



## PROMOTING OSTEOGENESIS IN ADIPOSE DERIVED STEM CELLS USING CRISPR-DCAS9

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### I. INTRODUCTION

For 1.9% of the population and 9% of specific age groups broken bone never fully heals from a fracture [1]. This nonunion of fractured bone can cause pain and discomfort as well as hamper natural movements [2]. Currently the only treatments include ultrasound stimulation or bone grafts. Patients undergoing the ultrasound stimulation may have to use the device several hours a day during the treatment for it to be successful [2]. A bone graft is taken from either the patient or a donor causing pain at the site of bone harvesting [2]. A new treatment is needed that would consume less time than the ultrasound stimulation and be less painful than the bone graft. This new treatment could be done using osteoblasts derived from adipose derived stem cells (ASCs) to treat large bone fractures that have not healed [3].

ASCs can differentiate into osteoblasts among other cell types. [4]. ASCs can be easily harvested from the patient's own fat supply and expanded *ex vivo*. [5]. After they are taken from the patient, the ASCs can be expanded and encouraged to differentiate [4]. Differentiation of ASCs into osteoblasts has been moderately successful [3]. The most prominent and successful method of differentiation is achieved using external growth factors [6]. Differentiated osteoblasts derived from ASCs are shown to be almost identical to those that naturally occur in bone tissue [7]. This makes osteoblasts differentiated from ASC a viable option in regenerative medicine [5]. Although external growth factors are a viable option for differentiation, they are expensive and provide little control over specific cells. Internal factors such as epigenetic modification can be less expensive and provide greater control of the differentiation process.

Epigenetic modification is known to drive differentiation in stem cells [8]. A known way to influence the epigenetics of stem cells is through a modified version of CRISPR-Cas9 [9]. An edited version of the Cas9 protein produces a nuclease deficient version of Cas9 known as dCas9. This dCas9 will still bind to a target segment of DNA but will not be able to cleave it [9]. dCas9 can be modified even further by the addition of proteins that mimic natural transcriptional initiation. The addition of the proteins VP64, p65 and Rta, collectively known as VPR, to dCas9 create a synthetic promoter of gene production [10]. When a site upstream of a gene is targeted with dCas9-VPR, an increased level of transcription of the gene is observed. This method enables the targeting of genes for upregulation.

Currently no upregulation of any gene is known to encourage osteogenesis in ASCs. The method of producing osteoblasts is limited by having to use specialized cell media with specific growth factors [7]. This provides limited control of the cells outside of this specialized media. Using internal rather than external methods to drive osteogenesis improves control of the process. Differentiation by gene upregulation is a possible internal driving force to promote osteogenesis. We hypothesized that some number of genes could encourage osteogenesis if they were upregulated.

To test which genes would promote osteogenesis we first created a cell reporter line. The reporter line has a gene that encodes for a fluorescent protein. This gene's promoter segment is identical to the gene that produces osteocalcin. Osteocalcin production by the cell is a late indicator for osteogenesis. If the cell is producing osteocalcin it also produces the fluorescent protein.

To test which cells would produce osteocalcin after gene upregulation, we first infected the cells with a CRISPR library. This library targeted five different locations upstream of each gene in the genome. We then grew these cells in normal stem cell media and allowed them to differentiate and fluoresce. Then we isolated cells undergoing osteogenesis based on fluorescence. Then we analyzed the fluorescent cells for gene upregulation content to see which genes played a role in osteogenesis.

Knowing which genes encourage osteogenesis is a major step in being able to quickly and successfully create osteoblasts from ASCs. Encouraging differentiation through internal methods allows greater control of the process. These internal methods of gene upregulation can promote osteogenesis improving the osteogenic potential of ASCs. ASCs are easy to harvest and with the right methods can become effective tools in regenerative medicine. This could lead to new treatments for large bone fractures that have not healed.

## II. METHODS

To prove that upregulation of some genes can cause osteogenesis the following methods were used. We had to separate the genetic editing of the cells we were testing into three areas, the first being the addition of dCas9-VPR to ASCs. The next being the osteocalcin reporter and finally the CRISPRa guides.

Plasmids for two separate virus vectors were sourced, CRISPRa guides from Addgene, and the osteocalcin reporter from Rodrigo Somoza at Case Western reserve University. Amplification, transfection, were performed for both viruses. Titration was performed for CRISPRa library virus. We used cells that had already been transduced with dCas9-VPR.

The dCas9-VPR cells were infected with the virus created from the plasmid designed by Somoza which contained a GFP fluorophore. The plasmid also contained a copy of the DNA sequence for the promoter region of osteocalcin but produced a D-Tomato fluorophore instead. Positive selection for GFP was done through flow cytometry and positive single cells were extracted and plated to create a clonal population.

This clonal group was tested for osteocalcin reporting reliability. A group of the clonal cells were grown in Mesenchymal Stem Cell Basal Medium for Adipose, Umbilical and Bone Marrow-derived MSCs (ATCC<sup>®</sup> PCS-500-030<sup>™</sup>) or ASC culture medium for a control group and another group were grown in osteogenic cell media. After 3 weeks the cells were examined through flow cytometry for D-Tomato fluorescence (the fluorophore linked to osteocalcin production).

After verification of the reporter cell line, cells from the reporter cell line were then transduced with the CRISPRa library virus. These cells were also analyzed by flow cytometry for positive BFP expression through flow cytometry. After 2 weeks pictures of the positively transduced CRISPRa cells using a fluorescent microscope were taken with GFP-BFP and D-Tom channels. ASCs were incubated at 37C and 5% CO<sub>2</sub>.

### *A. Creation of Osteocalcin Reporter Lentivirus*

First, we obtained the reporter virus plasmid from Somoza. To amplify the plasmid we thawed NEB Stable cells on ice and added the plasmid at 100 ng to 50ul of NEB E. coli cells. We then gently mixed the tube that contained the plasmid/bacteria. We then placed the mixture on ice for 30 minutes. Next, we heat shocked the mixture at 42C for 30 seconds without mixing. We then transferred tubes to ice for 2 minutes. We Added 950ul room temperature LB medium to the tube and incubated it at 37C while shaking for 2 hours. We Took 5ul of recovery, made serial dilutions and plated with beads to calculate transformation efficiency. We transferred 5ul of recovery to 995ul Outgrowth medium (1:200 dilution), 10ul of 1:200 dilution to 490ul Outgrowth medium (1:10000 dilution), 250ul of 1:200 dilution to 250ul Outgrowth medium (1:20000 dilution), 200ul of 1:20000 dilution to 200ul Outgrowth medium (1:40000 dilution), and 15ul of 1:40000 dilution to 135ul Outgrowth medium (1:400000 dilution). We plated 100ul of each dilution onto carb plates using beads. We added remaining recovery to 500ml LB+Carb grew overnight (16h) while shaking at 37C. We then harvested the cells and purified the reporter plasmid using multiple maxiprep columns.

#### *B. Creation of CRISPRa Lentivirus*

We obtained the CRISPRa Library plasmids from Addgene #83978. The plasmids came as a 20ul aliquot at 25ng/ul (500ng total). To amplify the CRISPRa library we thawed NEB Stable cells on ice and added the CRISPRa library plasmids at 100 ng to 50ul of NEB E. coli cells. We then mixed the tube that contained the plasmid/bacteria mixture. We then placed the mixture on ice for 30 minutes. Next, we heat shocked the mixture at 42C for 30 seconds without mixing. We then transferred tubes to ice for 2 minutes. We Added 950ul room temperature LB medium to the tube and incubated it at 37C while shaking for 2 hours. We Took 5ul of recovery, made serial dilutions and plated with beads to calculate transformation efficiency. We transferred 5ul of recovery to 995ul Outgrowth medium (1:200 dilution), 10ul of 1:200 dilution to 490ul Outgrowth medium (1:10000 dilution), 250ul of 1:200 dilution to 250ul Outgrowth medium (1:20000 dilution), 200ul of 1:20000 dilution to 200ul Outgrowth medium (1:40000 dilution), and 15ul of 1:40000 dilution to 135ul Outgrowth medium (1:400000 dilution). We plated 100ul of each dilution onto carb plates using beads. We added remaining recovery to 500ml LB+Carb grew overnight (16h) while shaking at 37C. We then calculated the successful transformation the next day. Since the efficiency was higher than 1000 colonies/sgRNA (100,000,000 colonies), we harvested the cells and purified the CRISPRa library using multiple maxiprep columns.

#### *C. Transfection and Lentivirus Collection (For both Somoza and CRISPRa library screen)*

We thawed and plated HEK293T cells and let them divide until we had approximately 7.5 million. We then plated them in a T175 in 30ml D-MEM high glucose medium without antibiotics. Using a 1.5ml tube, we mixed 1.3ml OptiMEM with 48ul Mirus and incubated for 5 minutes at room temp. In a separate 1.5ml tube, we mixed the lentiviral plasmid with packaging vectors. 7ug of psPAX2, 1ug of pMD2.G and 8ug sgRNA vector/Somoza. We combined the two vials and mixed them together by pipetting gently. We then incubated the combined vial for 20-30 minutes at room temperature. We added the contents of the vial dropwise evenly to the HEK 293T cells on the T175 plate. We let the HEK 293T cells produce virus for 72 hours. After which we pulled off the supernatant from the cells and filtered it using a 0.4um filter. We stored aliquots of the of the virus at -80C.

#### D. *Viral Titration*

We plated 47,500 ASCs per well in a 24-well plate and incubated for 18-20 hours. We prepared 5ml of ASC culture medium containing 8ug/ml polybrene at 2x concentration. We thawed the lentivirus stock at room temperature. We then prepared 0.3mL 2-fold serial dilutions ranging from 1x to 1:128 dilution in 1.5 ml tubes and mixed gently by inverting the tubes 10 times. We added 250 ul ASC culture medium containing polybrene (4ug/ml final concentration) to one well of ASCs as a control. Then we added 250ul of serial diluted viral media to the remaining wells of the 24-well plate. We then let the cells incubate at 37C. After 24 hours, we removed the medium containing virus from the wells and rinsed 3 times with PBS. We then replaced the media with 500 ul of ASC culture medium (without polybrene). We replaced the media every 2-3 days until BFP expression was noticed. When it was noticed we passaged the cells into cold PBS and submitted them for FACS analysis. We used the FACS analysis to determine the successful transduction of cells based on how many cells were fluorescing out of how many cells were sampled. We used a well that had between 1% and 20% of cells expressing BFP to determine titer. The formula we used for calculation was

$$\text{titer} = \{(F \times Cn) / V\} \times DF$$

Where F: The frequency of BFP-positive cells determined by flow cytometry;

Cn: The total number of target cells infected.

V: The volume of the inoculum.

DF: The virus dilution factor.

#### E. *Osteocalcin Reporter ASC Transduction*

We thawed and plated dCas9-VPR transduced ASCs onto a T75. We then passaged the cells after they approached confluency into a T175. We transduced with the same format as part D but at a MOI (multiplicity of infection) of .3 to ensure every cell received only one copy of the virus. We then incubated the cells for 24 hours. After 24 hours we removed the virus containing media and rinsed the cells 3 times with PBS. After expression of GFP was noted the cells were passaged and screened using flow cytometry. Positively transduced cells were separated, and a single cell was plated in each well of a 96-well plate. We used conditioned growth media in the 96-well plate (50% normal stem cell expansion media, 50% filtered expansion media pulled from other ASCs).

#### F. *Validation of Somoza cells*

We let the single cell lines grow in the 96 well plate and passaged colonies that survived into a 24-well plate. We then switched the cells to normal media and picked two clonal population lines to test for osteocalcin reporting. We expanded the cell lines into a 12 well plate and saved some from each line by freezing them. We froze them in a mix of 90% normal cell media containing 10% FBS and 10% DMSO. We plated a control group for each line in ASC culture medium and one in osteogenic medium composed of in volume 85.9% DMEM(No Glutamine, Low Glucose, Pyruvate, No Phenol Red) 10% FBS, 1% L-Alanyl-L-Glutamine (200mM) 2% Pen/Strep (5000ug/ml), .01% Dexamethasone (.1mM), .1% Ascorbic Acid (50mM), 1% Beta-Glycerophosphate (1M), and .1% Phenol Red. The cells were left to differentiate in the osteogenic media. Cell medium was changed 3x a week for 3 weeks at which point the cells were submitted to FACS for analysis.

### G. CRISPRa library ASC transduction

We thawed and plated the osteocalcin reporter cell line which had the best osteocalcin expression from part F. When the cells approached confluency, we passaged them and seeded 2 T175s at 5,000 cells/cm<sup>2</sup>. When the cells in those T175s approached confluency, we passaged the cells and seeded 10 T175s at 5,000 cells/cm<sup>2</sup>. We let those cells come to confluency. We calculated that with 100,000 guides in the CRISPRa library x 100-fold coverage / .3 MOI x 1.1 for human error we would need 36,666,667 cells total. We know that at confluency there are 25,000 ASCs/cm<sup>2</sup>. This times the 175 cm<sup>2</sup> in the T175 is 4,375,000 so we decided to use 9 of the 10 confluent T175 for the CRISPRa transduction. We transduced the cells in the 9 T175s using the same protocol as part D except at a known MOI of .3. After 24 hours the cell medium containing the virus was removed and the cells were washed 3 times with PBS. After observing the cells fluorescing under BFP light we passaged and submitted the cells for FACS analysis. Using flow cytometry, we separated the cells expressing BFP and plated them in a T175. We let the cells grow in ASC culture medium. After 2 weeks we used a fluorescent microscope to take pictures of cells using GFP (reporter positive), BFP (CRISPRa positive) and D-Tomato (Osteocalcin positive).

## III. RESULTS

After sending the osteocalcin reporter line through flow cytometry we received some positive results for the

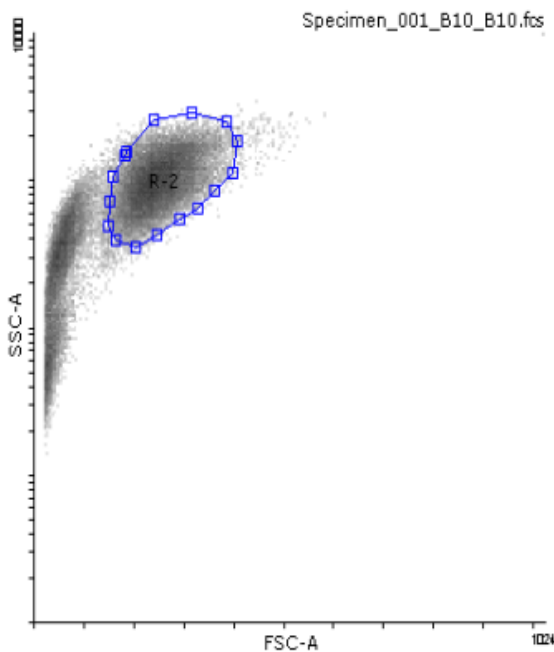


Fig. 2. X axis is cell size and Y axis is cell density. Presents the flow data for the cells grown in normal media. Each dot represents an individual

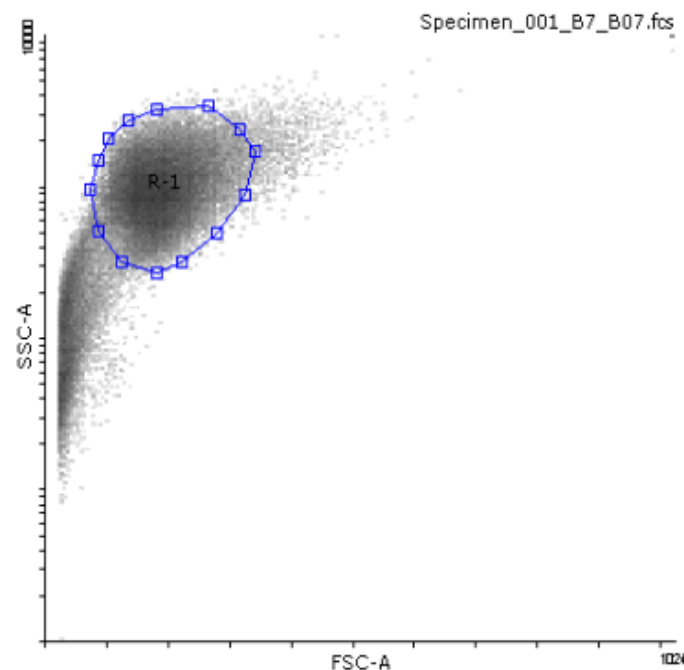


Fig. 1. X axis is cell size and Y axis is cell density. Presents the flow data for the cells grown in osteogenic media. Each dot represents an individual cell.

success of the reporter. Figures 1-3 are based on cell

morphology while figures 4 and 5 are based on fluorescence. Gating the cells involves setting parameters for the machine to search for. When we gated for morphology only as would be done in figure 3. It yields 14.18% of false positives, or cells that are not osteoblasts being recorded as osteoblasts by the flow machine. When combined with the information from figures 4 and 5 the false positive report goes down to 3.8%. This mixture of morphology and fluorescence is done by looking at which cells are outside the range for the bulk of undifferentiated ASCs but in the range of the bulk of the osteoblast. Next, we take those cells in the gated area of the bulk of the osteoblasts but not the ASCs and compare the fluorescence. There are already fewer undifferentiated ASCs because most are not in the same morphological range as the osteoblast. The ones that are do not fluoresce as much as the osteoblasts. Using these two gating techniques we can eliminate many false positives for osteogenesis. We used FlowJo for all calculations using flow cytometry data.

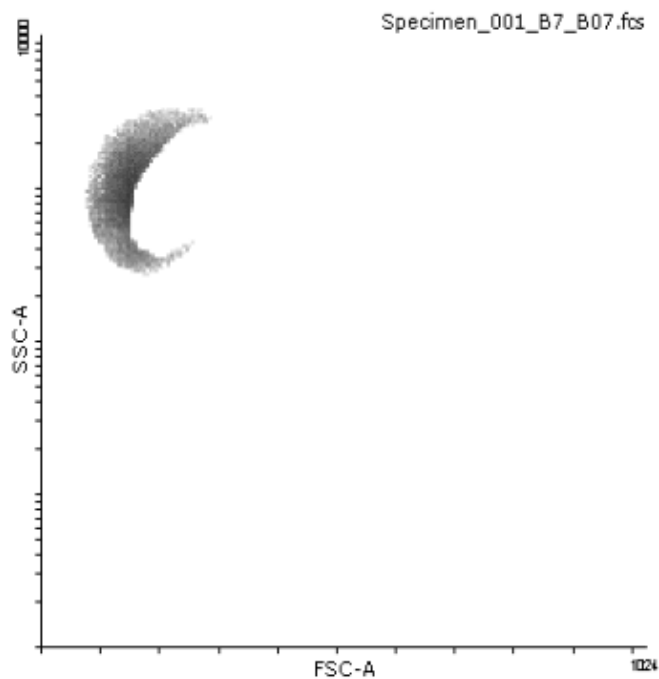


Fig. 3. This is the area of cells that fit the morphological change for osteoblast cells. It is found by taking R-1 and subtracting the field for R-2.

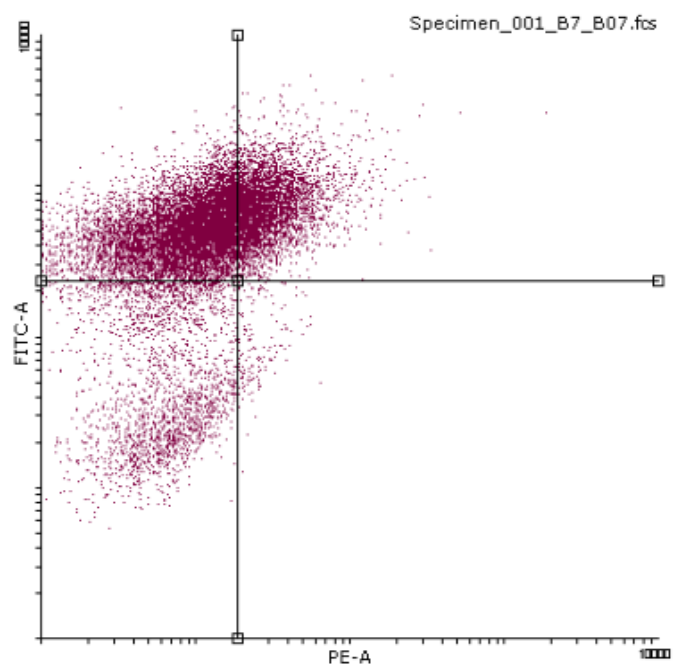


Fig. 4. X axis is the magnitude of fluorescence. This is the flow data from cells grown in the osteogenic medium that are the area of Fig. 3.

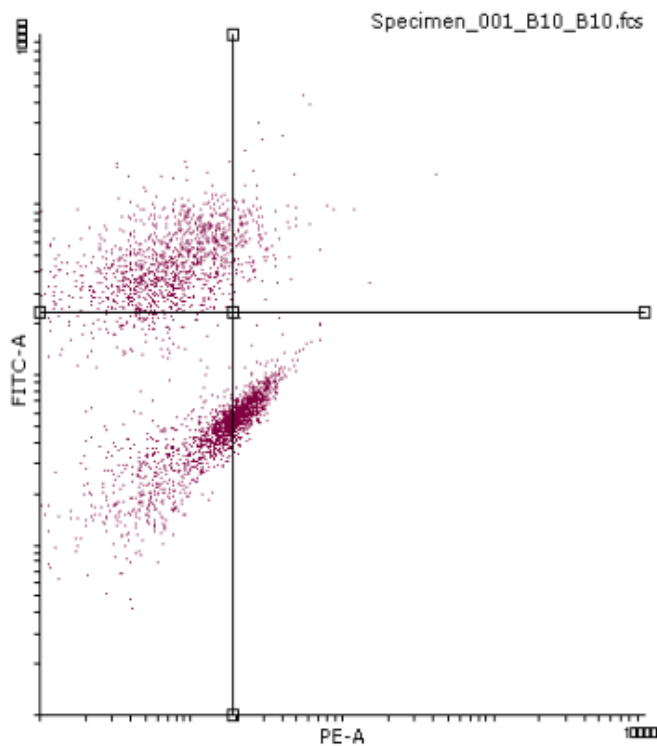


Fig. 5. X axis is magnitude of fluorescence. These are the cells grown in ASC medium that are in the area of fig. 3.

The final product of positively transduced ASCs grown in ASC culture medium have been photographed. When placed under a fluorescent microscope the different fluorophores tied to the different additions to the cells can be seen. Figure 6 shows the GFP fluorophore associated with positive transduction of the osteocalcin reporter virus. Many cells are seen. The same group of cells are photographed again under BFP fluorescent light in figure 7. Once again, the same cells can be seen, and it is evidence of the positive transduction of the CRISPRa library. Figure 8. Is different in that only one cell from the group can be seen. This means that this cell but none of the others is expressing osteocalcin. Since this cell has been positively transduced with the osteocalcin reporter as can be seen in figure 6 it is also producing a D-Tomato fluorophore. Other cells like this have been found scattered throughout the group of ASCs. This means that some gene that was upregulated in the cell in figure 8 is driving osteogenesis.

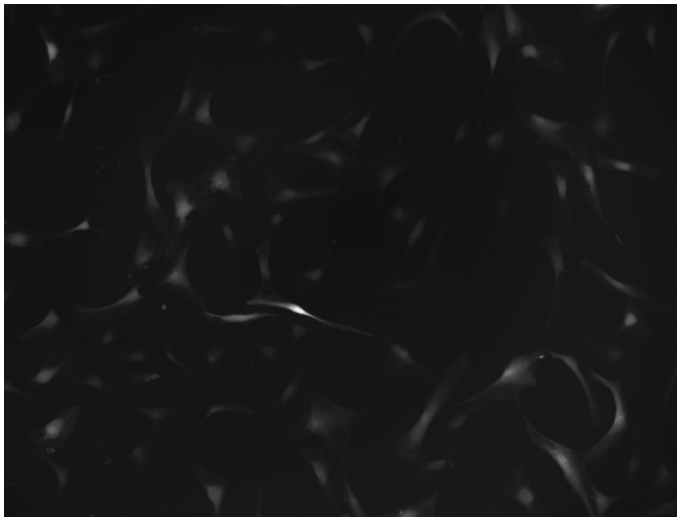


Fig. 6. Final cell line under GFP fluorescent light. Proof that these cells have been transduced with Somoza virus.

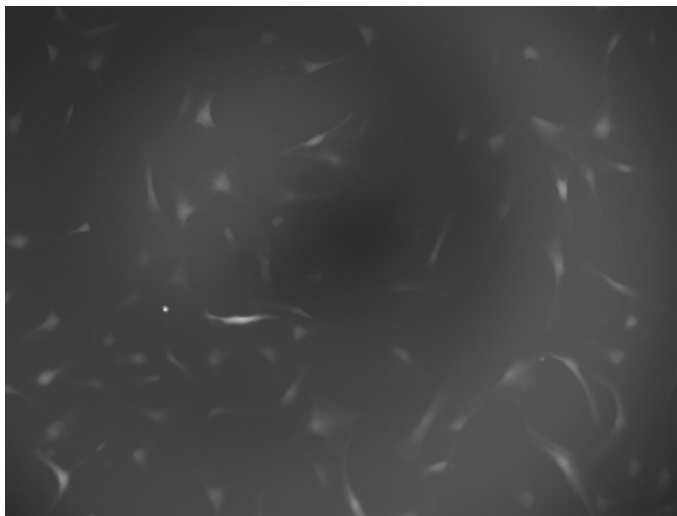


Fig. 7. Same cells from final cell line under BFP fluorescent light. Proof that these cells have been transduced with CRISPRa library virus.





Fig. 8. Same cells from final cell line under D-Tomato fluorescent light. Proof that some cells like the one pictured are producing osteocalcin despite being in normal growth media.

#### IV. Discussion

In adipose derived stem cells (ASC) changes in gene expression can lead to differentiation. Our goal was to test if bone cell differentiation or osteogenesis could occur through random gene upregulation. To do this we used a CRISPR library that targeted X number of genes and a nuclease deficient Cas9, known as dCas9. To promote upregulation the dCas9 was coupled with the proteins VP64, p65 and Rta (VPR). The CRISPR library was transferred to the ASCs via lentivirus transduction. Each cell infected with the CRISPR library had one of X genes upregulated. These ASCs had previously been engineered to report osteogenesis via fluorescence. After 3 weeks post transduction some of the cells were found fluorescing. This means that the upregulation of some gene by the CRISPR-dCas9-VPR system was successful in inducing osteogenesis.

Creation of the cell line that was tested was done through a lentivirus transduction. The lentivirus added a gene that produced a fluorophore when the cell was expressing osteocalcin. We decided to use osteocalcin as a reporter for osteogenesis because it is specifically expressed by osteoblasts [11]. In order to validate this cell line for positive reporting of osteogenesis we differentiated some positively transduced ASCs into osteoblasts. This was done using osteogenic media to drive osteogenesis in some ASCs [3]. Other ASCs were left in normal stem cell proliferative media.

The two cell lines were analyzed using flow cytometry. Results from the testing of the differentiated and undifferentiated cell lines are shown in figures 1-5. These results show the difference between the undifferentiated ASCs and osteogenesis induced ASCs. The data show a difference between cell types morphologically and levels of fluorescence. Using the parameters of morphology and fluorescence the reporter line works with a 5% false positive rate for distinguishing between cell types. This way we could isolate cells that had undergone osteogenesis and be certain of their differentiation.

We also used flow cytometry to select for positively transduced ASCs. The findings in figures 6-7 show the cells exhibiting a positive transduction via fluorescence. Figure 6 for the virus used to create the osteogenesis reporter cell line and Figure 7 for the lentivirus transduction of the CRISPR-dCas9-VPR system. Cells positive for both transductions were selected and grown in normal growth media. After three weeks we checked for osteogenesis.

The reporter cell line reports osteogenesis by a fluorophore. Figure 8 is the fluorescent activity of a cell expressing this fluorophore. Some cells fluorescing like the one shown in figure 8 were found. This means that whatever gene(s) was upregulated in those cells has also influenced them to undergo osteogenesis.

Gene expression modification has been done before. Generally, there is specific and nonspecific gene targeting. Targeting of a specific gene to change gene expression and promote osteogenesis was done in a study published in 2014. In the study the researchers targeted the Noggin gene for downregulation in adipose derived stem cells (ASC). The downregulation of the gene prevented a protein antagonist from being produced by the cell. This protein, thus unhindered, started a signaling cascade that drove osteogenesis in the cell [3]. This research is important in showing that gene expression can influence the differentiation of stem cells. This is similar to our research in that the goal is osteogenesis of ASCs. It is also similar because of the method of differentiation, gene expression modification. It differs from our study in two ways. First there is a specific gene chosen. Second the modification of gene expression is to downregulate expression of that gene. Our method of gene regulation was through gene upregulation and we were nonspecific on the gene chosen. Nonspecific gene targeting was done in a study using E.coli. They used CRISPR-dCas9 to target thousands of genes for downregulation to see which ones were essential for E.coli survival [12]. This experiment is similar in showing how CRISPR-dCas9 can be used to screen through several different genes and find ones of interest. The differences are the downregulation and E.coli cells instead of ASCs. Our study combined the ideas of both of these studies but with upregulation of gene expression versus downregulation. These two studies were helpful in showing success with CRISPR-dCas9 gene expression modification. This success was then also realized in our experiment.

Despite the success of seeing a cell positive for osteogenesis it is important to note the limitations of the study. As shown in the results the reporter cell line is not 100% accurate or comprehensive. There is overlap between the undifferentiated ASCs and the osteoblasts during cell sorting. This overlap was in the form of similar morphology and similar fluorescence levels. The implication of this overlap is that some differentiated cells will not be included after sorting. It also means some cells that were not differentiated were included in the final sorted group. In future work this indicates that some genes that did cause osteogenesis may not be discovered. It also means that some false positive upregulated genes may be presented.

However, knowing that upregulation of some genes plays a role in osteogenesis is useful knowledge for future tissue engineering research. Also, knowing that the CRISPR-dCas9-VPR system can be used to upregulate genes causing differentiation is useful for other areas of stem cell differentiation. We can speculate that the genes that were upregulated that caused the cells to undergo osteogenesis, if upregulated again would produce viable osteoblasts.

Future work would involve isolating these cells and determining which genes were upregulated. Then testing of the individual genes through gene specific upregulation would be needed to prove their involvement in osteogenesis. If they are verified these genes could be used as targets for any research involved in trying to differentiate ASC's into osteoblasts.

Being able to differentiate ASC's into osteoblasts would allow their use in future clinical work involving large bone defects. A sample of ASC's could be taken from the patient and expanded ex vivo and differentiated into osteoblasts. These osteoblasts can then be inserted in the bone defect and promote bone growth and healing. This could change the lives for people who deal with nonunion bone fractures by healing the bone defect without the use of a bone graft.

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