University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: **Grant** Forename: **Emma**

   E-mail address: 2032915g@student.gla.ac.uk

2. Supervisor:

   Surname: **West** Forename: **Adam**

   E-mail address: Adam.West@glasgow.ac.uk

3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Gene editing using RNA-guided nucleases in human cells

   3.2 Project Lay Summary (copied from application):

Many genetic applications require precise sequence changes to genes of interest. The use of targetable nucleases in living cells provides that capability. The DNA break made by these enzymes is rapidly repaired by cellular repair processes. The dominant repair mechanism in most cell types is inaccurate. Therefore targeting a nuclease to sequences encoding a gene of interest will often result in gene inactivation. Alternatively, user-supplied DNA can be used as template by alternative homology-directed repair processes to introduced desired sequence changes. This project will investigate the ability of the CRISPR nuclease system to edit genetic sequences in human cells.

3.3 Start Date: 26/05/14 Finish Date: 18/07/14

3.4 Original project aims and objectives (100 words max):

The West group already employs the CRISPR/Cas9 system for gene editing. This short project will test different guide RNA sequences for their ability to direct efficient genetic modification by Cas9. Specifically, we aimed to test whether the length of sequence complementarity between gRNA and the genome target affects cleavage efficiency of the same target. There is a theory that shorter guides, referred to as "tru-gRNAs" result in minimal off-target cleavage by Cas9.

3.5 Methodology: Summarise and include reference to training received in research methods etc. (250 words max):

The project initially involved training in the process of cloning. Three plasmids, pGem-T-easy, pRFP-turbo and pGFP-Cas9, were selected as vectors for the expression of guide RNAs. Both plasmid and guide sequences were digested with restriction enzymes, ligated and then transformed into DH5α *E.coli* cells. Using the pGem-T-easy vector I also received training in T-A cloning. Once colonies had grown and selected for (using X-gal screening for the pGem-T-easy plasmid) the DNA was extracted from the cells and sent for sequencing to ensure the desired guide sequences has been cloned into the plasmids without mutation. Guide sequences cloned into the backbone of pRFP-turbo were found to be correct and were used in experiments.

The project also involved the culturing of K562 human cells, a model for erythroid gene expression. The successful DNA plasmids containing the guide RNAs were transfected into K562 cells along with a plasmid containing the Cas9 nuclease. The fluorescence of EGFP (co-expressed with Cas9) and RFP-turbo (co-expressed with guide RNA) was measured by flow cytometry to determine the efficiency of plasmid transfection. Transfected K562 cells were cultured for 4 days allowing the Cas9 nuclease to cleave the target sequence and DNA repair to occur.

RFLP analysis was carried out on the genomic DNA extracted from these cells. This involved developing a PCR for the genomic target sequences. The PCR products were digested with a restriction enzyme that cuts at the same location as the CRISPR target sequence. CRISPR-mediated mutagenesis was scored by loss of restriction digestion.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

1. The cloning of synthesized CRISPR guide sequences into the pGem-T-easy and pRFP-turbo plasmids was successful. pGem-T-easy plasmids containing the 5 guide sequences used in this study were sequenced. Mutations were found in one guide, requiring preparation of other clones. Eventually all 5 guides were cloned accurately and then sub-cloned into the RFP-turbo plasmid.

2. Transfection of the pGem-T-easy and pGFP-Cas9 plasmids into the K562 wild type human cells was successful. This was determined by the measuring the percentage of GFP positive cells. See Image 1 and Table 1.
3. The RFLP assay for the 3 VEZF1 guides was unsuccessful due to problems with the PCR. There was too much background and unspecific bands when the PCR products were run on an agarose gel. See Image 2.
4. The RFLP assay of the 2 GATA1 guide RNAs was successful. A PCR protocol was optimised. When the GATA1 PCR was digested with restriction enzyme Nde1 and run on an agarose gel the results suggested that the CRISPR-Cas system had worked. See Image 3. This was confirmed by repeating the digest with another restriction enzyme BglII. See Image 4

3.7 Discussion (500 words max):

The CRISPR guide sequences in this study were synthesised within ~490 bp fragments that also contained the U6 RNA polymerase III promoter for RNA expression. There currently is no reliable assay to check that the ~100 base CRISPR guide RNAs are being expressed in cells during a genome editing experiment, making it difficult to troubleshoot why a CRISPR experiment might not be working. This project therefore incorporated three different plasmid options for the expression of guide sequences. The first option was to insert guide sequences into the backbone of the pGFP-Cas9 vector, allowing for an "all-in-one" vector. After several attempts to clone the guide sequences into pGFP-Cas9, we had to abandon this approach. We were able to produce some plasmids clones, but these had mutations so we focussed on alternative approaches.

The second approach was to clone guide sequences into the backbone of the pRFP-turbo plasmid, where co-expression of RFP could be used to monitor for the presence of the guide vectors. The cloning of guide fragments into pRFP-turbo was efficient in that there were plenty of transformant colonies after DNA ligation for all 5 guides. These were sent for sequencing but on receiving the results, it was soon clear that the guides contained an unnoticed additional site for the restriction enzyme used in guide fragment cloning, so the guide fragments had been cleaved in half. The cloning strategy was re-designed to use a different enzyme. All of the 5 guide fragment were successfully cloned into pRFP-turbo without mutation. It was decided to proceed with this plasmid to transfect in to K562 human cells along with pGFP-Cas9. Unfortunately, flow cytometry revealed that a substantial proportional of the transfected K562 were not viable. Unsurprisingly, the quality of genomic DNA from these cultures was too low to produce genomic PCR products for RFLP assays.

The TA cloning of guide fragments into the pGem-T-easy plasmid was straightforward. Firstly there were plenty of white colonies for each of the 5 guides. From the sequencing results all guides had been ligated in to the plasmid without mutation and so it was possible to proceed with the transfection in to the K562 cells. Again there were problems with cell death after transfection, which was only marginally better than the RFP experiment. It was decide to continue with the genomic DNA extraction for the RFLP assay. After several attempts to optimise a PCR for the *VEZF1* guides, including the use of Herculase, Taq and KAPA polymerases, and changing the protocol to a touchdown, it was not possible to produce a specific product. The *VEZF1* target locus is GC-rich, which may explain the difficulty with this genomic PCR. The PCR for the *GATA1* guides was successful

and so the PCR product was digested with restriction enzyme NdeI. Agarose gel electrophoresis showed that PCR products from CRSPR treated cells could no longer be efficiently cleaved by NdeI. The PCR products also became variable in size. These findings are presumably due to mutations at the targeted sequence.

4. Reflection by the student on the experience and value of the studentship (300 words max):

I thoroughly enjoyed my experience in Adam West's lab this summer. To have spent the summer studying the CRISPR-Cas system, with it being such a novel area of gene editing, I feel I really have witnessed and taken part in cutting edge research. I appreciated the time Ileana Guerrini spent training and helping me through this project, as she taught me many experimental procedures and techniques essential for molecular biology, including some helpful tips. I spent a lot of my time re-thinking experiments that did not give the intended result so I learnt various methods for optimising procedures and had to think creatively too. Often experiments are very temperamental and have to be repeated to ensure there are no undesired factors influencing the results. This proved to be one of the most surprising things I learnt about research.

I am very grateful for the opportunity to work in a professional lab setting as this is an environment that cannot be experienced within the university teaching. I have gained some invaluable experience in the procedures involved in cloning and PCR, and also why experiments may be unsuccessful. I also spent a lot of time studying cells and gels, giving me more confidence in data analysis and interpretation.

I also feel very privileged to have worked alongside many PhD students who are working on some invaluable research in the field of epigenetics. They have inspired me to pursue a career in research with their enthusiasm and determination to succeed.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

Presentation given to the Adam West group on completion of the placement.

6. Signatures: Supervisor Date Student Date 13/08/14

Adam West
2014.08.13
12:55:54 +01'00'

Image 1: Flow cytometry analysis of transfected K562 WT cells with pGem-T-easy and pGFP-Cas9.

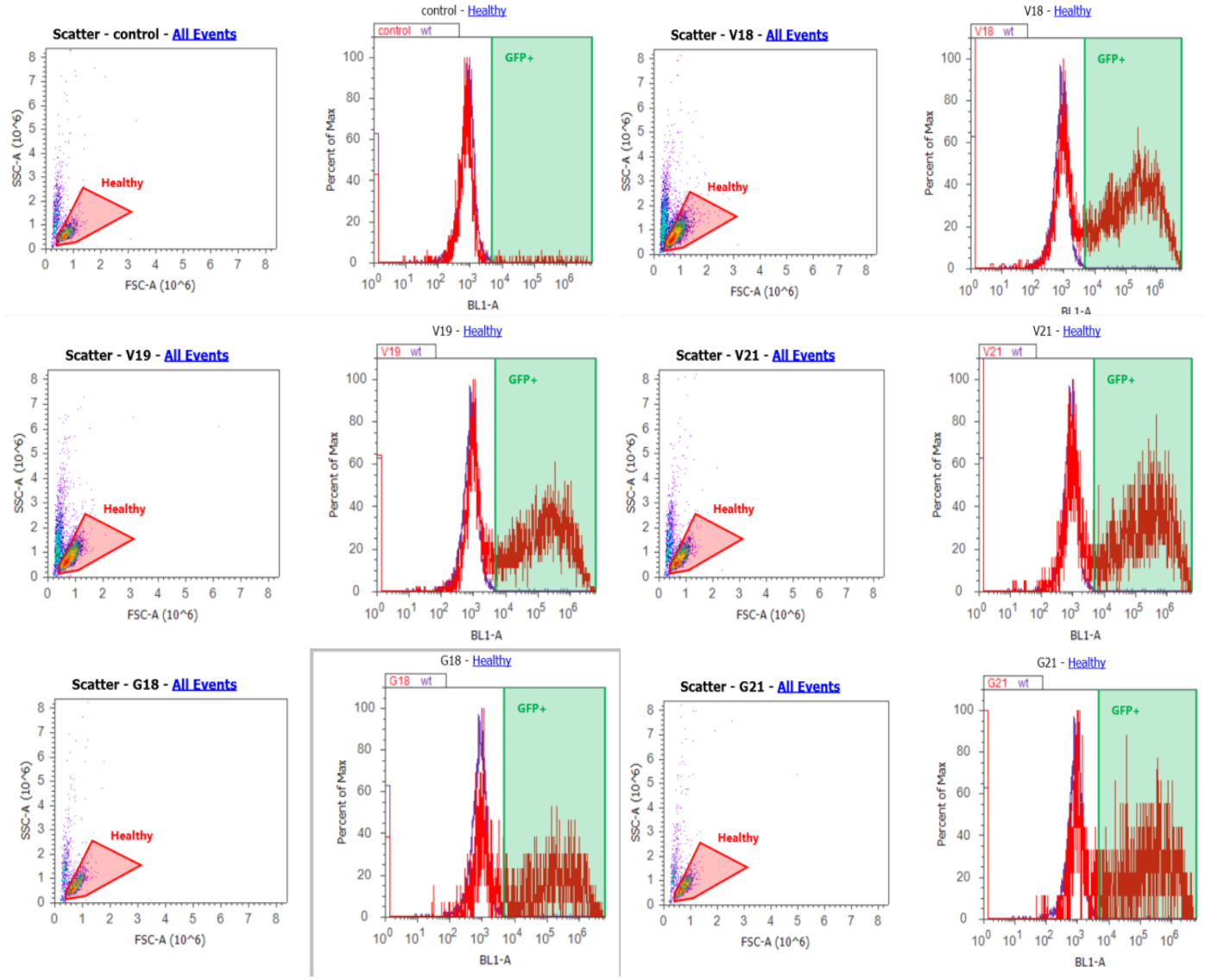

Table 1: Flow cytometry analysis of transfected K562 WT cells with pGem-T-easy and pGFP-Cas9.

Sample	% Alive cells	% GFP+ cells
Cells only	64	3
V18	75	66
V19	70	63
V21	70	66
G18	66	60
G21	67	66

Image 2: Samples of gels run of PCR on VEZF1 pGem-T-easy

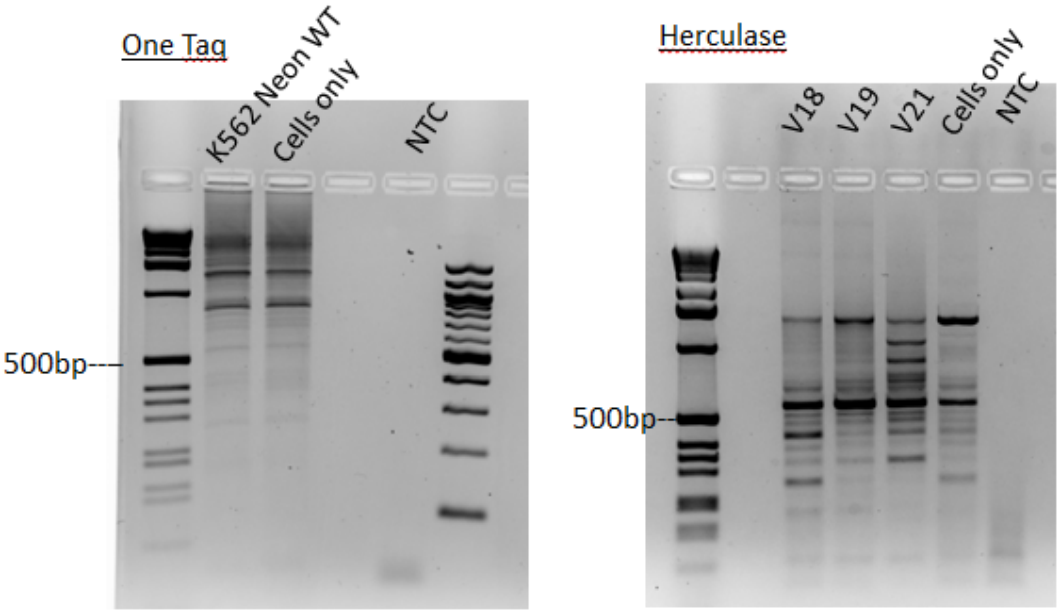

Image 3: RFLP assay of GATA1 pGem-T-easy guides (Nde1)

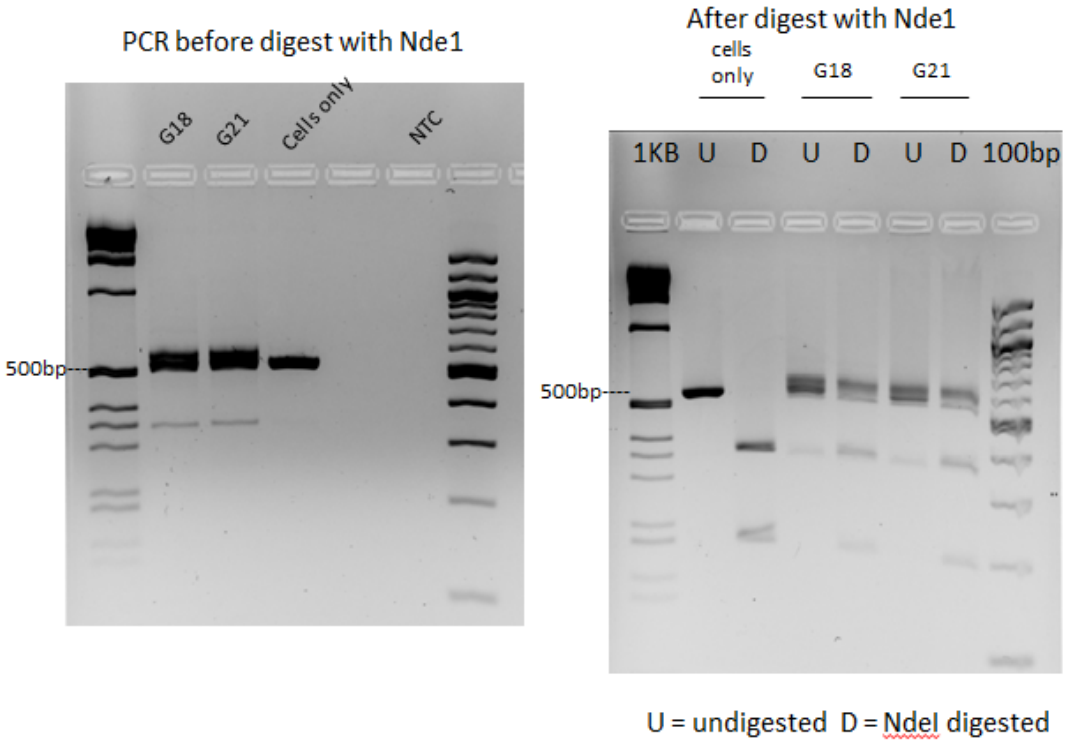

Image 4: RFLP assay of GATA1 pGem-T-easy guides (BglII)