Research Plan

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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

a. Rationale

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.

b. Research Question:

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

c. 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.

C . Procedure:

1. Transient Transfection of 293T cells for Lentivirus Production

I will 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. I will culture cells in DMEM 10% FBS (heat-inactivated) media for 24-30 hours to allow for growth, but never to confluence. I will 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 will be made up of a final volume of 450 uL with 0.1 XTE/dH20 (2:1). Finally, I will add 50 ul of 2.5M CaCl2 . Then, I will wait 5 minutes at room temperature. Then prepare one 15 mL tube for each of the two dishes. A plasmid with GFP will be used with an extra dish as transfection efficiency control. Next, a precipitate will be 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 I will immediately add precipitate to 293T cells. High magnification microscopy will 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 will settle to the bottom of the plate in large spaces between the cells. Following incubation, the media will be replaced with 8 mL of fresh media. 48-72 hours following transfection, the supernatants will be collected and filtered through a 0.2-micrometer filter to extract the virus. The 30 mL of filtered supernatant will then be placed in the ultracentrifuge at 20,000 rpm for 2 hours at 20 degrees celsius. Once this is completed, I will aspirate the media very carefully without removing the pellet. Then I will add 30 uL sterile PBS per tube to resuspend the cells and harvest the virus. Finally, I will create aliquots and freeze down at -80°C.

2. Lentiviral Titration

I will 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 I will change media and replace it with 1 mL IMDM 10% FBS + Polybrene 3 hours before the transduction. Polybrene will be added in each well at a final concentration of 8ug/mL. 5 uL of the concentrated virus from day 1 harvesting will be added to the first well, then a serial 10 fold dilution will be performed twice into two successive wells. This process will be repeated for the p57 lentiviral constructs, luciferase lenti-viral constructs and non-transduced control. The media will be 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, I will wash the cells with 2 mL of PBS. The greater the confluence the better as more genomic DNA can be isolated for qPCR. Then I will 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 I will add 1 mL PBS to each well to dilute trypsin. Take 1 mL of cells and centrifuge for genomic DNA (gDNA) preparation. Then I will 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, I will add a lysate is to be prepared in order to isolate the genomic DNA. This will be done by first adding Proteinase K and RNase A to the sample. They will then be vortexed to create a homogenous solution, and incubated at room temperature for 2 minutes. Then I will add Genomic Lysis/ Binding Buffer, forming the Lysate. They then will 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 will then be added to the spin column and spun down for a minute. The spin column will be placed into a new collection tube, where it will be washed with wash buffers to prepare for elution. An elution buffer will be added, and then the solution will be incubated at room temperature for a minute, and finally it will be spun down for a minute. The DNA will then be isolated, but the elution process will be repeated another time in a different tube in order to isolate more purified genomic DNA (gDNA). The gDNA from uninfected 3T3 cells will 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 I will 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 will ultimately yield 100 ng of gDNA per well in 4 uL of water. Then I will 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. The next step is to prepare a Mix for Psi and TaqMan. For one well and 8.5 uL mix, I will 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, I will load the first 3 rows with 8.5 uL of the TfR Mix. Then I will load the following ones with 8.5 uL of Psi mix. I will load the gDNA negative controls, copy number controls, samples, and water negative controls. The plate will then be ready for qPCR. For qPCR, both the SybrGreen and Psi Mix will be 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 will be correlated to the amount of mThfR (the control) while the readout of TaqMan in the Psi mix will 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 will be made in order to determine the final concentration of lentivirus.

4. Cell Transduction

CD34+ cells will be cultured in order to determine self numbers over the course of a few weeks. Serum-free culture with StemSpan SFEM will be used as a base media. Aliquots of the solution will be made and frozen until required. They will be thawed at room temperature when starting the culture. In order to make the SFEM media, the cytokines will be added and are responsible for sending signals to the cells, allowing them to grow. Concentrations of cytokines will be added to 10 mL of solutions. Stem Cell Factor (SCF) will be added at a concentration of 50ug/mL (20uL) . Interleukin 3 (IL3) will be added at a concentration of 50ug/mL (20 uL) . L-glutamine will be added at a concentration of 2mM (100 uL). Transferrin (Tf) will be added at 200ug/mL (40 uL). Specific cytokine concentrations will change depending on the phases. From days 0-7, Erythropoietin (Epo) will be added at concentration +0.5IU/mL (5uL). From days 7-11, Epo will be added at concentrations of +3IU/ mL (30 mL). From days 11-25 +3IU/mL of Epo (30 uL) will be added as well as +1mg/mL of Tf (200 uL). Cells will be thawed in a hot water bath until almost completely thawed. At that point, cells will be diluted by 10 mL of PBS and spun down gently to form a pellet. The reason for this dilution is the cells will be put in a solution with DMEM in order to aid in the freezing process. However, at room temperature the DMEM will be toxic to the cells at that concentration, and therefore needs to be diluted. These cells will then 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 will be effective transduction of lentivirus within the cell. I will prepare protein samples and measure the concentration (BCA kit). I will 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). I will boil the sample for 5 minutes for complete denaturation and do quick spin centrifugation in order not to lose the evaporated fraction. Samples will be frozen at -80 °C at this step if I don't need all of them (they will have to be boiled a second time after thawing). Next, the

SDS-Page itself will be prepared. First, I will wash the wells of the gel with distilled water. Then I will place the gel in an electrophoresis Tank with 1X Tris-Glycine SDS (TGS). 5 uL of prestained protein ladder will be loaded into the first well. Then, the boiled samples will be loaded at a volume of 30uL. The process of protein electrophoresis will now begin at 90-100V until the front migration line crosses the stacking gel (approximately 15 minutes). By doing so, better protein stacking will be achieved and will ultimately result in higher quality resolution. The voltage will 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. I will cut a Nitrocellulose membrane piece slightly smaller than the Biorad filter paper. Then, I will lace 2 pieces of the filter paper, the membrane, the gel and the black sponges in 1X cold TG buffer. A "sandwich" will then be made by placing down the black sponge, then stacking on top filter paper, gel, nitrocellulose membrane, filter paper, and black sponge. I will 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. I will 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 I will run the transfer at 95V for 1 hour. I will get the membrane, check the migration with a Red ponceau staining and wash with distilled water. I will perform a blocking with PBS 0.1% Tween 20 (PBST) 4% Milk, 1% BSA for 60-120min at RT. I will 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). I will wash 4-5 times for 5 minutes in PBST. I will incubate 1 hour at RT with the secondary Horseradish Peroxidase (HRP)-Antibody diluted in blocking buffer. I will wash 4-5 times for 5 minutes in PBST. I will incubate for 5 minutes with 4-5 mL of HRP substrate (ratio 1:1 of the 2 reagents). Once mixed, the substrate solution will be sensitive to light and must be covered with aluminum during incubation. The membrane will then be prepared for exposure and imaging.

6. Cell Proliferation Assay

At day 4 of the cell culture, the cells will be 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, will kill off any cells that have not been transduced by the lentivirus. Everyday cell counts will be 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 will be diluted in order to ensure the accuracy of hemocytometers results. When the daily cell count calculations are being made, the factor by which cell concentration will be diluted by will then be accounted for. This process will be 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 will be used in order to measure how differentiated cells are. For Flow Cytometry, only cell preparation and antibody compensations need to be made. First, I will wash 100,000 cells with filtered PBS 0.5% BSA. Then, I will add 22.5uL of PBS 0.5% + 2.5uL blocking buffer (PBS 4% AB+ human serum). I will 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 MgCl2. APC-Syto 60: same volume as Syto 16.

Data analysis

I will use western blots to analyze the effectiveness of lentiviral shRNA knockdown of CDKN1C. Next, I will use Cell Proliferation Assays in order to demonstrate the rate of cell proliferation when treated with the glucocorticoid Dexamethasone, with cells that have and have no CDKN1C knockdown. Finally, I will use Flow Cytometry to measure the state of differentiation of the same cell populations. Ultimately, these tests will look to prove the hypothesized phenotype of Dexamethasone in healthy peripheral blood cells.

Materials:

-DMEM 10% FBS (heat-inactivated)
-IMDM 10% Hyclone FBS
-packaging plasmids pCMV delta and pucMDG
-2x HBS solution
-Centrifuge
-Ultracentrifuge

-PBS and PBST

-6 wells COSTAR

-Polybrene

-Trypsin

-Proteinase K and RNase A

-Genomic Lysis/ Binding Buffer

-100% Ethanol

-Elution Buffer

-3T3 Cells

-qPCR grade water

-384 White Well Plate

-Forward mThfR Primer

-Power SybrGreen PCR Master Mix

-TaqMan Universal PCR Master Mix

-Forward Psi Gene

-CD34+ Peripheral Blood Cells

-Serum-free culture with StemSpan SFEM

-Stem Cell Factor, Interleukin-3, L-glutamine, Transferrin, Erythropoietin

-Dexamethasone

-BCA Kit for Western Blot

-Electrophoresis Tank

-1X Tris-Glycine SDS

-stacking gel

-nitrocellulose membrane

-BioRad filter paper

-Black Sponges

-1X Cold TG Buffer

-Red Ponceau

-Milk

-BSA

-Mouse Anti-Human p57

-Mouse Anti-Human GAPDH

-Blocking Buffer

-Horseradish Peroxidase antibody
-Puromycin
-Hemocytometer
-PBS 4% AB+ human serum
-APC-alpha 4 integrin
-PE GPA
-FITC-Band3
-PerCP Cy5 - 7AAD
-APC-CD34
-PE Cy 7 Annexin V
-FITC CD34
-FITC CD71
-FITC CD36
-PE-Cy7 IL3-R
-FITC-CD36
-PE-Beta1 int
-FITC-Syto 16
-APC-Syto 60

Risks and safety BSL-2

Chemical assessment
293TN Cells, Peripheral Blood Units, Lentiviral Particles

Safety: I will receive instruction in the safe use of biological agents and will complete instructional safety courses. Proper PPE will be worn at all times. All procedures will be carried out in a biological safety cabinet as necessary. Lentiviral particles will only be handled by designated supervisor. I will be instructed in the safe use of biological units. Biological agents will be inactivated with bleach and alcohol.

D. Bibliography

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