Characterisation of defects in erythroid differentiation as a result of GATA1short mutation

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Background:

Diamond-Blackfan Anaemia (DBA)-like syndrome is caused by rare mutations resulting in the expression of GATA1short (GATA1s), an N-terminally truncated isoform of the key erythroid transcription factor GATA1[1]. This condition is characterised by dyserythropoiesis and macrocytic anaemia. Current therapeutic treatments like blood transfusions and glucocorticoids face challenges such as iron overload or intolerance to long-term steroid treatment[2].

GATA1 is the master transcription factor of erythropoiesis and regulates all aspects of erythroid maturation and red cell functions. The GATA1 protein has three functional domains, including an N-terminal activation domain, which is absent in the GATA1s mutant protein, leading to impaired erythropoiesis[3]. Whilst this is known, the molecular basis for GATA1s dysfunction remains unclear. To investigate this, the Strouboulis lab has used gene editing in human proerythroblastic HUDEP-2 cells to create clones expressing only GATA1s.

The host lab has also worked on the analysis of a mouse erythroleukemic (MEL) cell line in which the Zfpm1 had been knocked out. Zfpm1 codes for FOG-1, a closely interacting co-factor of GATA1. Research showed that Zfpm1 knockout MEL cells are deficient in terminal erythroid differentiation. In addition, it was shown that cholesterol homeostasis, important for the cell membrane properties of red cells, was impacted by the Zfpm1 gene knockout in MEL cells.

The combined molecular characterisation of the cellular and mouse models will help elucidate the biological basis for GATA1s dysfunction and disease pathogenesis, which may lead to the identification of novel therapeutic targets and improve DBA diagnostic testing.

Aims:

This project aimed to:

(i) Investigate the molecular characterisation of the dyserythropoietic phenotype in the GATA1s cell lines. To do this I first concentrated on culturing and differentiating the cells and then carried out an assay for intracellular calcium levels.

(ii) Assess the expression levels of SREBP2 protein, a key transcription factor for cholesterol homeostasis in the Zfpm1 knockout MEL cells.

Materials and Methods:

HUDEP-2 Cell Culture – Cells were thawed and suspended in StemSpan media. The cells were then spun down at 1200 bpm for 7 minutes, resuspended in 1 mL of StemSpan and counted using a cell counter. A concentration of 1 x10^5 cells/ml was maintained in the StemSpan medium containing the cytokines 50 ng/ml SCF, 3 U/ml EPO, 1 uM dexamethasone and 1 ug/ml doxycycline. Total medium changes were performed every other day, keeping the concentration of cells the same. On Day 8 of expansion, the cells were put in the differentiation IMDM medium containing the cytokines 10 ng/ml SCF, 1 ng/ml IL-3, 3 U/ml EPO, 1 U/ml Pen/strep and 1 ug/ml doxycycline (only on D0-D4). Total media changes were performed every other day. FACs was used every other day to analyse early differentiation markers and detect apoptosis by Annexin V staining.

Calcium assay of HUDEP-2 cells – HUDEP-2 wild type (WT) and GATA1s clones 12 and 33 were taken on Day 0 and Day 4 of differentiation. A colorimetric calcium assay was carried out in duplicate using the ab102505 Calcium Assay Kit.

Western blot of FOG-1 knockout MEL nuclear and cytoplasmic extracts – Nuclear MEL extracts and cytoplasmic extracts were thawed, and samples were prepared with 25 ug of protein, 6x loading buffer and dH2O to a final volume 40 uL. After heating at 95C for 5 minutes, the samples were loaded onto gels with a molecular marker. The gels were run at 70V for 20 minutes, then at 110V until the molecular weight ladder reached the bottom of the gel. Then gels were transferred to nitrocellulose membranes and blocked in 5% BSA with Tris-buffered saline (TBST). Membranes were incubated overnight with control protein loading antibodies including anti-HDAC2 for nuclear extracts and anti-actin for cytoplasmic extracts. Western blots were developed by ECL mixture added to each membrane and imaged with the Biorad ChemiDoc. Then membranes were stripped and blocked in 10% milk with PBST, and re-probed with an anti-SREBP2 antibody overnight. Following anti-rabbit secondary antibody incubation, ECL mixture was added, and membranes imaged.

MEL Cell Culture and protein membrane extraction for western blot analysis – MEL cells were grown in DMEM medium and after 1 week of expansion, differentiated by DMSO induction for 4 days. Membrane protein extracts were collected at D0, D1, D2, D3 and D4 of differentiation using the mem-PER extraction kit. Then, these protein membrane extracts were used in a Western blot with anti-ABCA1 and anti-LDLR antibodies and the protein loading control anti-ATP1A1. Protein loading per lane was normalised to 100,000 cells per lane.

Results:

The flow cytometric profile of WT cells on Day 0 of differentiation showed only the proerythroblasts (Pro-e) to be present. On Day 2, there were Pro-e, early basophilic (EB) and late basophilic (LB) differentiation stages detected, indicating that differentiation had occurred (Figure 1A). The Annexin V data showed high apoptotic levels on Day 0, this may be due to stress on the cells from medium changes. By Day 2 apoptotic levels had decreased to 2.86% (Figure 1B).

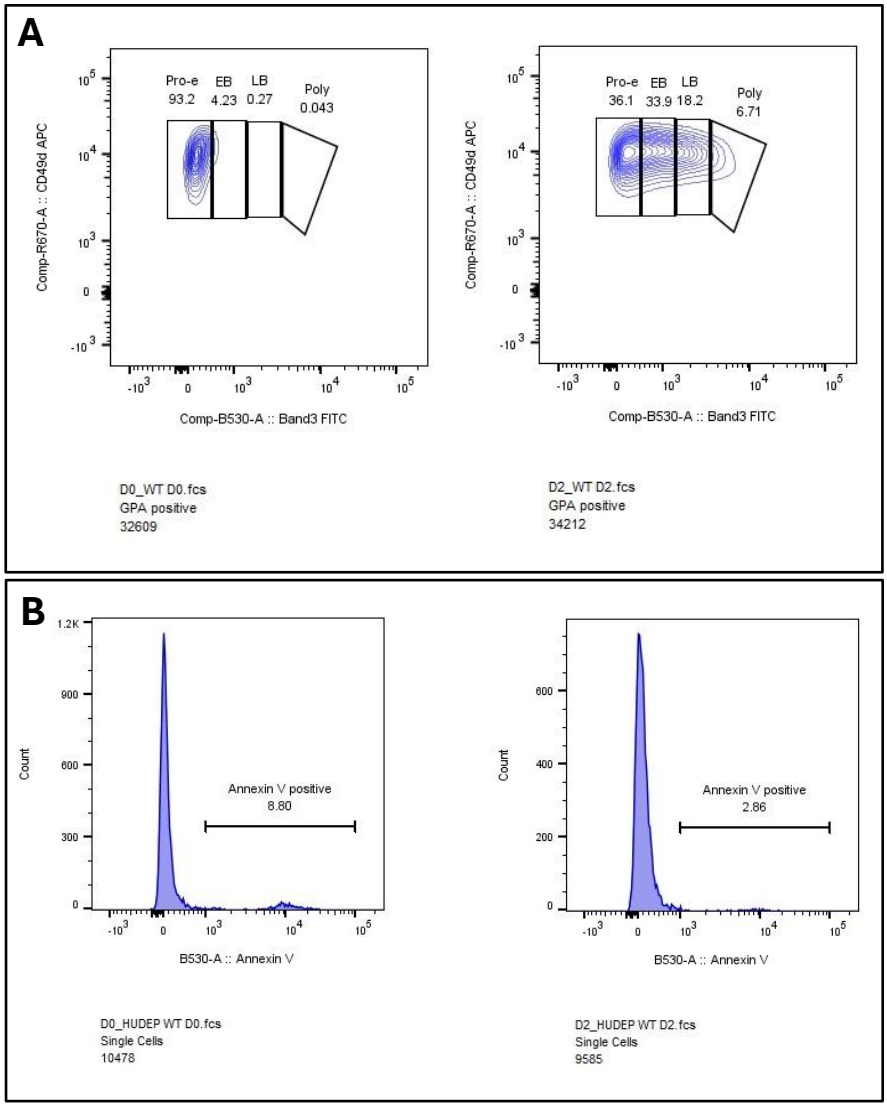

***Figure 1 A-B* – D0 and D2 Waterfall and Annexin V data from WT HUDEP2 cell culture.**

A: Waterfall data analysing early differentiation markers. On D0 there were only Pro-e detected. On D2 there were Pro-e, EB and LB stages detected.

B: Annexin V data detecting apoptosis. On D0, Annexin V positive is 8.8%. On D2 apoptosis levels reduced to 2.86%.

Calcium assay:

Calcium levels increased between Day 0 and Day 4 of differentiation. There was not a significant difference in calcium levels between the WT and GATA1s cell lines (Figure 2). The assay should be repeated with biological replicates instead of technical replicates.

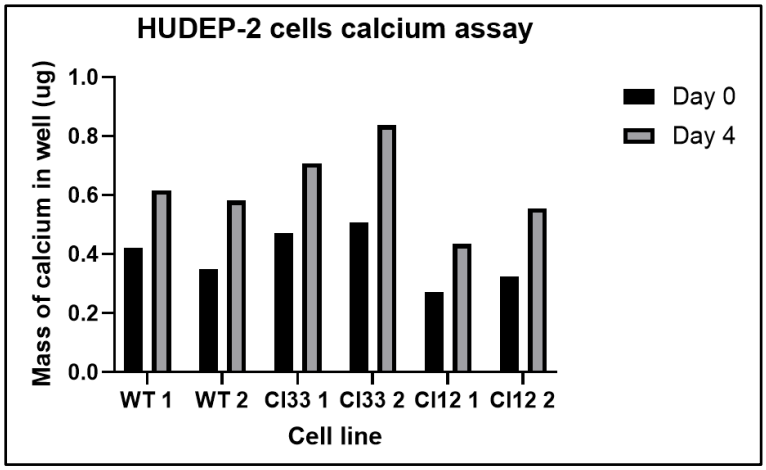

***Figure 2* – HUDEP-2 cell lines colorimetric calcium assay**

Graph shows quantitative levels of calcium in WT, Cl33 and Cl12 cell lines from Day 0 and Day 4 of differentiation in duplicate.

Western blots of FOG-1 knockout MEL cytoplasmic and nuclear extracts:

Cytoplasmic and nuclear extracts were obtained from WT MEL cells and the three FOG-1 knockout clones D3, C8 and G9, that were either non-differentiated (or non-induced) or induced to differentiate. Extracts were probed for SREBP2 (Figure 3). The cytoplasmic extract shows uneven loading for the actin control, which affected the normalised cytoplasmic 75 kDa SREBP2 levels. This may be due to FOG-1 knockout impacting actin levels, so this western blot should be repeated. All the western blots show multiple bands indicating SREBP2 protein is cleaved. The nuclear extracts show differences in SREBP2 levels between the WT and FOG-1 knockout clones (Figure 3B).

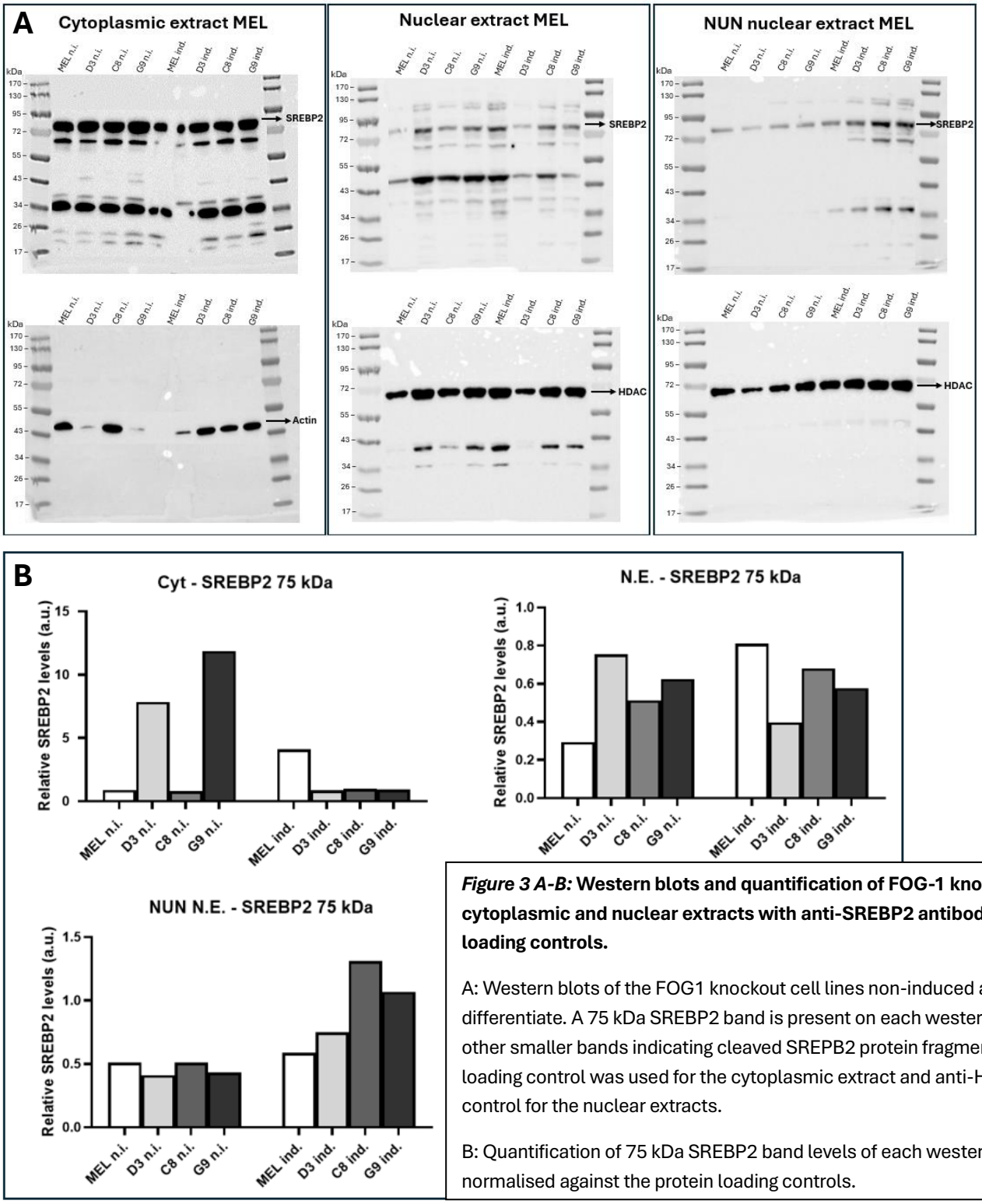

Figure 3 A-B: **Western blots and quantification of FOG-1 knockout MEL cytoplasmic and nuclear extracts with anti-SREBP2 antibody and protein loading controls.**

A: Western blots of the FOG1 knockout cell lines non-induced and induced to differentiate. A 75 kDa SREBP2 band is present on each western blot, and other smaller bands indicating cleaved SREPB2 protein fragments. Anti-actin loading control was used for the cytoplasmic extract and anti-HDAC loading control for the nuclear extracts.

B: Quantification of 75 kDa SREBP2 band levels of each western blot, normalised against the protein loading controls.

Impact and future directions of work:

This project addresses an unmet need to understand the molecular basis of DBA-like syndrome caused by mutations affecting the GATA1 transcription factor. This will have broader implications in understanding how transcription factor mutations give rise to disease and, in the long term, will also aid in developing targeted therapies as transcription factors are notoriously difficult to target. Ultimately, my project is crucial to further the research group's mission to improve patient outcomes by enhancing diagnostic accuracy and identifying novel therapeutic strategies for DBA and related disorders.

In addition, my work showed that SREPB2 is affected by the FOG-1 knockout in MEL cells, which may affect expression of downstream SREPB2 gene targets, thus affecting cholesterol homeostasis in the knockout cells.

Departures from original project plan:

Due to time constraints, I did not use CRISPR/Cas9 to knockdown or knockout proteins, instead I used cell pellets to make protein extracts to carry out Western immunoblot analysis on HUDEP-2 and MEL cell extracts.

Value of the studentship:

After two years studying the theory surrounding Biochemistry, this studentship has allowed me to gain hands-on laboratory experience. During this time, I have learnt and gained confidence in tissue culture, induction of terminal erythroid differentiation, protein extraction, Western immunoblot analysis, and phenotypic analysis by flow cytometry. Apart from acquiring these experimental skills, I also learnt to plan and troubleshoot experiments, reporting on my findings to the immediate supervisor and presenting to the PI in regular meetings, thus honing presentation skills.

Currently, I am deciding what I want to pursue after university, and this project presented the unique chance to delve into the practical application of my knowledge and explore life as a postgraduate research student first-hand. Collaborating with experts from a different university has exposed me to different perspectives and specialised backgrounds, enriching my understanding of the field.

Acknowledgements:

I am grateful to Professor John Strouboulis and the Lab at Kings college for supervising and supporting me during the six-week studentship. I especially thank Eranda Vokshi and Casey Ching for overseeing my work daily and teaching me new laboratory techniques. Completing this studentship has affirmed to me that I want to pursue a career in research.

I am grateful to the Biochemical Society for providing me the opportunity to gain hands-on-experience in a lab. The success of this programme contributes to achieving their strategic objectives which focus on promoting the importance of molecular bioscience.

References:

1. Sankaran VG, Ghazvinian R, Do R, Thiru P, Vergilio JA, Beggs AH, Sieff CA, Orkin SH, Nathan DG, Lander ES, Gazda HT. Exome sequencing identifies GATA1 mutations resulting in Diamond-Blackfan anemia. J Clin Invest. 2012 Jul;122(7):2439-43. doi: 10.1172/JCI63597. Epub 2012 Jun 18. PMID: 22706301; PMCID: PMC3386831.
2. Da Costa LM, Marie I, Leblanc TM. Diamond-Blackfan anemia. Hematology Am Soc Hematol Educ Program. 2021 Dec 10;2021(1):353-360. doi: 10.1182/hematology.2021000314. PMID: 34889440; PMCID: PMC8791146.
3. Gutiérrez L, Caballero N, Fernández-Calleja L, Karkoulia E, Strouboulis J. Regulation of GATA1 levels in erythropoiesis. IUBMB Life. 2020 Jan;72(1):89-105. doi: 10.1002/iub.2192. Epub 2019 Nov 25. PMID: 31769197.