fluorescent cells and limited patched transfection areas that are prevalent with the lower concentration ratios. Transfected cells are indicated by the bright green areas in the first column. The second column shows the brightfield image at that time.

![Figure 1.1: Results at 24 Hours](image-url)
**Transfection 2: Initial Cell Count Variations**

There is an increase in positive transfected cells with the initial cell count of $6.5 \times 10^5$ cells/μg. Though it shows the most success, there is still a very limited number of positive transfections in each well. Transfected cells are indicated by the bright green areas in the first column. The second column shows the brightfield image at that time.
**Figure 2.2: Results at 48 Hours**

<table>
<thead>
<tr>
<th>Transfection 3: Initial Cell Count Variations</th>
<th>6.0x10^5</th>
<th>6.5x10^5</th>
<th>7.0x10^5</th>
<th>7.5x10^5</th>
<th>8.0x10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfected cells are indicated by the bright green areas in the first column. The second column shows the brightfield image at that time.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Results below show wells seeded with low initial cell counts. This was in attempt to combat the excessive confluency and cell death from the previous transfections. There is a very limited number of successful transfected cells and little variation between the three experimental wells. I used “6.5” since it previously showed a greater number of transfected cells in Transfection 2.

**Figure 3.1: Results at 24 Hours**

<table>
<thead>
<tr>
<th>6.5x10^3</th>
<th>6.5x10^4</th>
<th>6.5x10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfected cells are indicated by the bright green areas in the first column. The second column shows the brightfield image at that time.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2: Results at 48 Hours

6.5x10^3 6.5x10^4 6.5x10^5

Transfection 4: pBABE/PEI JET Variation with Low Ratios

The final transfection, again, varied the ratio of pBABE and PEI JET in attempt to visualize whether or not a lower ratio constitutes a greater successful output. I cut the previously successful initial cell count to 3x10^5 in attempt to, again, counteract the initial cell death that occurs at high levels of confluency. There were three experimental wells with 1:6, 1:3, and 1:2 pBABE/PEI JET ratios respectively. Transfected cells are indicated by the bright green areas in the first column. The second column shows the brightfield image at that time.

Figure 4.1: Results at 24 Hours

1:6 1:3 1:2
V. Discussion

PEI-JET and pBABE ratios show a drastic difference in transfection efficiency since the Green Fluorescent Protein utilizes PEI-JET for its genetic deliverance. Results show that lower ratios promote successful transfections, indicated by the increased level of green fluorescence cells. Initial cell count plays a secondary role in transfection success with the TOV-112 cells. In the comparison of transfection 2 with transfection 3, though high levels of initial cell count resulted in a greater incidence of cell death at the 48 hour mark, vastly decreasing the original plate seeding resulted in confluence that was not optimal for success. With a low pBABE/PEI JET ratio and a slight decrease in original seeding to 3x10^5 cells/mg (reaching 100% confluency by 24 hours), there was an elevation in pBABE-transfected TOV-112 ovarian cancer cells. Thus, in responding to the initial thesis question and in hopes to regulate the transfection procedure for this type of carcinoma cell line, the results illustrate that success correlates with a low pBABE/PEI JET ratio and an initial cell count around 3x10^5 cells/mg. A revised procedure that take these results into account is as follows:

Grow TOV-112 ovarian cancer cells to 80% confluency on a 100-mL cell culture plate in 12-mL of 90% DMEM, 10% FBS, and 0.5% Penstrep media. Seed a 6-well plate with an initial cell count of 3x10^5 cells/mg. Add 2-mL of the 90% DMEM, 10% FBS, and 0.5% Penstrep media to each well. Incubate the cells for 24 hours. At this mark, add 200-μL of OptiMem media, 6-μg vortexed PEI-JET, and 3-μg of pBABE in a 1.5-mL conical tube. Repeat this for each experimental well. Vortex each tube for 10-seconds at T-0min, 5min, 10min, and 15min. Carefully replace the present media in the 6-well plate with 2-mL of 90% DMEM and 10% FBS in each well. Add the conical tube mixtures to their respective wells. Carefully
move the plate to swirl the mixture and incubate. Observe the cells at 24 and 48 hours.

While the experiments were successful in formulating a more accurate transfection procedure for TOV-112 cells, more transfection repeats could have been conducted for better result accuracy. Future work will focus on this repetition and utilize the detailed transfection procedure. Possible errors in experimentation may include working with two different concentrations of pBABE and therefore having a lack of control. Despite the different concentrations, however, calculations accounted for the variation. With this resulting information, there may be future experiments working with pBABE/MetLuc transfections with the introduction of 2-methoxyestradiol. MetLuc is an plasmid that contains the luciferase gene. This gene is under control by the NFkB promoter. NFkB is a transcription factor that is stimulated in response to cellular stress. Increased activity is correlated with increased inflammatory response. Ovulation, essentially, is an inflammatory process. If 2-methoxy attracts anti-inflammatory factors, the ovarian cancer over-inflammation stimulated by NFkB, may be reduced. 2-methoxyestradiol is a natural metabolite of estrogen. By co-transfecting the TOV-112 cells with pBABE and MetLuc, we can measure transfection efficiency. Using a Luciferase protein assay, the amount of NFkB activity on TOV-112 cells can be studied, since NFkB response is proportional to Luciferase activity. Introducing 2-methoxyestradiol to the cell plate allows for the analysis of its anti-inflammatory action on the ovarian cancer cells by observing the difference of NFkB activity over time. In conclusion, this study is a necessary step for larger ovarian cancer research and the regulation of this procedure will help to diminish possible errors with further experimentation.

VI. Acknowledgments

I want to thank Dr. Buck Hales for his support in the completion of the project and allowing the usage of his laboratory to conduct the experiments. Thank you to my colleague, Kara Starkweather, for initially showing the general method on how to complete transfections and others in Dr. Buck Hale’s lab who supported me throughout the semester.

VII. Calculations

*Transfection 1*

<table>
<thead>
<tr>
<th>Volume (μL)</th>
<th>Concentration (μg/μL)</th>
<th>Amount (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.47</td>
<td>0.94</td>
</tr>
<tr>
<td>2.5</td>
<td>0.47</td>
<td>1.25</td>
</tr>
<tr>
<td>3.0</td>
<td>0.47</td>
<td>1.43</td>
</tr>
<tr>
<td>3.5</td>
<td>0.47</td>
<td>1.75</td>
</tr>
<tr>
<td>4.0</td>
<td>0.47</td>
<td>2.00</td>
</tr>
</tbody>
</table>
**Transfection 2**
576 cells x \( \frac{3}{5} \times 10,000 = 2.3 \times 10^6 \) cells/mL
2.3\( \times 10^6 \) cells/mL = 6.0\( \times 10^5 \) = 0.261 mL cell suspension/well
2.3\( \times 10^6 \) cells/mL = 6.5\( \times 10^5 \) = 0.283 mL cells suspension/well
2.3\( \times 10^6 \) cells/mL = 7.0\( \times 10^5 \) = 0.304 mL cell suspension/well
2.3\( \times 10^6 \) cells/mL = 7.5\( \times 10^5 \) = 0.326 mL cell suspension/well
2.3\( \times 10^6 \) cells/mL = 8.0\( \times 10^5 \) = 0.348 mL cell suspension/well

**Transfection 3**
357 cells x \( \frac{2}{5} \) 10,000 = 1.43\( \times 10^6 \) cells/mL
1.43\( \times 10^6 \) cells/mL = 6.5\( \times 10^5 \) = 4.5 μL cell suspension/well
1.43\( \times 10^6 \) cells/mL = 6.5\( \times 10^5 \) = 45 μL cell suspension/well
1.43\( \times 10^6 \) cells/mL = 6.5\( \times 10^5 \) = 450 μL cell suspension/well
0.811 μg/μL pBABE x “x” = 2.0 μg/μL = 2.5 μL pBABE

**Transfection 4**
304 cells x \( \frac{2}{5} \) x 10,000 = 1.22\( \times 10^5 \) cells/mL x 3x10^5 cells/mL = 0.247 mL cell suspension/well
0.811 μg/μL pBABE = 3 μg/X μL = 3.7 μL pBABE
0.811 μg/μL pBABE = 2 μg/X μL = 2.5 μL pBABE
0.811 μg/μL pBABE = 1 μg/X μL = 1.2 μL pBABE

VIII. References


