Canterbury Medical Research Foundation

Final report for Project Grant 05

Grant recipient

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Grant details

GRANT TYPE	Project Grant	FUNDING ROUND	2018 Major Project Grant
GRANT REFERENCE	05	GRANT AMOUNT	$99,560

Final report

1. Report for the Scientific Assessing Committee

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CMRF final report.pdf
89.3 KiB

2. Brief summary

CMRF Project Final Report Dec 2020

Media summary

Acute myeloid leukaemia (AML) is a particularly aggressive form of blood cancer. Chemotherapy and bone marrow transplant can result in a period of clinical remission, but overall only 20% of patients survive for more than 5 years. Furthermore, many patients do not respond to chemotherapy at all, indicating that new treatment strategies are needed.

Many of the early genetic changes in the development of AML affect epigenetic regulation, with effects that are potentially reversible. A prime example is the epigenetic regulator TET2, a protein whose activity is affected in up to one third of AML patients. TET2 requires vitamin C (ascorbate) to function, and patients with decreased TET2 activity could benefit from ascorbate supplementation. Recent studies using mouse models support this hypothesis.

However, patients with TET2 mutations always have additional mutations, which can greatly affect response to treatment. The effective design of future clinical trials relies on targeting the right treatment to the appropriate combination of mutations. Therefore, we will develop AML cell lines that combine TET2 mutations with additional mutations seen in patients to investigate their response to treatment with ascorbate. These cell lines will be invaluable tools for identifying subgroups of AML patients that could respond to ascorbate.

Objectives

For the effective treatment of AML, new therapies are needed. Genetic analysis of AML patients has revealed that many different mutation pathways are possible, which can affect response to current treatment. Therefore, it is critical to match new therapies to the right patients. Our overarching goal is to bridge this gap by developing tools that will help predict response to ascorbate.

Our specific objectives are:

1. To develop novel cell lines where TET2 activity has been altered, as a platform to introduce further mutations that are found in AML patients
2. To introduce additional mutations that are already known to affect prognosis
3. To begin initial characterisation of these cell lines in order to determine the effect of ascorbate on survival, morphology, replating capacity and 5hmC levels.

By achieving these objectives, we aim to answer the following questions:

- Does ascorbate restore a normal stem cell phenotype in human cell models of AML?
- If a response is seen, is it TET2 dependant?
- How much intracellular ascorbate is required to see an effect?
- Does supplementation with ascorbate increase intracellular ascorbate content?
- Do different additional mutations affect response to ascorbate or the amount of ascorbate required?

Progress and results to date

Objective 1

As discussed in the grant application, Paul Pace has mentored me with respect to developing the CRISPR methodology required for this project. This has involved learning how to design and construct accurate sgRNA guides and related primers using a range of bioinformatic tools (online databases and associated software). Cas9 sgRNA guides, primers and other required materials were then purchased, after which electroporation/editing of TET2 was carried out. Cells were grown under a various conditions to determine optimal growth. We were able to successfully edit TET2 in human bone marrow stem cells, and this was confirmed using a DNA gel based restriction as well as DNA sequencing. We have also optimised the serial replating assay. One of the key changes we made was using IL-6 in the growth media, which aided in selection of the edited cells. The assay designed to detect efficiency of editing, as well as sequencing of the DNA provided confirmation that we have in fact edited the cells. Non-edited cells did not survive beyond three weeks of re-plating, whereas TET2 edited cells were viable up to 10 weeks. This increased growth potential correlates with what others have shown with mouse work as well as patients with clonal haematopoiesis. The increased growth potential allowed us to proceed with Objective 2 (adding additional mutations seen in AML patients).

Objective 2

Objective 2 Mutations that affect epigenetic modifiers such as *TET2* occur early in the development of AML, with additional mutations required for the full AML phenotype. Mutations in two genes, *NPM1* and *FLT3*, are included in current clinical prognosis estimates. Mutated *NPM1* without *FLT3* internal tandem duplications ($FLT3^{ITD}$) predicts a favourable response based on current chemotherapy regimens. On the other hand, $FLT3^{ITD}$ in the absence of *NPM1* mutations is associated with a worse outcome. Furthermore, *TET2* mutations along with either mutant *NPM1* mutations or $FLT3^{ITD}$ can be the sole changes detected in AML patients.

The original plan was to introduce NPM1 and FLT3 mutations using electroporation to deliver Cas9 RNPs and rAAV6 to deliver the HR donor. However, we experienced unexpected difficulties on two fronts. Firstly, the genetic constructs that were in place to carry out objective 2 needed to be redesigned and ordered commercially. This involved changing to an rAAV6 overexpression system. The design process took more time than expected to ensure the accuracy at our end. Secondly, delivery of the genetic constructs was interrupted by the global lockdown due to COVID-19. The upshot of this is that we now have developed the understanding and capability to work with rAAV6

as well as lentiviral delivery. Thanks to the time extension kindly provided by CMRF, we were able to continue with objective 2 and transfect TET2 mutant cells from objective 1 with the appropriate rAAV6s. We can confirm that these cells are viable, with altered morphology, but have yet to confirm the genetic modification by sequencing. We have ordered the required primers needed to confirm that transfection was successful, and will carry out sequencing in January 2021.

Objective 3

The original intent was to begin initial characterisation of these cell lines (from Objective 2) in order to determine the effect of ascorbate on survival, morphology, serial replating capacity and TET activity (5hmC levels measured using mass spectrometry). As mentioned in Objective 1 were able to optimise the serial replating assay. However, given the fact that the development of these cells has taken longer than expected, we proceeded with development of the required methodology for this objective using a commercially available cell line. Specifically, we used an AML cell line (SKM-1) that harbours a heterozygous *TET2* mutation in addition to a *TP53* mutation. In addition to enabling work on Objective 3 to continue, this will broaden the significance of our findings.

In addition to the serial replating capacity assay, we were also able to develop an assay for TET2 activity - using mass spectrometry to measure C, 5mC and 5hmC (a product of TET enzymes) in DNA. This is a challenging endeavour because 5hmC is only present at very low levels and requires fine tuning of HPLC and mass spec parameters. We were able to do this with the help of Dr Nick Magon (in our research group).

We are excited to report that we brought an honours student (Carlos Smith-Dìaz) on board this year. Specifically, Carlos was mentored by Andrew, Prof Margreet Vissers and Prof Mark Hampton to use some of the assays mentioned above on SKM-1 AML cells. With Andrew and Judy Mackenzie's help, Carlos also learnt the use of FACS to interrogate SKM-1 cell phenotype used a panel of cell surface markers.

Treatment with ascorbate decreased SKM-1 proliferation in vitro and promoted differentiation towards a more mature cell phenotype. We observed that growth in ascorbate-replete media significantly decreased SKM-1 cell numbers after six days. In cells treated with only ascorbate there was a dose-dependent decrease in cell numbers up to 500 µM ascorbate. However, it is well known that adding ascorbate to cell culture media can generate extracellular hydrogen peroxide via the reduction of free iron in solution. The accumulation of hydrogen peroxide causes the death of cancer cells grown in the media through apoptotic and necrotic pathways. To control for possible contributions to cell killing or growth inhibition by hydrogen peroxide generated in cell culture conditions, we used catalase at 20 µg/mL to catalyse the decomposition of hydrogen peroxide. When catalase was added to the cell culture media, cell numbers only decreased in a dose-dependent manner up to 300 µM ascorbate. These data indicate that ascorbate inhibits SKM-1 cell growth without promoting cell death in a hydrogen peroxide-independent manner at concentrations of ascorbate below 300 µM.

Moreover, a 4-fold increase in 5-hydroxymethylcytosine levels was measured 24 hours following 300 µM ascorbate administration, indicating that ascorbate was enhancing TET activity. We also discovered that ascorbate supplementation did not interfere with the activity of the novel anticancer agent Prima-1Met (APR-246) *in vitro*. These results highlight that ascorbate may act as an epigenetic therapeutic in context of AML characterised by decreased TET2 activity and TP53 loss of function. We have prepared a draft manuscript with these results and plan to submit it for publication in the first half of 2021.

Research outcomes

Training and development of scientists in Canterbury

I am grateful to Prof Margreet Vissers for her invaluable support as an experienced PI and mentor. Her advice and guidance along the way have enabled this project to get to where it is. Along with Prof Mark Hampton, they both helped me greatly in developing supervision skills. I am also very grateful to Dr Paul Pace for teaching me CRISPR methodology. The award of this grant and the skills I developed over the course of this project have greatly helped with ongoing grant applications. Namely, I have been awarded a 2-year John Gavin Fellowship by the CRTNZ (https://www.cancerresearchtrustnz.org.nz/stories/article/andrew-das). I will be taking up this fellowship at the Peter MacCallum Cancer institute in Melbourne in 2021. The long term goal is to develop epigenetic and bioinformatic expertise and return to Otago, Christchurch. As always, I am happy to be contacted to speak in a lay setting or share knowledge gained and details of my current research in whatever contexts CMRF deems appropriate. I look forward to working with CMRF again in the future.

Also, as mentioned above, we brought Carlos on board as an honours student this year. In addition to this being a great opportunity for him to develop lab skills, he was also instrumental in the writing of a review on this topic (see publications below). The work he carried out will go on to be part of the first research publication from this project (see results above under Objective 3). Carlos has successfully completed his honours project and has gone on to secure a PhD scholarship to continue research at Otago, Christchurch.

Publications

AB Das, CC Smith-Diaz, MCM Vissers. Emerging epigenetic therapeutics for myeloid leukemia: modulating demethylase activity with ascorbate. Haematologica, 2020. Accessible at: https://doi.org/10.3324/haematol.2020.259283

MCM Vissers, AB Das. Ascorbate as an Enzyme Cofactor, in *Vitamin C: New Biochemical and Functional Insights* 1, p71-98. Accessible at: https://doi.org/10.1201/9780429442025

Research Presentations

1. **Das AB**, Hampton MB, Vissers MCM. Creodes and Cancer. Epigenetics Meeting of QRW, Queenstown, NZ (**2019** September). Invited oral presentation.
2. **Das AB**. How to make leukaemia. CRISPR Hub Workshop, Dunedin, NZ (**2019** November). Invited oral presentation.
3. **Das AB**. Targeted therapy in myeloid leukaemia. C4 Workshop, Christchurch, NZ (**2019** November). Invited oral presentation.
4. **Das AB**. Ascorbate and the potential for targeted therapy in myeloid leukaemia. University of Otago Seminar Series, Christchurch, NZ (**2020** October). Invited oral presentation.

3. Photographs

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CFRR group photo 2020.JPG
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Lab photo.jpg
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5. Feedback

Publication

Date

30/06/2021