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

Head of College Scholars List Scheme
Summer Studentship 2019
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: maureen.bain@glasgow.ac.uk **within four weeks of the end of the studentship**.

1. Student
   Surname: Al-Mohammad Forename: Leen
   E-mail address: 2298202A@student.gla.ac.uk

2. Supervisor:
   Surname: Baillie Forename: George
   E-mail address: George.Baillie@glasgow.ac.uk

Project Title (maximum 20 words):

Disrupting the NADK-HSP20 protein-protein interaction in MCF7 cells.

Project Lay Summary (copied from application):

Proteins need to interact with one another for their activity to take effect. Protein-protein interactions (PPIs) are important steps in disease pathways – by using therapeutic peptides we can manipulate (enhance or disrupt) the way certain proteins in the body interact, and therefore their effects. Recent studies showed that this, if developed effectively, could be used to create drugs targeting a range of chronic disease pathways, including those of certain cancers. This project will evaluate the efficacy and efficiency of pre-identified candidate peptides (PPI enhancers/disruptors), using biophysical and cellular assays, to see which ones could be successfully developed for therapeutic treatments.

Start Date: 10/6/19 Finish Date: 2/8/19

Original project aims and objectives:

With this project, I aimed to analyse the efficacy of a candidate disruptor peptide on blocking the target PPI between HSP20 and NADK. First, by further validating that there is an interaction between these two proteins, followed by the main portion of the project, where I worked to assess the effect of the disruptor peptide and how it would affect proliferation and morphology in cancer cells. Thus, preventing the interaction between HSP20 and NADK in cancer cells could have potential therapeutic benefit. By using previous peptide array data in the Baillie lab, a cell-permeable disruptor peptide was designed and generated.

Methodology: Summarise and include reference to training received in research methods etc.:

The disruptor peptide intended to block this interaction was assessed using three approaches in MCF7 cells.

We used co-immunoprecipitation (co-IP) and western blotting to assess efficacy of the disruptor. Protein lysates harvested from MCF7 cultures treated with DMSO, control (scrambled) peptide and the disruptor peptide were set up to IP for either HSP20 or NADK (both directions of the PPI of interest). We then conducted Western blots with 700μg protein from each sample and their mock complexes. The blots were scanned to compare the prevalence of HSP20/NADK in the lysates following treatment with the disruptor peptide. This approach required training for techniques such as cell harvest, Bradford assay, gel electrophoresis, scanning membranes using Odyssey programming, and analysing blot results.

To attempt to further validate that endogenous HSP20 and NADK interact, proximity ligation assay (PLA), was used on MCF7 (breast cancer) cells. This included techniques such as fixation in 4% paraformaldehyde, immunostaining using primary antibodies, washes and use of fluorophore-labelled secondary antibodies, cell mounting and PLA imaging using a confocal microscope, followed by image analysis using ImageJ.

Finally, a colony formation assay (CFA) spanning 9 days, with MCF7 cells treated with DMSO (control for treatment media), control peptide (control for disruptor), and the disruptor peptide. These treatments were applied in 1:1000 concentration, in 3-day intervals, to 30,000 cells per well. The cells were photographed daily to monitor effects of disrupting the PPI on growth and morphology, and on their 9^{th} day were stained in crystal violet. This experiment included techniques such as pipetting, cell flask splitting/cell culture techniques, seeding density calculations, use of the light microscope, and crystal violet staining.

Results: Summarise key findings. Please include any relevant tables or images as an appendix to this report:

Figures 1 & 2 show Western blots following co-IP for either protein of interest. 'Input' lanes showed strong signals for HSP20 and NADK identified in the samples. 'Mock IP' lanes were controls for 'IP' lanes, though both showed the same faint bands at 50kDa (NADK) and 20kDa (HSP20), for all four blots.

MCF7 cells were treated for 2 hours with DMSO, the control peptide, and disruptor peptide, as well as the secondary antibody control (not shown), then PLA was performed to attempt to further validate the interaction between NADK and HSP20 and to observe effects of the disruptor on the PPI. As shown on figure 3, the green channel signals indicate cell distribution. The red channel signal, indicating NADK/HSP20 points of interaction in the cells, showed more interactions in DMSO and disruptor conditions, and fewer in control peptide treated cells.

The CFA yielded interesting results (figure 4). While the extent of growth of the disruptor-treated cells was not significantly hindered compared to the control peptide treated cells, they did appear less stable in their development of typical MCF7 morphology. In comparing images for either peptide treatment to DMSO control images - the cells here display said 'typical' MCF7 morphology - it is made clear that the health of the disruptor-treated cells is not stable throughout the 9 days. Further to this, crystal violet staining of the plate revealed that there was an overall difference in proliferation between the 3 treatments, with the disruptor treated well showing fewer colonies, while control and DMSO treated wells were similar (figure 5). For all light microscope images, a scalebar of 0.1mm is provided.

Discussion:

NADK phosphorylates NAD into NADP (nicotinamide adenine dinucleotide phosphate), which is then reduced to NADPH. Tedeschi et al. (2016) found that NADPH neutralises the high levels of reactive oxygen species (ROS) encouraging hyperproliferating cancer cells. HSP20, while its role in tumour progression is ambiguous, has been reported to be overexpressed in some cancers (Matsushima-Nishiwaki, et al., 2011). Previous research by Baillie lab found that NADK and HSP20 interact – exploring the effects of blocking this interaction will reveal more about either protein's role/significance in cancers.

Combining IP and Western blot was to see if treatment affected HSP20 and NADK within protein lysates isolated from MCF7 cells. If the disruptor succeeded in blocking the PPI, we would expect to see bands for

both proteins in input and IP, but a weaker signal for disruptor IP, and blank mock IP lanes. Towards the end of the project, we found that the primary antibodies had been faulty, which hindered our previous blots, as the light chain and heavy chain had hidden the protein bands. However, even after fixing this issue, we found that our NADK/HSP20 bands were still weak and non-specific binding was still prominent. To improve this in future repeats, a higher protein concentration, e.g. 900μg, should be prepared at the co-IP step, to give a clearer signal in blot scanning, and a longer blocking buffer (5% BSA) wash should be used.

The PLA allowed us to observe if the disruptor peptide could successfully block the PPI. The PPI (red dots) occurred less in control peptide treated cells than DMSO/disruptor treated cells, which does not support our hypothesis. Had the disruptor been effective, we would expect to see fewer red signals in disruptor-treated cells. Lack of results from PLA on MCF7 cells could be attributed to a number of practical factors at various steps, e.g. human error in following PLA protocol, antibodies may have been too old/stored for too long, etc. Despite this, we continued our investigation of the PPI via other approaches.

Typical morphology for untreated MCF7 adhered monolayer cultures, (figure 4), follow a relatively concentric growth pattern, producing close-formed colonies. Such MCF7 colonies also have even colouring under the light microscope, due to an even layer of cells. Regions 1 and 3 of disruptor-treated cells did not follow typical growth patterns, nor did they closely resemble healthy MCF7 morphology. Region 2 of control peptide treated cells also became less healthy, and by day 9 it bore little resemblance to healthy untreated cells. This suggests there may be other factors affecting cell development. Disruptor-treated regions 1 and 3 also clearly grew slower than DMSO and control peptide treated cells, based on size comparisons throughout. When culturing adhered cells, a sign of distress is bright spots, indicating detachment from the growth surface. Bright spots are consistently more frequent among disruptor peptide colonies. Crystal violet staining of the CFA plate showed promising results with regards to the disruptor's ability to hinder MCF7 development (figure 5). Overall, CFA results suggest the disruptor peptide may successfully interfere with typical cancer proliferation. However, further repeats of the CFA with multiple seeding densities are needed to confidently draw such conclusions.

4. Reflection by the student on the experience and value of the studentship:

This studentship was a brilliant introduction to working in research. I was given countless opportunities to learn and practice new techniques and tests, which I am sure will benefit me greatly in my studies, postgraduate research and future career prospects. It has broadened my modest understanding of Pharmacology and of what it is to work in translational research, as well as simply the inner workings of an academic lab. In running into hurdles in our research, and through watching postgraduate researchers, I learned the importance of curiosity, tenacity and teamwork in academic research.

As well as the theoretical and practical knowledge gained, working at the Baillie lab has also given me confidence in my ability to work independently in a lab setting. This is no doubt thanks to the entire Baillie team for their endless patience despite my mistakes (which I hopefully learned from), and the nurturing and friendly environment they created day to day – I am very grateful to have been made to feel so welcome.

The HOC Scheme and studentship has provided valuable experience from start to end; from first finding confidence to approach Professors to arrange a project, to preparing an application, adapting to a new setting and working hard there, to collating evidence and putting together a report – I would recommend embracing the opportunities the Scheme provides to anyone it is offered to. I have also come out of this experience with a reinforced drive to work in Pharmacology and to pursue postgraduate studies in this field.

Appendix:

IP HSP20 in MCF7:

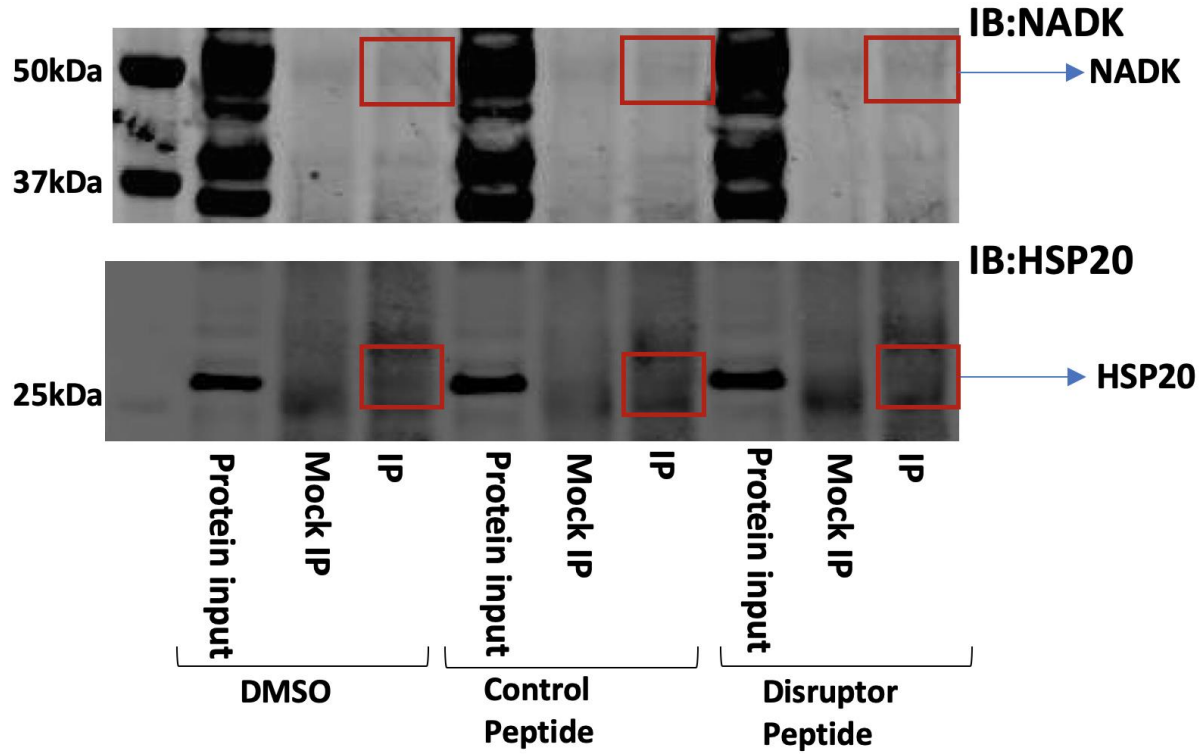

Figure 1: Presented here are 2 scans of the same co-IP samples for **HSP20**, where endogenous HSP20 was immuno-precipitated from MCF7 cell lysates. The first (top) scan shows NADK, and the second (lower) scan shows the HSP20 pulled down. NADK bands are found at approximately 50kDa, while HSP20 bands are found at approximately 25kDa. The first lane is the protein ladder. For each of the three treatment conditions, a protein input, mock IP (IgG only control) and IP lane was set up.

IP NADK in MCF7:

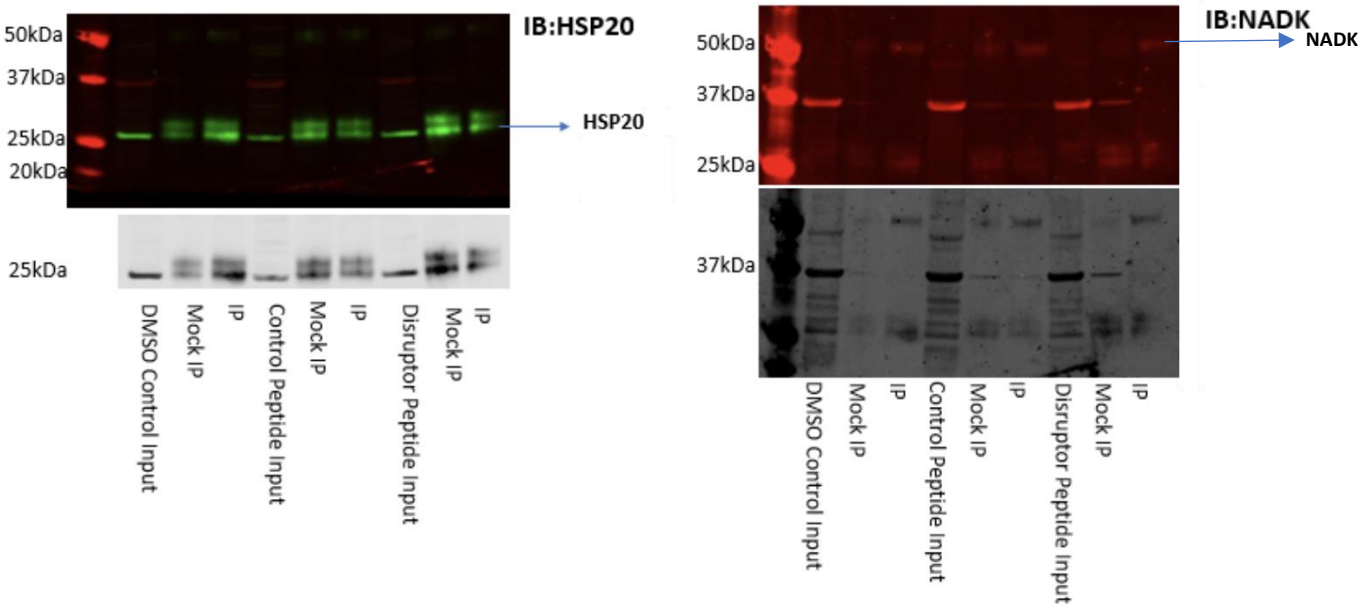

Figure 2: Presented here are 2 scans of the same co-IP samples for **NADK**, where endogenous NADK was immuno-precipitated from MCF7 cell lysates. The left scan shows the HSP20 protein in the samples, as highlighted. The scan to the right shows the NADK that was pulled down. NADK bands are found at approximately 50kDa, while HSP20 bands are found at approximately 25kDa. The first lane on either scan is the protein ladder. For each of the three treatment conditions, a protein input, mock IP (IgG only control) and IP lane was set up.

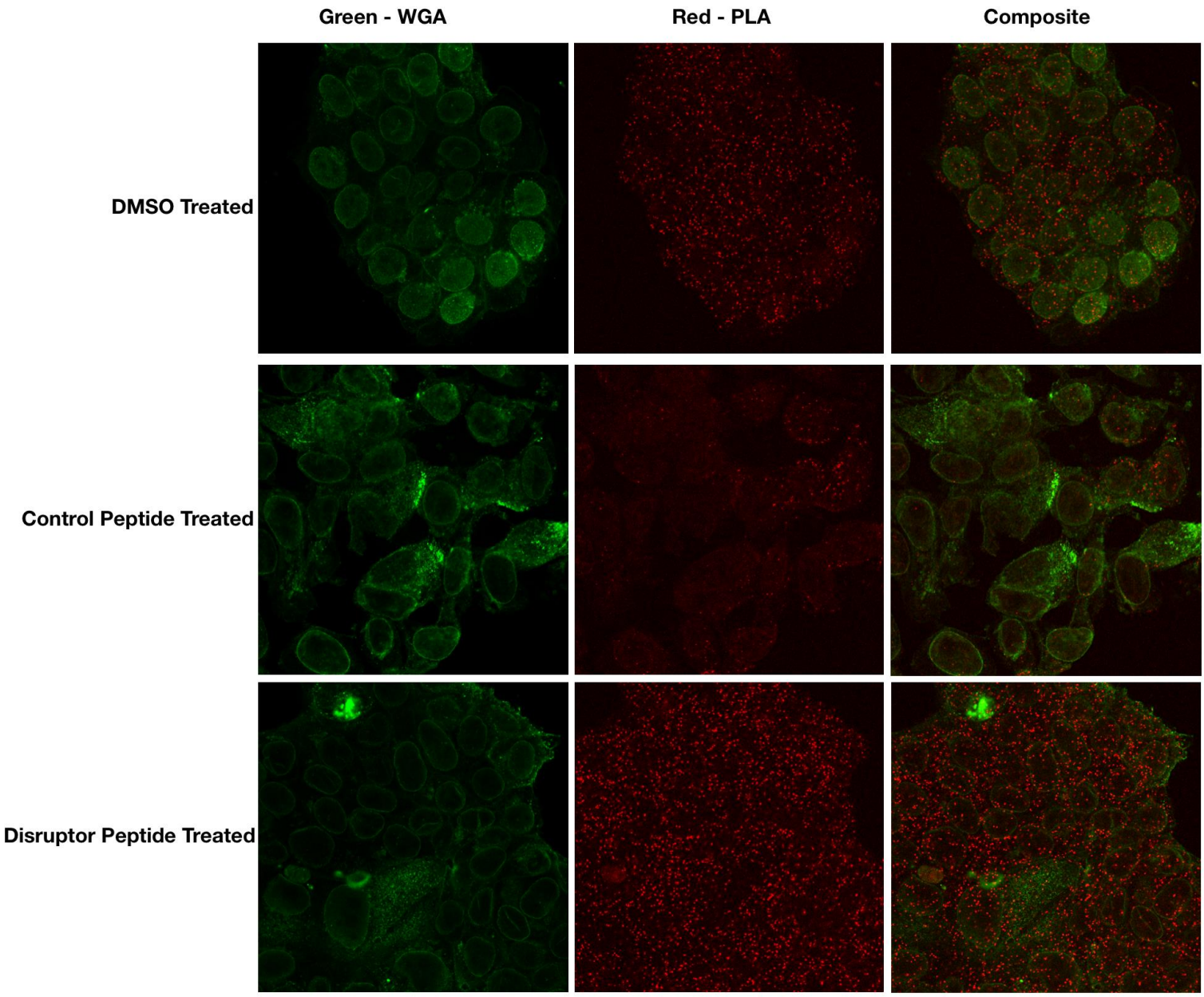

Figure 3: A comparison of the PLA images analysed using ImageJ, showing the subcellular distribution of endogenous HSP20 and NADK PPIs in MCF7 cells. Each set of images corresponds to different treatments; DMSO, Control Peptide, and Disruptor Peptide. The sets consist of green channel (membrane protein, stained for using Wheat Germ Agglutinin), red channel (PLA signals), and a composite image merging the two. In analysing these images, the brightness was increased equally to better express results.

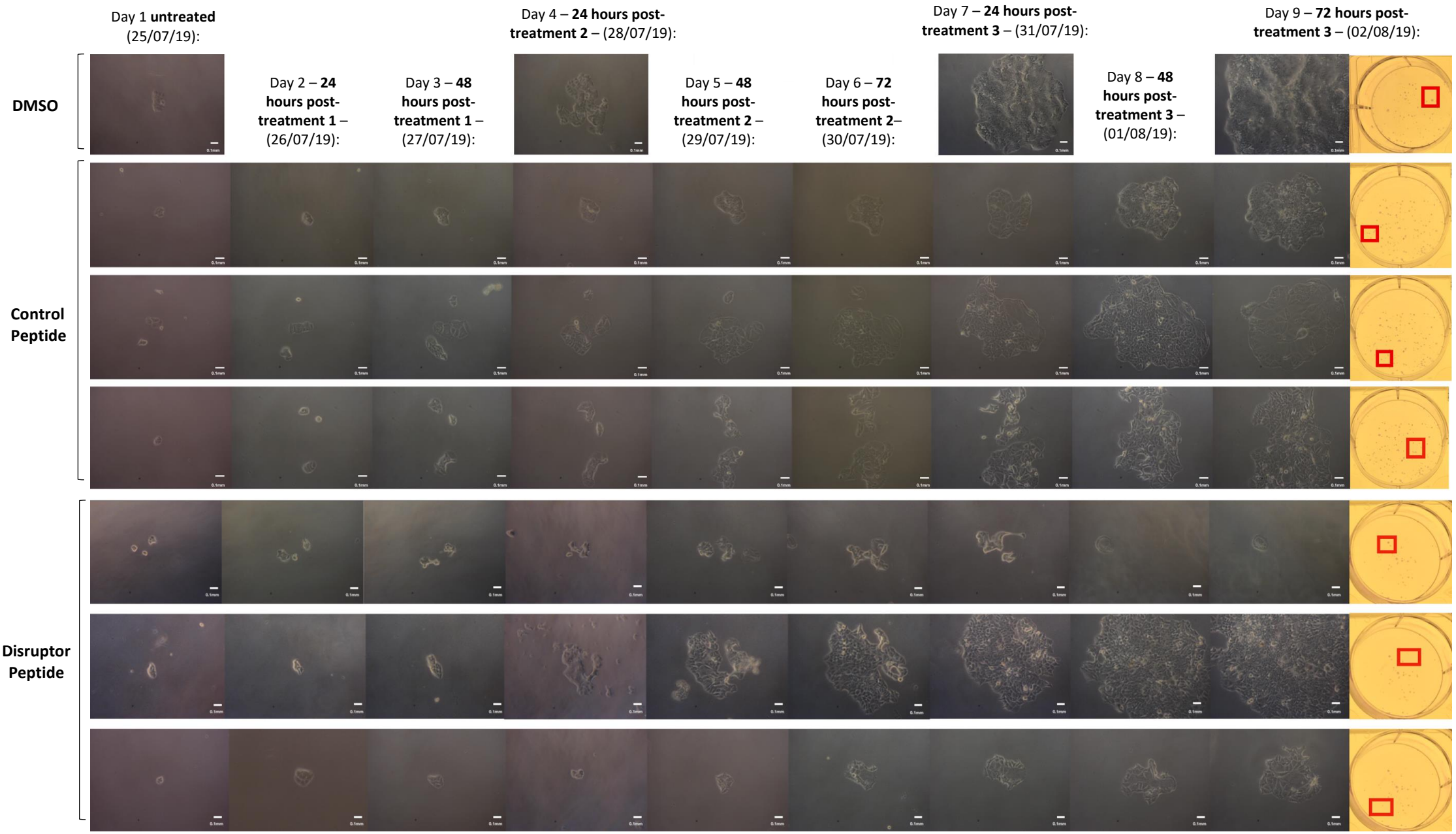

Figure 4A: CFA results for the effect of disrupting the HSP20/NADK PPI. Presented are results for DMSO, control peptide (x3 regions), and disruptor peptide (x3 regions). DMSO-treated cells were photographed 24 hours post each of the 3 treatments (where growth media was refreshed and DMSO treatment was reapplied), as well as on the day of staining (day 9). The DMSO well is used as a control for 'typical' MCF7 morphology. Below that are 3 regions of control peptide treated cells, which were photographed daily through the 9 days. The control peptide well is used as a control against the disruptor peptide. 3 regions of disruptor-treated cells were also photographed daily. A photo of each treatment well after crystal violet staining was taken on staining day, highlighting the region of cells each time. This experiment allowed us to monitor growth and any potential morphological effects in response to treatment.

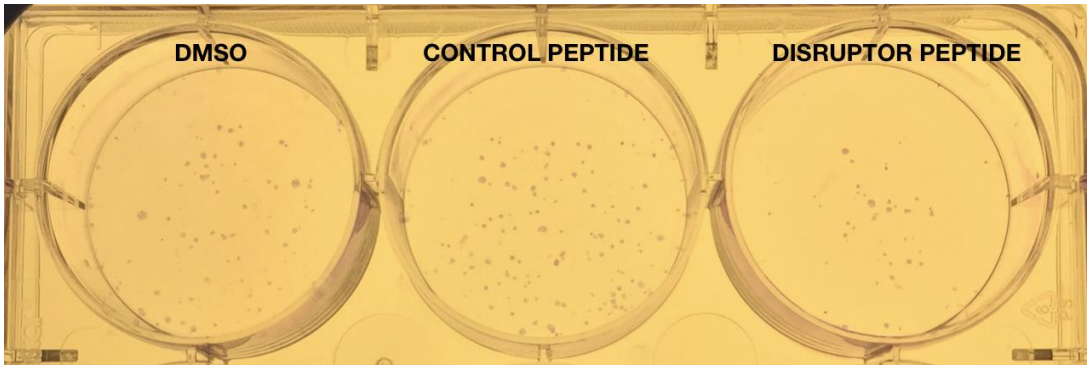

Figure 4B: This figure shows three treated wells as part of the CFA, showing different levels of colony formation of MCF7 cells. Labelled are the DMSO treated well, control peptide treated well, and the disruptor treated well. Each well was stained using crystal violet for screening cell viability. This allows for the effects of the peptides on MCF7 cell proliferation and survival to be compared.

References:

Tedeschi, P.M., Bansal, N., Kerrigan, J.E., Abali, E.E., Scotto, K.W., Bertino, J.R., 2016. NAD^+ Kinase as a Therapeutic Target in Cancer. Clinical Cancer Research, [e-journal] 22(21), pp. 5189-5195. https://doi.org/10.1158/1078-0432.CCR-16-1129

Matsushima-Nishiwaki, R., Adachi, S., Yoshioka, T., Yasuda, E., Yamagishi, Y., Matsuura, J., Muko, M., Iwamura, R., Noda, T., Toyoda, H., Kaneoka, Y., Okano, Y., Kumada, T., Kozawa, O., 2011. Suppression by Heat Shock Protein 20 of Hepatocellular Carcinoma Cell Proliferation via Inhibition of the Mitogen-Activated Protein Kinases and AKT Pathways. Journal of Cellular Biochemistry, [e-journal] 112(11), pp. 3430-3439. https://doi.org/10.1002/jcb.23270

Signatures:

Supervisor:

Date: 28th Aug 2019

Student:

Date: 24/8/19