Generation and initial characterisation of parental and A3A-knockout bladder cancer cell line clones for cancer drug resistance studies

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Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning

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Finally, I'd like to thank my family for their unending support.

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Abbreviations

AID	Activation Induced Deaminase
APO-B100	Apolipoprotein B100
APO-B48	Apolipoprotein B48
ATR	Ataxia telangiectasia and Rad3-related
DSB	Double stranded break
FBS	Foetal Bovine Serum
FRT	Flippase Recognition Target
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homologous Recombination
HSV-TK	Herpes Simplex Virus Thymidine Kinase
IMDM	Iscove's Modified Dulbecco's Media
KO	Knockout
LDL	Low Density Lipoprotein
PGK	PhosphoGlycerate Kinase
TAE	Tris-acetate-EDTA
TBP	TATA sequence binding protein
TCR	Transcription Coupled Repair
UGI	Uracil Glycosylase Inhibitor
UNG2	Nuclear Uracil DNA Glycosylase 2

Vif	Virion infectivity factor
WT	Wild Type

Abstract

The primary role of the APOBEC3 family of enzymes is within the innate immune system, acting to limit the infectivity of retroviruses through the deamination of their DNA intermediates by converting DNA cytosines to uracils, leaving behind a series of characteristic mutational signatures. However, over the years since the initial discovery of this family of enzymes and their roles, a series of landmark papers have been published that reveal the presence of these same signatures in a wide variety of human cancers, with APOBEC ranking as the second most commonly seen endogenous mutational signature across all human cancers. This discovery prompted investigations into the underlying mechanisms behind this and into the member of the APOBEC family most likely to be causing these endogenous mutations and its possible interactions with common chemotherapeutics, however results remain unclear and contentious with various studies by high-level labs and authors disagreeing and debunking one another.

This study aimed to investigate the effect that A3A may have on the pace of development of resistance to cis-platin in BFTC-905 bladder cancer cells. This was due to be completed by using single cell culture and transfection techniques to expand numbers of colonies of A3A KO BFTC-905 cells that were created by a previous MSc-R student using CRISPR-Cas9, followed by a series of MTT assays and drug-sensitisation protocols on these A3A KO cells compared to their BFTC-905 WT equivalents. We were able to expand on the numbers of both the A3A KO and WT

BFTC-905 cells and verify their A3A status using qPCR but were unable to perform the MTT and drug-sensitisation due to COVID-19 and the resultant lockdown period.

Introduction

A brief history of the APOBEC's

The most common type of endogenous mutational signature in humans is the spontaneous deamination of methyl-cytosine bases that lead to C-to-T mutations – associated with age (Alexandrov et al., 2013). After this, the cytosine deamination activity of the APOBEC-family of enzymes is the second largest endogenous mutation source across all human cancers (Alexandrov et al., 2013). Despite this large mutagenic potential, there are 7 variants of the APOBEC3 enzyme found in humans and non-human primates (APOBEC3A, 3B, 3C, 3D, 3F, 3G and 3H) with fewer present in lower mammals, suggesting that this gene cluster may have arisen from an advantageous amplification event (Jarmuz et al., 2002) (Figure 1). Since DNA mutators pose an obvious threat to organisms with DNA genomes, the presence of a

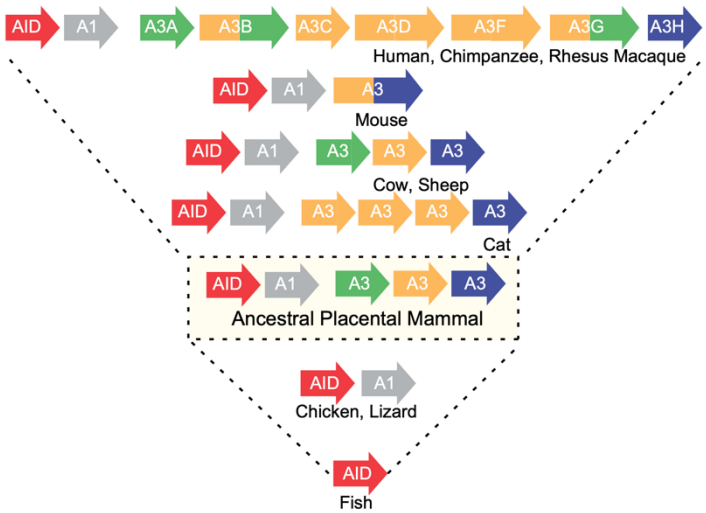

Figure 1 - Diagram showing the evolution and distribution of the APOBEC family of enzymes across several current mammals above the most likely common ancestor and current non-mammalian vertebrates. Adapted from Harris et al - 2015).

large number of variants of APOBEC3 enzymes suggests that they must also confer some evolutionary advantage which must outweigh the risk of not possessing these enzymes at all.

There are 11 members of the APOBEC (Apolipoprotein B mRNA Editing Catalytic Polypeptide-like) protein family, and all share a conserved zinc-dependent deaminase sequence motif that sits at the core of a catalytic cytidine deaminase domain. Variations in the secondary surrounding structures dictate cellular localisation, substrate specificity and the general function of each member of the APOBEC family. Of the APOBEC3 members all have a single catalytic site, but APOBEC3A, C and H are single-domain enzymes and ABOBEC3B, D, F and G are all double domain enzymes. The catalytic domains of APOBEC3A and APOBEC3B (the main subject of this thesis) share 92% amino acid sequence identity, and the proteins as a whole weigh approximately 23kDa and 46kDa respectively.

APOBEC3 enzymes play an important role in the innate immune system by inhibiting retroviruses through the deamination of retroviral DNA intermediates (Harris &

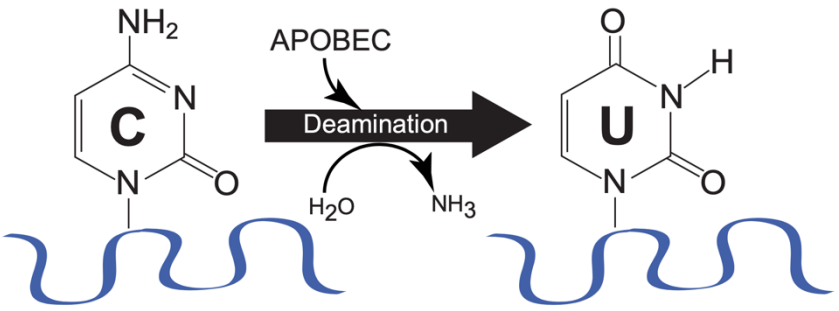

Figure 2 - Schematic of the cytosine deamination reaction catalysed by APOBEC enzymes. Adapted from Harris (2015).

Liddament, 2004). The first fundamental hallmark of APOBEC enzymes is their deamination activity of DNA cytosines to uracils. This deamination takes place through a zinc-mediated hydrolytic mechanism where water is de-protonated by glutamic acid, and the resultant hydroxide ion attacks the 4-position of the cytosine base. This results in the replacement of the amine group with a carbonyl group which ultimately results in the conversion of a cytosine into a uracil (Figure 2). The second hallmark feature is their preference for the DNA sequence surrounding the cytosine on which they act. Activation-Induced cytosine Deaminase (AID) prefers the target cytosine to be preceded by a purine, APOBEC3G prefers it to be preceded by another cytosine, and APOBEC1 along with APOBEC3A, 3B, 3C, 3D, 3F and 3H all prefer it to be preceded by a thymine ((Harris & Dudley, 2015) and references within). Whilst APOBEC1 and AID have roles in relation to RNA editing and antibody diversification respectively, APOBEC3 enzymes have roles relating to restriction of a multitude of viruses as well as endogenous transposable elements. The first member of the APOBEC family to be linked to viral restriction was A3G, at the time referred to as 'CEM15' after the CEM-SS cell line that was found to be permissive to HIV/Δ*Vif* infections during this early work.

Two classic studies began to reveal the existence of the anti-retroviral action of some 'factor' within certain types of cells. They describe this action as "a previously unrecognised form of cellular resistance to viral infection" (Simon et al., 1998). Initial work had already divided certain cell lines into groups based on whether or not they

can support the replication of HIV-1 strains that were deficient in *Vif*, termed HIV-1/Δ*Vif*. Those that can support replication of HIV-1/Δ*Vif* were termed 'permissive', and those that cannot support it 'non-permissive' (von Schwedler et al., 1993). Permissive cells produced Δ*Vif* viruses that had infectivity levels indistinguishable from wild-type viruses, whilst the non-permissive cells produced Δ*Vif* viruses that were 10-100X less infectious than the wild-type variant. This work lends itself to two possible explanations for this phenomenon; either permissive cells produce some activity or factor that can compensate for *Vif*, or non-permissive cells have some type of activity that can inhibit HIV-1 infectivity when in the absence of *Vif*.

The first of the two classic studies to build on this idea and gain traction was that by Simon et al, 1998 (Simon et al., 1998). Their work revolved around attempting to experimentally prove one of these hypotheses by investigating the infectivity phenotype of HIV-1/Δ*Vif* that had been produced by heterokaryons formed from permissive (CEM-SS, 293T) and non-permissive (HUT78) cells. Their work showed that these heterokaryotic cells retained the non-permissive phenotype with the produced virions being 10x less infectious when the host cells were infected with HIV-1/Δ*Vif* compared to the wild type virus. This meant that there must have been some dominant activity present in the non-permissive cell lines that was not present in the permissive lines, and that at least one of the purposes of *Vif* was to counteract whatever this dominant activity may be.

The second case study was published by Sheehy *et al*, 2002 (Sheehy et al., 2002), whose work built directly on that done by Simon *et al*. In order to identify possible genes that fit the criteria set out in this previous work, this study used a cDNA subtraction strategy on a pair of genetically related cell lines that exhibited different abilities in supporting HIV-1/Δ*Vif* replication. These were the non-permissive CEM cell line, and the permissive CEM-SS cell line (a sub-clonal isolation of the CEM line). Subtracted cDNA's were then used as probes in northern analyses of RNA's extracted from a panel of permissive and non-permissive cells, revealing the presence of cDNA from a previously unknown gene that was termed *CEM15*. This gene was easily detected in the non-permissive cells in the panel, but only very minimally or not at all in the permissive cell lines (Figure 3).

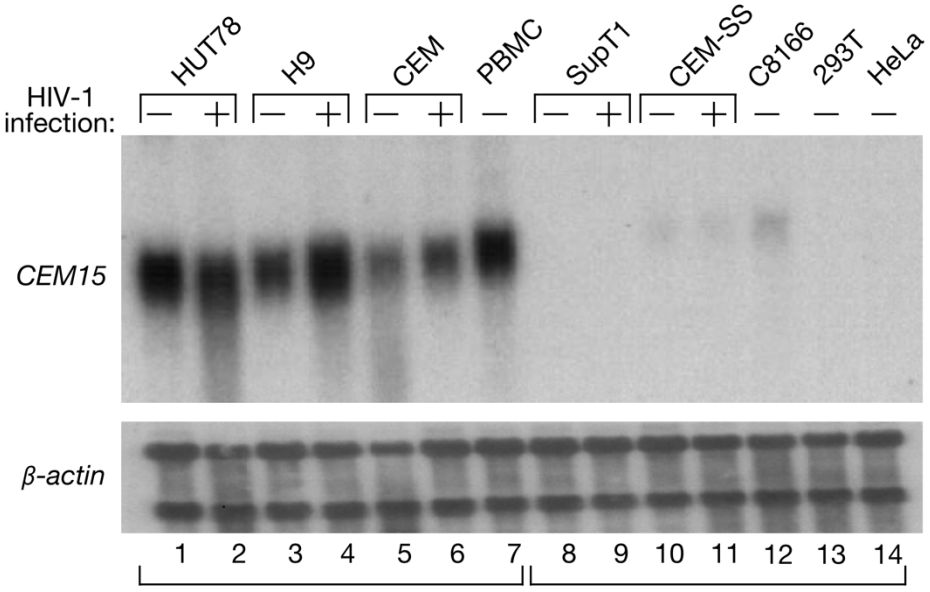

Figure 3 - Northern analysis of CEM15 expression non-permissive and permissive cell lines (Sheehy et al, 2002).

Further testing of the effects of CEM15 on HIV-1 virus particle production showed that compared to the wild type virus, which was mostly unaffected by CEM15 expression, the Δ*Vif* virus had extremely low levels of infection at any dose of CEM15 tested in a 293T transiently transfected cell line. A database search of CEM15 at the time did reveal a record of a previously recognised gene, but with no known description or function. Further analysis of CEM15 showed certain portions that had high similarity to APOBEC-1, and the paper even reports the presence of a zinc-binding domain (a critical component for the proper function of APOBEC enzymes) that it recognises has been previously identified in cytosine deaminases and states its importance in their catalytic activity. However, it did not explicitly state that CEM-15 could be a member of the APOBEC family although this link was made by others in the years after this landmark paper was published.

The huge reduction in the infectivity of HIV-1/Δ*Vif* that was shown to exist when in the presence of this previously unknown factor revealed its enormous possible potential to contribute to the treatment of HIV-1 infections. These two studies paved the way for future research into how this factor works, and how it could be leveraged for real world use.

Prior to this initial beneficial link between certain members of the family of APOBEC enzymes and their retroviral restriction properties, the very first APOBEC to be

discovered (APOBEC-1) was accidently found to be a possible oncogene. One study was initially testing whether the overexpression of the newly found APOBEC-1 in the livers of transgenic mice and rabbits would cause a reduction in the expression of apo-B100 and LDL's, both thought to be its primary target through mRNA cytidine deamination and creation of an early stop-codon, which would lead to a truncated version of apo-B100 (apo-B48) being formed (Yamanaka et al., 1995). Instead, they unexpectedly found that all of the transgenic mice and one of the transgenic rabbits had liver dysplasia and that many of the transgenic mice has hepatocellular carcinomas. The livers of the transgenic mice were all at least twice as large, and some more than ten times larger than their non-transgenic litter mates with one liver weighing 40% of the entire weight of the mouse it was harvested from. They found that these hepatic abnormalities depended specifically on the overexpression of the apo-B mRNA editing activity rather than any secondary side effects such as insertion effects of the transgene. In short, this study concluded that the significant oncogenic effects that they were seeing in the APOBEC-1 overexpressing, transgenic animals were due to aberrant mRNA editing by APOBEC-1 rather than DNA editing – an important difference.

It was not until 2002 in Harris *et al's* and Petersen-Mahrt *et al's* papers (Harris et al., 2002; Petersen-Mahrt et al., 2002) that it became clearer that members of the APOBEC family had the capability to act on and deaminate genomic DNA as well as RNA. They showed that the expression of AID in E. coli gave rise to a mutator

phenotype of nucleotide transitions at dC/dG pairs (which are only found in DNA and not RNA) and also that the AID mutator phenotype is enhanced when there is a deficiency of uracil DNA glycosylase (which normally removes DNA uracils), further indicating that AID must be deaminating dC residues in DNA.

Fuel for cancer

A chance to act on the human genome

There is now a wealth of evidence from cancer genome sequencing studies implicating off-target APOBEC3 activity in generating mutations in cancer cells. APOBEC3's act only on ssDNA and not the dsDNA that makes up the human genome (Roberts & Gordenin, 2014). Whilst this specificity would in principle allow the APOBEC3's to act on and deaminate only viral ssDNA whilst not harming the dsDNA human genome, in reality this does not take into account the small but regular presence of human ssDNA when genomic dsDNA is replicating or undergoing transcription or repair. This leads to the possibility for the human genome becoming deaminated, which can in turn lead to mutations and ultimately the formation and accelerated evolution of a wide variety of cancers.

Mutational signatures, Kataegis and a case for attention on A3A

Cancer has long been known to occur as a result of mutations in proto-oncogenes and tumour suppression genes. Historically, not much was known about the types of signatures that could be the cause of the myriad of different cancers that exist, but more recent sequencing analysis has revealed a trail of ‘mutational signatures’ that consistently appear in a vast variety of different cancers, suggesting that the action of a relatively small number of substances can cause the wide variety of cancers seen in patients every day. These signatures have been characterised, with either just one signature or a variety of signatures being found in most human cancers. The

pioneering study detailing such signatures found that whilst most signatures are unique to a small number of specific human cancer types, signatures 2 and 13 are found in a large variety of cancers and are attributable to the activity of the APOBEC family of cytidine deaminases (Alexandrov et al., 2013).

Before these APOBEC signatures were ever first formally identified and catalogued (Alexandrov et al., 2013), they were seen in a multitude of cancers years earlier before the underlying mechanism was fully understood. For example, one of the TpC signatures that was later attributed to APOBEC activity was initially found on multiple protein kinase genes in breast cancer samples (Stephens et al., 2005), and also in lung and ovarian cancers (Greenman et al., 2007). Further whole genome analysis of 21 breast cancers showed that this same TpC mutational signature was present across the whole genome of the samples, and also that this single mutational signature accounted for up to 90% of all detected mutations across the cancer genome for some patients (Nik-Zainal et al., 2012). Finally, these mutations were found in clusters and were highly strand co-ordinated, with many of the substitutions characterized by the same distinctive C>T transitions at TpC dinucleotides as was found in Stephens *et al* and Greenman *et al* (Nik-Zainal et al., 2012). A 'rainfall plot' was constructed, showing inter-mutational distance against mutation number. This revealed a general macro-mutational signature across much of the genome of one of the breast cancer samples with a central hyper-mutated micro-mutational signature, shown as the lower red coloured cluster of dots on the diagram (Figure 4). This

localised hypermutation was termed 'Kataegis' (Greek for "thunderstorm"), and the study concluded that the APOBEC family was likely responsible for this phenomenon (Nik-Zainal et al., 2012).

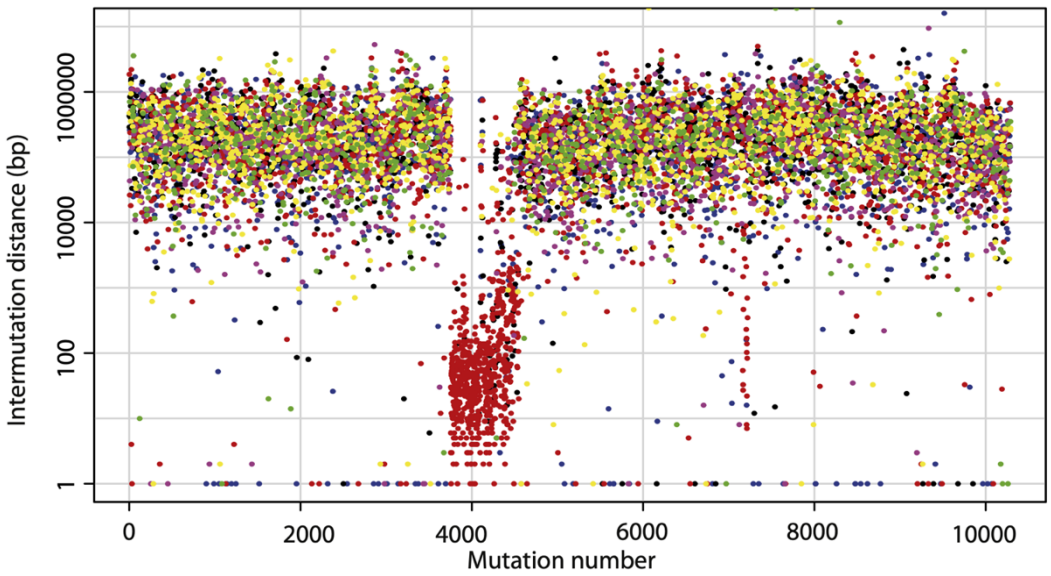

Figure 4 – A rainfall plot showing the macro and micro hyper-mutational signature patterns identified in a PD4107a breast cancer cell line with a BRCA1 germline mutation. Mutations are ordered on the x axis from the first variant on the short arm of chromosome 1 to the last variant on the long arm of chromosome X. The distance between each mutation and the one prior to it (the inter-mutation distance) is plotted on the vertical axis on a log scale. Adapted from Nik-Zainal et al, 2012.

Initially the general consensus among many different studies on the APOBEC enzymes placed a relatively equal level of blame for the cancer-causing members of the enzyme family on A3A and A3B. For example, *Burns et al. 2013* analysed gene expression data and mutation patterns from over 4,800 exomes and 1,000,000 somatic mutations and concluded that A3B was likely the cause of a large fraction of

dispersed and clustered cytosine mutations in six different types of cancer, including bladder cancer (Burns, Temiz, et al., 2013). The seminal paper by *Alexandrov et al 2013* linked the presence of the APOBEC mutational signatures to the action of APOBEC1, A3A or A3B over any other member of the APOBEC family (Alexandrov et al., 2013), and *Taylor et al, 2013* found that mutation data derived from yeast revealed that A3A and A3B were the only deaminases whose target specificity matches the mutational and kataegic patterns found in breast cancer, concluding that they are both very likely to be involved in breast cancer hypermutation. This was in agreement with another study by *Burns et al 2013* (Burns, Lackey, et al., 2013) which came to the same conclusion.

It wasn't until 2015 that this consensus was challenged in a study by *Chan et al*. They definitively showed that the mutational signatures left behind by A3A and A3B, which were previously thought to be too similar to reliably distinguish from one another, were in fact statistically distinguishable. The study also found that in five different cancer types there were more than 11 times more A3A-like mutations than A3B-like mutations, and that BRCA breast cancer patients with a relatively common APOBEC3B germline deletion still had A3A mutational signatures present, as well as at levels higher than that of patients without the A3B deletion confirmed by Fisher's exact tests (Chan et al., 2015). Taken together their results would suggest that, contrary to previous data, A3A is in fact the more predominant mutagenic deaminase in cancers. This alternative hypothesis was significantly backed up later in 2019 in

Cortez et al's paper which found that the A3B targeting shRNA that was used in *Burns et al's* 2013 seminal study to claim that A3B activity is the main culprit behind cytidine deamination (Burns, Lackey, et al., 2013), actually also decreased endogenous A3A mRNA levels by up to 13.8-fold. This means that *Burns et al's* study is fundamentally flawed, as there is no way of knowing from their data whether the 30-70% reduction in genomic uracil loads (APOBEC-induced mutations) was truly to do with the reduction in expression of A3B, or if it was actually down to a reduction in expression of A3A. By selectively depleting A3A and A3B with different shRNA's, *Cortez et al* showed that the levels of APOBEC-induced mutations present in BRCA cell lines linearly correlates with A3A expression, but not with A3B expression. In fact, in the BT474 APOBEC-mutated cell line in which the A3B mRNA transcript levels were on average 243-fold higher than A3A mRNA transcript levels, knockdown of A3A expression eliminated cytidine deaminase activity whilst knockdown of A3B expression had no effect, clearly showing that A3A is the dominant cytidine deaminase in these BT474 cells. Similar results were seen in the CAMA-1 and MDA-MB-453 cell lines, where their cytidine deaminase activity was almost entirely dependent on A3A expression (Cortez et al., 2019).

Taken together, these recent papers give a solid argument for the focus of research to be turned towards A3A, rather than A3B. It remains contentious as to where endogenous A3A localises, with some studies claiming it remains in the cytoplasm and is thus non-genotoxic (Land et al., 2013) and others that it has a pan-cellular

localisation and so would have access to cellular DNA (Bogerd et al., 2006; Mussil et al., 2013). However more recent papers such as that from *Chan et al 2015* and *Cortez et al 2019* give solid evidence to suggest that not only does A3A have direct access to genomic DNA, but through their novel methodologies they have also shown that A3A is a significantly more potent cellular DNA deaminase than A3B.

Cause and effect – mutation mechanism in detail

Despite their potent innate anti-viral activity, studies such as *Nik-Zainal et al* and others are more clearly coming to show that APOBEC's can cause somatic mutations, likely contributing to cell mutagenesis and formation of cancers within certain types of tissues.

If the immediate product of A3A deamination is uracils and DNA uridines base-pair like thymidines and template the insertion of adenosines, then it would follow that the uracil products of A3A action would amplify by PCR to result in C/G to T/A transition mutations. One study used 3D-PCR to detect uridine-containing DNA intermediates in HEK-293T cells transfected with a reporter plasmid and either A3A or A3AE72A (a catalytically inactive mutant of A3A) expression plasmids, whilst in the presence of UGI (a uracil DNA glycosylase inhibitor). The 3D-PCR technique is based on the principle that DNA with fewer interstrand hydrogen bonds (i.e. more A/T rich regions, which under normal conditions are on average scarce and scattered in the human genome (Woynarowski et al., 2001)) will preferentially amplify at lower

denaturation temperatures. The study also used UGI to inhibit the UNG2 DNA glycosylase which would normally function to repair genomic uracils. This allows for the detection of otherwise short-lived uridine-containing DNA intermediates.

Their results show that at higher denaturation temperatures, PCR products were seen in both the A3A and A3AE72A expressing cells, but at lower temperatures PCR products were only seen in the A3A expressing cells, suggesting that A3A does edit transfected plasmid DNA, leaving behind uracil-containing DNA intermediates (Stenglein et al., 2010).

Other studies have reported increased presence of DNA damage response signalling in response to upregulated A3A expression in various cell lines. Increased A3A expression resulted in an increase in ɣH2AX (indicative of DSB's being present) and ATR (indicative of a high level of ssDNA being present and protects against DNA replication stress) in Human U2OS and HepaRG cell lines (Green et al., 2016; Landry et al., 2011), with another study showing inhibition of ATR leading to replication catastrophe in cells expressing high levels of A3A (Buisson et al., 2017).

Accelerating mutation formation

More recently, there is strengthening evidence and support for the 'just right' hypothesis for tumour diversity (Swanton et al., 2015). This states that if there is too little diversity the tumour will fail to respond and adapt to selection pressures, and too much diversity will lead to genomic instability and cell lethality. Therefore, a 'just right sweet spot' of diversity may exist, where there is just enough genetic diversity to overcome the selection pressures that the tumours face, such as cancer treatment drugs, whilst not diverging so much that they fail to properly propagate genetic information to their daughter cells (Figure 5). Cancer cells may also have to attenuate APOBEC mutagenesis and enhance DNA repair mechanisms to ensure cell survival (Venkatesan et al., 2018). Given the mutation rates that APOBEC enzymes can cause, it is plausible that they may be able to foster this 'just right' level of diversity within tumour cell populations given the right circumstances. For example, one study looked at 100 surgically

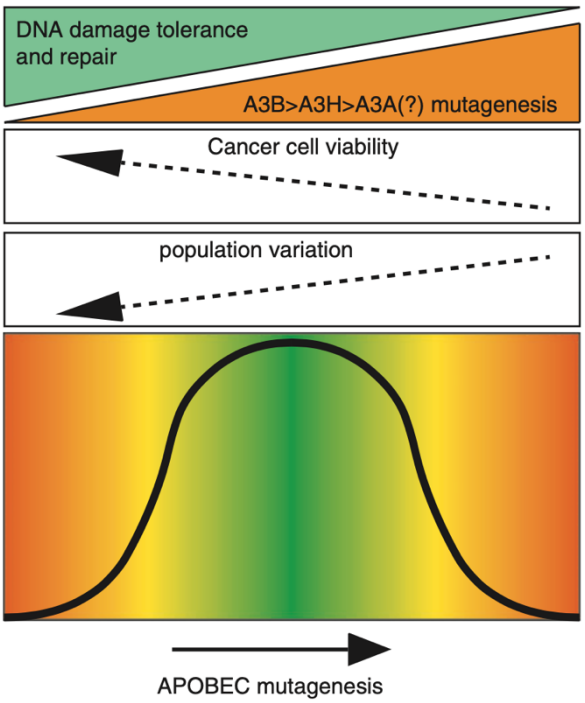

Figure 5 – Diagram illustrating how as APOBEC mutagenesis increases, the chance of lethal levels of mutagenesis within the cell populations also increases. However, a trade-off between lethal mutagenesis and cell population variation can create a optimal range where APOBEC mutagenesis can increase population fitness. Adapted from Venkatesan et al, 2018.

resected and untreated small-cell lung cancers and found a strong correlation between frequencies of APOBEC mutational signatures and overall numbers of sub-clonal mutations (Jamal-Hanjani et al., 2017), and another study found that this kind of increased intra-tumour heterogeneity was shown to correlate with a shorter progression-free survival in some cancers (Andor et al., 2016).

The formation of a 'just right' state for cancer cells with an APOBEC3 mutational load is unlikely to result solely from the presence of APOBEC3-induced mutations – APOBEC3's require the presence of an ssDNA substrate which under most conditions is only present in very low quantities in any human cell. Rather, it's more likely that a number of different circumstances come together in such a way that can promote the long-term survival of these tumours.

Take urothelial carcinomas, which most often originate in the bladder (Mayo Clinic, n.d.). Bladder cancers are amongst the most mutated types of cancer overall (Lawrence et al., 2013), are associated with high morbidity and mortality rates (Kamat et al., 2016) and show the second highest levels of APOBEC-induced mutations of any cancer, only behind cervical cancer (Roberts et al., 2013). They are currently and commonly treated with cisplatin (Figure 6) or a combination of cisplatin with other chemotherapies (e.g. gemcitabine) or invasive procedures (e.g. resection) in muscle-invasive cases (Kamat et al., 2016) yet the prognosis generally remains

poor for the patient if not diagnosed early on during disease progression (Stenzl et al., 2012).

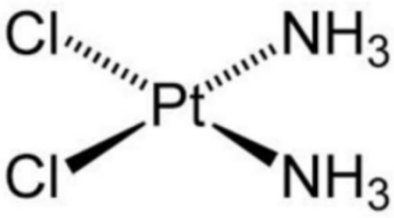

Figure 6 - chemical structure of cisplatin. Adapted from Dasari & Tchounwou, 2014.

The mechanism behind the action of cisplatin is based on its chloride atoms being displaced by water molecules, creating a hydrolysed product and potent electrophile which allows it to react with nitrogen donor atoms on nucleic acids. In the context of DNA, cisplatin bonds to purine residues causing several different variations of intra-strand and inter-strand crosslinks. The most common variation is the 1,2-intra-strand d(GpG) adduct, accounting for 90% of all intra-strand adducts attributed to the action of cisplatin (Dasari & Tchounwou, 2014). Depending on exactly where the adducts are formed and the form in which they take, they can cause a significant change in the physical structure and shape of the DNA which is recognised by a variety of proteins involved in a number of different DNA repair or cell-cytotoxicity mechanisms. For example, since intra-strand crosslinks are the most common type of DNA lesion caused by cis-platin, they are primarily repaired by the NER DNA repair system, but cisplatin related damage can also recruit the TCR and HR repair systems

and all of their related proteins (Basu & Krishnamurthy, 2010; Borst et al., 2008; Damsma et al., 2007).

Cisplatin has historically been used to treat a wide variety of cancers for decades, but the more subtle interactions that it may have in cancers with specific mutational signatures could alter its effectiveness in treating the patient. These types of interactions couldn't have previously been identified and studied before the drug was cemented as one of the most used in the world to treat many types of cancers, as the idea of mutational signatures wasn't properly fleshed out and catalogued until 2013 in *Alexandrov et al's* seminal paper.

In 2016 the idea of a relationship between the presence of mutational signatures and the use of certain chemotherapies was investigated in one study involving urothelial carcinomas. The study by *Faltas et al* collected urothelial carcinoma tumour samples from a number of patients before and after cisplatin-based chemotherapy, with a smaller subset of these matched to be from the same parental tumour (Faltas et al., 2016). This gave an insight into the common genetic mutations that cancerous cells can go through as a result of the direct or indirect effects of such chemotherapy. Through whole exome sequencing they found that only 28.4% of mutations were shared between pre-chemotherapy and post-chemotherapy samples, suggesting that there could be some mutational action at play occurring after initial application

of cisplatin chemotherapy to the patients which results in increased clonality after the fact without fully killing off the tumour cells.

Upon further examination they found significant enrichment of C>G mutations at the characteristic TCW motifs (where W is either A or T) that the APOBEC3's tend to act on in the post-chemotherapy tumour samples, as well as the presence of mutational signatures that looked remarkably similar to Sanger signatures 2 and 13 – highly indicative of APOBEC mutations (COSMIC, 2015). Further analysis of the favoured motif context of the mutations suggested that, specifically, A3A (and to a lesser extent A3B) could be the driving and dominant APOBEC3 culprit behind the majority of the mutations found in the post-chemotherapy tumours. Bearing in mind that only 28.4% of the mutations were shared between pre-chemotherapy and post-chemotherapy samples, these data suggest that the cisplatin-based treatment used here could be modifying or enhancing the effect that APOBEC3 enzymes have on the genome of the cancer cells, providing them with a higher amount of inter-clonal genetic diversity and therefore also the chance for surviving tumour cells to become resistant to the therapy and thus also become recurrent in the future. For example, it's possible that the ssDNA generated during the repair of cisplatin-induced DNA breaks may act as a substrate for A3A, therefore making it more active than normal which would result in the A3A-specific mutational signatures seen in the post-chemotherapy tumours. However, without further work it is impossible to say whether or not the remaining 71.6% of mutations that were not shared were present

because of ongoing APOBEC activity during the chemotherapy, or whether the chemotherapy simply selected for minor sub-clones that existed before the treatment.

Taken together, the extensive presence of A3A-induced mutations in post-chemotherapy, metastatic urothelial carcinoma tumours shows that A3A could have the capability to not simply induce one time 'fire and forget' somatic mutations that might contribute to the formation of the initial cancer, but can could play a significant role in the evolution of advanced cancers. This can occur through the promotion of the clonal expansion of chemotherapy-resistant and possibly metastatic clones, perhaps due to an increased level of ssDNA made available to A3A as a result of the action of the cisplatin-based chemotherapy that is initially applied to treat the cancer. However, functional studies are needed in order to properly test this hypothesis.

Project outline

The culmination of existing work has highlighted the potential danger that the family of APOBEC enzymes, namely A3A, could pose to the continued effectiveness of platinum-based chemotherapy. Since *Alexandrov et al's* paper detailing the discovery of mutational signatures and that two of these signatures can be attributed to one of the sub-families of the APOBEC enzymes, there have been a large number of studies that look into which member of this sub-family is the most likely to contribute to these mutational signatures and cause the most significant damage to the human genome. It is hard to ignore *Cortez et al's 2019* discovery that the *Burns et al's 2013* seminal paper claiming that A3B is the main APOBEC culprit is based on data that can no longer be held as truly reliable (which was cited by at least 604 other papers at the time of writing). *Cortez et al* puts forward a convincing argument that it is indeed A3A that causes the most significant damage to the human genome, bringing together a wealth of new information to back this up.

Studies such as that by *Faltas et al* provide a basis for the idea that cisplatin could be providing a novel route for bladder cancer cells into significantly higher levels of inter-clonality and thus also into a higher chance of recurring in the future than was ever thought to be the case. However, without a sample of bladder cancer cells with a full knockout of A3A (and ideally A3B) in order to A/B test and compare the timescales for development of resistance to cisplatin to a sample of WT bladder cancer cells, it remains difficult for this hypothesis to stand on solid ground. It is

entirely possible that the cisplatin treatment is simply selecting for minor pre-existing subclones that have higher levels of APOBEC3 expression which existed before the rounds of cisplatin treatment, rather than actually contributing to some novel method of mutagenesis such as one that provides cells which have normal levels of APOBEC3 expression significantly more ssDNA substrate on which to act. If this is the case, then inhibition of APOBEC3 alongside cisplatin treatment would not be a viable method to prevent resistance to chemotherapy. It is also possible that both of these mechanisms may be at play, as has been shown to happen in EGFR-mutant non-small-cell lung cancers to EGFR inhibitors, where drug-resistant cancer cells were shown to both pre-exist and evolve from drug-tolerant cells (Hata et al., 2016).

Given the fact that at present there is no other drug that has similar levels of efficacy to cisplatin on such a wide variety of cancers (Dasari & Tchounwou, 2014), it is important to consider how best to enhance the activity and reduce the negative effects of the use of cisplatin in the interim period before a newer, more appropriate treatment option inevitably becomes available. Whilst still an endeavour, modulation of existing tried and tested treatment options with other known compounds will likely take less time and expense to research, licence and produce than the development of an entirely new treatment option.

The aim of this project was to functionally test whether A3A plays a role in chemotherapy resistance through knockout experimentation. No study involving the

full knockout of A3A from a cell line has been published as of yet. Given the high relative levels of APOBEC mutations found in bladder cancer cells and the mounting evidence that APOBEC mutations are present in a large number of different cancers (albeit in varying levels), it would follow that bladder cancer would form a good base on which to begin testing if there is a relationship between the presence of the APOBEC signatures and prolonged effectiveness of cisplatin treatment.

The possibility for this type of relationship was due to be studied by creating a number of qPCR verifiable clonal populations of wild-type BFTC-905 cells and their A3A KO equivalents using CRISPR-Cas9 and cell culture techniques such as single cell cloning, and then performing MTT cis-platin drug sensitivity assays over a number of weeks. This would allow for a comparison of the length of time it takes for the A3A KO cells to develop resistance to cisplatin compared to wild-type equivalents (if at all). The results of this study could give a use case for using APOBEC3 inhibitors alongside traditional chemotherapy treatment to enhance the recovery prospects for patients with cancers that have evidence of APOBEC mutational signatures. The idea to inhibit APOBEC3's may be seen as detrimental due to their regular anti-viral activities within the cell, but it has been noted by others that there is likely a therapeutic window that exists for APOBEC3 inhibition, where "benefits will greatly outweigh the potential detriments of inhibiting only one component of the body's expansive innate immune system" (Olson et al., 2018).

I had taken over the work of a previous MSc-R student who had utilised CRISPR-Cas9 and transfection techniques to successfully create a homozygous A3A knockout clone from the urothelial carcinoma cell line, BFTC-905 (Dooner, 2019). My plan was to focus on the homozygous A3A BFTC-905 knockout; creating, screening and verifying further clones from the pool of cells that the previous MSc-R student had produced. I would then have performed the MTT cisplatin drug sensitivity assays as mentioned above, followed by data analysis of the results. However, the extraordinary circumstances of COVID-19 and the resultant lockdown period led to me not having access to the lab just after I had developed and verified three parental wild-type BFTC-905 cell lines and two additional A3A KO BFTC-905 clones and performed preliminary growth tests, but before I could begin MTT drug sensitivity assays. My results and data are therefore limited compared to what should have been achieved during the time period.

Materials & Methods

Tissue culture

The human bladder cancer cell line BFTC-905 was maintained in IMDM with added L-glutamine and 25mM HEPES supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were cultured at 37°C and 5% CO_2 in an incubator and grown to 80-90% confluency before being passaged.

Cells were frozen down in 1mL aliquots in their normal growth medium and supplemented with 10% DMSO for longer term storage. They were initially stored in a -80°C freezer, before being moved into a nitrogen freezer after 2-5 days for indefinite storage. Revival of nitrogen-frozen cells was completed by rapid thawing in a 37°C water bath followed by dilution into 9mL of fresh warmed media and a spin down and resuspension in a further 5mL of IMDM, in order to remove any remaining DMSO.

Once cells had been grown to a high enough volume and confluency, cell pellets were made at 500 RCF for 10 minutes and frozen in a -80°C freezer for later DNA extraction.

Single cell cloning

Cells were grown to 80-90% confluency before being serially diluted to a final concentration of 5 cells/mL. They were then plated into 96 well plates at 100µL per well to give 0.5 cells per well (one cell in every other well) in order to allow clonal

colonies to form. These plates were then screened for the presence of successful colonies under a microscope, and actively growing successful colonies were passaged into increasingly larger flasks after becoming confluent and given unique names before being frozen in growth medium containing 10% DMSO in liquid nitrogen as per the method above.

DNA extraction

One of two methods of DNA extraction were used depending on the available stock at the time:

- A QIAGEN QIAamp DNA Mini Kit (Cat. No 51306) was used to extract DNA from cell pellets, completed according to the manufacture's guidance.
- A KAPA Express Extract kit (Cat No. KK7100) was used to extract DNA from cell pellets, according to the manufacturer's guidance. This method was followed by an ethanol DNA precipitation to increase DNA purity.

Extracted DNA was kept frozen at -20°C until required for use in PCR.

Ethanol DNA precipitation

0.1 vols of 3M Sodium acetate and 3 vols ice cold 100% ethanol was added to the extracted DNA (from the KAPA Express Extract Kit method above) and vortexed to mix thoroughly. This solution was precipitated at -20°C for 1 hour and then centrifuged at full speed using a microcentrifuge at 4°C for 30 mins. The resulting

pellet was washed twice with 0.5mL ice cold 75% Ethanol, spinning at full speed at 4°C for 10 mins each time. The ethanol was then removed, and the pellet spun for 10 seconds at full speed to remove any trace amount of Ethanol. Finally, the pellet was air dried and resuspended in an appropriate volume of nuclease free water depending on the size of the pellet.

RNA extraction

A Monarch Total RNA Miniprep Kit (Cat. No T2010S) was used to extract RNA from cell pellets, completed according to the manufacturer's guidance. Extracted RNA was converted into cDNA for use in qPCR, with RNA stored at -80°C and cDNA stored at -20°C.

cDNA Synthesis

A New England Biolabs LunaScript RT SuperMix Kit (Cat. No E3010) was used to synthesise cDNA from RNA for use in a two-step RT-qPCR workflow, completed according to the manufacture's guidance. A concentration of 1,000ng/µL cDNA was initially achieved, then synthesised cDNA was diluted to a concentration of 5ng/µL for later use in qPCR, and stored at -20°C.

PCR

PCR was completed using a KAPA HiFi HotStart ReadyMix PCR Kit (Kit Code: KK2601) according to the manufacturer's instructions. Forward and reverse A3A primers with codes TRF497 and TRF498 were used (see Table 1 below).

Agarose gel & PCR genotyping

1% agarose gels were prepared with either 0.75g agarose to 75mL 1X TAE buffer for smaller running gels, or 1g agarose to 100mL 1X TAE buffer for larger running gels if more wells were needed. The 1% agarose mixture was microwave heated until the agarose had dissolved. When cooled down but still warm to the touch, ethidium bromide solution was added at 2.5µL ethidium bromide per 50mL agarose gel solution and the solution thoroughly mixed. This gel mixture was then poured into the appropriately sized gel cast with a gel comb and allowed to cool and set before the comb was removed.

After 6X blue/purple loading dye was added and mixed with PCR products to make a final solution of 1X concentration, the set gel was placed into the gel tank and covered with 1X TAE buffer. PCR products mixed with loading dye were added to the gel wells alongside a Thermo 1Kb Plus DNA ladder (Cat. No 10787018) and electrophoresis was conducted at 90V for 30 minutes. The following PCR primers were used:

Primer code	Primer sequence	Description
TRF497	TGAGCTCACACCAGAACCAC	A3A forward
TRF498	TAGAGCCCAGAGAAGGTCCC	A3A reverse

Table 1 – A3A genotyping PCR primer codes and functions

qPCR

An Applied Biosystems PowerUp SYBR Green Master Mix (Cat. No A25741) was used in the two-step qPCR workflow, according to the manufacturer's guidance, set up for a 10µL total reaction. Wells were set up in duplicate for each sample and no-template controls were also set up in duplicate for each sample in order to identify any contamination. The following primers were used:

Primer code	Primer sequence	Description
TRF566	GAGAAGGGACAAGCACATGG	A3A forward
TRF567	TGGATCCATCAAGTGTCTGG	A3A reverse
TRF568	GACCCTTTGGTCCTTCGAC	A3B forward
TRF569	GCACAGCCCCAGGAGAAG	A3B reverse
TRF589	CCCATGACTCCCATGACC	TBP forward
TRF590	TTTACAACCAAGATTCACTGTGG	TBP reverse
TRF596	GTCATCCATGACAACTTTGGTA	GAPDH forward
TRF597	GGATGATGTTCTGGAGAGC	GAPDH reverse

Table 2 - qPCR primer codes and functions

Transfections

FuGENE HD transfection reagent (Cat No. E2311) was used to transfect clonal pre-flippase (cells in which the PGK-Puro-delta TK selection cassette was still present in intron 1 of A3A (figure 7)) A3A KO cell lines with a Flippase-GFP plasmid and an empty GFP plasmid as a negative control according to the manufacturer's guidance. The concentration of the Flippase-GFP plasmid was determined using a UV spectrometer (Nanodrop) and had a concentration of 677.3ng/µL. 26µg of DNA was needed for the transfection according to the manufacturer's guidance, therefore 26,000ng/677.3ng = 38.38µL of Flippase-GFP plasmid was added to 1202.62µL of optimem medium to make a total of 1241µL transfection solution (as required by the manufacturers' guidance, indicated for the three T25 flasks). Transfections were later checked for efficacy by viewing under a LumaScope to check for the presence of GFP within the cells.

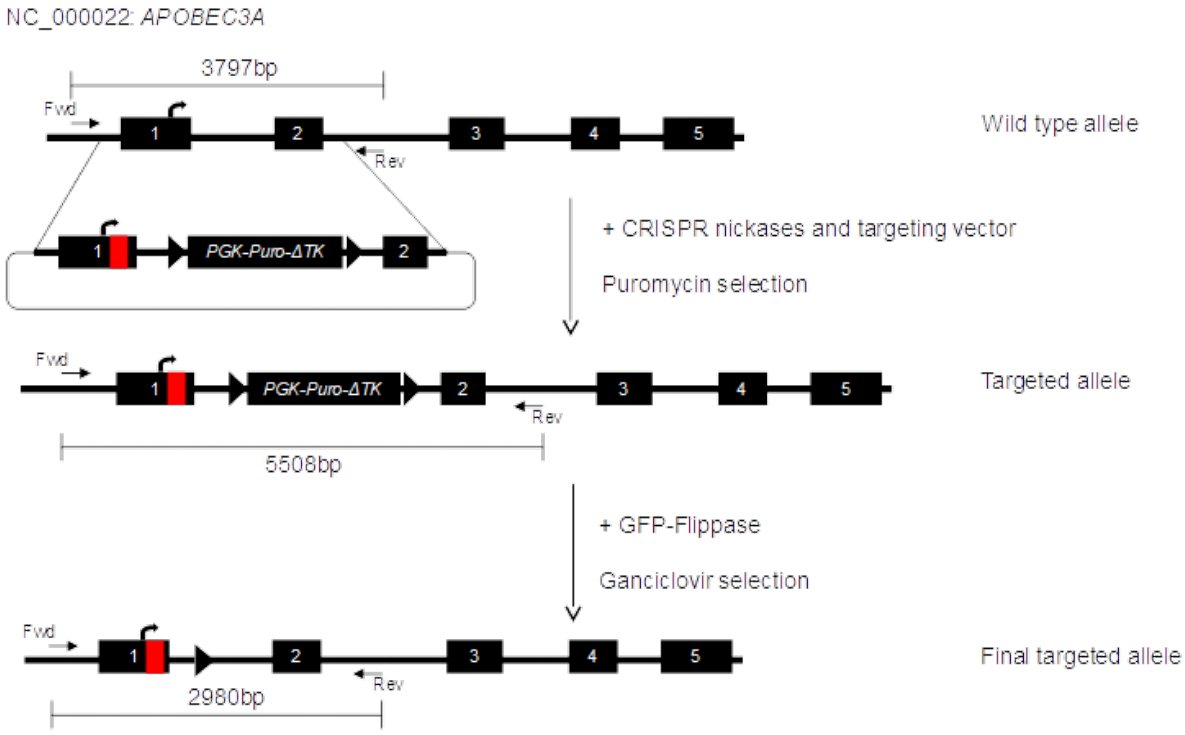

Figure 7 -This figure illustrates the A3A targeting vector used by the previous student in this study. The vector contains a puromycin resistance gene and an HSV-TK gene which together form the selection cassette. The puromycin resistance gene allows for positive selection after inserting the vector, and the HSV-TK gene confers ganciclovir sensitivity which allows for negative selection after the vector has been removed by flippase (from the Flippase-GFP plasmids) at the FRT sites, marked as triangles. The red section in intron 1 of the vector represents a stop codon which, after inserted into the WT allele, knocks out A3A giving A3A KO cells. The estimated length of the wild type A3A allele is 3797bp, the targeted allele is 5508bp and the final targeted allele with the selection cassette removed is 2980bp. The difference in lengths allows for genotyping of cell populations to ensure effective targeting at each stage.

Successfully transfected cells were selected for using ganciclovir to remove unsuccessfully transfected cells still containing the selection cassette and therefore also the HSV-TK ganciclovir susceptibility conferring gene. Transfected C9, C28 and KO 2.1 cells were plated in 10mL of media with 5µL of 20µM ganciclovir at a low density on a Friday and left to grow over the weekend.

MTT assays

For each cell line, five 96 well plates were set up, each with 6 rows with a length of 10 wells of cell concentrations of:

- 500 cells per well
- 1,500 cells per well
- 2,000 cells per well
- 3,000 cells per well
- 4,000 cells per well
- 5,000 cells per well

Surrounding these rows were wells filled with IMDM media to minimise evaporation of media within the central wells containing the cells. Each of the five plates for every cell line were grouped and allocated to be read on a plate reader on each day out of a consecutive period of five days. This allows growth data to be obtained every day for five days for every concentration of cell listed above, for all cell lines. Every day, MTT was added to every row in each plate for each cell line allocated to the following day and left to incubate overnight. The next day SDS was added to that days set of plates and read to measure growth rates. MTT was then added to the following days set of plates and the process repeated until all day's plates had been processed.

Results

PCR & genotyping

PCR and subsequent genotyping were carried out on the parental WT, A3A KO pre-Flippase and A3A KO post-Flippase + selection cell lines in order to verify each populations' A3A status (Figure 8). This is possible due to the different molecular weights of the WT A3A allele (3797bp), the targeted A3A allele with the selection cassette inserted (5508bp) and the final targeted A3A allele with the selection cassette removed (2980bp) (Figure 7).

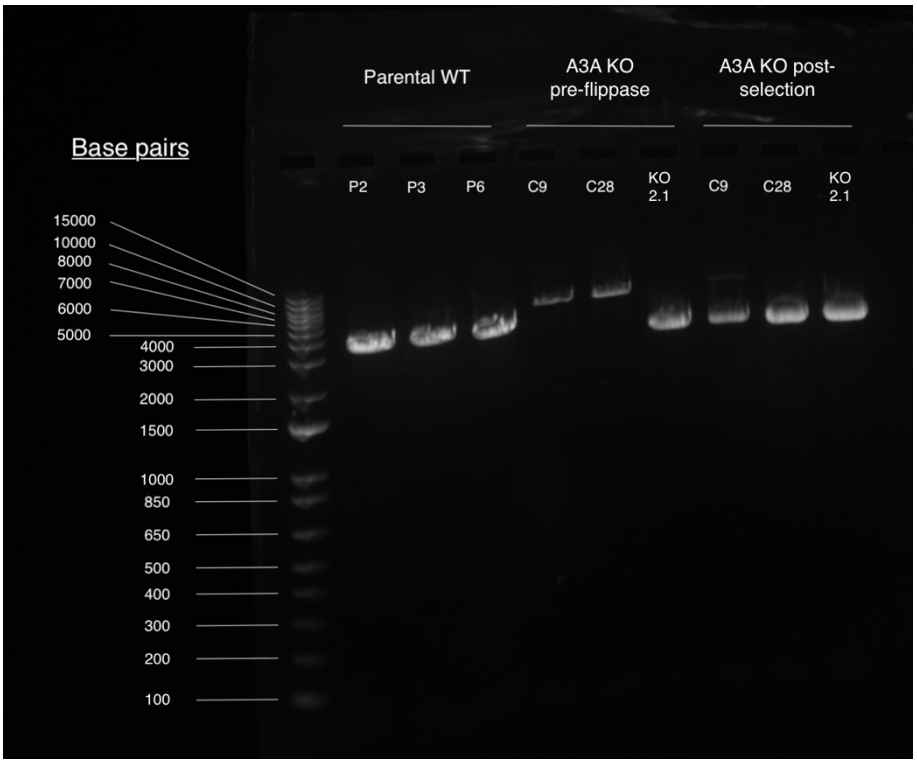

Figure 8 - Genotyping analysis of WT, pre-Flippase and post-Flippase + selection cell lines.

The A3A KO pre-Flippase KO 2.1 cell line's band is the same molecular weight as the band for the post-Flippase and selection of the same cell line as, unknowingly at the

time, the pre-Flippase KO 2.1 cell line had already had its selection cassette removed by the previous MSc-R student. Ideally a repeat of this gel would have been completed to improve its clarity and reduce the ambiguity of the image as this was a first attempt, however this was not possible due to lockdown restrictions.

Growth curves (MTT assays)

Cell viability (MTT) assays were performed over 5 days to determine the optimum plating densities for drug sensitivity assays and to generate growth curves for each WT and A3A KO clone. This would reveal the optimum starting concentration for each of the cell lines taken forward to the MTT drug sensitivity assays in the future. This method also allows for the comparison of the growth characteristics of the cells not only to each other over time, but also to each other after the removal of the selection cassette and the addition of any drug. Please note that due to the COVID-19 lockdown, I was only able to generate data for two A3A KO clones and none of the parental (WT) clones.

The resultant data showed similar growth rates for all cell lines, growing largely linearly before beginning to peak and level off upon reaching day 5:

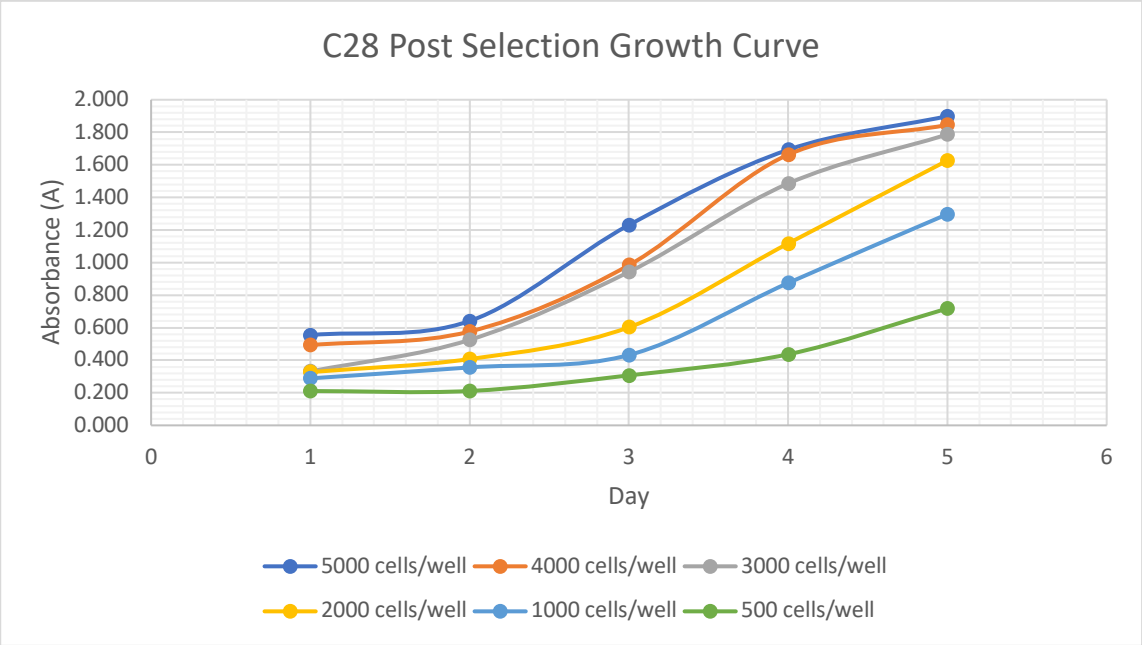

Figure 9 – Preliminary MTT growth assay for the C28 cell line at various starting concentrations over a five-day period.

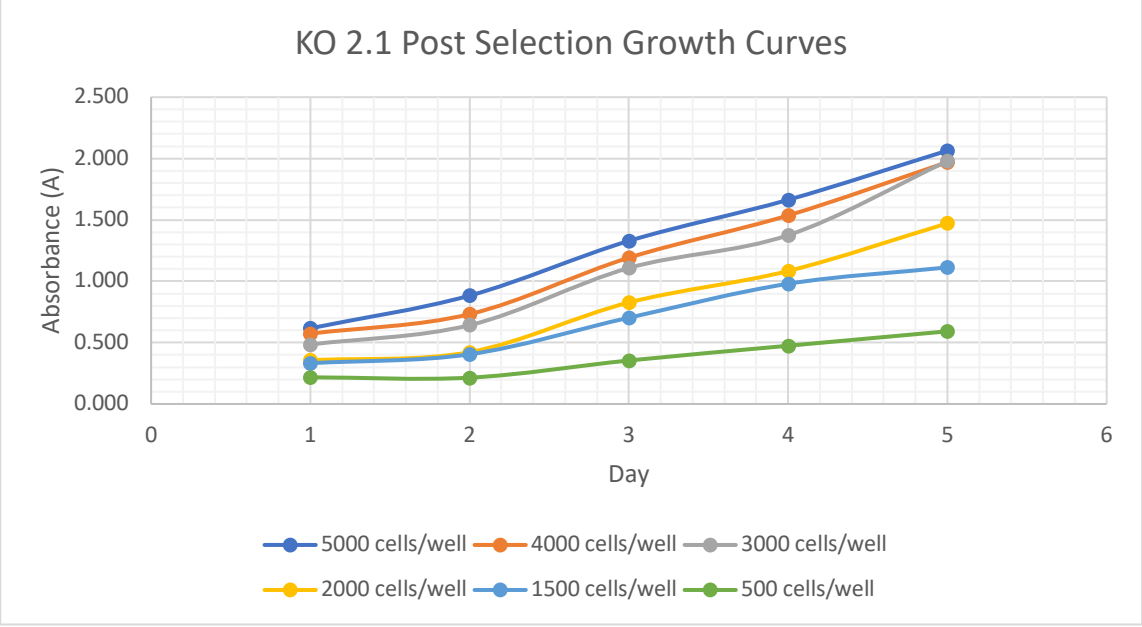

Figure 10 - Preliminary MTT growth assay for the KO 2.1 cell line at various starting concentrations over a five-day period.

FuGENE HD plasmid transfection

After growing an extensive stock of A3A KO SSC's, adding to the single existing A3A KO clone that the previous master's student had generated, the selection cassette had to be removed in order to mitigate any interference between the selection cassette and the surrounding genes, notably A3B.

The selection cassette also contains a ganciclovir susceptibility gene (Herpes Simplex Virus Thymidine Kinase - **HSV-TK**) which confers ganciclovir sensitivity. By using flippase to remove the selection cassette and therefore also the HSV-TK gene, it becomes possible to select for the cells that have had the cassette successfully removed by plating the resultant cell populations out with ganciclovir. The required cells that have had the cassette and the HSV-TK removed will survive the ganciclovir treatment, whilst the cells that have had an unsuccessful transfection die off upon inoculation with ganciclovir. A GFP tag was included within the plasmid in order to visualise successful transfection and transcription of the GFP and therefore also the flippase within the cells.

Transfections were successful with GFP appearing within the nuclei of cells when viewed under a Luma-Scope, indicating high efficiency of transfection and expression of the Flippase-GFP fusion protein (Figure 11).

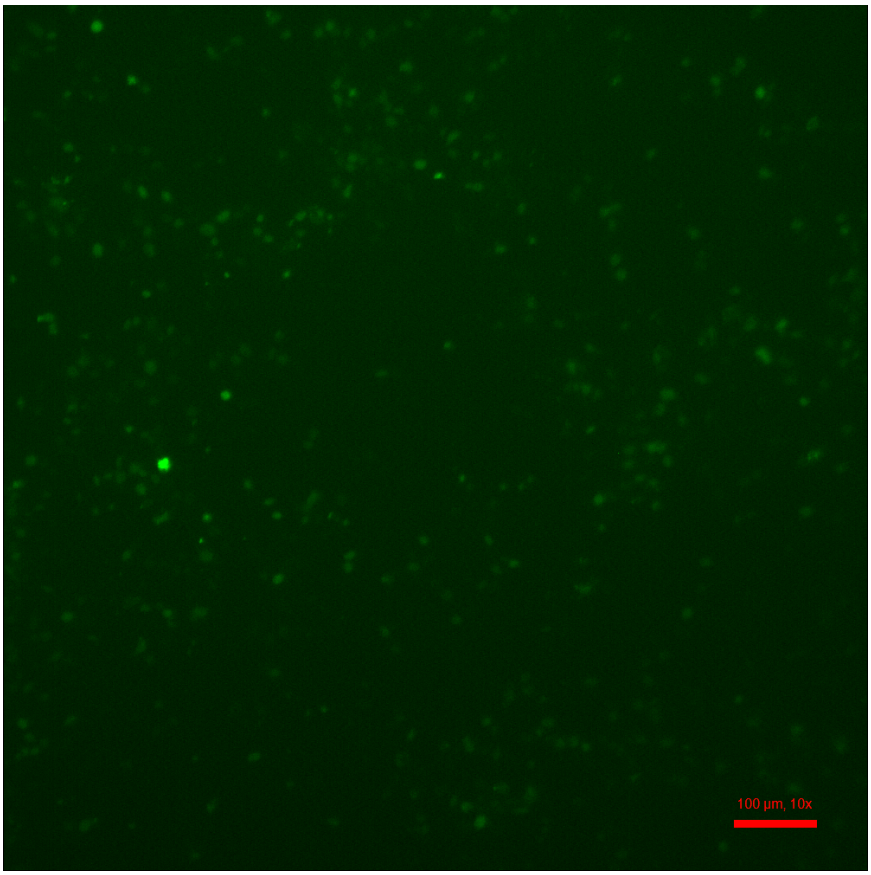

Figure 11 - Luma-Scope image of successful transfection of Flippase-GFP plasmid into C28 cells

Validation of A3A KO clones

Following the single cell cloning and Flippase transfection protocols, it was necessary to check that the resultant populations of cells were in fact truly A3A KO or WT cells. To do this, qPCR was utilised with APOBEC3A primers to check that there was no expression of the A3A gene in the A3A KO cell lines and normal levels of A3A expression in the WT cell lines. A3B primers where also used to check that knocking out A3A did not interfere with expression of A3B, along with primers for the housekeeping genes TBP and GAPDH to compare amplification data with (Table 2). Housekeeping genes are recognised as essential cell maintenance genes with relatively stable expression levels across sample or treatments groups. This means that expression data for other genes can be compared to the stably expressed housekeeping genes in order to normalise for any differences in input cDNA quantity (Turabelidze et al., 2010).

qPCR melt curve data clearly shows a strong peak for the parental cell lines that indicates the presence of A3A (Figure 14), and much weaker and non-specific peaks for the A3A KO cells, indicating a lack of A3A (Figure 15). These non-specific peaks are likely due to contamination as none are close to the melting temperature of A3A seen in the parental cell melt curves. The variation in expression of A3A and A3B seen in the parental cells could be due to the cells being in different stages of the cell cycle or at different levels of confluency when being harvested.

The raw qPCR amplification data also shows that the three parental cells lines carried forward all display similar levels of amplification of A3A relative to each other, as expected. Both of the suspected A3A knockouts show amplification only at very late cycles if at all, consistent with a total lack of A3A mRNA in these clones (Supplemental data – figures 16, 17, 18 & 19). qPCR data was converted into copy number data using standard curves and was then normalised to the TBP housekeeping gene. The resultant data shows a drastic reduction in the levels of A3A copy numbers seen in the A3A KO C28 and KO 2.1 cell lines compared to the parental cell lines (Figure 12). Importantly, the levels of A3B expression were similar in the parental and A3A KO cell lines (Figure 13), meaning that the A3A knockout process could not have significantly affected the expression of A3B in the C28 and KO 2.1 cell lines.

Please note that the final A3A knockout that was created and due to be carried forward, C9, was not able to be qPCR verified due to COVID-19 and not having access to the lab. Biological repeats should also have been completed in an attempt to remove or reduce the non-specific peaks seen in the data and to increase its reliability, however they were not able to be performed for the same reason.

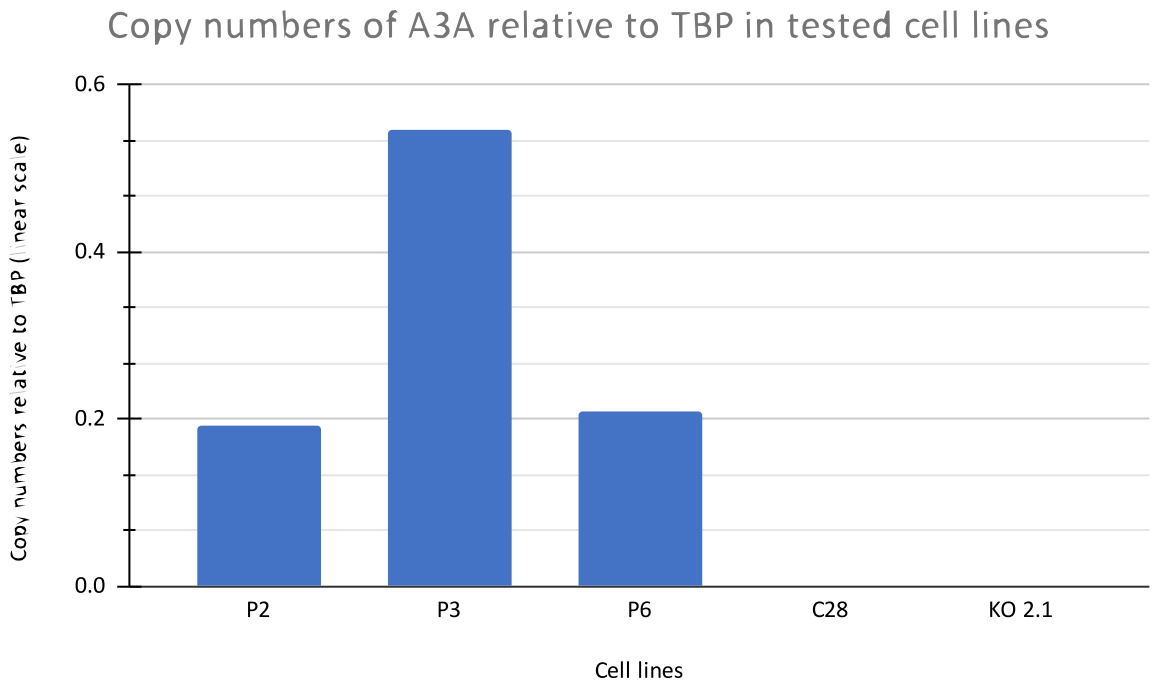

Figure 12 - Chart showing the decrease in copy numbers of A3A seen in the C28 & KO 2.1 cell lines compared to the parental cell lines on a linear scale.

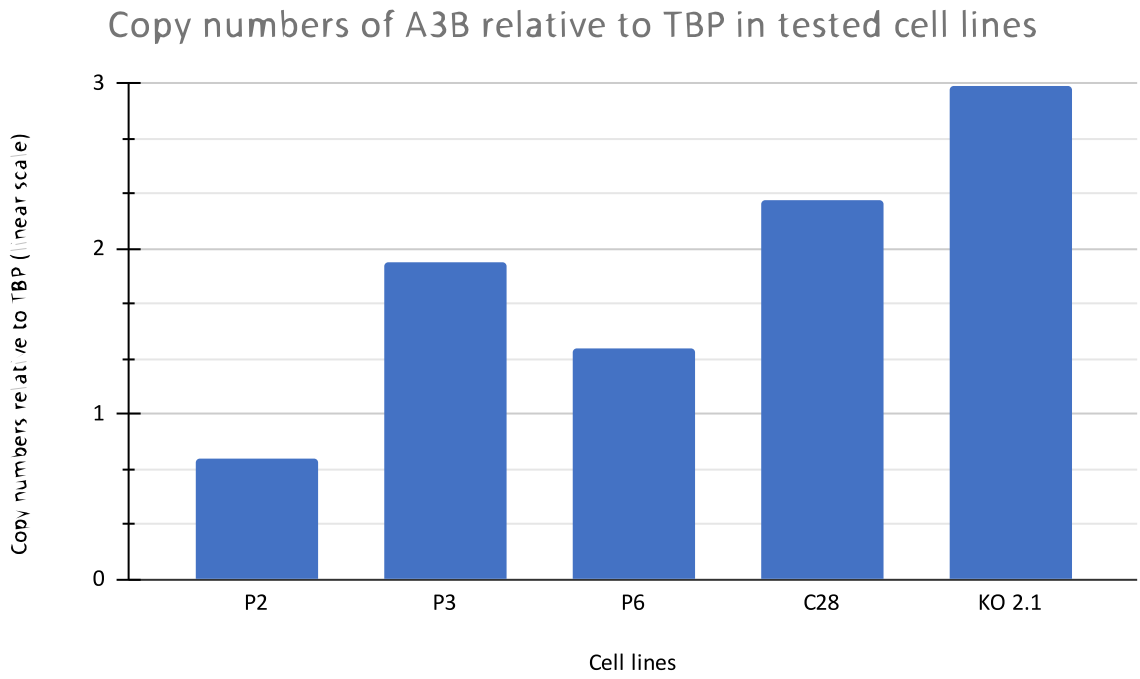

Figure 13 - Chart showing similar levels of copy numbers of A3B in the C28 & KO 2.1 cell lines compared to the parental cell lines on a linear scale. The similar levels of copy numbers seen here would suggest that the process of knocking out A3A from the C28 & KO 2.1 cell lines did not affect the expression of A3B.

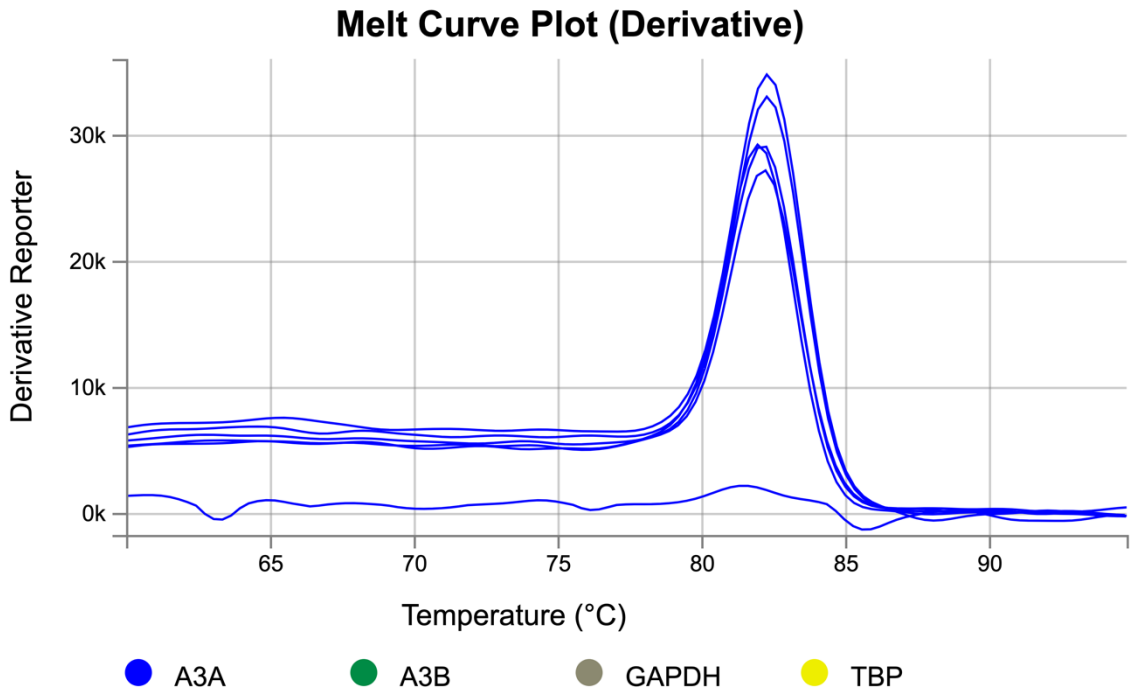

Figure 14 - qPCR melt curve for A3A primers in parental cells (P2, P3 & P6)

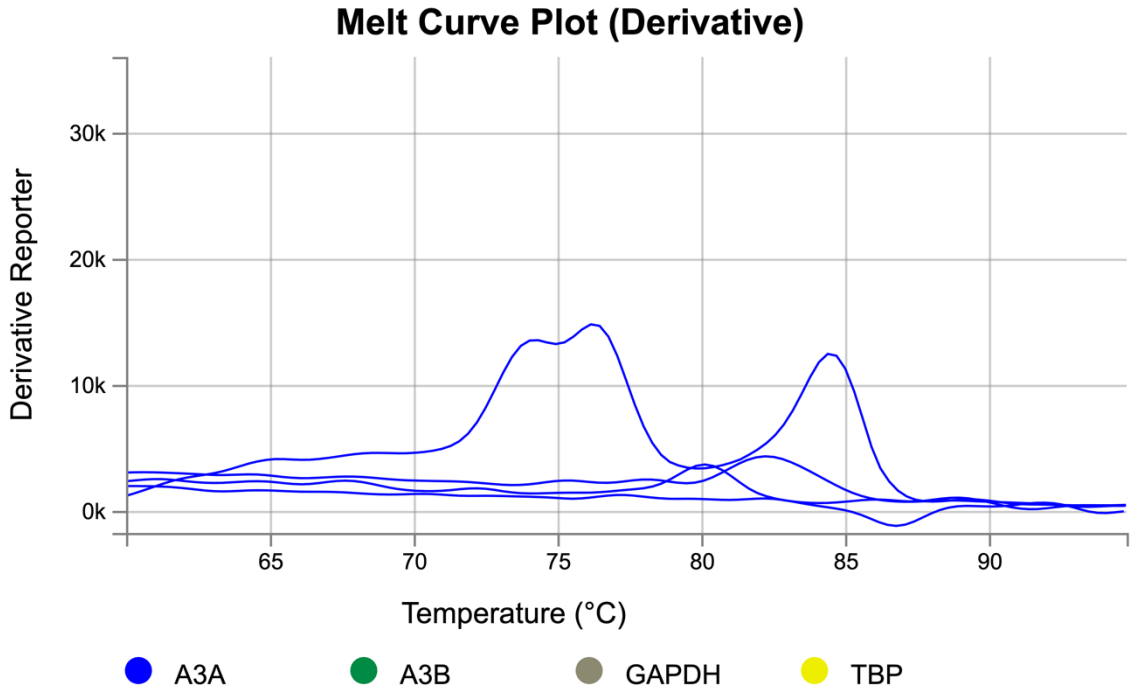

Figure 15 - qPCR melt curve for A3A primers in A3A KO cells (C28 & KO 2.1)

Discussion

Preface

Unfortunately, due to the COVID-19 outbreak I had to leave the university labs and was unable to complete MTT drug sensitivity assays on the qPCR-verified A3A KO and WT cell lines that I had cultured during my time at the lab, and to initiate the long-term drug-sensitisation protocol on these clones. When looking at the whole project, this is likely to have been the most important part and I am very disappointed to not have been able to complete it. It would likely have given me a large dataset to have worked with and analysed, potentially giving meaningful insights into how A3A may interact with bladder cancer cells, perhaps giving evidence for a viable use case for the use of A3A inhibitors alongside traditional cisplatin chemotherapy as a way to prolong the usefulness of the drug and preventing, or at the least delaying, the onset of cisplatin resistance within the bladder cancer cells.

However, I can still elaborate on what I would have expected to happen given the other ongoing research in the area. Therefore, this discussion will include information both on the experiments that I completed myself and what they mean, as well as what I think is most likely to have happened in the final stages of my project had I been able to complete it based on some relevant information on other studies in the same or similar areas.

Growth curves

I conducted 5-day growth curves on all but one of the cell lines carried forward (P2, P3, P6, C28 & KO 2.1). This was done so that when I would have started the MTT cisplatin sensitivity assays I would have had a good idea as to what the optimum starting concentrations for each of the cell lines would have been. I was looking for the highest possible startling cell concentration that didn't level off up to the 5th day of growth. This would mean that, come the 'real' MTT assay, the cells would have the best possible chance of establishing themselves in the wells of the 96 well plate whilst not slowing down growth before the last day of the experiment.

The results show that in most of the cell lines growth doesn't begin to level off at all, even up to the final day and highest cell concentration I tested (5,000 cells/well). However, as it is important that all cell starting concentrations would have been the same in the MTT drug sensitivity assay, I would have the chosen 2,000 cells/well starting concentration, as it is the only concentration tested that did not show any levelling off at any point in any of the cell lines (Figures 9 & 10).

Validation of cells using qPCR

The amplification levels corresponding to the A3A primers were very clear in the parental cells and virtually non-existent in the A3A KO cells. (Figures 14 & 15). This data confirms that all preceding processes leading up to this point to knock out the A3A gene from the selected cells had been successful. Importantly the cycle count at

which the amplification levels of the A3A primers in the A3A KO cells become significant are above 30, with some as high as ~37 (supplemental data, figure 16). According to a paper detailing qPCR guidelines, it can be considered safe to disregard qPCR results with C_q values that are as high as this, instead classifying them as 'noise' (Bustin et al., 2009). All parental cell lines and all knockout cell lines show broadly similar levels of A3B amplification, indicating that knocking out A3A did not affect the expression of A3B and meaning the A3A KO cells can be reliably compared to wild type cells when undertaking drug sensitivity assays.

Predictions

MTT drug sensitivity assays

As talked about in the 'Accelerating mutation formation' section of this thesis, studies have shown that there is an extensive presence of the APOBEC3 mutational signature in a large number of different cancers and particularly in bladder cancer. More recent studies have shown that the exact favoured motif context of the mutations would suggest that it is A3A, and not the long-thought A3B, which is the main contributor to these mutational signatures. Finally, it has also been shown that the commonly used cisplatin chemotherapy used to treat patients with certain types of bladder cancer (amongst many other types of cancer) may be interacting with APOBEC3's to enhance their mutational effects on cancer genomes, causing them to mutate at an even greater extent than normal. This could contribute to the 'just right' hypothesis of cancer evolution, whereby the possible interaction between the

cisplatin and A3A could be causing levels of mutation in the cancer genome that are just high enough to evade selection pressures (such as the cisplatin itself), but not so high that the cancer cells begin to fail to propagate information properly down to their daughter cells.

Having taken everything together, I would hypothesise that the A3A KO cells that have been developed so far would show both lower levels of survival and a longer period of time to become resistant to cisplatin in the MTT assays compared to the WT parental cells. However, both the WT parental and A3A KO cells would eventually become resistant to the cisplatin over time if a long enough MTT assay were to be run. This is because although the evidence does point towards A3A as being a potent mutator, it is far from being the only endogenous mutagen that could cause such an effect within the cancer cells.

Conclusions

To summarise, I was able to create two more clonal populations of the BFTC-905 A3A KO cells (in addition to the single clonal population that was created by the previous student), and three separate clonal populations of the parental WT BFTC-905 cells using SSC techniques. I was also able to successfully prove using qPCR that the transfection protocols were successful and that there were cells present in each A3A KO population that had had their selection cassettes removed and A3A gene knocked out.

Due to the exceptional circumstances surrounding COVID-19 and the lockdown period, I was unable complete the original plan of using these cells in MTT drug sensitivity experiments. The large bulk of work involving the creation of the BFTC-905 A3A KO cells using CRISPR-Cas9 (completed by the previous student) and time-consuming culture of multiple clonal cell populations of both the A3A KO and WT BFTC-905 cells has now been completed. The important MTT assay results for this work are now just around the corner and could give an idea as to whether or not A3A is playing a significant role in the formation of cisplatin resistance in these BFTC-905 bladder cancer cells. The data from these experiments could give a reason to further investigate the effects of inhibition of A3A on the formation of drug resistance in other cell lines as well as further elucidate the specific drug interactions that A3A may contribute to.

To date, there have been no other studies that have looked into the effects of A3A using full knockout rather than knockdown or inhibition experimentation methods. Fully knocking out A3A from the cell line and performing A/B test MTT assay experiments will give a much more solid idea as to the function of A3A in cancer cells – in many other studies referenced in this thesis that look into the APOBEC3's, it is impossible to tell for certain whether the results they are seeing are truly to do with the action of A3A (or the lack thereof) or secondary effects that arise from the application of inhibitory, knockdown or other experimentation methods.

Supplemental data

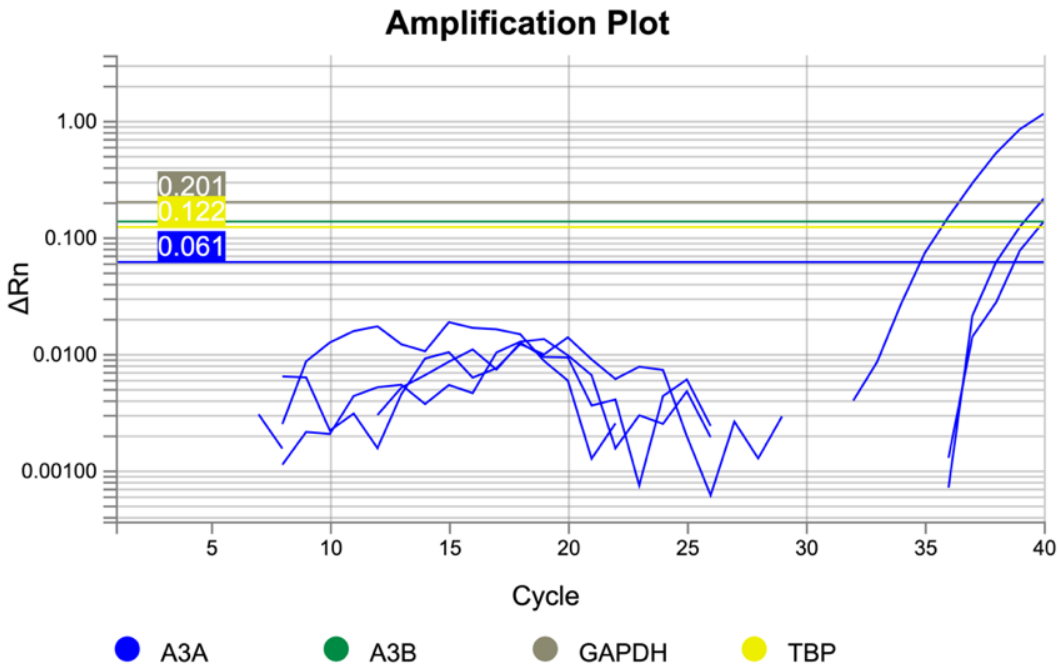

Figure 16 - A3A qPCR amplification plot for the C28 and KO 2.1 A3A knockout cell lines

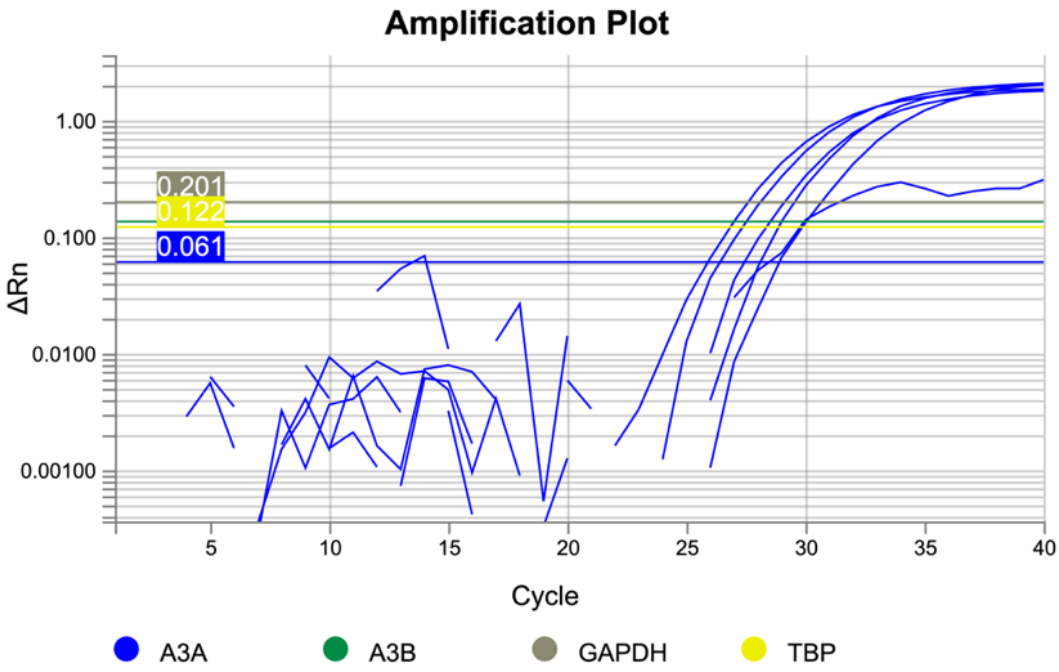

Figure 17 - A3A qPCR amplification plot for the parental P2, P3 & P6 cell lines

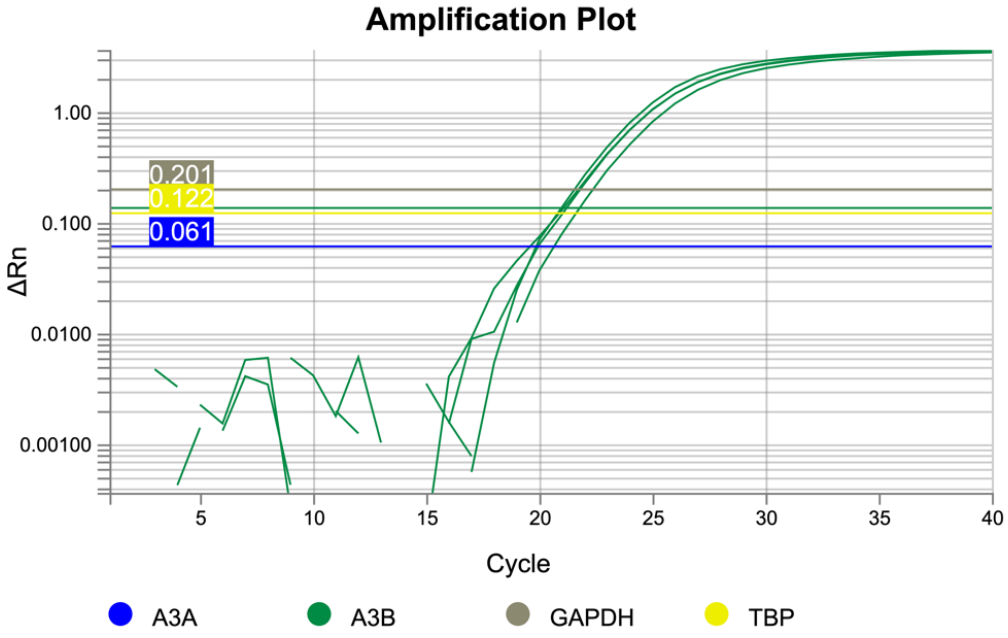

Figure 19 - A3B qPCR amplification plot for the C28 and KO 2.1 A3A knockout cell lines

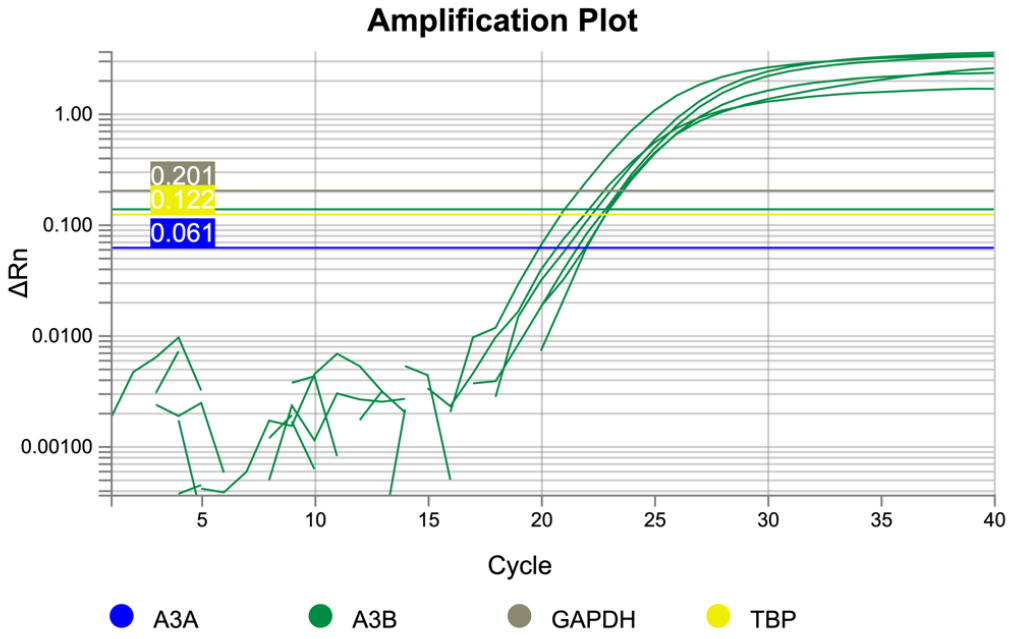

Figure 18 - A3B qPCR amplification plot for the parental P2, P3 & P6 cell lines

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