# The Effects of $P57^{KIP2}$ Down Regulation via Lentiviral shRNA Knockdown of CDKN1C ($P57^{KIP2}$ Expression Gene) on the Glucocorticoid Dexamethasone's Function in Culture Peripheral-Blood Derived CD34+ Cells

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**Abstract:**

In theory, healthy peripheral blood cells, when treated with the glucocorticoid Dexamethasone (Dex), will experience greater rates of cell proliferation. The reason for this is Dex upregulates a protein called $P57^{KIP2}$. This protein is able to slow down the differentiation process, which allows the cells to have greater time to proliferate. In the erythroid lineage, cells can be classified as progenitors and precursors. During the progenitor stage, cells will experience high amounts of proliferation, while in the precursor stage, proliferation becomes less common. $P57^{KIP2}$ acts primarily in the final parts of the progenitor stage, allowing for increased proliferation rates. This treatment is especially useful in diseases such as Diamond Blackfan Anemia (DBA), where red blood cells have a difficult time proliferating. However, in DBA, there is a patient population that has become resistant to glucocorticoid treatment. This leads to the theory that there may be a problem with the $P57^{KIP2}$ pathway in DBA cells. However, before that conclusion is made, further studies had to be conducted on the effects $P57^{KIP2}$ had on Dex's ability to increase proliferation rates in healthy peripheral blood cells. This was tested, by creating two lentiviral constructs, that would knock down CDKN1C (gene responsible $P57^{KIP2}$ expression). These lentiviruses were transduced into healthy peripheral blood-derived CD34+ cells and treated with and without Dex to test its effects.

A luciferase lentivirus was also created and transduced into additional CD34+ cells as a transducing control. This lentivirus was designed to knockdown luciferase expression, however, humans don't have this gene, and therefore no changes in $P57^{KIP2}$ expression should be expected. In order to create these lentiviral constructs, 293T cells were transfected. They were then titrated and placed through Quantitative Polymerase Chain Reaction (qPCR) to determine concentrations. Once cells were transduced, some cells from each of the shp57 transduced populations were taken for Western Blotting, to ensure effective transduction of lentivirus. In preparation for the cell proliferation assay, that would be used to measure proliferation rates, all lentiviral transduced cells are split into two cell groups, Dex treated and untreated cell groups. Cell counts were taken each day until day 16, at which point all cells were observed using flow cytometry to determine their differentiation states.

Results showed that $P57^{KIP2}$ downregulation plays a significant role in Dex's ability to increase cell proliferation rates. The shp57 groups showed very similar growth rates in Dex treated and untreated cell groups. The shluc group showed significantly higher proliferation rates in the Dex treated cell than the untreated cell group. Furthermore, the shluc group treated with Dex were less differentiated than the untreated group. While the shp57 groups showed similar differentiation states in both Dex treated and untreated populations.

**Introduction:**

Diamond Blackfan Anemia (DBA) is a genetic disease that causes the body's bone marrow to fail to produce red blood cells. DBA is a very rare disease, with only 25 to 35 new cases per year in the United States and Canada (Vlachos, Adriana et. Al. 2010). There is an estimated total of just 5000 DBA cases worldwide (Vlachos, Adriana, et. Al. 2010). As of right now, it is believed that DBA results from ribosomopathies (genetic ribosomal mutations). However, this classification has been called into question recently, as other non-ribosomal genotype mutations have been identified to cause the same DBA phenotypes. The most common treatments for DBA patients are blood transfusions and corticosteroids (Vlachos, Adriana, et. Al., 2010). There is also an option for stem cell (bone marrow) treatment, however, this option is typically riskier. DBA is a rather difficult disease to predict or diagnose as there is essentially no genotype-phenotype correlation (Vlachos, Adriana, et. Al., 2010). Parents can't be identified as carriers for a gene mutation that would result in DBA, as there are a variety of different outcomes that would result in this specific bone marrow failure. The current scientific knowledge of DBA is limited, and obtaining cell samples from patients is very difficult. Patients' bodies produce very limited amounts of red blood cells and there is a very limited number of patients.

This experiment began with the goal of understanding the mechanism of corticosteroids in the treatment of DBA. One of the main mechanisms in regulating cell cycle is the protein $P57^{KIP2}$. $P57^{KIP2}$ is a part of the family of Cyclin-Dependent Kinase Inhibitors (CDK inhibitors), which regulate the speed of the cell cycle (Hwang, Yung, et. Al., 2017) Depending on the phases of the cell cycle, these CDK inhibitors will bind with CDK's to prevent these cyclins and kinases from speeding up differentiation (Hwang, Yung, et. Al., 2017). While the upregulation of CDK inhibitors slows down the cellular differentiation process, it ends up increasing the quantity of fully developed cells. Erythroid cells stop proliferating once they exit the progenitor phase and transition into the precursor phase. The reason for looking at $P57^{KIP2}$ is because it acts primarily during the progenitor phase of differentiation (Hwang, Yung, et. Al., 2017). So, if the differentiation process is slowed down, the quantity of progenitor cells (Specifically end-stage BFU-E and early-stage CFU-E) increases by providing more time for proliferation, resulting in a greater quantity of Red Blood Cells. However, in DBA cells this is not always the case. While many DBA patients are responsive to treatment with corticosteroids, there are some that are classified as corticosteroid resistant (Narla, Anupama, et. Al., 2011). While the reason for this resistance is unknown, it is a likely possibility that DBA resistant cells may potentially have an issue with the $P57^{KIP2}$ pathway, ultimately leading to the exploration of $P57^{KIP2}$ in the context of DBA.

Previous experiments on mice have shown that $P57^{KIP2}$ was increased when treated with the corticosteroid Dex (Samuelsson, Magnus, et. Al, 1999). This explained the Dex phenotype of slowing down differentiation in erythroid cells during the end of Burst Forming Unit Erythroid (BFU-E) and the start of Colony Forming Unit Erythroid (CFU-E) stages. In a more recent study conducted on humans, healthy peripheral blood samples treated with Dex experience a subsequent increase in their $P57^{KIP2}$ levels as well. However, not too much is known about the effects of $P57^{KIP2}$ on red blood cells when treated with and without Dex in a human model. So, this experiment was designed to study the effects of Dex following knockdown of CDKN1C (the gene that expresses $P57^{KIP2}$) on red blood cell proliferation and differentiation.

**Question:**

Does $P57^{KIP2}$ reduction via lentiviral shRNA knockdown of CDKN1C reduce the effects of Dexamethasone in cultured peripheral blood-derived CD34+ cells?

**Hypothesis:**

$P57^{KIP2}$ reduction should reduce the effects of Dex as the mechanism of action is perturbed. Since Dex is unable to upregulate $P57^{KIP2}$, there should be no significant delay in differentiation. As a result of this, there should be no significant difference in cell proliferation rates as well, as the cells don't have extended time to proliferate.

**Procedure:**

1. Transient Transfection of 293T cells for Lentivirus Production

Seed and incubate 4.5 x $10^6$ (all incubations at 37°C) low passaged 293TN cells in a 10 cm dish about 23-30 hours before transfection. Culture cells in DMEM 10% FBS (heat-inactivated) media for 24-30 hours to allow for growth, but never to confluence. Change media to IMDM 10% Hyclone FBS 2 hours before transfection. Create a Plasmid DNA mix by adding 6.5 ug packaging plasmid pCMV delta R8.9, 3.5 ug pucMDG and 10 ug - 15 ug of gene transfer plasmid together. The plasmid solution is made up of a final volume of 450 uL with 0.1 XTE/dH20 (2:1). Finally, a 50 ul of 2.5M CaCl2 is added. Wait 5 minutes at room temperature. Then prepare one 15 mL tube for each of the two dishes. A plasmid with GFP can be used with an extra dish as transfection efficiency control. Next, a precipitate is formed by a dropwise addition of 500 uL of 2x HBS solution to the 500 uL DNA-TE-CaCl2 mixture created previously, while vortexing 1400 rpm. Then immediately add precipitate to 293T cells. High magnification microscopy should reveal a very small granular precipitate of the CaPi-precipitated plasmid DNA, initially above the cell monolayer. Following 14-16 hours of incubation at 37 degrees celsius, the precipitate should settle to the bottom of the plate in large spaces between the cells. Following incubation, the media should be replaced with 8 mL of fresh media. 48-72 hours following transfection, the supernatants should be collected and filtered through a 0.2-micrometer filter to extract the virus. The 30 mL of filtered supernatant should then be placed in the ultracentrifuge at 20,000 rpm for 2 hours at 20 degrees celsius. Once this is completed, aspirate the media very carefully without removing the pellet. Add 30 uL sterile PBS per tube to resuspend the cells and harvest the virus. Finally, create aliquots and freeze down at -80°C.

2. Lentiviral Titration

Plate 5 x 10^4 3T3 cells per well a day before in 6 wells COSTAR on a tissue culture treated well plate in DMEM 10% FBS media, ensuring no large aggregates form on the plate (The day after there should be 10^6 cells). Incubate (all incubations at 37°C), then the next day change media and replace it with 1 mL IMDM 10% FBS + Polybrene 3 hours before the transduction. Polybrene is added in each well at a final concentration of 8ug/mL. 5 uL of the concentrated virus from day 1 harvesting is added to the first well, then a serial 10 fold dilution is performed twice into two successive wells. This process is repeated for the p57 lentiviral constructs, luciferase lenti-viral constructs and non-transduced control. The media is changed after 24 hours and they're then incubated for 48-72 hours until approximately 60%+ confluence is reached. Once this level of confluence is reached, wash the cells with 2 mL of PBS. The greater the confluence the better as more genomic DNA can be isolated for qPCR.

Then add 200 uL of trypsin to the cells to remove them from the surface of the well plates and incubate for approximately 2-5 mins (or until detached from the surface). Then add 1 mL PBS to each well to dilute trypsin. Take 1 mL of cells and centrifuge for genomic DNA (gDNA) preparation. Then add 200 uL of media to the rest of the cells just in case the process needs to be repeated.

3. Real-Time qPCR (Quantitative Polymerase Chain Reaction)

First, a lysate is to be prepared in order to isolate the genomic DNA. This is done by first adding Proteinase K and RNase A to the sample. They are then to be vortexed to create a homogenous solution, and incubated at room temperature for 2 minutes. Then add Genomic Lysis/ Binding Buffer, forming the Lysate. They are then to be incubated at 55°C for 10 minutes in order to aid in protein digestion. 96-100% ethanol is added and vortexed briefly to homogenize again. This solution is then added to the spin column and spun down for a minute. The spin column is then placed into a new collection tube, where it is washed with wash buffers to prepare for elution. An elution buffer is added, and then the solution is incubated at room temperature for a minute, and finally spun down for a minute. The DNA has now been isolated, but the elution process can be repeated another time in a different tube in order to isolate more purified genomic DNA (gDNA). The gDNA from uninfected 3T3 cells must be prepared as a control and infected cells with different dilutions of the virus. Specifically a 5 uL, 0.5 uL, and 0.05 uL dilutions. Then prepare a 384 white well plate, in which 0.7 ug of each gDNA sample and control with nuclease-free water at a final volume of 28 uL PCR grade water. This should ultimately yield 100 ng of gDNA per well in 4 uL of water. Then prepare a mix for the housekeeping gene mThfR with SybrGreen. For 1 well and 8.5 uL mix, 6.25 uL Power SYBR Green PCR Master MIx and 0.25 uL of forward mThfR Primer at 10 uM + 0.25 uL of the reverse mThfR Primer at 10 uM and 1.75 uL PCR grade water. Now the next step is to prepare a Mix for Psi and TaqMan. For one well and 8.5 uL mix, combine 6.25 uL of TaqMan Universal PCR Master Mix, 0.375 uL of forward psi primer at 10 uM, 0,375 uL of the reverse psi primer at 10 uM, 0.5 uL of psi probe at 5 uM and 1 uL of PCR grade water. Now that the second mix is made, the plate can be prepared as follows. Load the first 3 rows with 8.5 uL of the TfR Mix. Load the following ones with 8.5 uL of Psi mix. Then load the gDNA negative controls, copy number controls, samples, and water negative controls. The plate is now ready for qPCR. For qPCR, both the SybrGreen and Psi Mix are used as fluorescent indicators of DNA replication. SybrGreen binds to all amplified DNA and the TaqMan construct in the Psi Mix only binds to the Psi gene specifically. The fluorescent readout of SybrGreen can be correlated to the amount of mThfR (the control) while the readout of TaqMan in the Psi mix can be correlated to the Psi gene to later calculate the amount of each of these DNA sequences in our

samples. From this information, a complicated series of calculations are made in order to determine the final concentration of lentivirus.

4. Cell Transduction

CD34+ cells have to be cultured in order to determine self numbers over the course of a few weeks. Serum-free culture with StemSpan SFEM base media. Aliquots of the solution are made and frozen until required. They can be thawed at room temperature when starting the culture. In order to make the SFEM media, the cytokines are added and are responsible for sending signals to the cells, allowing them to grow. Concentrations of cytokines were added to 10 mL of solutions. Stem Cell Factor (SCF) added at concentration of 50ug/mL (20uL) . Interleukin 3 (IL3) is added at a concentration of 50ug/mL (20 uL) . L-glutamine is added at a concentration of 2mM (100 uL). Transferrin (Tf) is added at 200ug/mL (40 uL). Specific cytokine concentrations change depending on the phases. From days 0-7, Erythropoietin (Epo) is added at concentration +0.5IU/mL (5uL). From days 7-11, Epo is added at concentrations of +3IU/ mL (30 mL). From days 11-25 +3IU/mL of Epo (30 uL) is added as well as +1mg/mL of Tf (200 uL). Cells have to be thawed in a hot water bath until almost completely thawed. At that point, cells should be diluted by 10 mL of PBS and spun down gently to form a pellet. The reason for this dilution is the cells are put in a solution with DMEM in order to aid in the freezing process. However, at room temperature the DMEM is toxic to the cells at that concentration, and therefore needs to be diluted. These cells can now be divided into a 6 well plate with approximately 1 mL of solution in each well. By doing so this allows for 2 wells of luciferase lentiviral transduced cells as well as 2 wells for each of the p57 lentiviral transduced cells. One of the wells in each of the transduced groups will be treated with Dex, while the other will receive no drug treatment. Every time there needs to be a change in cytokine concentrations, the media of the cells will be changed with concentrations of cytokines adjusted accordingly.

5. Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-page) western blot is a procedure that was used to verify that there was effective transduction of lentivirus within the cell. Prepare protein samples and measure the concentration (BCA kit). Mix protein sample or ghosts at the desired protein amount up to a maximum volume of 30uL ratio 1:1 with loading buffer (usually 15ug ghosts protein is enough). Boil the sample for 5 minutes for complete denaturation and do quick spin centrifugation in order not to lose the evaporated fraction. Samples can be frozen at -80 °C at this step if you don't need all of them (they will have to be boiled a second time after thawing). Next, the SDS-Page

itself must be prepared. First, wash the wells of the gel with distilled water. Then place the gel in an electrophoresis Tank with 1X Tris-Glycine SDS (TGS). 5 uL of prestained protein ladder can be loaded into the first well. Then, the boiled samples are loaded at a volume of 30uL. The process of protein electrophoresis can now begin at 90-100V until the front migration line crosses the stacking gel (approximately 15 minutes). By doing so, better protein stacking is achieved and will ultimately result in higher quality resolution. The voltage can then be increased at 130-140V (should take between 45-90 minutes). The electrophoresis should be stopped once the front line migration is at the bottom of the gel. Cut a Nitrocellulose membrane piece slightly smaller than the Biorad filter paper. Place 2 pieces of the filter paper, the membrane, the gel and the black sponges in 1X cold TG buffer. A "sandwich" can then be made by placing down the black sponge, then stacking on top filter paper, gel, nitrocellulose membrane, filter paper, and black sponge. Put the sandwich into the "cassette" with the membrane towards the cathode as the proteins will migrate to the cathode and therefore to the membrane. Place the sandwich in the Tank filled with cold 1X TG and an ice block, and place the tank in an ice bucket with water (to keep the buffer refrigerated during the transfer). Then run the transfer at 95V for 1 hour. Get the membrane, check the migration with a Red ponceau staining and wash with distilled water. Perform a blocking with PBS 0.1% Tween 20 (PBST) 4% Milk, 1% BSA for 60-120min at RT. Incubate with Primary Antibody (Mouse Anti-Human p57 or Mouse Anti-Human GAPDH) diluted in Blocking buffer ON at 4OC (or 2-3h at RT). Wash 4-5 times for 5 minutes in PBST. Incubate 1 hour at RT with the secondary Horseradish Peroxidase (HRP)-Antibody diluted in blocking buffer. Wash 4-5 times for 5 minutes in PBST. Incubate for 5 minutes with 4-5 mL of HRP substrate (ratio 1:1 of the 2 reagents). Once mixed, the substrate solution is sensitive to light and must be covered with aluminum during incubation. The membrane is now prepared for exposure and imaging.

6. Cell Proliferation Assay

At day 4 of the cell culture, the cells are undergoing a puromycin selection. This is a chemical that is toxic to regular cells, however, all lentiviral constructs used have a resistance gene to this toxin. This, as a result, kills off any cells that have not been transduced by the lentivirus. Everyday cell counts were taken using a device called a hemocytometer. 10 mL of the cells from each of the wells will be placed into the hemocytometer to be measured. The hemocytometer consists of 4 grids. By counting the total amount of cells in each of the 4 grids, dividing that total number by 4, and multiplying by a factor of $10^4$, a fairly accurate estimate of the daily cell count can be determined. Lastly, once cell concentrations reached a level greater than 100,000 cells/mL, they were diluted in order to ensure the accuracy of

hemocytometers results. When the daily cell count calculations being made, the factor by which cell concentration was diluted by will then be accounted for. This process is repeated over the course of 16 days and will be conducted on each of the 6 wells.

7. Quantification of Cellular Differentiation Using Flow Cytometry

Cells from the final day of growth are used in order to measure how differentiated cells are. For Flow Cytometry, only cell preparation and antibody compensations need to be made. First, wash 100,000 cells with filtered PBS 0.5% BSA. Then add 22.5uL of PBS 0.5% + 2.5uL blocking buffer (PBS 4% AB+ human serum). Add antibody and incubate for 10 to 15 minutes at RT and wash with 700uL PBS BSA, resuspend in 200-300uL PBS-BSA solution. The following are concentrations for compensations that must be made in order to gate desired cell concentrations. APC-alpha 4 integrin (CD49d): 1.5uL PE GPA : 1uL of the 1:100 dilution. FITC-Band3: 2.5uL of the 1:10 dilution. PerCP Cy5 - 7AAD: 5uL in 100uL (without washing). APC-CD34: 5uL. PE Cy 7 Annexin V: 0.8uL in Annexin buffer. FITC CD34: 5uL. FITC CD71 : 0.7uL. FITC CD36: 0.75 uL. PE-Cy7 IL3-R: 2ul. FITC-CD36 :0.75uL. PE-Beta1 int: 2uL. FITC-Syto 16 : 0.5ul of 1:200 dilution in PBS 5uM $MgCl_2$. APC-Syto 60: same volume as Syto 16.

**Western Blot Results:**

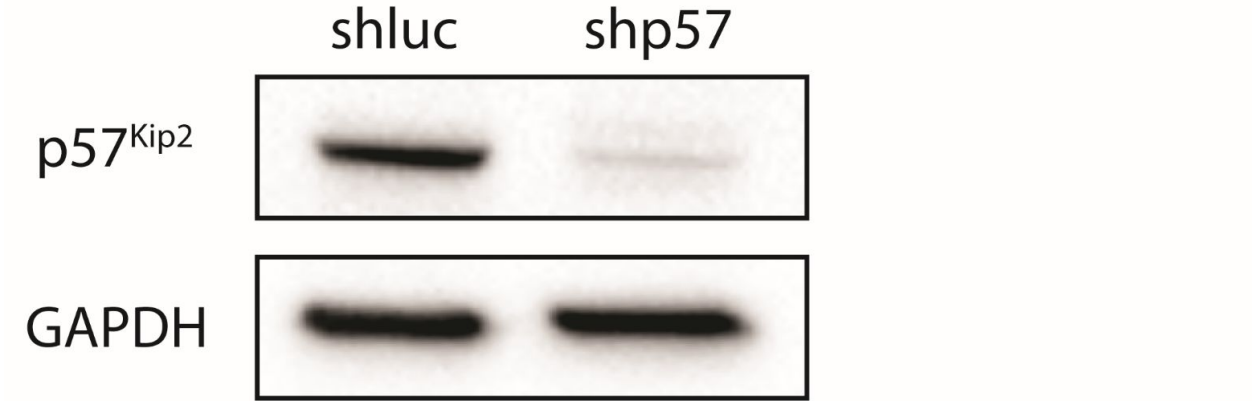
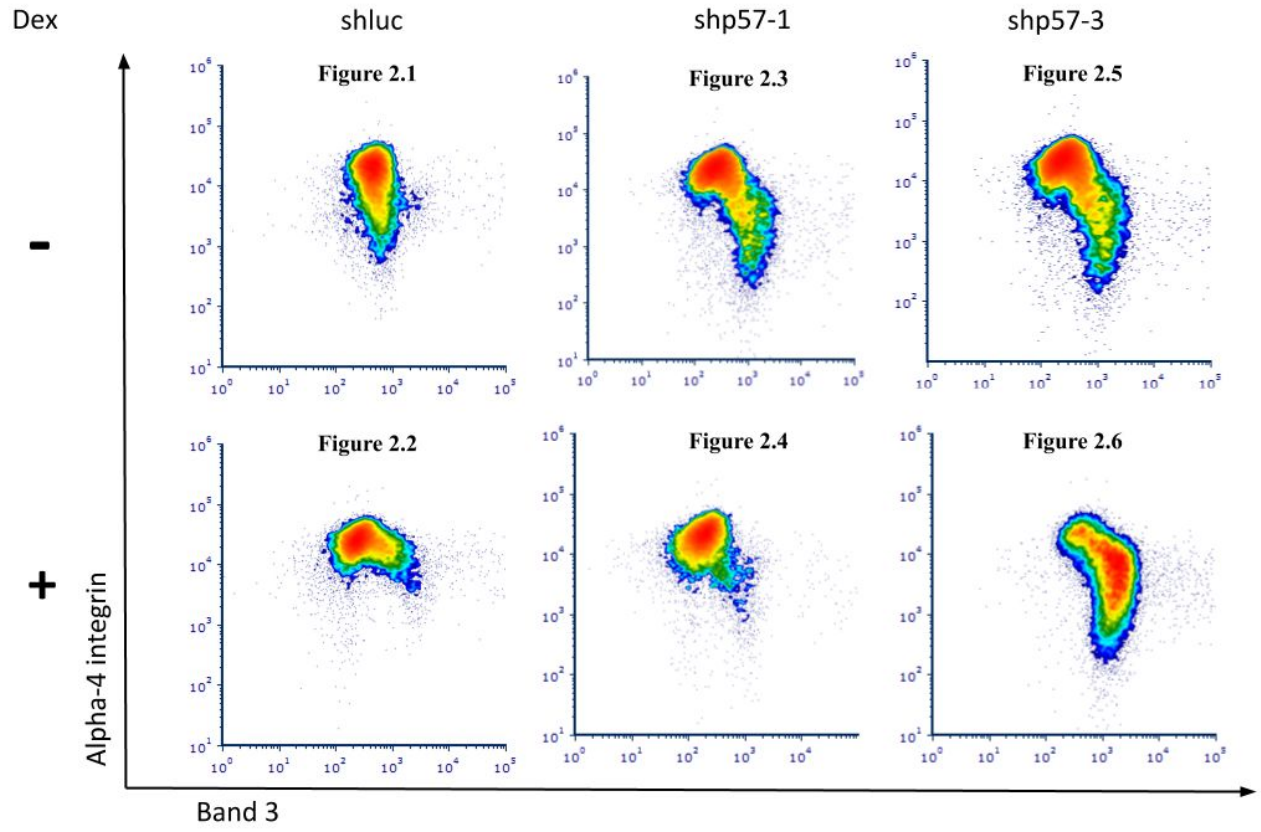



**Figure 1.1**

**Flow Cytometry Results:**



**Cell Proliferation Assay Results:**

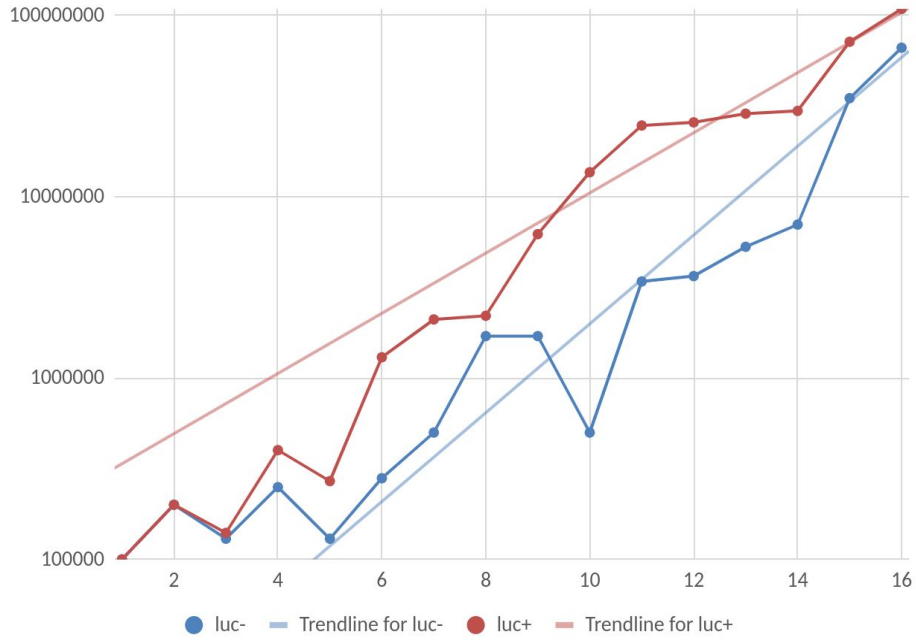



**Figure 3.1**

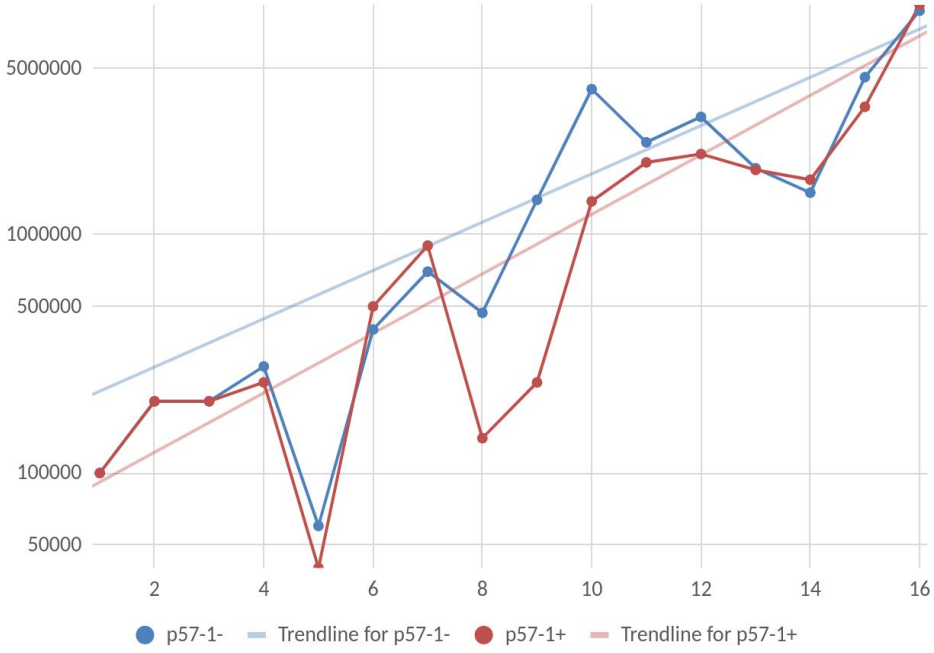



**Figure 3.2**

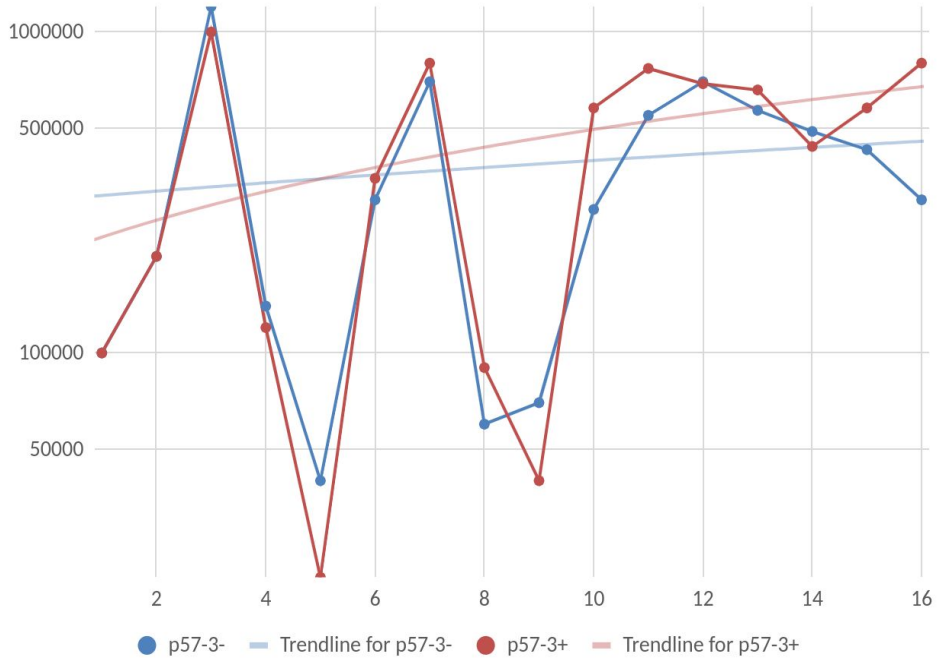



**Figure 3.3**

**Discussion:**

The first part of the data collection process was the Western Blot. The Western Blot served as a method of confirmation for successful lentiviral transduction. This was done by measuring $P57^{KIP2}$ levels in the shp57 transduced cell population. It also served as confirmation that there was no change in $P57^{KIP2}$ levels in the shluc transduced cell population. As a loading control, GAPDH was measured in both cells populations to prove that samples have been loaded equally across all wells and confirms that effective protein transfer has taken place. GAPDH was used because it is involved in many crucial aspects of basic cellular function. In **Figure 1.1**, GAPDH expression appears to be very similar in both cell populations, which indicates that wells were loaded equally. This is significant as it means that the $P57^{KIP2}$ levels in both cell populations are comparable. As seen in the shluc transduced cell population, there is a clear expression of $P57^{KIP2}$, which was expected. However, in the shp57 transduced cell population, there is a very minimal expression of $P57^{KIP2}$, which was also expected. Ideally, if the lentivirus was successfully transduced, the shRNA will bind to its target mRNA. In the case of the shp57 lentivirus, the shp57 should bind to specific CDKN1C mRNA. CDKN1C is the gene that expresses $P57^{KIP2}$, preventing target CDKN1C mRNA from transcription and resulting in low $P57^{KIP2}$

expression. In the case of shluc lentivirus, the shRNA are targeting mRNA that belong to the gene that expresses luciferase. Human cells do not have this gene, therefore this lentivirus serves as a transducing control. Since it did not contain shRNA designed to target CDKN1C, it ideally should have no effect on $P57^{KIP2}$ expression. As evident by the western blot results, this proved to be the case.

The second part of the data collection process was the cell proliferation assay. Daily cell counts were taken and recorded from days 1 through 16. These graphs are intended to show the rate of cell growth as opposed to cell counts themselves, and are therefore graphed exponentially. Since the shluc transduced cell population should have no effect on $P57^{KIP2}$ expression, the Dex treated population should experience greater rates of cell growth than the untreated population. In the shp57 transduced populations, it was expected that $P57^{KIP2}$ expression has decreased significantly. This means that there should be no significant effect on cell proliferation rates when treated with Dex. While there were instances when cell proliferation rates had experienced sudden decreases in proliferation rates, these decreases are consistent across all cell populations. These drops in growth rates can most likely be attributed to parts of the procedure that heavily influenced the cells environment. For example, at day 4, cell proliferation rates experience a universal decrease. But, this was the same day as the puromycin selection, which places high amounts of stress on the cells, and is not the optimal environment for proliferation.

In **Figure 3.1**, the results agreed with the predictions. The shluc population treated with Dex experienced significantly higher cell growth rates than those untreated. In **Figure 3.2** and **Figure 3.3**, The results also agreed with the predictions, however, the shp57-3 transduced cell population was more similar to the predictions. In **Figure 3.2**, it showed that cell proliferation rates of Dex treated and untreated populations became more similar as time progressed. However, around day 16 is when $P57^{KIP2}$ levels tend to play a less significant role in proliferation. This indicates that there may have been something wrong with this population. In **Figure 3.3**, cell proliferation rates seemed to be very similar across both treated and untreated populations at day 4, the peak of $P57^{KIP2}$ expression. But over time, cell growth rates appeared to steadily differ. This is closer to the predictions, as over time $P57^{KIP2}$ progressively should play a less significant role in cell proliferation. In addition, **Figure 3.3** agrees with **Figure 3.1**, as they both demonstrate that $P57^{KIP2}$ plays a less significant role over time. While not all the data agrees with the predictions, the data certainly indicates that $P57^{KIP2}$ reduction has an effect on Dex's ability to increase proliferation rates.

The last part of the data collection process was Flow Cytometry. Flow Cytometry served as a method for testing the effects of Dex's role in differentiation. In Flow Cytometry, previously determined

markers, along with cells physical characteristics are used to gate the true cell population. Gating cells will exclude any dead cells or cells that don't belong to the erythroid cell lineage from this measurement. Once cells are gated, specific markers can be examined and graphed at in order to get an idea of the cell differentiation state. In peripheral blood cells, decreasing alpha-4 integrin expression, and increasing band-3 expression indicate further cellular differentiation. On these differentiation graphs, colors that are "warmer" (closer to red) indicate higher concentrations of cells while colors that are "cooler" (closer to violet) indicate lower concentrations of cells. In shluc transduced population, it was expected that Dex treated cells will be less differentiated than the untreated population. However, in the shp57 transduced populations, it was expected that there should be no significant changes in differentiation. Dex should upregulate $P57^{KIP2}$ expression as a means for slowing down differentiation. By doing so, it extends the time cells are in the progenitor stage (the stage in which the majority of proliferation occurs), providing more time for cells to proliferate.

**Figure 2.1** and **Figure 2.2** demonstrate what was expected in the shluc populations. In the Dex treated group (**Figure 2.2**), there is greater alpha-4 integrin expression and less band 3 expression than the untreated group (**Figure 2.1**), indicating that it is less differentiated. On the other hand, the results of **Figure 2.3** and **Figure 2.4** (shp57-1 group) do not completely agree with its predictions. The untreated group (**Figure 2.3**) has a higher quantity of cells that express more band 3 and less alpha-4 integrin, thus making them appear further differentiated. This would make sense, as the shp57-1 transduced group also experienced a slightly different result in the proliferation assay. In both the treated and untreated groups, the vast majority of cells appear to be in the same region, indicating that both populations are very similarly differentiated. A similar problem occurred in the shp57-3 populations (**Figure 2.5** and **Figure 2.6**). The Dex treated population (**Figure 2.6**) expresses higher quantities of Band 3 and lower quantities of Alpha-4 Integrin than the untreated group (**Figure 2.5**), indicating that it is further differentiated. However, the cause of this is likely the same as the reason for the sudden difference in proliferation rates observed at day 16 of the shp57-3 proliferation assay.

**Conclusion:**

This experiment demonstrates that $P57^{KIP2}$ plays an integral role in Dex's ability to successfully upregulate cell proliferation rates. The results have indicated that when $P57^{KIP2}$ is downregulated, Dex is not able to optimally increase cell proliferation rates. This data is very significant, as this may indicate an issue in the $P57^{KIP2}$ pathway in DBA patients resistant to corticosteroids. Moving forward, this pathway can be studied in DBA cells in order to better understand the mechanisms of this disease. If a treatment for this pathway is developed, it would ensure a reliable treatment method for all those who have DBA. While this study only focuses on specific stages of erythroid differentiation cycle, it occurs during the time frame in which Dex is most active, which means fixing the $P57^{KIP2}$ pathway with alternative treatment should theoretically yield the same results. Not only does this study provide new areas of exploration in DBA, but further studies in healthy red blood cells as well. This study, while very similar to the hypothesis, showed that there may be alternative mechanisms by which Dex acts to increase proliferation rates in later stages. **Figure 3.3** is a perfect example of this. While at first, it appeared the Dex treated population had been proliferating at very similar rates to the untreated population, towards day 16, there appears to be an increase in the Dex treated population proliferation rates. This may be significant, as it may imply that Dex acts on other mechanisms that allow for this to occur. Further evidence for this can be seen in **Figure 2.5** and **Figure 2.6**. $P57^{KIP2}$ upregulation leads to the slowing down of differentiation, so that cell proliferation rates can increase. However, what is seen here is the Dex treated group being further differentiated, which means there is a possibility that Dex uses another mechanism to achieve an increase in proliferation rates at different stages of erythroid differentiation cycle.

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