

Investigating the enzymatic properties of endometrial DPPIV in the molecular adhesive pathways at implantation using a co-culture model.



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Introduction

In the UK, 1 out of 6 couples are infertile and 10% of them suffer from recurrent implantation failures. The clinical success rate has been stagnant at 25-30% per cycle for a few decades. The knowledge of molecular events at the embryo implantation remains limited, hindering further increase in clinical pregnancy success rates.

Identifying epithelial endometrial candidates from published differentially expressed genomics datasets of human endometrial biopsies sets priorities for in vitro assays using a model of embryo implantation.

A transmembrane receptor, Dipeptidyl peptidase-IV (DPPIV), was one of the identified endometrial targets involved in cell-cell adhesion at Implantation (Shimomura, Y. *et al.*, 2006). Complementing the interesting function complexity, DPPIV contains an enzymatic domain which we identified to be active, *in vitro*, in Ishikawa cell lysates by a series of DPPIV inhibitor concentrations and aim to further investigate.

Aims of the project

The project was designed to identify new insight into the adhesive pathway at Implantation at the embryo-maternal interface by investigating the role of DPPIV enzymatic function in relation to fibronectin using Diprotin A. Project aims:

- 1. Localise DPPIV in endometrial Ishikawa cells and attachment sites
- Determine the impact of the inhibition of DPPIV enzymatic function on endometrial cell adhesion
- 3. Identify the role of DPPIV enzymatic function in an *in-vitro* model of implantation

Description of work

Cell lines The human endometrial epithelial adenocarcinoma Ishikawa cell line and trophoblastic choriocarcinoma BeWo cell line were bought from the European Collection of Authenticated Cell Cultures (ECACC, UK). Both cell lines were cultured in DMEM:F12 medium (10% Fetal Bovine Serum and Glutamax) at 37 °C and 5% CO2.



Figure 1: BeWo spheroids attached onto a monolayer of Ishikawa cells. Phase contrast light microscopy

Spheroids generation In 25 μ L drop of complete cell culture medium, 3,000 cells of BeWo cell lines were plated onto the lid of a sterile petri-dish. A total of five Petri dishes with 30 drops per Petri lid were incubated at 37 °C and 5% CO2 for 72 hours.

Co-culture assay One hour before the co-culture assay, half of the well were pre-treated with $50\mu g/mL$ of Diprotein A, inhibitor of DPPIV. BCECF-AM-Fluorescent spheroids were resuspended again in 1% BSA serum-free culture medium, to be finally co-cultured onto the Ishikawa monolayer (8 spheres per well) at 5% CO2 and 37 °C. Spheroid attachment was assessed after 1h of co-culture and capturing phase-contrast images using Inverted Microscope.

Assessment of results and outcomes of studentship



Result 1: DPPIV was identified in Ishikawa cells and at BeWo spheroid attachment site

Immunocytochemistry (ICC) using an anti-DPPIV antibody of Ishikawa cells showed DPPIV to be detected at the lateral and apical cell membrane (Fig. 2A). In confluent monolayer of endometrial cells, positive cells were more present but still heterogenous (not shown). IgG from mouse serum and secondary antibody alone were used as controls for ICC. From preliminary observations, highly DPPIV-positive region displays in the endometrial cell membrane where spheroids attachment took place (Fig. 2B).

Result 2: Inhibition of DPPIV using Diprotin A significantly decreased endometrial cell attachment

Using endometrial single cells, the PolyL-lysine treatment was not as high as expected (expiry to be investigated). However, the fibronectin coating (DPPIV ligand) induced further endometrial cell attachment compared to BSA (basal level).

The specific endometrial attachment to Fibronectin coat was fully inhibited in presence of 50μ g/ mL of Diprotein A, back to unspecific basal cell attachment level (BSA).

Diprotein A, enzymatic inhibitor of DPPIV, inhibited endometrial cell attachment to DPPIV ligand, linking both separate enzymatic and adhesive functions of DPPIV.

Figure 2: Localisation of endometrial DPPIV in Ishikawa cells and at BeWo spheroids attachment A= Immunocytochemistry of PFA-fixed Ishikawa cells using DAPI (nucleus, blue), an anti-DPPIV antibody (red), and phalloidin (actin filaments, green). B= Z-stack image of Ishikawa monolayer and spheroid oc-culture using an anti-DPPIV antibody (red), DAPI (blue), and phalloidin (green), with transverse cut seen below. Circled area of z-stack shows the site of attachment between spheroid and endometrial cell monolayer. Arrows show highly DPPIV-positive endometrial

Result 3: Attachment trophoblast spheroids was impaired by the inhibition of endometrial DPPIV

In supplement to the single endometrial cell attachment assay, the treatment 50 µg/ mL of Diprotin A of endometrial cells was tested in the coculture. From preliminary data, after 1h of co-culture, approx. 40% of spheroids in treated monolayers were not attached. Since BeWo cells do not express DPPIV, only endometrial DPPIV was inhibited and was responsible for the lack of attachment.

Outcomes

In this study, combined with its subcellular localisation at spheroids attachment sites, we demonstrated that the inhibition of the enzymatic activity of DPPIV had a negative impact on the DPPIV-specific binding to fibronectin in Ishikawa cells, leading to the preliminary data on the impairment of the trophoblast/endometrial attachment in-vitro. These data set the scene for the investigation of DPPIV enzymatic activity and its prescribed inhibitors at implantation.

With these outcomes, Monica presented her results at the PABS Research Seminar (see figure 3). Moreover, these results were combined with the lab results into an abstract which has been accepted at Fertility 2023 conference, January, Belfast.



Future directions in which the project could be taken

Sitagliptin and vildagliptin, currently prescribed DPPIV inhibitors, could be used to investigate potential in-vivo inhibition at embryo implantation. To determine whether the inhibition of DPPIV has an impact on the adhesion, the co-culture model used could be carried out to complement the initial dataset with Diprotin A.

In addition to the inhibition, a siRNA-mediated knockdown of DPPIV was successfully carried out to decrease the expression of DPPIV at the cell surface membrane. However, this technique has not yet been tested in our in-vitro co-culture model.

Value of studentship to the student and to the lab

Working in Berneau's lab team, I have developed many lab skills such as cell culture, spheroids generation, and fluorescence microscopy. I have also enriched many transferable skills such as time management: an essential skill in the lab due to the timed experiments I was carrying out. Due to these experiments, I was also able to develop my independence and organisational skills, making decisions regarding the project, planning tasks, and prioritising appropriately. I also improved my scientific communication skills in weekly meetings with the Berneau lab team.

Completing this project has allowed me to experience life in the lab as a researcher and has reinforced my decision on pursuing academic research as a career.

The studentship was very valuable to the labs as my data complemented the ongoing research in Berneau's lab team, moreover, these data were included in an international conference poster.



Figure 4: Image of Monica pipetting spheroids into the co-culture assay and an image of Dr Stéphane Berneau and Monica Smithies in the lab

References

Shimomura, Y. et al. (2006) Possible involvement of crosstalk cell-adhesion mechanism by endometrial CD26/dipeptidyl peptidase IV and embryonal fibronectin in human blastocyst implantation. Molecular human reproduction. [Online] 12 (8), 491–495.

Biochemical Society Summer Vacation Studentship Report 2022



Using novel enzyme fusion technology to control protein secretion in clostridia

Student: Evelyn Thesia, Loughborough University Supervisor: Dr Mandy Nicolle, Biocleave

Introduction

Biocleave is a company that studies and utilises clostridia to produce difficult-to-express protein. They specialise in generating proteins from protozoal and parasite organisms. By using endogenous CRISPR-Cas technology, CLEAVETM, they were able to engineer non-pathogenic clostridia as a recombinant expression host to produce, high purity, active recombinant protein.

The company's focus on studying Clostridia is what piques my interest in having my placement here. The company having over 15 years of experience in engineering, adapting and fermenting clostridia, I expect to learn more about this bacterium I have not heard a lot before and get an in-depth study on a specific subject of interest. In the beginning, I also hoped to gain technical skills in microbiology, molecular biology, and biochemistry. I will also have the opportunity to gain insight into the industry outside of academia.

Aim

Biocleave has identified a native protein (Protein A) that is efficiently secreted at high yields and by fusing new target molecules to this protein, we aimed to establish a method for improved isolation of the target molecule from outside of the cell. Success would open up new research opportunities.

This project aimed to make fusion proteins that will secrete proteins normally produced intracellularly, extracellularly. This project will investigate the possibility of fusing the different targets (T1, T2) at different positions (Fusion 1,2,3,4) within the coding sequence of Protein A and transform them into clostridia for testing.

Methods

The methods used in this project were largely molecular biology related. I generated DNA fragments from genomic DNA and cloned them using standard techniques such as PCR, a-tailing, ligation and colony screen using IPTG and X-gal. I also had the opportunity to transform the plasmid to E-coli by heat shock. While I was not able to generate and screen the clostridial recombinant strain myself, I had the opportunity to shadow members of the tech team doing small-scale fermentation, SDS-PAGE and Western blotting to check if they got the desired protein.

Result and Discussions

By the end of the studentship, I successfully generated the cloning parts to make Fusion 1 with Target 1. However, this did require redesigning the constructs to optimise the cloning strategy. Direct cloning of this into clostridia was done after the studentship ended. The sequencing for Target 1 on Fusion 2,3,4 was also successful. This was transformed into clostridia however no colonies were observed. If it had worked, the intention was to complete bottle screening to grow the strain, and SDS-PAGE to check for any protein present. Western blotting would be

applied to identify if the specific target protein is produced and secreted successfully. Due to the fact we got no colonies for Target 1, the decision was made with my supervisor, to clone and test an alternative target, Target 2. I successfully generated Target 2 to be used for Fusions 1-4. Direct cloning of these into clostridia was done after the studentship ended.

Future directions in which the project could be taken

Different targets could be fused on fusion protein to express different proteins that are normally difficult to express with traditional methods. Due to the timeframe of the project, my supervisor provided me with the promoter part she recommended I start with. As no colonies were observed with Target 1-Fusion 2, 3 and 4, I could have reviewed this and used a different promoter sequence, for example, if the expression level under the first promoter was too high, we could use a lower one.

Departures from original proposal

A total of 6 strains instead of 4 were made using 'parts' from the cloning library. And instead of using signal peptides for secretion, I constructed full fusions as my supervisor thought it would generate more optimal conditions for secreting the Target proteins. However, there was not enough time for the characterisation of strains to establish growth rates, and detection of target enzymes using Western blotting and assay for enzyme activity.

Value of studentship

Student: Overall, it was a very enjoyable and informative studentship. I have gained technical skills in handling and measuring nucleic acid products as well as transforming them to host vectors. I was expected to carry out experiments that I have not encountered before, and it challenged my problemsolving and organisational skills. I also get to experience what life could be like after graduating and opened myself to more options in the future. In addition, it has also better prepared me to start my final year project at university.



Supervisor: We really enjoyed having Evelyn join our team over the summer. She was enthusiastic, picked up techniques quickly and her project output has made a significant contribution to one of our research streams. It was a great opportunity for us to showcase an alternative career option and give Evelyn a sense of how exciting it can be to work for a biotech company.



Child Health Research Center

CENTER FOR CELL FATE AND THERAPEUTICS



The Role of Gata3 in Renin Cell Identity

Student: Jesus Samuel Nevra Supervisor: Maria-Luisa Sequeira Lopez, Ariel R. Gomez, Silvia Medrano

Background & Aims:

Renin cells are highly specialized cells crucial for the regulation of blood pressure and fluid-electrolyte homeostasis. During development, renin cells act as precursors for other cell types in the kidney (i.e. juxtaglomerular, smooth muscle, and mesangial cells) and show high plasticity not only during embryonic development but also in postnatal life in response to homeostatic threat (1). The mechanisms responsible for renin cell identity and plasticity are not well understood. ScRNA-seq studies conducted in our lab revealed that the dual-zinc finger transcription factor (TF) Gata3, which is important for cell lineage commitment and differentiation in several tissues, is expressed in mouse renin cells under normal conditions when homeostasis is threatened. Moreover, we found an enhancer associated with Gata3 in renin cells suggesting that this TF may be involved in the control of renin cell identity. The goal of this study is to:

1) Investigate whether GATA3 regulates renin cell identity during different stages of development and in adult life. 2) Understand how GATA3 impacts Renin expression in renin cells.

We hypothesized that GATA3 regulates renin expression and the morphological structure of the kidney

Materials & Methods:

Generation of Mice with Conditional Knockout (cKO) of Gata3 in Renin Cells:

We crossed *Gata3* floxed mice with mice expressing Cre recombinase under the control of the Ren1^d promoter. Mice were studied at 2 and 4 months of age.

Physiological Threat:

To induce an increase in the number of renin expressing cells in the kidney, Gata3 ^{fl/f}; Ren1^{d/Cre} and control mice were subjected to a low salt diet (0.05% NaCl) and Captopril treatment in the water (0.5mg/L) one week before sacrifice and harvesting of tissues.

Histological Analysis and Immunohistochemistry:

Immunohistochemistry for Gata3 was performed to

demonstrate efficient deletion in Gata3 cKO mice. Gata3 cKO showed a significant reduction of expression in the mesangial, juxtaglomerular cells, and the arterioles (Figure 2).

immunostaining, renin-expressing cells appeared very thin

compared to the normal plump shape in control mice. Renin

signal was abnormally localized to the Bowman's capsule in

some glomeruli, and Acta2 signal was misplaced in the

mesangium, interstitium, and Bowman's capsule for

Tissues were fixed in Bouin's, 4% Paraformaldehyde, or 10% Formalin solution. Paraffin-embedded and sectioned kidney tissue sections (5 µm) were immunostained for Ren1, Acta2, and Gata3. Hematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS), and Masson's Trichrome stainings were performed to analyze kidney structure.

Real Time - Quantitative PCR:

Results & Outcomes:

RNA was extracted from kidney cortices using Trizol. Oligo-dT priming and M-MLV reverse transcriptase was used to generate cDNA. RT-qPCR for Ren1 and Akr1b7 was performed using Sybr Green and Go Taq DNA Polymerase (Promega). RT-qPCR data was normalized to RPS14. Data was expressed as a means ± SD. Statistical significance was determined by Student's t-test. P< 0.05 was considered significant.

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Figure 1. Gata3 cKO Mice Carry a deletion of Gata3 in Juxtaglomerular, Mesangial, and Vascular Cells





Figure 2. Immunostaining for Gata3 of adult kidney sections. A,C. Control Mice. Gata3 is expressed in mesangial cells (asterisk), Juxtaglomerular cells (arrow), and arterioles (bracket). B,D. Gata3 cKO mice. A marked reduction in Gata3 staining is observed in all areas mentioned in control mice, demonstrating the highly efficient deletion of Gata3 in renin lineage cells. Bar: 100 µm.



Figure 3. Immunostaining for Renin and Acta2 of sections of adult kidneys from cKO and control mice under basal conditions and physiological threat. A-D. Renin Immunostaining. Under basal conditions, Gata3 cKO mice showed a reduction in the number of juxtaglomerular areas containing renin positive cells (C) compared to control mice (A). JG cells in cKO mice appeared flattened (arrow) compared to the plump and rounded morphology of control mice (arrow). Glomeruli in cKO mice showed abnormal morphology that appeared flattened with misplaced renin staining in the Bowman's capsule (circle). Under physiological threat, Gata3 cKO mice failed to reexpress renin along the arterioles (D) in comparison to control mice (B) (asterisks). E-H. Acta2 Immunostaining. In Gata3 cKO mice. Acta2 was present in the intraglomerular mesangium (arrow), interstitium (bracket), and Bowman's capsule (circle) in addition to the regular localization in smooth muscle cells. Bar: 100 µm



Figure 4. Histological Analysis of Adult Kidney Mice. A-D. Hematoxylin and Eosin staining. *Gata*₃ cKO mice showed fusion of the glomeruli (bracket). Mice under physiological threat showed a greater degree of glomerular fusion. There was presence of perivascular infiltration (arrow). E-H. **Periodic Acid-Schiff staining.** Cubical epithelial cells were absent in Bowman's capsule (bracket), and abnormal dilation of the capillaries were present in the glomeruli (arrow). There was further evidence of the dilation of the distal tubules (asterisk). Glomeruli appeared smaller in size in mice subject to physiological stress (**H**). **I-L. Masson's Trichrome Staining.** We observed infiltration of blood and dilation of the capillaries in glomeruli of *Gata*₃ cKO mice (**K**,**L**) indicative of aneurysms (arrow). In addition, we observed Increased collagen deposits depicted by blue colored regions in *Gata*₃ cKO mice (bracket), indicating glomerular fibrosis. **Bar: 100 μm.**

Gata3 cKO mice. Distal tubules showed dilated morphology with visible intraluminal casts (Figure 3). H&E, PAS, and Masson's Trichrome reactions demonstrated increased glomerular fusion, absent cubical epithelial cells in Bowman's capsule, intraglomerular aneurysms, tubular dilations, and fibrosis. (Figure 4).

Gata3 cKO mice had a significant increase in BUN ($43\pm6.5mg/dl$) compared to control mice (BUN: $19.5\pm3.6mg/dl$, ***p=0.0005) under basal condition. Under physiological threat, *Gata3* cKO mice had a BUN of 78.6±7.4mg/dl and control mice $68.0\pm12.1mg/dl$, p=0.17. Elevated BUN in *Gata3* cKO mice was suggestive of hypovolemia and/or compromised renal function (Figure 5). *Gata3* cKO mice exhibited lower *Ren1* and *Akr1b7* mRNA levels compared to control mice. *Gata3* cKO mice under physiological threat showed a significant decrease in *Ren1* (1.78 ± 0.51 vs 12.96 ± 3.17, **p=0.0079) and *Akr1b7* (0.87 ± 0.18 vs 4.18 ± 1.61, *p=0.044) mRNA levels (Figure 6).

The conditional deletion of Gata3 in renin cells caused:

- 1) a significant decrease in renin expressing cells
- 2) a change in renin cell localization and morphology
- 3) a decrease in Ren1 and Akr1b7 mRNA levels in the renal cortex

 morphological renal abnormalities with a decrease in kidney size and impaired kidney function

Departures from Original Proposal:

Instead of performing an in situ hybridization for *Gata3*, we performed an immunohistochemistry analysis to measure protein expression and localization. In place of studying mice at 2 and 3 months, we studied mice at 2 and 4 months.

Future Directions:

Analysis of mice at 4 months of age is currently ongoing. Renal cortex samples fixated in 3% glutaraldehyde will be sent for Electron microscopy imaging. We plan to study *Gata3*^{fl/del}; Ren1^{d/Cre} and *Gata3*^{fl/del}; Ren1^{d/CreERT2} model mice. We will use the same methods described above. Single Cell isolation and fluorescent labeling will also be performed to assess the transcriptome of Gata3 deficient single cells. Glomerular filtration rates and assessment for proteinuria will be performed. I plan to continue investigating the effect *Gata3* has on renin expression and the morphological structure of the kidney at different stages of development. I will submit a manuscript to *The American Journal of Physiology-Renal Physiology* later this year with the findings of this study.

Value of Studentship for the Student and Lab:

Working on this project has given me immense insight and exposure to biomedical research. Prior to becoming a member of the Gomez-Sequeira Lopez lab under the Child Health Research Center at the University of Virginia School of Medicine, I had no prior experience conducting medical research. It has been an honor to help the lab discover which genes are vital for the development and differentiation of Renin Cells, and for Renin's impact on the Renin-Angiotensin-Aldosterone System. This study received an invitation for oral presentation at the American Physiological Society: Control of Renal Function in Health and Disease Conference 2022 at Charlottesville, VA and a poster presentation at the American Heart Association: Hypertension Scientific Sessions 2022 at San Diego, CA. Due to my newfound interest in conducting biomedical research, I plan to pursue a Medical Scientist Program (MD/Ph.D.) after my undergraduate studies.

C) Figure 5. BUN and Kidney/Body Weight Ratio under basal conditions and physiological threat in adult mice. A-B) Gata3 cKO mice showed a significant increase in BUN under basal conditions D) *p<0.001). **C-D)** There was a Physiological Threat ogic significant reduction in Kidney/Body Weight ratio in Gata3 cKO mice under both basal state and physiological threat (*p<0.05). A) B) Ren1 Akr1b7 expression ANA





Kidney/Body Weight

Figure 7. Jesus Samuel Neyra Imaging 2 Month Old *Gata3* cKO Mice Kidney Sections

References:

 Sequeira López ML, Pentz ES, Nomasa T, Smithies O, Gomez RA. Renin cells are precursors for multiple cell types that switch to the renin phenotype when homeostasis is threatened. Dev Cell. 2004 May;6(5):719-28. doi: 10.1016/s1534-5807(04)00134-0. PMID: 15130496.





Biochemical Society Summer

Studentship 2022 Dissecting the Role of SGTA Domains in Membrane Protein Quality Control

Student: Isa Nadim Supervisor: Dr Yvonne Nyathi

1. Experimental Background



Jake Hill (helping PhD student), Dr Yvonne Nyathi (supervisor) and Isa Nadim. From right to left

Protein quality control mechanisms are important for maintaining organismal health and preventing aggregation of proteins often seen in neurodegenerative diseases such as Alzheimer's disease. SGTA is a key player in the quality control of mislocalised membrane proteins (MLPs) due to its ability to interact with key degradation pathways. overexpression of SGTA was shown to stabilise MLPs in a range of previous studies, while knockdown of SGTA destabilises MLPs¹ (Bennaroch, et al, 2019). Its' role is intrinsically linked to its structure which includes an N-terminal UBL-domain which interacts with the BAG6 complex, a central TPR-domain, which interacts with Hsp70s, and a highly disordered, C-terminal domain containing NNP motifs, and a Q-rich region thought to bind hydrophobic substrates² (Martínez-Lumbreras et al, 2018). However, we still do not know how these domains work together in modulating MLP quality control. In this project, a mutagenesis approach was used to gain insights into how SGTA works with its binding partners. Double mutants of SGTA were generated by combining mutants in the TPR, UBL, NNP and Q-rich regions of SGTA and evaluating their effects on the stability of OP91 [a model substrate]. Mutants were transfected into an inducible stable cell line expressing OP91 and analysed by quantitative Western blotting.

2. Aims of the project

(1) To generate single and double mutants of the SGTA co-chaperone protein. (2) Observe the effects of the mutants on the substrate OP91. (3) Form conclusions on how SGTA domains can work collaboratively to ensure quality control.

3. Description of work

PCR-based site-directed mutagenesis was used to generate the SGTA mutants, using mutagenic primers. PCR products were transformed into *E. coli* XL10 gold cells, followed by plasmid isolation and DNA sequencing to confirm the mutants. For cell biology, inducible HeLa T-REx -cells expressing OP91 [a model substrate] were transfected with the mutants followed by induction of OP91 expression. Cells were harvested after 24 hours and analysed by quantitative western blotting using the LI-COR detection system. Western blotting was also carried out after cell lysates were subjected to centrifugation at 16000g for 30 minutes to separate the soluble and the insoluble fractions [containing aggregates]. An MTT assay was used to investigate how expression of SGTA mutants affect cell viability.

Assessment of Results



Figure 1: Hsp70 and the BAG6 complex are required

for the stabilisation of MLPs. Western blot (A) and quantification of OP91 signal (B) and SGTA signal (C). SGTA double mutants with exception of UBL/TPR mutant show statistically significant increase in stabilisation of OP91 than single mutants. The NNP mutants show increased stabilisation, but this is reduced when combined with a TPR mutation [lane 6], suggesting that Hsp70 plays a key role in MLP quality control. Additionally, the concentration of SGTA, remains stable throughout (C), suggesting that the observed effects are not due to differences in the expression of SGTA across all the mutants.



Figure 2. Overexpression of SGTA mutants promote aggregation of MLPs.

C-terminal domain mutants (notably NNP), have a higher level of OP91 in the pellet fraction suggesting more aggregation compared to the N-terminal mutants (A & B). Comparing the proportion of OP91 in pellet and supernatant for each mutant show that in most mutants a higher proportion of OP91 is the pellet fraction except for the TPR/NNP mutant which has more OP91 in the supernatant, suggesting Hsp70 interaction with SGTA is required for the stabilisation of substrates.

Discussion and outcomes of studentship

In this study we investigated the effect of combining various domain mutants of SGTA on the stability of OP91 [a model MLP]. We found that SGTA stabilises OP91, but this effect is dependent on the ability of SGTA to interact with both Hsp70 and the Bag6 complex. The NNP and UBL mutants act synergistically in stabilising OP91, whilst the TPR mutant reduces the stabilising effect of the NNP mutant when combined. We also find that SGTA mutants promote aggregation of OP91, but this is largely not accompanied by a reduction in cell viability [data was not shown]. Our results highlight the importance of Hsp70 and the Bag6 complex in modulating proteasomal degradation and aggregation of MLPs, consistent with previous studies which demonstrate their role in degradation of neurodegenerative proteins.

Future directions in which project could be taken

1.Use domain mutants of SGTA to dissect the interplay of SGTA, Bag6 and Hsp70 in MLP quality control by studying how the interactome of SGTA changes. **2**.Use fluorescence microscopy to visualise aggregates in cells and follow their fate in presence of SGTA mutants. Look at effect of mutants of cell viability over longer periods [48-72 hours].

Departures from original proposal: There were no departures from the original plan

Value of studentship to the lab. This project generated key mutants which will drive the research on protein quality control. Also provided preliminary data which suggest an interplay of SGTA, Hsp70 and Bag6 in protein quality control that warrants further investigation. The data will be part of a grant application, Also, suggest aggregates induced by SGTA are non-cytotoxic over 24 hours, the lab will focus on dissecting what this means and whether cell have benign aggregates and if this induces cellular fitness.

Value of studentship

The studentship provided immense value to me as it has provided me with a stronger motivation to pursue a career in research. I performed lab techniques in molecular biology [PCR and DNA sequencing], bioinformatics skills including primer design, Clustawl Omega multiple sequence alignment to visualise mutations and cell biology techniques such as growing and transformation of bacteria, culturing mammalian cells, transfection of plasmid DNA into the cells, induction of protein expression and analysis of cell viability in mammalian cells that will greatly benefit me in my final year project enabling me to get a first class degree which I need in order to pursue a career in research. I learnt how to use GraphPad Prism as statistical analysis of data. Additionally, the first-hand experience within a lab has allowed me to develop vital skills such as confidence, time-management, problem solving and working collaboratively with others, that would be invaluable in a research role. Furthermore, my writing skills have improved in a scientific context through writing the lab report and blog posts. Additionally, my presentation and network skills were improved through a network skills were improved through a function and network skills were improved through a scientific context through writing the lab report and blog posts. Additionally, my presentation and network skills were improved through a scientific context through writing the lab report and blog posts. Additionally, my presentation and network skills were improved through the presentation and network skills w



Me, Isa Nadim presenting my poster at the NECB conference.

through presenting my poster at the North of England Cell Biology [NECB] forum 2022, held at the University of Manchester. This conference allowed me to see what other aspects are involved in a research career.

Imperial College

Results and Analysis



Discovering Antibodies for Post-translationally Modified Amyloid-beta Oligomers

Student: Jielei Wang

Supervisors: Dr Francesco A Aprile and Dr Ying Ge, Department of Chemistry, Imperial College London

Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia, affecting over 55 million people worldwide.¹ A hallmark of AD is the accumulation in the brain of insoluble deposits called plaques, whose main constituents are fibrillar self-assemblies, i.e., amyloids, of the amyloid-beta peptide (A β). The self-assembly of A β starts when abnormal monomers nucleate into oligomers, which can then mature into amyloids. Among these forms, oligomers are believed to be the major species causing neurotoxicity.² Notably, several types of post-translational modifications (PTMs) have been reported in amyloids isolated from patients. Particularly, phosphorylation of Ser8 (pSer8) has also been found to both promote the formation of A β oligomers and increase neurotoxicity.^{3,4}

Aims and objectives

The overall aim of this project was to set up a platform to generate antibodies to investigate the role of pSer8 on A β oligomer formation. In the first stage, a modified peptide with phosphorylated cysteine pC8, a phosphoserine pSer8 mimic, was generated via a chemical mutagenesis approach developed in the lab. Then, two libraries of synthetic nanobodies were screened against the modified peptide epitopes via ribosome display.

Methods

Production of Purified Aβ40-pC8 Peptide

Point mutation S8C was introduced to pT7-His-NTS-TEV-A β 40 plasmid via site-directed mutagenesis, which was then used to transform chemically competent *E. coli* BL21 cells for protein expression. The expressed fusion proteins (Silk Domain-A β 40-S8C) were purified and the site was chemically modified into dehydroalanine (Dha) then phosphocysteine. The silk domain (SD) was then cleaved by incubation with TEV protease, and the sample was subjected to size exclusion chromatography (SEC) to afford purified A β 40-pC8 peptide. The above steps, relevant mass spectra and Thioflavin T (ThT) aggregation assay were all carried out following a protocol published by the group.⁵

Screening of Antibodies

Starting with two synthetic nanobody libraries of concave and loop surfaces,⁶ three rounds of ribosome display were carried out according to a published protocol⁷, against biotinylated peptide epitope RHD{pSer}GYEV. The successful library after round 3 was cloned into pSb_init vector and transformed using electrocompetent BL21 cells. Single colonies from the transformation were induced to express antibodies, which will be subjected to ELISA to find hits with high affinity and specificity for the modified fulllength peptide. The steps have been taken from published protocols.⁷



Figure 1. Mass spectra of (A) purified SD-Aβ40-S8C fusion protein, (B) the Dha intermediate, (C) the phosphorylated product, and (D) monomeric peptide Aβ40-pC8 after TEV proteolysis. *Cal.* stands for calculated mass and *Obs.* stands for observed mass.

The observed mass shift after the introduction of Dha was -34 Da as expected. When phosphocysteine was introduced, the observed mass shift was +78 Da compared to S8C, also in agreement with the expected mass shift of +80 Da.

After the chemical modification was in place, the modified fusion protein SD-A β 40-pC8 was subjected to TEV cleavage and purification via SEC (Figure 2). For comparison, the same treatment was also carried out for wild type SD-A β 40. The identity and purity of SEC products were confirmed by SDS-PAGE, and fractions containing A β were collected for subsequent studies.



Figure 2. A representative SDS-PAGE of Aβ40 (wild type or pC8 variant) showing the components after TEV cleavage, before and after size exclusion chromatography.

<u>Comparison of the Aggregation Behaviours of Aβ40 and</u> <u>Aβ40-pC8 via ThT Aggregation Assay</u>

Compared to wild type A β 40, the pC8 variant also showed concentration dependence in aggregation while the normalised data showed a much slower initial increase in fluorescence intensity (Figure 3A), thus slower fibril formation. This was not in accordance with literature,³ likely due to the epimerisation effect: the planar conformation of Dha intermediate leads to a mix of L- and D- conformers in the final product,⁵ therefore epimeric control should be included in the future.



Figure 3. Normalised data from ThT aggregation assay, with fluorescence of 20 μ M ThT in buffer alone subtracted as background signals. (A) 10 and 8 μ M wild type A β 40 (blue and pink), and A β 40-pC8 (orange and gray). (B) All samples contain 10 μ M A β 40-pC8, but with varying concentrations of DesAb-O.

When A_{β40}-pC8 variant was incubated with DesAb-O, an antibody known to have high specificity and affinity towards oligomers,² amyloids formation was delayed as predicted, compared to the control (Figure 3B). However, no concentration dependence was observed, and the error bars were quite significant. Moreover, unnormalised data from DesAb-O containing samples showed higher absolute fluorescence signals than the negative control, indicating formation of more fibrillar species at the end. It is therefore worth repeating to confirm whether the antibody made the peptide variant more aggregation-prone. Another test carried out was an SDS-PAGE of aggregation samples (10 μ M wild type and variant) at different time points to detect the forms (monomer, dimer, oligomer etc.). Unfortunately, the peptides used were not fresh and no valid conclusion could be made, but this investigation would benefit us with insights into the kinetics of nucleation species.

Ribosome Display and Expression of Hits

Following 3 rounds of ribosome display, it appeared that the concave sybody library was better in targeting the phosphorylated epitope (Figure 4), thefore the purified cDNA was cloned into pSB_init vector and transformed via electroporation.



Figure 4. PCR amplification product of complimentary DNA (cDNA) from round 3 of ribosome display. Left: Concave; Right: Loop. The bands at 300-400 bp (highlighted in blue) were the desired product.

During electroporation, an arc event occurred, possibly because of impurities or salts present in sample, a too high temperature for electroporation, or even defective cuvette. Transformation was still successful overall, despite at a lower transformation rate where around 150 colonies were observed while at least 500 should be expected according to the paper.⁷ This transformation could be performed again with optimised conditions to generate more colonies for characterisation, in case there has been a loss in diversity. Nonetheless, 95 single colonies were picked and induced alongside a negative control with no DNA, but owing to the limited time frame, the project stopped at the stage where periplasmic extracts of the cultures were ready to be screened via the ELISA procedure, against both phosphorylated and unphosphorylated epitope RHDSGYEV.

Future Directions

Top hits from ELISA will be sent for sequence analysis, and subjected to transformation and purification processes in order to produce the unique hits. The antibody will be used in ThT assays to compare the aggregation behaviours of A β 40-pC8 with and without the antibodies, from a kinetic and more quantitative point of view. SDS-PAGE of aggregation samples at different time points could be helpful in determining whether the antibodies could prevent the formation of oligomeric species. Moreover, since it is only the phosphoserine mimic being used, all assays involving the hit antibodies could be done with phosphoserine to see if there is difference in behaviours.

Departures from original proposal

Yeast surface display was originally proposed, but ribosome display was adopted instead as a more timeefficient *in vitro* screenign method. The initial proposal also intended to produce the Aβ42-pC8 variant, but attempts to transform the plasmid were unsuccessful, despite using chemical transformation and electroporation, as well as varying conditions and DNA concentrations.

Value of Studentship to the Student

This studentship made this opportunity possible. This experience strongly suits my research interest and has equipped me with both useful research techniques and valuable transferrable skills like problem solving, therefore immensely boosted my confidence in independent research and in pursuing a PhD in chemical biology. The unsuccessful transformation was frustrating but it helped me realise the persistence I have in me to find ways around the problem.

Value of Studentship to the Lab

Jielei's intership would have not been possible without the support of the Biochemical Society, as our Department allows summer interships only if students are supported by a bursary.

Jielei Wang and Dr Francesco A Aprile

Jielei worked incredibly hard in the laboratory. Thanks to this experience, we made substantial progress towards an antibody discovery procedure to target pSer8 of $A\beta$.



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Biochemical Society Summer Vacation Studentship 2022 Taming Hippos in the Spiny Mouse

Student: Marcin Krzysztof Maniak

INTRODUCTION:

Vertebrates present a wide spectrum of regenerative abilities. For instance, zebrafish can fully regenerate multiple adult organs, while mammals respond to injury with fibrotic scarring [1]. In humans, fibrosis contributes to 45% of all deaths in the United States [2]. Recently, however, rodents within the genus *Acomys* referred to as spiny mice, have been shown to fully regenerate several adult organs without fibrosis. For example, they rapidly re-epithelialize adult skin wounds, subsequently regenerating hair follicles and sebaceous glands, which does not occur in house mice (*Mus musculus*) [3].

This high regenerative capacity of spiny mice has often been attributed to their peculiar tissue biomechanics - their skin is weak and tears easily upon the attack of a predator, allowing for swift escape [4]. The extracellular matrix (ECM) of spiny mouse wounds also has a different composition to that of Mus [5]. Since mechanical forces lead to myofibroblast differentiation and excessive collagen production [6], the ECM of the spiny mouse could represent a proregenerative matrix whose biomechanics prevents scar formation. Interestingly, blocking the interaction between Yap, the component of the Hippo signalling pathway responsible for mechanotransduction, and the TEAD family of transcription factors in spiny mice has been shown to inhibit regeneration by prolonging myofibroblasts persistence in the wounded tissue [7]. **AIMS OF THE PROJECT:**

We aimed to compare fibroblasts isolated from spiny mice and house mice in terms of their biophysical properties and Hippo-Yap/Taz signalling. We set out to isolate and culture fibroblasts from both species, confirm their identity using fibroblast markers and validate whether antibodies used can recognise proteins from both house mice and spiny mice.

METHODS:

<u>Fibroblast isolation:</u> Fibroblasts were isolated from the skin and lung by collagenase digestion, whereas the crawl-out method was more efficient for the heart and kidney as described previously [8].

Supervisors: Nancy Hui & Dr Carsten Gram Hansen

<u>Cell culture:</u> Cells were cultured in a humidified incubator at 37°C with 5% CO². Isolated fibroblasts were cultured in DMEM/F12 (Gibco), while HEK293A cells were cultured in DMEM (Gibco) supplemented with 2 mM L-glutamine (Gibco); both media were supplemented with 10% heat-inactivated FBS (Gibco) and 1% (v/v) penicillin–streptomycin (Gibco). All cells were passaged using 0.05% Trypsin-EDTA (Gibco) before reaching 80% confluence. Passage number was tabulated before experiments. Cells were used between passages 2 and 6.

<u>Western blotting:</u> Lysates were prepared from harvested cells with lysis buffer consisting of pH 6.8 50 mM Tris buffer, 2% (w/v) SDS, 0.025% (w/v) bromophenol blue, 40% (v/v) glycerol and 5% (v/v) β mercaptoethanol. Samples were segregated using SDS-PAGE (70-120 V) and subsequently transferred to a PVDF membrane. Proteins were immunodetected with enhanced chemiluminescence (Millipore), and the signal was developed onto X-ray films.

Immunofluorescence: Cells were plated on μ -Slide VI 0.4 channels (ibidi) and after 24 hours - fixed, rinsed, blocked in permeabilization buffer, and incubated with primary, rinsed, and then secondary antibodies [9]. The nuclei were labelled with Hoechst (H3570; Thermo Fisher Scientific, 1:2,000). Cells were imaged using a 10× air objective on a Leica TCS SP8 MP confocal microscope.

RESULTS:

Initially, we examined whether the fibroblast markers, α SMA and PDGFR β , are expressed in isolated cells by Western blotting (**Fig. 1A**). α SMA is strongly expressed in all lung and skin samples from *Acomys*. PDGFR β is strongly expressed in some lung samples but also appears to be visible in others upon longer exposure. Neither is expressed, as expected, in non-fibrotic HEK 293A cells. To confirm our observations, we visualised each protein using immunofluorescence (**Fig. 1B**). Again, α SMA and PDGFR β were only present in cells isolated from spiny mice. When we measured the mean fluorescence intensity, it was robustly higher in the isolated cells (**Fig. 1C**). To conclude, we were able to isolate spiny mice fibroblasts. We have also confirmed that our antibodies recognise fibroblast markers and Yap and Taz in the spiny mouse.

DEPARTURES FROM ORIGINAL PROPOSAL:

The proposed use of Digital Holographic Microscopy [9] was unsuccessful as fibroblasts turned out to be too flat to be studied with this technique. Secondly, the immortalization of fibroblasts was not completed due to their slow growth, which meant we postponed the planned knock-down studies.

FUTURE DIRECTIONS:

Fibroblasts from mice and spiny mice could be compared in terms of their morphology using the Fluorescence eXclusion method (FXm) [10]. Secondly, further underlying molecular mechanisms should be investigated to understand how cellular properties differ between wild-type fibroblasts and those with their Hippo pathway components' expression altered. Lastly, differences between fibroblasts isolated from various organs could be assessed to understand underlying organ-specific differences.

VALUE OF STUDENTSHIP TO THE STUDENT:

I had a very fulfilling and enjoyable time in the Gram Hansen group. I mastered the techniques of cell culture, Western Blot, and microscopy, as well as experimental data analysis and scientific communication. The studentship has made me confident in my plans to pursue a PhD in biochemistry. I am grateful for the continuous support of my group and funding I received from the Biochemical Society.

VALUE OF STUDENTSHIP TO THE LAB:

Marcin shows enthusiasm, is inquisitive and takes initiative in learning various techniques. Marcin, at a very early stage in his career, shows that he is well cut out for a career in the Sciences. It was enjoyable to host Marcin in the lab, and it is exciting to follow Marcin's research career. The whole team wish Marcin well for his future career. www.gramhansenlab.com



Figure 1. Confirmation of isolated cells' identity and validation of antibodies.

A. Western blot probed for fibroblast markers, α SMA and PDGFR β , reveals their expression in isolated cells. Antibodies used successfully recognise the transcriptional Hippo pathway components, Yap and Taz. L – lung, S – skin sample from Acomys. **B**, **C**. Cells were analysed by immunofluorescence and labelled for Hoechst (green), YAP (yellow), and α SMA or PDGFR β (magenta). Expression of α SMA and PDGFR β indicates isolated and expanded cells are indeed fibroblasts. Antibodies used likely recognise Yap and Taz. Knock-down studies are necessary to ensure the specificity of the signal. Scale bar = 15 μ m. Error bars represent mean ± 95% CI. Mann–Whitney U test. ***p < 0.0001.

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