Studies on Generation and Characterisation of Monoclonal Antibodies

A thesis submitted for the degree of MSc

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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This thesis is dedicated to my late father, Jean-Pierre.

Carpe Diem

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Abbreviations

%	Percentage
Ab	Antibody
ADC	Antibody Drug Conjugate
ADCC	Antibody dependent cell mediated cytotoxic activity
Ag	Antigen
ATCC	American Tissue Culture Collection
BCR	B Cell Receptor
CDC	Complement dependent cytotoxic activity
DCU	Dublin City University
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ECACC	European Collection of Authenticated Cell Cultures
ECL	Enhanced Chemiluminescence
F(ab)	Fragment antigen binding
FBS	Fetal Bovine Serum
Fc	Fragment, crystallizable
HAT	Hypoxanthine-Aminopterin-Thymidine
HIER	Heat Induced Antigen Retrieval
HPRA	Health Products Regulatory Agency
HRP	Horseradish Peroxidase
HT	Hypoxanthine-Thymidine
ICC	Immunocytochemistry
ID	Identification
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-6	Interleukin-6

IMS	Industrial Methylated Spirit
IP	Immunoprecipitation
kDa	Kilo Dalton
mAb	Monoclonal Antibody
Min	minute
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
NK	Natural killer
NCTCC	National Cell & Tissue Culture Centre
NICB	National Institute for Cellular Biotechnology
PBS	Phosphate Buffer Saline
PD-1	Programmed cell death protein-1
PDAC	Pancreatic Ductal Adenocarcinoma
PD-L1	Programmed cell death protein ligand-1
PDX	Patient Derived Xenograft
PEG	PolyEthylene Glycol
PMSF	phenylmethylsulfonyl fluoride
PNPP	p-Nitrophenyl Phosphate
RIPA	Radioimmunoprecipitation assay buffer
RO	Reverse Osmosis
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SCID	Severe Combined ImmunoDeficient
SFM	Serum Free Medium
TME	Tumour Micro Environment
UHP	Ultra High Purified
v/v	Volume/volume
WB	Western Blot
w/v	Weight/volume

Abstract

Justine Meiller-Fay. Studies on Generation and Characterisation of Monoclonal Antibodies

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive and chemotherapeutic resistant disease, which is virtually incurable with current treatment modalities. Unlike other solid cancers, pancreatic cancer treatment has not improved significantly in the last thirty years. With limited molecular targets, there is an urgent need to discover new therapies to improve the outcome of the patients. We proposed to develop novel monoclonal antibodies through hybridoma technology to identify candidate proteins associated with PDAC that may represent potential molecular therapeutic targets. Using a combination of established PDAC cell lines, novel patient derived xenograft (PDX) material and patient tumours as immunogenic material, we successfully established hybridomas reacting to their respective immunogens. One lead hybridoma candidate, mAb 4A5 raised against PANC-1 and PIN 127 PDX derived cells, secreted an IgG-kappa monoclonal antibody. It was characterised and found to react with a protein potentially in higher abundance in PDAC of approximately 60 kDa. In regards to the potential functional effects of mAb 4A5, our study demonstrated that it has no effect on the proliferation of PANC-1 and PIN 127 cells.

Despite advances in the field of hybridoma generation, fusion technology is still the preferred method for monoclonal antibody development. BriClone™ is a well-established hybridoma growth supplement, added at the post-fusion step in order to improve formation of hybridoma colonies. Whilst very effective, this product contains fetal bovine serum and hence can show batch-to-batch variation. The second strand of the thesis focused on the development of a serum-free hybridoma growth supplement for enhancing the generation of hybridoma post fusion. A robust and reliable product was developed, which demonstrated similar to better performances compared to BriClone™.

Thirdly, in order to eliminate the use of mice in the quality control testing of BriClone™, it was proposed to create an *in vitro* cell based assay that would display the potency of BriClone™. While no effective reliable assay was achieved, there is plenty of scope for more research to find an adequate test.

1. Introduction

The immune system is a complex structure of defences involving many types of organs, cells and molecules, vital for protecting oneself. There are two subsystems within the immune system known as the innate (non-specific) and the acquired (specific) systems. The innate system is a simple defence mechanism that acts as the first barrier against pathogens, by providing an immediate but non-specific response. It involves physical barriers such as epithelial barriers and mucosal barriers, and the production of antimicrobial peptides such as the alpha- and beta-defensins (Lehrer *et al*., 2005), and phagocytic cells that can ingest and destroy large particles such as microorganisms (Rosales & Uribe-Querol, 2017). The acquired immunity is supported by T cell lymphocytes involved in cell-mediated immune responses and by B cell lymphocytes that mature and are then capable of becoming activated (plasma cells) and produce large amounts of antibodies against specific antigens. Under the clonal selection theory (Burnet, 1976), the immune system is composed of multiple different lymphocyte clones. For example, the binding of an antigen by a particular B cell will lead to its expansion and activation. A clone tends to persist after the disappearance of the antigen, which provides provides long lasting protection through an immunological memory. In contrast to the innate system, the acquired immunity is a more sophisticated form of response, which is highly specific to the pathogen.

1.1 Antibody structure and function

First identified by Nobel Prize winners Emil von Berhing (*The Nobel Prize in Physiology or Medicine 1901*, no.date.) and Paul Erlich (*The Nobel Prize in Physiology or Medicine 1908*, no.date.), in recognition on their works on immunity, antibodies (Abs) are glycoproteins fundamental to the acquired immune response in vertebrates. These molecules, also referred to as immunoglobulins (Ig) arise from the activation of mature B lymphocytes, known as plasma cells, which have encountered a foreign molecule, in order to neutralise them. These foreign extracellular substances, known as antigens (Ag), can be pieces or substances generated by microrganisms (bacteria, viruses), pollens or chemicals. Immunoglobulins can be secreted by B cells or be membrane bound (B cell receptor); they circulate in the blood and other tissue fluids (Kapingidza *et al*., 2020). Most mammals, including humans, produce five classes of Abs: IgA, IgD, IgE, IgG, and IgM. Each class is dependent on a specific antigen recognition, has different biological properties and plays a different role in the immune response.

1.1.1 Immunoglobulins G

Due their extended half-life in serum, immunoglobulins G (IgG) are the most prevalent of Ig in the human blood, accounting for 75% of all serum antibodies (Tiller & Tessier, 2015). They are heavy proteins of around 150 KDa, structured in a Y-shape that consists of four polypeptides: two identical heavy chains (HCs) and two identical light chains (LCs). A disulphide bond, forming a heterodimer, links one HC and one LC. The two heterodimers are also connected through the HCs by a disulphide link, forming a tetramer (Chiu *et al*., 2019). At the top ends of the Y, both the LC and HC form a variable region, known as the paratope or F(ab) region, that specifically reacts with an epitope present on the antigen. The lower end of the Y shape, known as the constant or Fc region, serves as a less specific binding site with molecules located on the membrane of an immune cell, or with another antibody. There are four subclasses of IgG named IgG1, IgG2, IgG3 and IgG4, each possessing a unique capacity in the immune response. IgG1 is usually the most prevalent subclass, as its production is induced by soluble and membrane proteins. IgG2 antibodies are usually produced after exposure to polysaccharides from encapsulated bacteria. IgG3 antibodies are effective in pro-inflammatory responses and have a limited life. Finally IgG4 tend to be generated by the body after multiple and continuing exposure to an antigen (Vidarsson *et al*., 2014). Although each class possess defined heavy chains, only two types of light chains, kappa (κ) and lambda (λ) are commonly found in all five classes. The most prevalent are the kappa light chains (Goulet & Atkins, 2020).

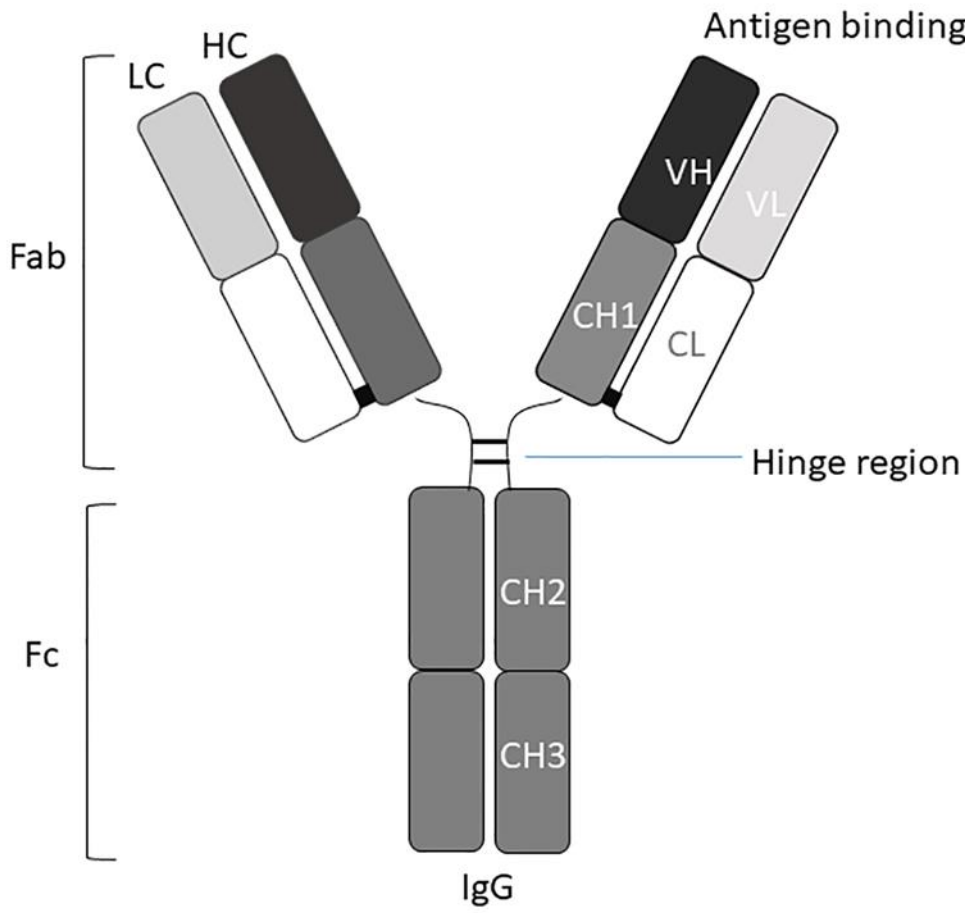


Figure 1-1: Schematic structure of an immunoglobulin G (IgG) molecule consisting of two heavy chains and two light chains of either κ or λ type linked by disulphide bridges (Maibom-Thomsen *et al*., 2019).

1.1.2 Other immunoglobulin classes

IgM are the first class to be secreted during the maturation of B cells and are observed during the primary immune response. They are made of five units, all linked together by disulphide bridges in the constant region. IgAs are dimers found in abundance in secretions, such as breast milk and saliva, and prevent the attachment of bacteria and viruses on the surface of mucosa. IgEs are monomer immunoglobulins associated with allergic reactions and hypersensitivity, they are implicated in the release of histamine and inflammatory inducing molecules. IgDs have a monomeric structure and can be found in the circulation or in a membrane attached form. Although their function is not fully understood, it is thought IgDs act as antigen receptors for activating B cells differentiation (Schroeder & Cavacini, 2010).

1.2 Monoclonal antibodies

Monoclonal antibodies (mAbs) are man-made antibodies produced by identical clones of B-cells, and have a high monovalent affinity for their antigen (Lipman *et al.*, 2005). They are intended to mimic natural antibodies, and so can help combat illnesses. Each mAb has a specificity for a single epitope. The field of biomedicine relies heavily on the use of these "magic bullets", as they can help in the diagnosis and the treatment of human diseases. MAbs can be produced in large quantities, for either analytic or therapeutic applications (Monoclonal Antibodies, 1999).

1.2.1 Monoclonal antibodies as a diagnostic tool

MAbs are widely used in laboratory settings for research and *in vitro* diagnostic analysis as they are generated to react to almost any given substance. Immuno-based assays depend on an antigen-antibody reaction to allow detection and/or quantitation of a distinct analyte (Darwish, 2006). For examples, mAbs can be used to detect the presence of an antigen in body fluids, such as saliva or urine, helping in the quick and reliable diagnosis of an infectious disease. They can be employed in various methodology including immunohistochemistry (IHC), immunocytochemistry (ICC), Western blotting (WB) and Enzyme-Linked Immunosorbent assays (ELISA) (Demlie *et al.*, 2020). For many years, mAbs have enabled the creation of rapid and precise tests, replacing the need for long analytical methods in the detection of infectious illnesses (Nowinski *et al.*, 1983). The sensitivity and specificity of mAb based tests means they are likely to perform better and can replace other form of less sensitive techniques (Chard, 1992). More recently, mAbs have been essential in the development of immunoassays for the detection of SARS-CoV-2 in serological tests (Hwang *et al.*, 2022).

1.2.2 Monoclonal antibodies as therapeutics

Over the last thirty years, monoclonal antibodies have successfully translated from the bench to the clinical setting. The first therapeutic mAb, muromonab-CD3 (Orthoclone OKT3), was granted approval by the FDA back in 1986. Used as an immunosuppressant post-transplant, it is directed against the CD3 receptor of T-cells (Norman & Leone, 1991). MAbs are known to have an effect on a variety of natural mechanisms. They can neutralise the physiological

function of their target-signalling pathway, resulting in activation of pro-apoptotic signals, or inhibition of cell multiplication. First produced as murine (“omab), monoclonal antibodies have been engineered to become chimeric (“ximab), containing 60 to 70% of human sequences, to humanized (“zumab) with a minimum of 10% of mouse sequences, and eventually fully human (“umab) (Kerpel-Fronius, 2018). The humanisation of antibodies (Figure 1-2) can be made by having the entire constant region sequence and most of the variable region sequence as human with only the grafting of murine complementary determining regions (CDRs), i.e the amino acids sequence directly in contact with the antigen. Humanised mAbs are produced in wild type mice hence bearing the mouse Ig locus; whereas fully human mAbs are obtained from mice that are genetically engineered using phage display to express the human Ig locus.

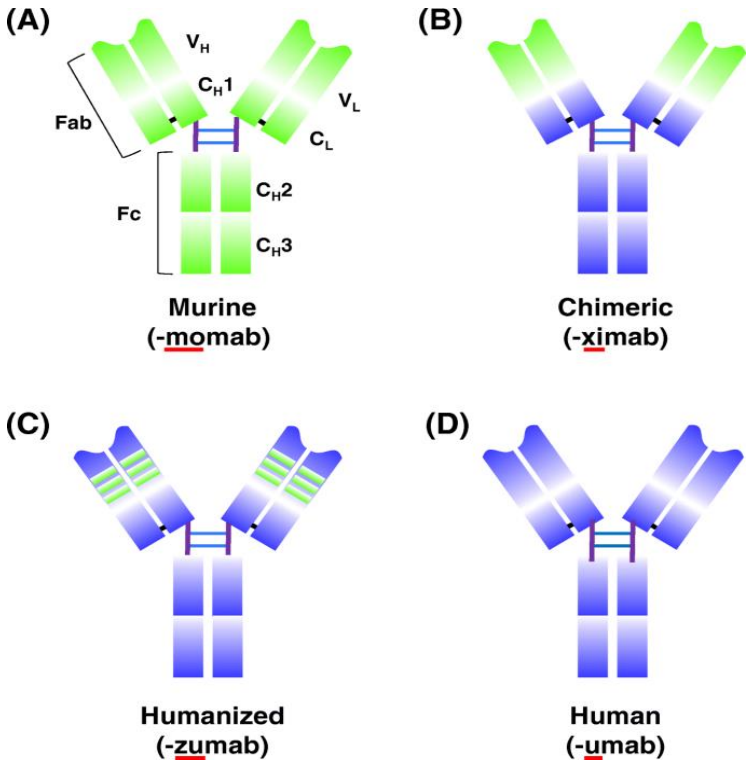


Figure 1-2: Illustration of the process of monoclonal antibody humanisation from murine to human. a) Murine monoclonal antibody. B) Chimeric monoclonal antibody, c) Humanized monoclonal antibody, d) Human monoclonal antibody. The regions of murine origins are in green, and the human regions are in blue (Lu *et al.*, 2020)

Monoclonal antibodies are usually well tolerated by patients, although some reactions can develop, such as fever, skin reactions and flu-like symptoms. In some rare severe cases, potentially fatal anaphylaxis can occur (Hansel *et al.*, 2010). Since the discovery and use of muromonab-CD3, almost 80 mAbs have been approved by the FDA and 30 of these

biologics are used to treat malignancies (Lu *et al.*, 2020). In oncology, rituximab was the first mAb to be used for the treatment of relapsed/refractory non-Hodgkin's lymphoma. Approved in 1997, rituximab is directed against the CD20 marker, expressed on 95% of the B-cells of the lymphoma . Another mAb with proven clinical success is trastuzumab, targeting the HER-2 protein and allowing growth inhibition of aggressive malignant breast and ovarian tumours overexpressing this receptor (Shepard *et al*., 2017).

Antibodies can target cancerous cells and bind to membrane receptors leading to change in growth signalling pathways, with major effect on cellular proliferation (Redman *et al*., 2015). Antibody-dependent cell-mediated cytotoxic activity (ADCC) relies on the capacity of antibodies to target a cell, and triggering cell death via effector cells, such as natural killers (NK). Monoclonal antibodies can also provoke complement-dependent cytotoxic activity (CDC) leading to lethal cell lysis (van Egmond & Bakema, 2013). New derivatives of therapeutic mAbs have become a promising category in the treatment of cancer. Antibody drug conjugates (ADCs) selectively deliver cytotoxic agents to targeted tumour cells. To overcome the monospecificity of IgG antibodies, new formats of therapeutics can also be engineered. Bispecific/ multispecific antibodies and antibody fragments can bind to two or more targets, and even have the capabilities to target two or more epitopes within the same target, offering promising therapeutic potential (Sawant *et al.*, 2020).

Some of these new formats of therapeutics and their mechanisms of actions are summarised in Figure 1-3.

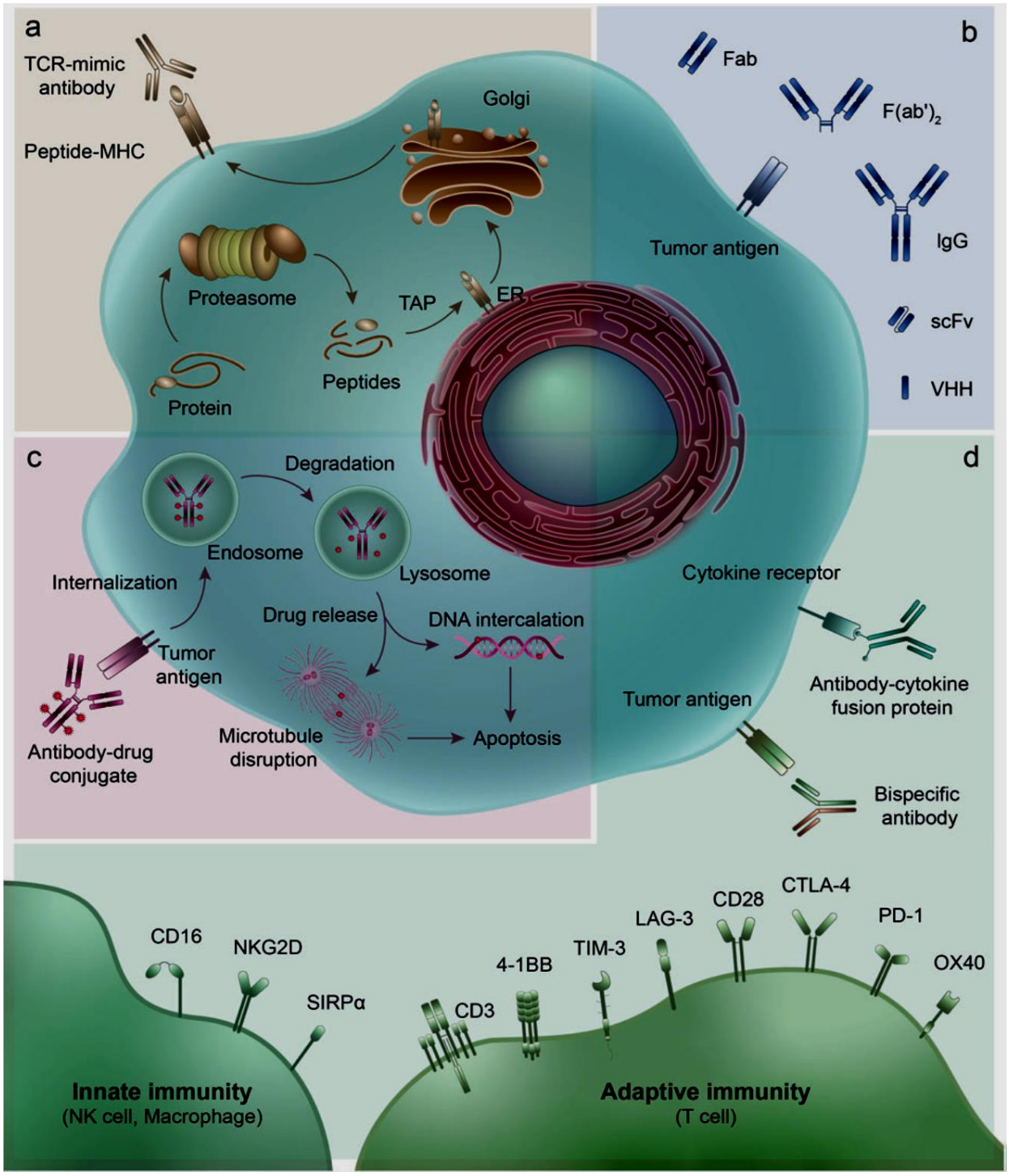


Figure 1-3: Representative therapeutic antibodies and their derivatives. a) TCR-mimic antibody; b) IgG antibody and antibody fragments; c) antibody-drug conjugate (ADC) and its mechanism of action; d) multifunctional antibodies, such as bispecific antibodies, immunocytokine (antibody-cytokine fusion protein) (Jin *et al.*, 2022).

1.2.2.1 Antibody drug conjugates

Antibody drug conjugates (ADCs), know a “biological missiles” have opened a new door in the field of targeted therapies, and represent a fast growing class of anti-cancer drugs. By binding to specific tumour antigens, ADCs heighten tumour selectivity and offer a larger therapeutic window comparing to chemotherapeutic agents. A key element of an ADC is its target. The ideal antigen is overexpressed by the cancer cells, and has low to no expression in the surrounding normal cells (Khongorzul *et al.,* 2020). The protein target needs to be localised on the membrane of the cells, and should not be secreted to avoid binding of the ADC outside the tumoral spot. The binding of these cell surface proteins with the antibody triggers endocytosis, and the ADC is internalised by the cells. ADCs are linked to potent molecules capable of inducing cell death. Upon internalisation via an endosome, the highly effective payload, i.e the cytotoxic compound linked to the antibody, is released following cleavage of the linker. The endosome degrations releases drug which is then transported within the cytosol and finds its molecular target, leading to cell death (Kitson *et al.*, 2013). As of 2021, 14 ADCs were approved by the Food and Drug Administration (FDA) as anti-cancer therapeutics (Fu *et al*., 2022). While some solid malignancies, such as breast cancer, can benefit from these market available ADCs, these therapeutics have an impact mainly on haematological malignancies as seen in Table 1-1. It appears that the difficulties in developing ADCs for solid tumours come from their lack of accurate tumour specificity, and the target selection continues to be an obstacle (Criscitiello *et al*., 2021).

Table 1-1: Summary of antibody–drug conjugates approved for market worldwide for clinical use, as of December 2021 (Fu *et al.*, 2022)

Drugs (Company)	Trade Names	Target antigens	Payloads	Approved Countries	Approved Date	Approved indications
Gemtuzumab ozogamicin (Pfizer)	Mylotarg®	CD33	N-acetyl-γ-calicheamicin	FDA/EMA/PMDA	2000/5/17; 2017/9/1	Acute myeloid leukaemia.
Brentuximab vedotin (Seagen)	Adcetris®	CD30	MMAE	FDA/EMA/PMDA/NMPA	2011/8/19	Systemic anaplastic large cell lymphoma.
Inotuzumab ozogamicin (Pfizer)	Besponsa®	CD22	N-acetyl-γ-calicheamicin	FDA/EMA/PMDA	2017/6/28	Acute lymphoblastic leukaemia.
Moxetumomab pasudotox (AstraZeneca)	Lumoxiti®	CD22	PE38	FDA/EMA	2018/9/13	Hairy cell leukemia
Polatuzumab vedotin (Roche)	Polivy®	CD79B	MMAE	FDA/EMA	2019/6/10	Diffuse large B cell lymphoma
Belantamab mafodotin (GSK)	Blenrep®	BCMA	MMAF	FDA/EMA	2020/8/5	Multiple Myeloma
Loncastuximab tesirine (ADC Therapeutics)	Zynlonta®	CD19	PBD dimer (SG3199)	FDA	2021/4/23	large B-cell lymphoma
Ado-trastuzumab emtansine (Roche)	Kadcyla®	HER2	DM1	FDA/EMA/PMDA/NMPA	2013/2/22	HER2- breast cancer.
Enfortumab vedotin (Seagen)	Padcev®	Nectin-4	MMAE	FDA	2019/12/18	urothelial cancer
Fam-trastuzumab deruxtecan (Daiichi Sankyo)	Enhertu®	HER2	DXd	FDA/EMA/PMDA	2019/12/20	HER2- breast cancer
Sacituzumab govitecan (Immunomedics)	Trodelvy®	Trop-2	SN38	FDA	2020/4/22	Triple negative breast cancer
Cetuximab sarotalocan (Rakuten Medical)	Akalux®	EGFR	IRDye700DX	PMDA	2020/9/25	head and neck squamous cell carcinoma
Disitamab vedotin (RemeGen)	Aidixi®	HER2	MMAE	NMPA	2021/6/8	gastric cancer (
Tisotumab vedotin (Genmab/Seagen)	Tivdak®	TF	MMAE	FDA	2021/9/20	cervical cancer

1.3 Production of monoclonal antibodies

Monoclonal antibodies are one of the most important type of biologicals for therapeutic and diagnostics application. Their market was evaluated to be $186 billion in 2021, with a value forecast at $490 billion in 2030. MAbs are generated in laboratories using mammalian cells which growth are stable and continuous.

1.3.1 Hybridoma technology

In 1975, Cesar Milstein and George Kohler published their work that led to their Nobel prize triumphant work (Medicine and Physiology in 1984) that established a successful method to generate mAbs *in vitro* in continuous cultures (Köhler *et al.*, 1976). Laboratory animals (mice, rats, rabbits, etc…) are inoculated multiple times with an antigen over a period of time. The animals are humanely sacrificed and their spleen is used to extract the splenocytes containing a population of B cells. These cells are fused with immortalised non-secreting Ab mouse myeloma cells (e.g. Sp2/0-Ag14) using polyethylene glycol (PEG) and the resulting fused cells are grown in a selecting medium containing HAT (Hypoxanthine-Aminopterin-Thymidine). Due to their lack of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene, the myeloma cells B cells are sensitive to the medium containing HAT. Aminopterin is an enzyme inhibitor that binds to dihydrofolate reductase resulting in no tetrahydrofolate synthesis. This leads to no synthesis of nucleotides, and the unfused myeloma cells die, due to lacking HGPRT and subsequent no production of nucleotides. If not removed, the myeloma cells outgrow the hybridomas. The unfused splenocytes do not survive due to their short life, meaning only the hybrid cells can keep growing and replicate indefinitely. The hybrid cells survive because although aminopterin blocks the ability to synthesise nucleotides, the medium contains a source of purines (hypoxanthine) and pyrimidines (thymidine), providing a supply of nucleotides via the salvage pathway. Hybridoma colony formation is monitored and each colony screened for their reaction against the desired antigen.

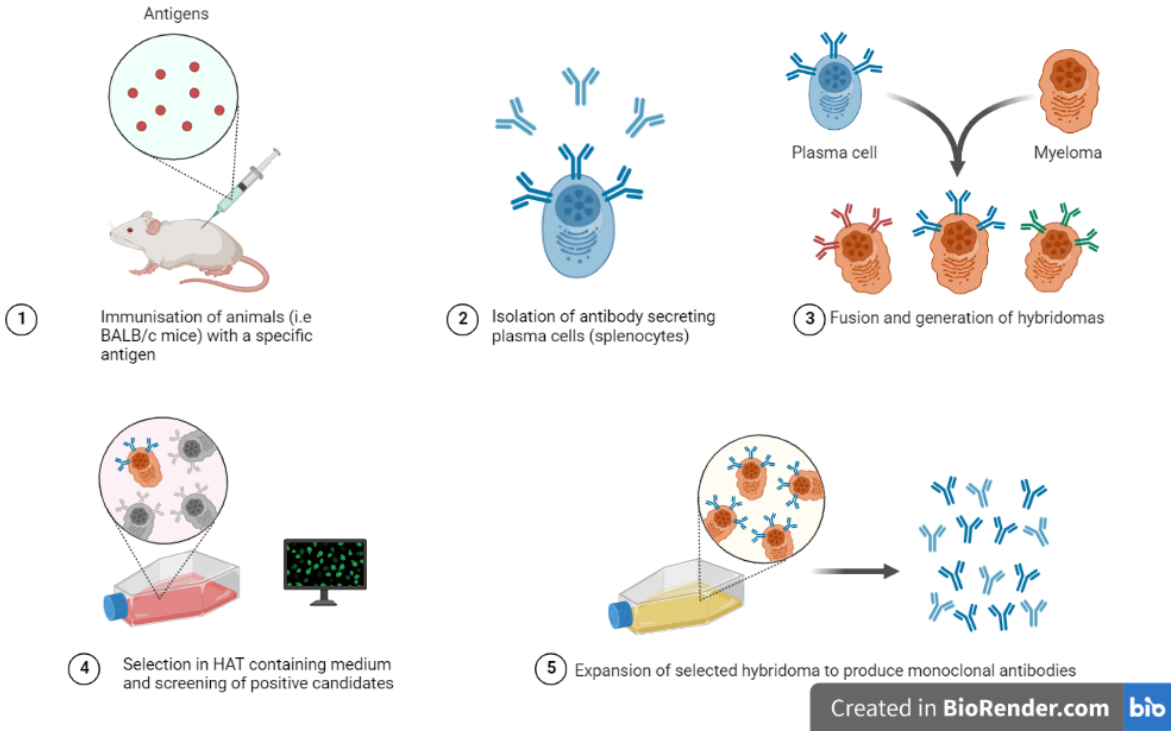


Figure 1-4: General representation of the fusion technology for the generation of hybridomas

The hybridoma technology as described above and summarised in Figure 1-4 remains a popular method amongst researchers for the generation and development of monoclonal antibodies. Some of the advantages of this technology are that the antigens are not required to be pure for immunisation of the animals and that once stable, the hybridomas provide an endless source of homogeneous mAbs. This platform has established the large scale, unlimited production of mAbs. This approach remains the traditional method for generation of monoclonal antibodies due to reliability in obtaining stable hybridomas in a cost effective manner (Mitra & Tomar, 2021). The hybridoma platform permits the preservation of the natural pairing of antibodies. The mAbs produced demonstrate low level of immunogenicity since they bear natural coupling of heavy and light chains, having gone through a maturation process (Parray *et al.*, 2020). Genetic modifications are a necessary process for antibody secretion; first secreted as IgMs, the antibodies usually undergo a switch in class to become IgGs, which is the class of most diagnostics and therapeutics mAbs. The hybridoma technology, which relies on the host animal immune system, favours the generation of non-engineered mAbs (Zaroff & Tan, 2019).

Most recently, this key methodology has permitted the discovery of some of the most successful and FDA approved mAbs, such as nivolumab (anti-programmed cell death protein 1; anti-PD-1) and atezolizumab (anti-programmed cell death protein ligand 1; PD-L1) (Moraes *et al.*, 2021).

1.3.2 Limiting dilution cloning

Once cloned, the clonal population of hybridomas will produce and secrete mAbs recognising one specific target. Since they are immortal, hybridomas can be expanded indefinitely and thus the mAbs produced can be continually harvested. The isolation of clones is critical in the production of monoclonal antibodies. Commonly used for its ease of application, cell cloning by limiting dilution is achieved by serial dilutions of a concentrated heterogenous cell population, resulting in single cell isolation. Although cells might need to be cloned twice, the final clones would stably express the antibody of interest (Greenfield, 2019).

1.3.3 Recombinant technologies

Technical advances in the field of mAbs generation have succeeded in their quick and efficient production and for use in a clinical setting. Monoclonal antibodies can nowadays be produced in a full human form, decreasing the risk for mouse-human reactions in the patients. A summary of the different technologies used for the creation of monoclonal antibodies is summarised in Figure 1-5.

1.3.3.1 Transgenic mice

Transgenic animals can serve as a platform for mAb production. These mice, through genetic manipulations and subsequent breeding are not able to produce mouse antibodies, but only human immunoglobulins.(Chen & Murawsky, 2018). However, it was reported that the human mAbs produced through the use of engineered animals may not compare to wild type antibodies (Sanlav *et al.*, 2020). Another disadvantage in regards to transgenic mice is the high costs associated and the length of time it takes to establish them (‘Transgenic Animals’, 2016).

1.3.3.2 Phage display technology

First described by GP Smith (Smith, 1985), phage display consist of the insertion of a fusion protein into bacteriophages, viruses that can infect and replicate in bacteria cells only. Vectors encoding for antibody fragments are inserted within the phages .Upon infection of a bacterium, the phages presenting the desired antibody on their surfaces, are amplified. The phage display technique relies on the construction of an antibody library. Genetic material is extracted from either splenocytes of a pre-immunised mouse or hybridomas. Using the messenger RNA (mRNA) of hybridoma cells results in the cloning of fragment of the parent mAb. Following reverse transcription polymerase chain reaction (RT-PCR) and complementary DNA establishment, primers are used to amplify the heavy and light chains variable genes. The amplified genetic material is cloned into the phage vectors for subsequent transformation of a bacteria (Daly, 2001). The library is then added to an immobilised support for antigen presentation. The highest affinity antibodies bind their epitopes, and following washes and enzymatic elution, confirmation of binders is carried out by titration (Ledsgaard *et al.*, 2018).

As of 2020, nine fully human monoclonal antibodies generated through the phage display methodology were approved for use in treatment by the FDA (Lu *et al*, 2020). However, antibody phage display has its limitations. The pairing of the heavy and light chains may be different to the pairing in patients. During screening ,some relevant clones can be lost due to the small size of the samples resulting from the affinity enrichment of antigen-binding clones (Hammers & Stanley, 2014).

1.3.3.3 Single B cell

B cells are isolated from the donor (human or animal) via various procedure such fluorescence-activated cell sorting (FACS) or magnetic beads. Single cell cDNA synthesis is then performed.and full-length Ig gene transcripts are amplified by RT-PCR. Forward primer complementary to the corresponding Ig heavy chains and Ig light chains and a single reverse primer specific complementary to the constant region sequence are commonly used. The rearranged heavy and light chain genes are combined, and subsequently cloned into a vector for the production of the monoclonal antibody, which is then submitted to screening (Tiller, 2011).

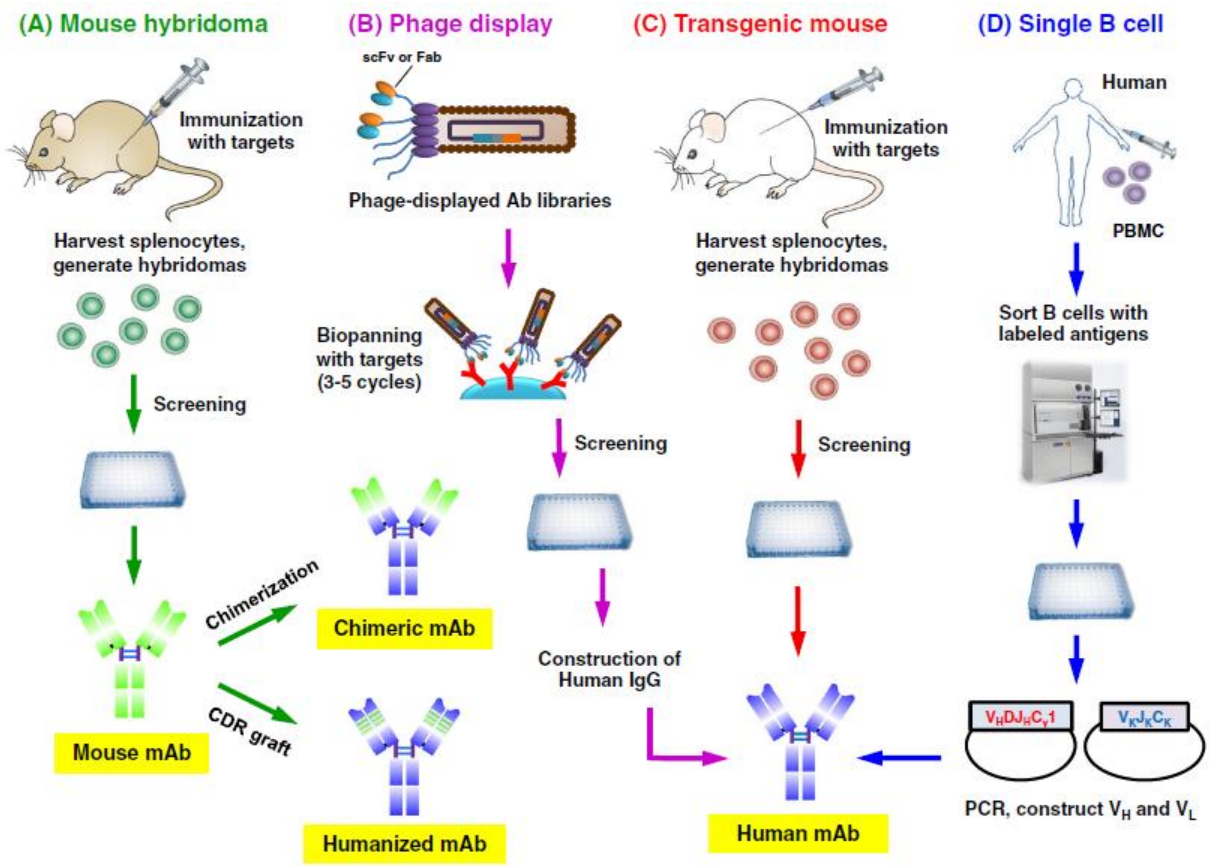


Figure 1-5 Approaches for the development of therapeutic antibodies. A) The traditional mouse hybridoma technique. B) Phage display. C) Transgenic mouse. D) The single B cell technique. (Lu *et al*, 2020)

1.3.4 Improving the growth of freshly fused hybridomas

Newly generated hybrid cells are fragile, sometimes unstable and extra care to improve their growth post fusion and at the single cloning stage is necessary. This can be helped with the use of feeder cells. These growth-arrested cells are not able to replicate and do not overgrow the hybridomas, yet due to being live, they can secrete many factors leading to an improvement in hybridoma colony formation. Whilst effective, this method has its limitations; it is time consuming and can lead to cross contamination between the cells lines (Hnasko *et al.*, 2018). Conditioned media harvested from established cell cultures have long proven to enhance the growth of freshly fused hybridomas (Walker *et al.*, 1986), mainly by containing growth factors and other cytokines (Ukaji *et al.*, 2014) (Apiratmateekul *et al.*, 2012).

In the 1990s, the National Cell and Tissue Culture centre (NCTCC) at Dublin City University, as part of the Irish National Biotechnology Program developed a hybridoma growth supplement for its own in-house use in its hybridoma programmes, since commercially-available supplements were very expensive. The central marketing group of Bioresearch Ireland, based in Enterprise Ireland, saw the commercial potential of this in-house supplement as a product, named it BriClone™ (BRI for BioResearch Ireland), and identified international distributors. For the past 30 years, BriClone™ has been rigorously manufactured at DCU, and sold worldwide. (https://nicb.ie/briclone/)

BriClone ™ derives from the conditioned medium of proprietary human cell line and helps overcome the difficulties in generating competent colonies (Pandey, 2010). It has been cited in numerous peer reviewed scientific publications, in which the development of mAbs was made possible through the addition of this supplement. The application of these mAbs was extremely wide, from cancer research (Moran *et al.*, 1992), to toxicology (Morita *et al.*, 2018), and neuroscience studies (Yoshikawa *et al.*, 2000) to name but a few.

BriClone™ contains interleukin-6 (IL-6), secreted by the producer cells into the conditioned medium, in unknown, yet potent quantities. It was previously described that IL-6, originally named B cell stimulatory factor II (Kishimoto, 1987), is a soluble cytokine that bears a mosaic of effects on the blood cell production, on the immunity and the response to inflammation.. Due to its multifaceted biological functions, IL-6 is involved in many diseases and disorders as well as normal physiology. Homeostatic plasma levels of IL-6 are low, of 1-5 pg/mL, but can increase dramatically in the mg/mL range when the human body is confronted to injuries or infections (Jones *et al.*, 2018). Upon its activation, IL-6 assists in the maturation of B cells into antibody secreting plasma cells (Muraguchi *et al.*, 1988). It is also involved in the promotion of myeloma and plasmacytoma growth, two malignancies originating from dysfunctional plasma cells (Hirano, 1991). IL-6 demonstrated promotion of expansion and increased mAb expression of murine hybridomas (Zhu *et al.*, 1993).

1.3.5 Replacing the use of animals in life science

In 1959, Russel and Burch published *The Principles of Humane Experimental Technique* where they introduced the concept of alternative methods to improve the treatment of animals used in research and yet promoting advances in the quality of scientific and medical

research and testing (Tannenbaum & Bennett, 2015). To fall in line with the concept of the 3Rs, which encourages the Reduction, Replacement and Refinement of live animals for scientific purposes (*The 3Rs | NC3Rs*, no.date.), scientists are encouraged to change the way they perform their research. The 3Rs terms are described in Article 4 of EU Directive 2010/63/EU as (*Directive 201063EU of the European Parliament an.Pdf*, no.date.)

- Replacement: using alternative methods that don't require live animals
- Reduction: using the most appropriate number of animals
- Refinement: minimising suffering and improving animal welfare

BriClone™ is the resulting product of a cell line growing in a complete medium. This medium contains Fetal Bovine Serum (FBS), which benefits have long been demonstrated *in vitro* cell cultures. However this highly potent additive is challenged by numerous controversies, be it the risk of contaminant contents or animal welfare concerns. Some of the disadvantages of FBS are its unknown composition, batch-to-batch variations leading to unreproducible data and the potential bearing of mycoplasma, viruses, endotoxins or prion proteins (Chelladurai *et al.*, 2021). In some instance, FBS can be detrimental to the growth of difficult cells, such as primary cell lines. Another barrier in the use of FBS are the ethical issues surrounding the probable suffering of the calf fetuses when their blood is collected (Mellor *et al.*, 2005).

The development of *in vitro* processes that could replace the use of live animals in quality control testing is also an important field to consider in regards to animal welfare. Many *in vitro* tests were successfully created and approved by regulatory authorities, such as for the release of cosmetic products (Spielmann *et al.*, 2008). Assays to test for the potency of a product should be reliable and relevant to the ensure accurate representation of the biological activity of the compound tested (Moreira *et al.*, 2020). Amongst other parameters, the choice of the cell lines to use must be representative of the intended application to test, the cell density employed, growth conditions and time in culture must be optimised (Hirsch & Schildknecht, 2019).

1.4 Pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC), known as the silent killer, is one of the most lethal solid malignancies, characterised with a very poor prognosis. Only 9% of patients will live 5 years post diagnosis, with this figure having changed very little over the last decade. It is projected that by 2030, PDAC will be the second cause of cancer related death in Europe and the United States of America across men and women. This is due to a lack in adequate screenings that could detect pre-malignanices, and effective treatments (Rahib *et al*., 2021). Pancreatic ductal adenocarcinoma is the most common form accounting for 90% of cases of neoplasms of the pancreas (Hidalgo *et al.*, 2015). Due to the late onset of symptoms, patients are often diagnosed at an advance stage of the disease. PDAC can be categorised into four groups: resectable, borderline resectable (BR), locally advanced (LA) and metastatic (Clementine *et al.*, 2019).

1.4.1 Conventional therapies for the treatment of PDAC

1.4.1.1 Surgery

Surgery for PDAC is the only curative option that can give patients a potential chance of long-term survival. Unfortunately, only a small fraction (15 to 20%) of patients are eligible for surgery at time of diagnosis as the remaining patients present with stage IV disease, which means the disease has spread to neighbour organs (Hackert, 2018). Pancreaticoduodenectomy is a highly complex surgery, but major advances in the field and management of the disease in high volume centres have reduced the mortality rate to below 3% (Ansari *et al*., 2016). Surgery on its own is rarely a viable option and resectable tumours often have to be treated with adjuvant chemotherapy for the patients to achieve an overall survival (OS) of between 24 and 58 months (Fong *et al*., 2019). Furthermore, the recurrence rate is very high at 80% with the disease relapsing locally or distantly from the original tumour (Moletta *et al*., 2019).

1.4.1.2 Radiation

Radiotherapy in the treatment of PDAC is used for early stages disease and most often in combination with chemotherapy. It is rarely employed for late stage tumours and if so, only has a palliative role, to reduce the pain caused by metastases (Koay *et al*, 2018). In the

adjuvant setting, chemoradiotherapy (CRT) following radical pancreatectomy shows an improved overall survival (OS) when the radiation is delivered at the right dose (between 50 and 55 Gy). If radiotherapy is administered at low doses, the treatment is almost ineffective (Morganti *et al*, 2019).

1.4.1.3 Chemotherapy

Systemic chemotherapeutic agents are usually utilised as primary treatments for patients presenting with resectable and borderline resectable malignancies. FOLFIRINOX (FOLinic acid, Fluorouracil, IRINotecan, OXiplatin), gemcitabine and gemcitabine with capecitabine, are commonly administered to such patients after surgery, if their overall health is satisfactory (Lo *et al*., 2022). Neoadjuvant therapies are also considered for resectable disease, with gemcitabine and cisplatin showing an OS of 26.5 months. In combination with radiotherapy, gemcitabine showed a 5 year OS of 57% for resectable patients (Sahani *et al*., 2013). For patients diagnosed with non-resectable tumours and metastatic disease, gemcitabine + nab-paclitaxel (GnP) and FOLFORINOX would be the first choice of treatment, allowing for a resection rate at 61% and an OS of 16 months (Oba *et al*., 2020). Even though these treatment regimens can increase the life of PDAC sufferers, the severe side effects experienced by some patients pose a problem. Symptoms such as fever, anorexia, diarrhoea, vomiting, neuropathic pain are commonly observed with chemotherapy treatments, and can only disappear with the discontinuance of the treatment. Furthermore, multidrug therapies, due to their increased toxicity, can lead to severe adverse effects even amongst the fittest patients (Lohse and Brothers, 2020). Another concern associated with chemotherapeutics treatments for tumour shrinkage is that they are frustratingly met with the development of chemoresistance (Adamska *et al*., 2018). This resistance can develop at molecular or genetic levels. Likewise, the tumour microenvironment of pancreatic tumours, acting as a barrier, can reduce the delivery of the drugs however, some therapies targeting the stroma have demonstrated increased survival rates (Du *et al*., 2020).

1.4.2 Immuno-oncology for pancreatic ductal adenocarcinoma

Over the last decades, huge improvements have been made in the treatments of some solid malignancies, such as breast, lung, head-and-neck, renal, urothelial, and gastric cancers and melanoma thanks to the development of immunotherapies (Nixon *et al*., 2018). In order to fight cancer, development of treatments that take advantage of the body's immune system

are taking effect. However, such therapies are yet to show complete effectiveness in the treatment of PDAC but hope remains.

1.4.2.1 Immune checkpoint inhibition

Blocking the immune checkpoint has shown great effect in the treatment of cancers such as melanoma and lung Zhou *et al*., 2021) Checkpoint inhibitors targeting represent an hopeful avenue, and antibodies are currently on trials using multiple receptors linking the tumour cells to lymphocytes, helping in lymphocyte-mediated cell destruction. The targeting of the transmembrane protein Programmed death-ligand 1 (PD-L1) has shown clinical success in recent years. In regards to PDAC, several pre-clinical and clinical trials are currently ongoing. Envafolimab, a bispecific PD-L1/CTLA-4 antibody, is currently undergoing phase II trial in combination with gemcitabine and nab-paclitaxel in the advanced setting, where almost 89% of patients have achieved complete response, partial response and stable disease (Kole *et al*., 2022). As of 2022, most ongoing clinical trials in PDAC employ a combination of toxic agent and mAbs targeting programmed cell death protein 1 (PD-1) (Turpin *et al*., 2022).

Figure 1-6: US trials in progress in PDAC patients with a combination of drugs and monoclonal antibodies (Turpin *et al*., 2022)

Combined agent	Immunotherapy	Type	Phase	NCT
Plerixafor	Cemiplimab	Anti-PD-1	II	NCT04177810
G-nab-P	Camrelizumab	Anti-PD-1	II	NCT04498689
G-nab-P or FOLFIRINOX	BsAb	PD-L1/CTLA4	I/II	NCT04324307
Single agent, 2d line	Retifanlimab	Anti-PD-1	II	NCT04116073
FOLFIRINOX	Not precised	Anti-PD-1	III	NCT03977272
Manganese, G-nab-P	Not precised	Anti-PD-1	I/II	NCT03989310
mFOLFIRINOX	Not precised	Anti-PD-1	III	NCT03977272
G-nab-P	SHR-120	Anti-PD-1	I	NCT04181645
SX-682 (CXCR1/2)	Nivolumab	Anti-PD-1	I	NCT04477343
CXCR4 antagonist,G-nab-P	Cemiplimab	Anti-PD-1	I	NCT04543071
Taladafil (PDESi), vancomycin	Nivolumab	Anti-PD-1	II	NCT03785210
Autologous TIL	Pembrolizumab	Anti-PD-1	II	NCT01174121
ABBV-927 (anti-CD40), FOLFIRINOX	Budigalimab	Anti-PD-1	Ib/II	NCT04807972
LYT-200 (targets galectin-9)	Not precised	Anti-PD-1	I/II	NCT04666688
Epacadostat (IDO1i, CRS-207 (listeria monocytogenes), CY/GVAX)	Pembrolizumab	Anti-PD-1	II	NCT03006302
Siltuximab (anti-IL-6)	Spartalizumab	Anti-PD-1	I/II	NCT04191421
Aldoxorubicin, cyclophosphamide	Not precised	PD-L1 t-haNK*	II	NCT04390399
CDX-1140 (CD40Ab), CDX-301 (FLT3L)	Pembrolizumab	Anti-PD-1	I	NCT03329950
Olaparib	Pembrolizumab	Anti-PD-1	II	NCT04548752
Niraparib	TSR-042	Anti-PD-1	Ib	NCT04673448
Anlotinib (TKI)	Toripalimab	Anti-PD-1	II	NCT04718701
Cabozantinib (TKI)	Pembrolizumab	Anti-PD-1	II	NCT04820179
Anlotinib (TKI), 2d line	Pembrolizumab	Anti-PD-1	II	NCT05218629
Cabozantinib (TKI)	Pembrolizumab	Anti-PD-1	II	NCT05052723
Vaccine, imiquiomod, sotigalimab (APX005M) (CD40 agonist)	Pembrolizumab	Anti-PD-1	I	NCT02600949

IL, interleukin; PDAC, pancreatic ductal adenocarcinoma.

1.4.2.2 Targeting the tumour microenvironment

The tumour microenvironment (TME) of PDAC plays a huge role on the ineffectiveness of checkpoint inhibitors immunotherapies. The TME of pancreatic tumour is rich of immune engaged cells, such as macrophages and monocytes, leading to immunosuppression (Sally *et al*., 2022). It was observed that targeting one mechanism at a time led to failure of the studies. There is a necessity to use combinations of cytotoxic therapies and immunotherapies to block immune checkpoints and optimise the tumour microenvironment (Bockorny *et al*., 2022). Multitudes of cells in the TME secrete tumour-promoting factors, all interacting with

each other and therefore the need for combination therapies is prevalent (Robatel & Schenk, 2022).

1.4.2.3 The need for novel targets in PDAC

Although the development of monoclonal antibodies targeting PDAC in progressing, the knowledge around specific cancer targets related to this cancer is not extensive. Identifying monoclonal antibodies targeting the surface of PDAC cells, or discovering new cellular membrane targets offer a new perspective in the treatment of PDAC (Sorbara *et al*., 2022). Furthermore, in anticipation of the development of resistance, there is a constant need to discover new targets and keep immune based therapies as a key treatment of PDAC (Panchal *et al*., 2021). In recent times, pre-clinical studies were conducted with antibodies targeting a range of targets, offering new hindsight and possible avenue for treating this cancer. Some of this studies are summarised in Table 1-2.

Table 1-2: Recent pre-clinical studies employing monoclonal antibodies in PDAC

Antibody	Target	Authors
Anti-BAG3 H2L4	BAG3	(Dufrusine *et al*., 2022)
NEO-201	CEACAM-5 and CEACAM-6	(Tsang *et al*., 2022)
Anti-CD40	CD40	(Lau *et al*., 2022)
^{64}Cu-NCABoo1ipPET	EGFR	(Matsumoto *et al*.,
N2E4Ab	NRP2	(Wang *et al*., 2021)
A1-PE24X7	Mesothelin (MSLN)	(Li *et al*., 2021)
hRabMab1	Spliced Tissue factors	(Lewis *et al*., 2021)
pcmAb-60	PODXL	(Kaneko *et al*., 2020)
Anti-GPC-1 conjugated	Glypican-1	(Nishigaki *et al*., 2020)
TROP2-IR700	TROP2	(Nishimura *et al*., 2019)

1.4.3 Discovery of novel targets for PDAC

1.4.3.1 Established cell lines

Two-dimensional cell lines are a useful tool for discovery and proof of concept studies. Several cell lines representative of PDAC have been developed over the years (Deer *et al*., 2010). The use of established cell lines has previously been described in the development of novel monoclonal antibodies where the mAb candidates were selected through a phenotypic screening approach (Rust *et al*., 2013). A novel antibody, 7B7, targeting the Ku70/Ku80 heterodimer was generated through the use of hybridoma technology from mice inoculated with an invasive clonal variant of Mia PaCa-2 PDAC cell line. The targeting of the Ku70/Ku80 heterodimer resulted in a decrease in invasion in the immunogenic cell line and a lung cancer cell line (O'Sullivan *et al*., 2014) . As recently reported, two mouse antibodies were generated using a similar approach, by employing the human PDAC cell line CFPAC-1 as the immunogen. These two mAbs KU44.22B and KU44.13A were found to target integrin α3 and CD26 respectively (Arias-Pinilla *et al*., 2020)).

1.4.3.2 Patient-derived Xenograft

Patient-derived xenografts (PDX) are another commonly used model for the study of PDAC and were used to identify biomarker of this neoplasm (Jimeno *et al*., 2010). PDX are useful *in-vivo* pre-clinical models as they preserve the molecular and cellular heterogeneity of the cancer to study. To generate PDX models, tumour specimens are implanted into immune-deficient hosts, such as severe combined immunodeficient (SCID) mice, which lack B cells, T cells and natural killers (NK) functions (Genta *et al*., 2022). This immunodeficiency allows for new tumour growth that bear an accurate representation of the patient tumour (Jung et al., 2018). The tumour fragments are implanted either in a different site than the organ where the cancer originates from (heterotopical) or into to the corresponding site (orthotopical). The first engraftment is designed as F0, and subsequent grafts can be expanded in further generations named F1, F2, Fn etc…Most studies are carried out with early passage generations (Tentler *et al*., 2012).

Over a period a five years, researchers at the National Institute for Cellular Biotechnology (NICB) have established a PDX biobank from tumours of PDAC patients implanted subcutaneously into severe combined immunodeficient (SCID) mice (Roche *et al*., 2020). A robust proteomic profiling of these PDX material identified membrane targets associated with PDAC with an increased expression in PDX tumours comparing to adjacent normal tissue. These promising leads could serve as candidates for the development of monoclonal antibody targeted therapies (Coleman *et al*., 2018).

1.5 Aims of thesis

The aims of this thesis were as follows:

- To generate novel monoclonal antibodies directed against pancreatic ductal adenocarcinoma (PDAC) by using fusion technology and hybridoma generation. The immunogens are a combination of established PDAC cell lines and novel patient derived xenograft (PDX) material (cell line and tumours) and patient tumour.

- To characterise one lead newly developed monoclonal antibody designated mAb 4A5 through immune-based techniques in order to study the expression of the 4A5 target antigen in PDAC cells/ tissues.

- To investigate the effect of Mab 4A5 on the proliferation of its immunogen cell lines, PANC-1 and PIN 127 cells.

- To develop a formulation for a new hydridoma growth medium supplement that contains no animal derived products, which demonstrates comparable potency to its parent product BriClone™.

- To create an *in vitro* cell based assay for the quality control of BriClone™ for the replacement of live animals, in line with the 3Rs principles, which aim to enhance the welfare of animals used in testing.

2. Materials and Methods

2.1 Cell Culture and reagents

2.1.1 Aseptic techniques

All cell culture works were carried out aseptically in a class II laminar airflow cabinet. The laminar airflow cabinet was turned on for 15 minutes prior to starting the work and left to clear for 15 minutes between each cell line to avoid cross contamination. Before and after working with the cells, the laminar airflow cabinet was cleaned with 70% Industrial Methylated Spirit (IMS), and every item brought into the cabinet was sprayed with 70% IMS.

The laminar airflow cabinets were cleaned every fortnight with the disinfectant Virkon for 15 minutes, then rinsed with reverse osmosis (RO) water and sprayed with 70% IMS.

2.1.2 Cell culture media and reagents

All cells were maintained under standard culture conditions, in an incubator with 5% CO2, 95% humidity at 37°C. The cells were fed every 3-4 days with their respective growing media. Adherent cell lines were detached from their plastic support using TrypLE™ Express Enzyme (Gibco). All basal media were from Sigma-Aldrich except DMEM Glutamax™ supplement (Gibco). The Fetal Bovine Sera (FBS) and other additives were from Gibco.

The cell lines used in this thesis and their corresponding growing media are listed in Table 2-1.

Table 2-1: List of the cell lines employed in this thesis and their corresponding growing media

Cell line	Supplier number	Origin	Growing media
Caco-2	HTB-37™, ATCC®	Colorectal adenocarcinoma	DMEM, 10% FBS, 1% LGlutamine
BxPC-3	CRL-1687™, ATCC®	Pancreatic ductal adenocarcinoma	RPMI-1640, 10% FBS
HCC 1419	CRL-2326™, ATCC®	Breast ductal adenocarcinoma	RPMI-1640, 10% FBS
IGROV-1	The Netherlands Cancer Institute	Ovarian adenocarcinoma	RPMI-1640, 10% FBS
LOPRA-1	ACC 778, DSMZ	Multiple myeloma	RPMI-1640, 20% FBS**, 10 ng/mL human IL-6
MCF7	HTB-22™, ATCC®	Breast ductal adenocarcinoma	RPMI-1640, 10% FBS
MIAPaca-2	CRL-1420™, ATCC®	Pancreatic ductal adenocarcinoma	DMEM, 10% FBS, 1% LGlutamine
OAW42	ECACC 85073102	Ovarian adenocarcinoma	RPMI-1640, 10% FBS

Cell line	Supplier number	Origin	Growing media
PANC-1	CRL-1469™, ATCC®	Pancreatic ductal adenocarcinoma	DMEM 10% FBS, 1% LGlutamine
PIN 99*	NICB	Pancreatic ductal adenocarcinoma	DMEM/F-12, 10% FBS
PIN 127*	NICB	Pancreatic ductal adenocarcinoma	DMEM/F-12, 10% FBS
Sp2/0-Ag14	Immune Systems Ltd	Mouse myeloma	DMEM GlutaMAX™, 10%** FBS
SW1990	CRL-2172™ , ATCC®	Pancreatic ductal adenocarcinoma	DMEM 10% , FBS, 1% LGlutamine
ZR-75-1	CRL-1500™ , ATCC®	Breast ductal carcinoma	RPMI-1640, 10% FBS
7-TD-1	ACC 23, DSMZ	Mouse multiple myeloma	90% RPMI 1640 + 5% FBS**, 10 ng/ml IL-6

*The term PIN refers to the Patients Identification Number

** heat inactivated FBS

2.1.3 Sub-culturing of cell lines

2.1.3.1 Adherent cell lines

The waste cell culture medium was removed from the tissue culture flask to a sterile bottle. The flask was rinsed with 2mL of TrypLE™ Express Enzyme (1X) solution to ensure the removal of any residual media. 2mL of TrypLE™ Express Enzyme was then added to the flask and incubated at 37°C for the required period of time until all cells were detached from the inside surface of the culture flask. The enzyme was deactivated with an equal volume of complete media. The cell suspension was removed from the flask and placed in a sterile universal container and centrifuged at 200 g for 5 minutes. The supernatant was discarded from the universal and the pellet was suspended in complete medium. A cell count was performed using a haemocytometer. An aliquot of cells was then used to re-seed a flask at the required density with fresh media.

2.1.3.2 Semi-adherent cell lines

The cells were lifted off their support by mechanical action, using a 10mL pipette. The cell suspension was removed from the flask and placed in a sterile universal container and centrifuged at 200 g for 5 minutes. The supernatant was discarded from the universal and the pellet was suspended in complete medium. A cell count was performed using a haemocytometer. An aliquot of cells was then used to re-seed a flask at the required density with fresh media.

2.1.4 Cryopreservation of cells

Cells for cryopreservation were harvested in the log phase of growth. Cell pellets of adherent cell lines were resuspended 1 mL of cold 10% DMSO/ 90% FBS solution and transferred in a cryovial. The Sp2/0-Ag14 cells were frozen with 0.5mL of their conditioned medium and 0.5 mL of HyCryo (Cytiva). The cryovials were placed in the vapour phase of a liquid nitrogen container, which was equivalent to a temperature of -80°C. After a period of three hours, vials were removed from the vapour phase and transferred to the liquid nitrogen phase (-196°C). for storage.

2.1.5 Thawing of cryopreserved cells

A volume of 5mL of fresh warmed growth medium was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen tank and thawed rapidly at 37°C. The cells were removed from the vials and transferred to the aliquoted media. The resulting cell suspension was centrifuged at 200 g for 5 minutes. The supernatant was removed and the pellet resuspended in fresh culture medium. Thawed cells were then added to a T25cm^2 tissue culture flask with a suitable volume of fresh growth media.

2.2 Monoclonal antibody generation

2.2.1 Immunogens

2.2.1.1 Patients' tumours and PDX tumours

Patients tumour PIN 113 and first generation (F1) generated PDX tumour PIN 65 were obtained from Dr Sandra Roche (Roche *et al*., 2020). These tumours had been snap frozen in liquid nitrogen, and stored at -80ºC as soon after arrival in the laboratory. A chunk of tumour was retrieved from the freezer and reduced to powder using a mechanical grinder under constant cold conditions. The powdered tumour was resupended in PBS and stored at -80ºC until use.

2.2.1.2 Cell lines

PIN 99 and 127 cell lines were generated by Dr Sandra Roche as described (Roche *et al*., 2020).

PANC-1, PIN 127, PIN 99 and BxPC-3 cell pellets were resuspended in PBS and stored at -80ºC until use.

2.2.1.3 Groups of immunogens

Three groups of immunogens were investigated to generate the mAbs. Established cell lines and/or tumours were combined to increase the likelihood of an immunological reaction.

- Group 1: PIN 127 cells + PANC-1 cells
- Group 2: PIN 99 cells + PIN 65 F1 PDX Tumour
- Group 3: PIN 113 Patient Tumour + BxPC3 cells

2.2.2 Immunisation Regime

The inoculations of animals were carried out under HPRA Project Authorisation AE19115/P018.

Each group comprised of two female BALB/c mice (Charles River UK), first inoculated at 6 week old and then every three weeks for a total number of six inoculations. Fusion assays were carried three days after the last booster.

For each inoculation, 130 µL of each immunogen were pooled together and mixed with 260 µL of lipopeptide adjuvants Pam3Cys-SKKKK (EMC microcollectors). 150 µL of that mix was injected subcutaneously in the neck of each animal using an 18G needle and a 1mL syringe. The animals were observed for 1 hour after injection and the grimace scale recorded (*Grimace Scales | NC3Rs*, no date). The mice welfare was monitored weekly throughout the immunisation schedule with their weight and overall wellbeing recorded.

2.2.3 Fusion technology

The fusion procedure used for the production of monoclonal antibodies was a modification of the protocol outlined by Kohler and Milstein (Köhler *et al*., 1976).

For the fusion procedure, the media referred to are:

- Complete medium: DMEM Glutamax™, 10% heat inactivated FBS, 1% Penicillin/Streptomycin.
- Serum free medium (SFM): DMEM Glutamax™
- Plating medium (PM): DMEM Glutamax™, 10% heat inactivated FCS, 5% BriClone™ (NICB, Ireland), 1% Penicillin/Streptomycin, 1% HAT (Hypoxanthine, Aminopterin, Thymidine)

- Growing medium: DMEM Glutamax™, 10% heat inactivated FBS, 1% Penicillin/Streptomycin, 5% BriClone™.

2.2.3.1 Sp2/0-Ag14 cells

A frozen vial of Sp2/0-Ag14 was revived a week to 10 days prior to fusion and grown in complete media.

Prior to fusion, 4 xT75cm^2 flasks, (two per mouse; a minimum of 1 x 10^7 cells per mouse are required for this procedure) of Sp2/0-Ag14 myeloma cells were harvested. The cells were centrifuged at 200 g for 5 minutes and resuspended in SFM. This step was repeated twice and the cell suspensions were kept at 37ºC until fusion.

2.2.3.2 Splenocytes

For each group, two animals were sacrificed humanely by cervical dislocation, swabbed with 70% IMS and their spleen removed aseptically in a laminar flow cabinet with sterile instruments. A single cell suspension was obtained by mashing up the spleen in 3 mL of SFM through a sterile 100 µm cell strainer using the plunger of a sterile 10 mL syringe .This cell suspension was transferred into a 30 mL universal tube and the volume adjusted to 10 mL. Large clumps of cells were allowed to pellet at room temperature for 2-3 minutes. The supernatant was then transferred to a fresh tube and centrifuged at 200 g for 5 minutes.

2.2.3.3 Fusion of cells

The Sp2/0-Ag14 myeloma and the splenocytes cell suspensions were mixed in a universal and centrifuged at 300 g for 5 minutes before being resuspended in SFM. This step was repeated twice. Following the final washing step, 1 mL of polyethylene glycol (PEG) pre-warmed at 37°C, was added to the cell pellet with a Pasteur pipette using a gentle swirling and aspirating action for 30 seconds. After 30 seconds the aspiration was discontinued and gentle swirling was kept until 75 seconds after the start. Plating medium (0.5 mL) was added slowly down the side of the universal while continuing to swirl. 1 mL of plating media is added at every minute and after 5 minutes, the final 5 mL of plating medium was added. The cell suspension was centrifuged at 50 g for 5 minutes, supernatant was removed and the cells were resuspended in 10 mL of plating medium and incubated at room temperature for 15 minutes.

The fused cells were diluted in plating media (1 mL into 45 mL) and 800 µL was delivered into each well of 48-well plates. The plates were wrapped with parafilm and incubated for 10-12 days undisturbed at 37ºC before assessment.

2.2.4 Subculture of hybridomas

Hybridoma colonies were sub-cultured to 6 well plates in plating medium and gradually weaned off HAT to HT (Hypoxanthine and Thymidine), and eventually, within 2-4 weeks, hybridoma clones were weaned off HT and fed with complete media.

2.2.5 Single cell cloning by limiting dilution

Using a multi-channel pipette 100 µL of DMEM growth medium was pipetted into each well of a sterile 96-well tissue plate. 100 µL of cell suspension from a confluent culture of hybridomas was added to the top left hand well and mixed by pipetting. 1 in 2 doubling dilutions were performed down the left hand row of the plate (8 wells, 7 dilution steps) and mixed by pipetting, ensuring to change the pipette tip each time. 1 in 2 dilutions were also performed across the plate using a multi-channel pipette. Plates were incubated for 7-10 days at 37°C, 5% CO2. Wells containing a single colony were chosen and the cells transferred into 6-well plates. The selected clones were screened by immunofluorescence (section 2.3.1) further expanded, and frozen.

2.2.6 Isotype analysis

Isotyping was carried out using the Isostrip™ Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics GmbH).

150 µL of hybridoma supernatant was mixed with the isotyping beads in glass tube with a vortex for 30 seconds. The strip was then inserted in the tube for 5 to 10 minutes, allowing the liquid to travel up by capillarity, until the control band appeared. The strips were kept for further reading by cutting the black end.

2.2.7 Purification of monoclonal antibody

Small-scale affinity purification of mouse IgG mAb was carried out using NAb™ Protein A Plus Spin Columns (Thermo Scientific™).

Column and buffers were brought to room temperature (RT). Every centrifugation step was set to 1,000 × g. The column was equilibrated by performing 2 x 1 minute washes with 2 mL of binding buffer (0.1M phosphate, 0.15M sodium chloride; pH 7.2). The sample was added onto the column and incubated with end-over-end mixing for 10 minutes at RT. The flow through was collected and kept for assessment. The column was washed 3 times with binding buffer. Three eluate fractions were collected by applying 1 mL of IgG elution buffer (Pierce) onto the column. Each eluate was neutralised with 100 µL of neutralisation buffer (1M Tris at pH 8-9).

Purification was checked by protein gel coomassie staining (section 2.6.3 and 2.6.4)

2.2.8 Dialysis of monoclonal antibody

Dialysis of mAb was carried out using the Slide-A-Lyzer 10K cassette. 12 mL was injected in the device and left to float in PBS for 2 x 2 hours at RT followed by an overnight dialysis at +4°C in order for buffer exchange to take place.

2.3 Immunofluorescence studies

2.3.1 Immunofluorescence on unfixed cells

A day prior to analysis, cells were seeded onto multiwell glass slides and incubated at 37ºC overnight in a humidified chamber. The following day, the slides were gently rinsed twice with Live Cell Imaging Solution (Gibco), and the hybridoma supernatants to be tested were added on the layer of cells and incubated for 2 hours at 37ºC. After the incubation the slides were gently rinsed twice and the secondary antibody goat anti-mouse alexa fluor 488 (1:2,000) was applied onto each well for 1 hour at RT. In some instances, NucBlue™ Live ReadyProbes™ Reagent (Invitrogen) was added for 40 minutes at RT. Slides were rinsed with Live Cell Imaging Solution and observed immediately using a fluorescent microscope.

2.3.2 Immunofluorescence on fixed cells

A day prior to analysis, cells were seeded onto four compartments polymer 35 mm dish (Ibidi®) incubated at 37ºC overnight in a humidified chamber. The cells were rinsed once with PBS and fixed with 4% formaldehyde for 1 hour at RT, followed by two washes in PBS with 0.5% v/v Triton X-100 was applied for 30 minutes at RT for permeabilisation, followed by incubation with Image-iT™ FX Signal Enhancer (Invitrogen™) for 30 minutes at RT. After three washes in PBS the cells were blocked with BlockAid™ Blocking Solution (Invitrogen™). The primary Ab was applied overnight at +4°C. After three rinses in PBS, the secondary Ab Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Invitrogen™) was applied for 1 hour. After a final wash in PBS, glass slides were mounted with ProLong™ Gold Antifade Mountant (Invitrogen™), and analysed by Dr Finbarr O'Sullivan using MetaMorph software.

2.4 Histological analysis

Staining was carried out using the Dako Autostainer. The Dako REAL EnVision Detection Systems, Peroxidase/DAB, Rabbit/Mouse (Agilent) was used as per manufacturer's guidelines. All steps were carried out at RT. Blocking was performed using the Dako REAL Peroxidase-Blocking Solution for 10 minutes. The primary Ab was incubated for 1 hour, and the secondary Ab for 30 minutes. Two 5 minutes exposures with the DAB chromogen were executed followed by a final staining with hematoxylin for 5 minutes. Following dehydration through graded washes of ethanol and final wash in xylene, glass coverslips were mounted onto the slides with DPX mountant (Sigma Aldrich).

2.4.1 Immunocytochemistry

An area was marked with a wax pen onto Superfrost Plus glass slides (ThermoScientific). Freshly detached cells were seeded onto slides and left to attach overnight at 37ºC. The following morning, all slides were rinsed twice with PBS and put in the incubator for 3-4 hours to dry. After incubation, the slides were wrapped individually in tin foil and stored at -80ºC until use. On the day of the staining, the slides were fixed for 5 minutes with 4%

paraformaldehyde and then rinsed three times for 3 minutes in PBS followed by immediate staining with the Dako Autostainer (see section 2.4)

2.4.2 Immunohistochemistry

Formalin fixed and paraffin embedded tumours (performed by Dr Fiona O'Neill) were cut to 5 µm tissue section. Antigen retrieval was performed by paraffin dewaxing through graded washes in xylene and ethanol, or by heat induced epitope retrieval (HIER) using Citrate Buffer pH 6 (Abcam) or Tris-EDTA Buffer, pH 9 (Abcam), followed by immediate staining with the Dako Autostainer (see section 2.4).

2.4.2.1 Paraffin embedded cells

Cells were cultured in 1 x T175cm flasks to 80% confluency. The cells were detached from their support, resuspended in 10 mL of medium and centrifuged at 150 g for 3 minutes. The supernatant was removed, and the cell pellet was washed once with PBS. The centrifuged pellet was resuspended with 5 mL of 10% Neutral Buffered Formalin (NBF) added very slowly, and left overnight at room temperature. Following fixation, the cells were spun down at 150 g for 3 minutes and washed cells in 10 mL PBS. An 0.8% w/v agarose solution was prepared in PBS, (heated in microwave for 1 min) and 200 µL was added to the cell pellet and transferred to the tapered end of 2.5 mL Eppendorf. The cell-agarose mix was allowed to cool and solidify, then removed from the Eppendorf. The cell block was placed in an embedding cassette and submitted to dehydration. For such, the cassette was placed under running tap water for 1 hour and then placed in graded baths of ethanol and xylene.

- 50% Alcohol for 1 hour
- 70% Alcohol for 1 hour
- 90% Alcohol for 1 hour
- 100% Alcohol for 1 hour
- Xylene for 1 hour twice

The dehydrated cassettes were then incubated into melted paraffin for 2 hours. Following infiltration of paraffin, the block were left to cool and harden before being cut into 5 µm

sections. The sections were placed on Epredia™ SuperFrost Plus™ Adhesion slides and baked for two hours at 60°C.

2.5 Western Blot analysis

2.5.1 Preparation of cell lysates

Cells were grown to 80-90% confluency in 90 mm Petri dishes. Media was removed and the cells layers were rinsed twice with ice cold PBS. 300 µL of RIPA buffer (Sigma- Aldrich) containing 1X Protease Inhibitor cocktail (Calbiochem), 2 mM PMSF (Sigma), and 1 mM sodium orthovanadate, was added and cells were incubated on ice for 10 minutes. Cells were scraped and lysis buffer was collected, centrifuged at maximum speed (12,000 g) for 10 minutes at +4ºC. Supernatant were collected and stored at -80°C. Protein quantification was carried out using a bicinchoninic acid (BCA) quantification kit (Pierce) as per manufacturer's protocol.

2.5.2 Immunoblotting

Thirty µg of protein was electrophoretically resolved on Bolt™ 4 to 12%, Bis-Tris polyacrylamide gels (Invitrogen™). The proteins were transferred for 10 minutes to a nitrocellulose membrane using iBlot™ Transfer Stack and the iBlot transfer system (Invitrogen™). Ponceau S (Sigma) was used to confirm protein transfer and the membrane was blocked with Intercept® (PBS) blocking buffer or NET buffer (see Appendix I) for 1 hour at RT. The primary antibody was probed on the membrane at 4°C overnight. Membranes were washed three times for 30 minutes with PBS, 0.1% Tween-20 followed by 1 hour incubation in secondary antibody IRDye® 800CW Goat anti-Human IgG (H + L) (Li-Cor) or AffiniPure Goat Anti-Mouse IgG (H+L)-HRP (Jackson ImmunoResearch) at RT and three washes for 30 minutes with PBS, 0.1% Tween-20. Blots were visualised with ECL reagents (Amersham) for 4 minutes at room temperature. Following this, the solution was tapped off, the blot laid between two sheets of cling film, and exposed to Amersham HyperfilmTM, chemiluminescence film (GE Healthcare) for 4 minutes and processed using standard x-ray developing procedures. Dried film was aligned with the pre-stained molecular weight marker (ProSieve color marker, Lonza) for molecular weight determination.

2.6 Direct immunoprecipitation analysis

2.6.1 Ultrafiltration

4 mL of test antibody (mAb 4A5) and Sp2/0-Ag14 conditioned medium control were concentrated 8 times on a 30K MWCO Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore) for 30 minutes at 4°C at 3,000 g.

2.6.2 Immunoprecipitation

Proteins were isolated from 3 x 175cm^2 flasks each of PANC-1 and PIN 127 cell lines, as described in section 2.5.1. 250 µL cell lysates were pre-cleared by 2 x 2 hours incubation with 50 µL of protein-G agarose beads (Pierce). All samples were incubated 2 x 2 hours at 4°C on a rocking platform. Supernatants were transferred to clean eppendorf tubes and 50 µL of antibody, Sp2/0-Ag14 conditioned medium or mouse IgG control (used at a concentration of 22 µg/mL) (Sigma Aldrich) was added and incubated overnight at 4°C on a rocking platform. The following day, in order to precipitate the antibody-antigen complex, 50 µL of protein G agarose was added to the samples incubated at 4°C for 4 hours on a rocking platform and the supernatant discarded. The beads were then washed for 3 x 15 minutes periods with IP wash buffer (Pierce) and pelleted. Following the final wash and spin, as much liquid as possible wasremoved from all samples. The resin beads were resuspended in 7.5µL of reducing agent (Invitrogen™) and 18.75µL of loading buffer (Invitrogen™) and denatured for 10 minutes at 70°C

All centrifugation steps were performed at 500 g for 10 minutes at +4°C.

2.6.3 Gel electrophoresis of immunoprecipitated proteins

Immunoprecipitated proteins were separated on 4-12%, Bis-Tris electrophoresis gel as described in section 2.5.2. Following electrophoresis, gels were either stained using GelCode™ Blue Safe Protein Stain (Pierce), or Western blot analysis was carried out as outlined in section 2.5.2.

2.6.4 Gel protein staining

The gels were washed 3 x 5 minutes in ultra high purified (UHP) water, followed by 1 hours staining in GelCode™ Blue Safe Protein Stain. The gels were left to destain in UHP overnight at +4°C.

2.7 Proliferation assays

2.7.1 Acid Phosphatase assay

Cell lines were seeded in 96-well plates at a cell density of $5x10^4$ cells/mL. After 24 h, the cells were treated with mAb 4A5 or negative controls. After three days' incubation, proliferation was measured using the acid phosphatase assay. Medium was removed and cells were washed three times with PBS. 100 µL of acid phosphatase substrate (10 mM p-nitrophenol phosphate (Sigma)in 0.1 M sodium acetate (Sigma), 0.1% Triton X-100 (BDH, pH 5.5) was added to each well and the plate was then incubated at 37°C for 1 hour. The reaction was halted with the addition of 50 µL of 0.1 M sodium hydroxide. The absorbance was then read at 405 nm and at 620 nm as a reference on a plate reader. Percentage growth was calculated relative to an untreated control.

2.7.2 XTT assay

50 µL of cells were seeded at $5x10^4$ cells/mL in 96-well plates. Following a 24hours incubation, the cells were treated with 50µL of 2X respective conditions. After three days' incubation, proliferation was measured using the CyQUANT™ XTT Cell Viability Assay. Per plate, one bottle of XTT Reagent and one vial of the Electron Coupling Reagent were mixed and used immediately. 70 µL of the mix was added to each well and the plate was incubated at 37°C for 4 hours. After incubation, the absorbance was read at 450 nm and 660 nm. Percentage growth was calculated relative to an untreated control.

3. Results Section

3.1 Generation of monoclonal antibodies against pancreatic ductal adenocarcinoma

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive disease, which is virtually incurable with current treatment modalities. Unlike other solid cancers, pancreatic cancer treatment has not improved significantly in the last thirty years and has limited molecular targets. As such, there is an urgent need to identify novel targets for pancreatic cancer, ideally located on the membrane of the cells for potential access by therapeutic antibodies and Antibody Drug Conjugates (ADCs). By using well-established pancreatic cancer cell lines, novel patient derived xenograft (PDX) cell-lines, PDX tumours and patient tumours, this research aimed to exploit these valuable models as immunogens for the development of novel monoclonal antibodies (mAbs).

This section of the thesis describes the work to generate novel antibodies directed against PDAC using a combination of patients' tumours, patient derived xenograft (PDX) tumours, PDX derived cell lines and a panel of well characterised established PDAC cell lines. Three groups of BALB/c mice (n=2 per group) were inoculated with different combinations of immunogens (Table 3-1) to achieve a wider immune response. Two immunogens were administered per group of animals as detailed in section 2.2.1 and 2.2.2. The three groups were not inoculated at the same time. An interval of 6 weeks was introduced between the start of each group. The inoculations were carried out with freshly thawed materials, mixed with the adjuvant a few minutes prior to the inoculation.

Table 3-1: Details of the immunogenic material administered for the immunisation of BALB/c mice group 1, 2 and 3.

Group	Immunogen 1	Immunogen 2
1	PANC-1 cells	PIN 127 DPX derived cells
2	PIN 99 PDX derived cells	PIN 65 F1 PDX tumour
3	BxPC-3 cells	PIN 113 Patient tumour

3.1.1 Animals monitoring and welfare criteria

The animals were left to settle in their environment for a week prior to commencing the immunisation regime. Daily monitoring was carried out throughout the immunisation schedule by the staff at the Bio-Resource Unit, Dublin City University. A weekly check was performed by the research personnel, which involved a weighing of each animal and overall inspection of their wellbeing. All observations were recorded.as per HPRA authorisation AE19115/P018 requirements. Any adverse reactions were recorded as per Table 3-2.

Table 3-2: Adverse events recorded post injection and over the schedule of immunisation.

Group	Reaction post inoculation	Side effects over the course of the immunisation schedule
1	none	none
2	Mice were sleepy 15 minutes post injection but recovered after 1 hour	Skin necrosis at the site of injection that would heal between boosters
3	none	Slight skin necrosis at the site of injection

3.1.2 Fusion and screening

3.1.2.1 Selections of positive candidates post fusion

Fusion assays were performed as described in section 2.2.3. Two laminar flow cabinets were used for each group, with two researchers manipulating one mouse each. Dr Sandra Roche kindly helped in the smooth completion of this step of the work.

The fused cells were seeded in 48-well plates. Each group comprised of 12 plates combined between mouse 1 and mouse 2.

After 10 days incubation in HAT selecting medium, the plates of fused cells were screened for wells where the growth of colonies was observed. The observation of positive wells was visual, with chosen wells defined as ones where the medium had change colour from pink to orange/yellow. 500 µL of all positive hybridoma supernatants were harvested, placed into sterile Eppendorfs and kept at +4°C. The wells were fed with 600 µL of fresh HAT selection medium and the same was repeated at days 11 and 12.

Table 3-3: Details of the number of positive hybridoma wells chosen and corresponding fusion efficiencies from group 1, 2 and 3

	Group 1	Group 2	Group 3
Total wells seeded	576	576	576
Number of positive wells selected	403	398	490
Resulting fusion efficiencies	70%	69%	85%

3.1.2.2 Screening for positive candidates

The harvested conditioned media (CM) i.e., positive yellow hybridoma supernatant from the relevant wells, were used for the primary screening for each of the three fusions.

The screenings were performed by immunofluorescence on unfixed cells as described in section 2.3.1. A negative control of Live Cell imaging Solution in place of a secondary antibody was incorporated in all experiment.

The cell lines used for primary screenings were

- **Group 1: PANC-1 and PIN 127 PDX derived cells**
- **Group 2: PIN 99 PDX derived cells**
- **Group 3: BxPC-3 cells**

It should be noted that as group 2 and group 3 were inoculated with tumour material (PDX tumour for group 2 and patient tumour for group 3), using a second cell line for primary screening was not possible.

Screening of positive hybridomas from group 1

Following the primary screening for reactivity of the 403 hybridoma CMs against PANC-1 and PIN 127 cells, 30 supernatants reacted against the cells. Only the supernatants displaying a strong immunoreactivity (i.e. strong green signal from the Alexa Fluor 488 fluorophore) against the PANC-1 and PIN 127 cells were transferred from the 48-well plates to 6-well plates. These 30 candidates were further fed, let to grow and were tested on a second screening, which resulted in six candidates that showed a strong reaction with the two immunogenic cell lines. These six hybridoma cultures were gradually weaned off HAT and allowed to proliferate in complete growing medium. They were transferred from the 6-well plates into T25cm^2 flasks. They were subsequently cloned by limiting dilution (section 2.2.5), and the chosen clones (i.e. the ones showing one colony per well) were again screened by IF on unfixed cells. The cloned hybridomas that still showed immunoreactivity on the two cell lines were subsequently cryopreserved.

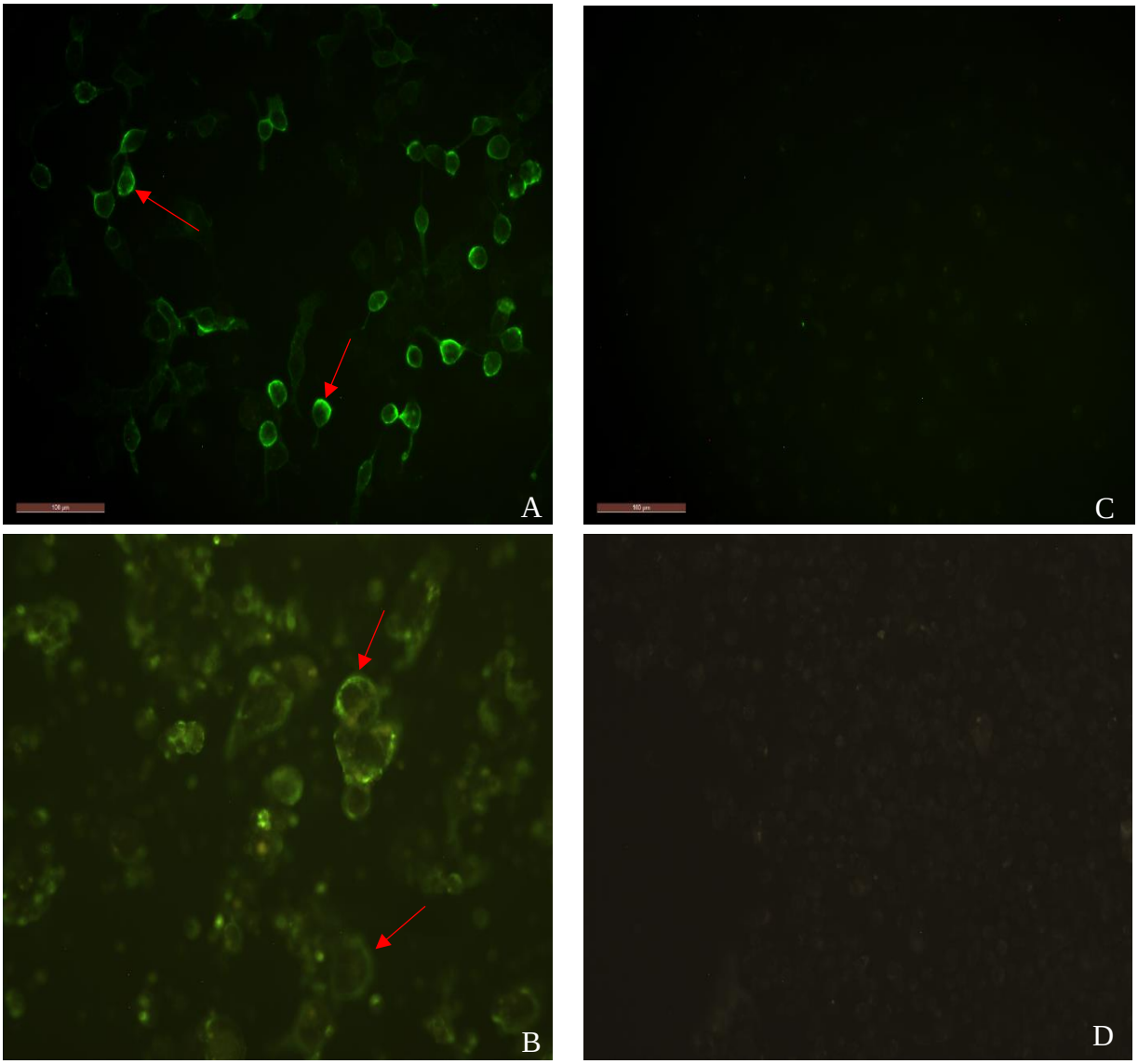


Figure 3-1: Representative microscopic images of immunofluorescent staining of 4A5-D8 hybridoma candidate from group 1 on unfixed A) PANC-1 cells, B) PIN 127 cells and their respective negative controls C) and D). The immunoreactivity appears to be membrane localised in the immunogen cell lines, as shown by the red arrows. Magnification 20x.

As observed in Figure 3-1, mAb 4A5 strongly binds to the cell lines PANC-1 (A) and PIN 127 (B) where the punctate like immunoreactivity (indicated by green fluorescence from Alexa Fluor 488) appears to be located on the membrane (indicated by the red arrows) and in the cytoplasm of the cells. The negative controls (C and D) where no staining was observed, indicated specific staining of mAb 4A5-D8.

Screening of positive hybridoma from group 2

The only immunogen of this group that could be exploited for screening were the PIN 99 PDX derived cells. At the time of this study, no cell line has been derived from the PIN 65 F1 tumour, hence the positive clones were screened against the PIN 99 cells only.

As per section 2.3.1, the supernatants were applied to PIN 99 cells grown on multiwell slides. The first screening resulted in 34 hybridomas giving a positive, but not striking, signal by IF (results not shown).

A second screening on PIN 99 cells resulted in the selection of 12 hybridomas which reacted with the cells. Following a third screening, six hybridomas showed consistent reactivity were gradually weaned off HAT and allowed to proliferate in complete growing medium. They were transferred from the 6-well plates into T25cm^2 flasks, cloned under limiting dilutions and re-screened. This screening led to three final hybridoma candidates, which were expanded and cryopreserved.

- 2.7A1-Clone 2D10
- 2.6D7-Clone 2F7
- 1.4F1-Clone F10

Hybridoma 1.4F1-Clone F10 led to a signal on PIN 99 cells by IF however, the produced mAb was unstable. CM of clone F10 tested inconsistently on IsoStrips ™ isotyping (section 2.2.6). When the mAb in the CM was tested by IsoStrips ™, the resulted varied with either no mAb detected or the detected mAb showed a wide variation of classes (G, M and A)

Both 2.6D7-2F7 and 2.7A1-2D10 gave mixed isotyping results, IgM and IgG3 combined, suggesting a mixed population of hybridomas. Both were re-cloned and clone F11, which arose from 2F7, was the only clone which still reacted with PIN 99 cells, although the signal

was weak. The characterisation of this mAb was paused, and the expanded cultures were cryopreserved for future investigations.

Screening of positive hybridomas from group 3

As equally with group 2, only one of the immunogens inoculated, the BxPC-3 cells were used for screening. At the time of this work, no cell line was derived from PIN 113 tumour, hence the positive clones were screened against the BxPC-3 cells only.

The first screening resulted in 19 hybridomas candidates, although the signal by IF was not strong (not shown). A second screening on BxPC-3 cells resulted in the selection of only three hybridomas which reacted with the cells. These were gradually weaned off HAT and allowed to proliferate in complete growing medium. They were transferred from the 6-well plates into T25cm^2 flasks, cloned under limiting dilutions and re-screened. This screening led to two final hybridoma candidates, which were expanded and cryopreserved. No further studies were performed with the hybridomas from group 3.

3.1.3 Summary of the generation of monoclonal antibodies

The generation of monoclonal antibodies from mice inoculated with clinically relevant material from a combination established PDAC cell lines, PDAC PDX derived cell lines, PDAC PDX tumour and PDAC patient tumour was successful. Three groups of animals were employed to produce hybridomas against PDAC. After seven months of antibody generation, several hybridomas of interest were selected and cryopreserved. Due to its distinct reaction with its two immunogens, hybridoma 4A5-D8 has been chosen for further characterisation to elucidate its specificity. For the remaining of this thesis, it will be designated as mAb 4A5.

3.2 Characterisation of monoclonal antibody mAb 4A5

Following the generation of hybridomas secreting monoclonal antibodies against pancreatic ductal adenocarcinoma (PDAC), it was decided to solely focus on the most promising candidate for logistical reasons, despite other candidates produced. MAb 4A5 generated from the inoculation of BALB/c mice with PANC-1 cells and PIN 127 PDX derived cells. This mouse anti-human mAb was subsequently characterised through a range of immuno-based techniques and its potential anti-cancer activity tested for possible effects on cell proliferation.

3.2.1 Isotype determination

In order to confirm the isotype of immunoglobulin mAb 4A5 is, a rapid isotyping was repeated through the use of an IsoStrip™ kit (section 2.2.6). The kit contains latex beads where anti-mouse kappa and lambda chains antibodies are immobilised. These beads are incubated with the CM containing mAb 4A5 for 30 seconds. A strip, consisting of immobilised anti-mouse antibodies of all classes, is then placed in contact with the beads-mAb complexes. The complexes travel up through the strip by capillary action until binding with their corresponding antibody class. Within 5 to 10 minutes, a blue line develops where the binding has formed indicating the subclass and light chain type of the mAb. The strip possesses two sides, each bearing a blue positive control band indicating complete development of the reaction.

Figure 3-2: Image of typical result obtained with isotyping of mAb 4A5 on IsoStrip™ showing a) subclass of immunoglobulin, b) type of light chain

Figure 3-2 demonstrates the results of the immunotyping of mAb 4A5 by IsoStrip™. The band in a) shows that mAb 45 was determined to be an **IgG1,** with the light chain determined as **kappa** from the blue band seen in b).

3.2.2 Immunofluorescence studies of mAb 4A5 on unfixed cells

Following on from initial screenings, further immunofluorescence (IF) on unfixed cells was carried out with mAb 4A5. Staining on unfixed live cells will allow for the determination of surface localisation In addition the use of a fixative can result in the loss of antibody antigen binding or incorrect localisation of the antigen.

The mAb was applied on a panel of cancer cell lines, PDAC and other cell lines representing a range of cancer types. In this setting, the method used was the same as described as in section 2.3.1 except a NucBlue™ (DAPI) counterstained was added during the incubation with the secondary Ab to stain the nuclei of the cells. Pictures were superposed using the Metamorph software.

Pancreatic ductal adenocarcinoma cell lines

In addition to the immunogens PANC-1 and PIN 127, the cell lines MiaPACA-2, SW1990 and PIN 99 PDX derived cells were tested for their potential expression of an antigen of mAb 4A5.

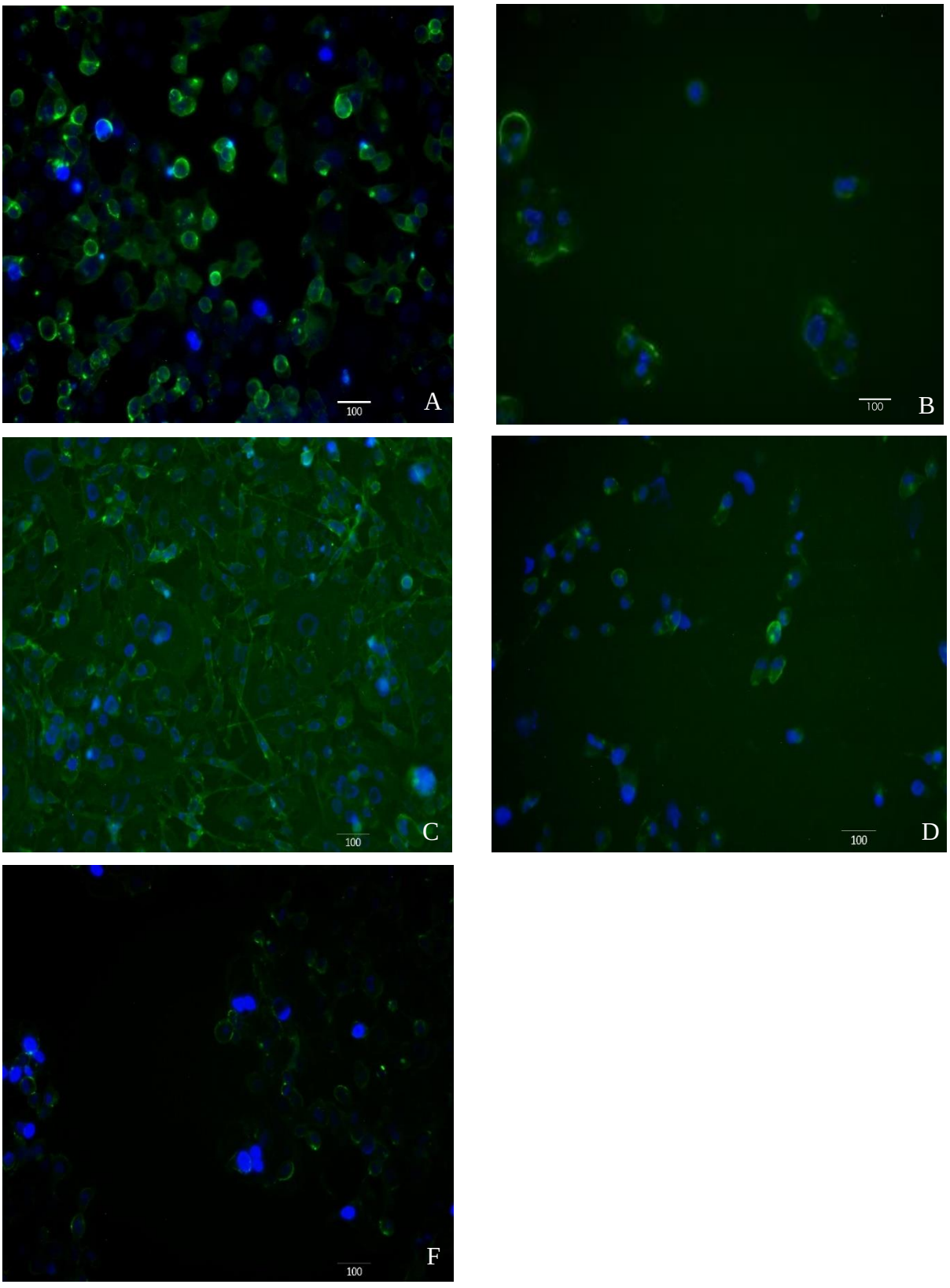


Figure 3-3: Representative microscopic images of immunofluorescent staining of mAb 4A5 on unfixed PDAC cell lines.A) PANC-1, B) PIN 127, C) MiaPACA-2, D) SW1990, E) PIN 99. Magnification 20x.

Figure 3-3 shows the fluorescent signal obtained after incubation of mAb 4A5 with PDAC cell lines PANC-1 (A), PIN 127 (B), SW1990 (C), Mia-PACA-2 (D), PIN 99 (E). The reaction of mAb 4A5 exhibited a green signal in all cell lines, visualised under a fluorescent microscope, with what appears to be membrane staining. The cell lines PANC-1 (A), PIN 127 (B), and SW1990 (D) gave a stronger staining than Mia-PACA-2 (D) and PIN 99 (E).

Cell lines representing other cancer types

Cells from various neoplasms were also incubated with mAb 4A5. The colorectal cancer Caco-2 cells, ovarian cancer IGROV-1 and OAW42 cells, breast cancer MCF-7, HCC 1419 and ZR-75-1 cells were investigated for their potential reaction with mAb 4A5.

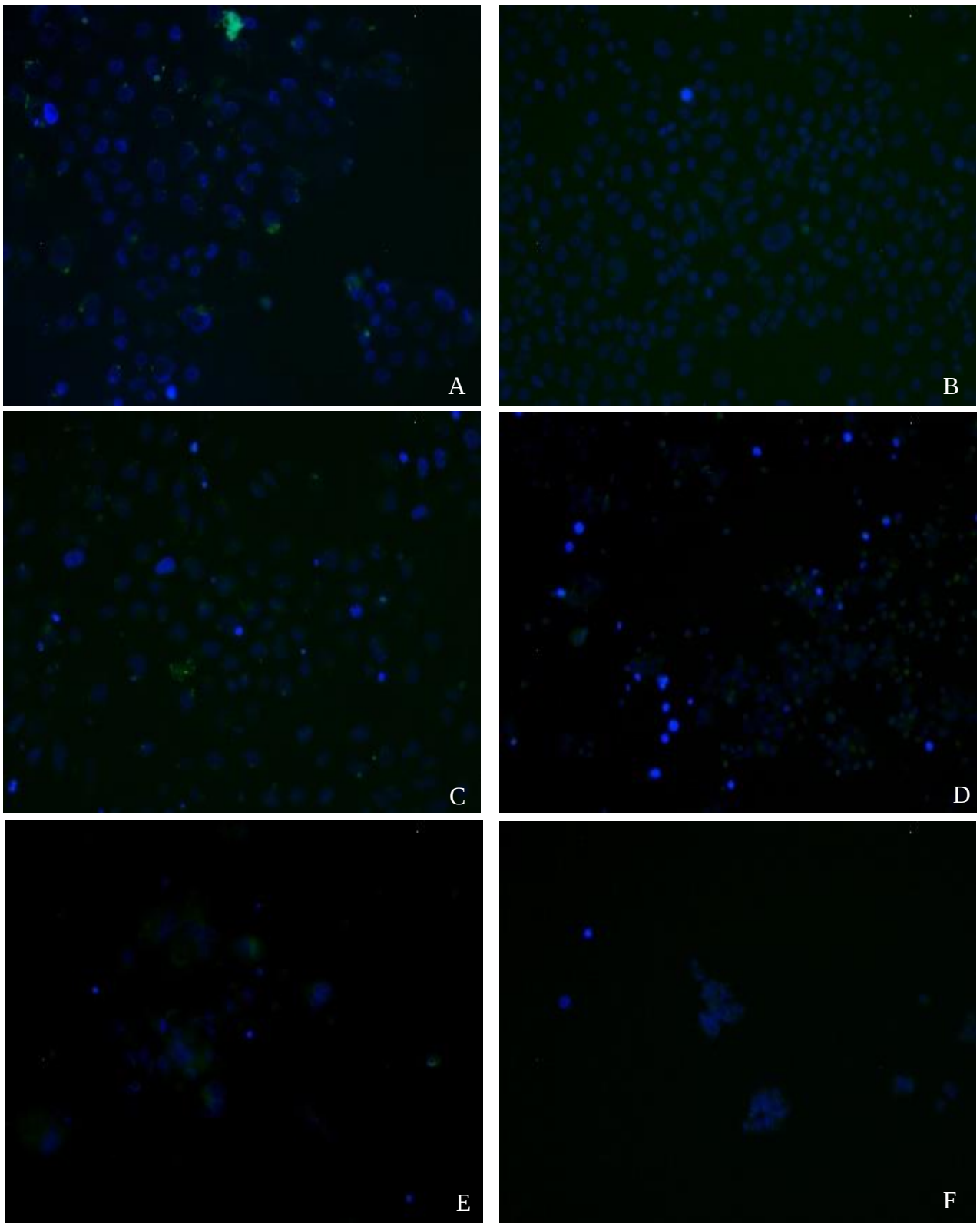


Figure 3-4: Representative microscopic images of immunofluorescent staining of mAb 4A5 on various non-PDAC cancer cell lines. A) Caco-2 cells, B) IGROV-1 cells, C) OAW42 cells, D) MCF-7 cells, E) HCC 1419 cells, F) ZR-75-1 cells. Magnification 20X.

Figure 3-4 indicates that there is very weak staining observed on the cell lines Caco-2 (F), IGROV-1 (G), OAW42 (H), MCF-7 (I), HCC 1419 (J) and ZR-75-1 (K), where almost no green fluorescence of Alexa Fluor 488 fluorophore is observed.

These findings indicated that mAb 4A5 reacted with a membrane protein potentially overexpressed in pancreatic ductal adenocarcinoma cells. The findings are summarised in Table 3-4.

Table 3-4 summary of observed immunocytochemistry staining of mAb 4A5 against a panel of established cancer cell lines

Cancer type	Cell line	Immunofluorescence staining on unfixed cells
Pancreatic ductal adenocarinoma	PANC-1	Strong, cytoplasmic and membrane reactivity
Pancreatic ductal adenocarinoma	PIN 127	Strong, cytoplasmic and membrane reactivity
Pancreatic ductal adenocarinoma	SW1990	Strong, cytoplasmic and membrane reactivity
Pancreatic ductal adenocarinoma	MiaPACA-2	Strong, cytoplasmic and membrane reactivity
Pancreatic ductal adenocarinoma	PIN 99	Strong, cytoplasmic and membrane reactivity
Colon adenocarcinoma	Caco-2	Weak
Ovarian adenocarcinoma	IGROV-1	Weak
Ovarian adenocarcinoma	OAW42	Weak
Breast ductal adenocarcinoma	MCF-7	Weak
Breast ductal adenocarcinoma	HCC1419	Weak
Breast ductal adenocarcinoma	ZR-75-1	Weak

3.2.3 Immunofluorescence studies of mAb 4A5 on fixed cells

In order to study the expression and localisation of mAb 4A5 antigen, immunofluorescence studies of mAb 4A5 was performed on fixed cells. In this setting, a fixation step was added to avoid possible cell degradation that can develop when staining unfixed cells. The fixation also preserves the structure and morphology of the cells. A permeabilisation step was also trialled to allow internalisation of the mAb and to check for its binding with its antigen within the cells (*Fixation and Permeabilization in ICC IF*, no date). The optimised protocol is described in section 2.3.2. The cells used were the PANC-1, PIN 127 and another well characterised PDAC cell line, SW1990.

The method of fixation was tested, comparing either 4% formaldehyde for 1 hour at RT or ice-cold methanol for 10 minutes. The optimal reagent used should conserve the structural part of the cells and tissue integrity (Man, no date). Methanol appeared too stringent on the cells, with final observations showing weaker staining than observed with 4% v/v formaldehyde, and therefore 4% v/v formaldehyde was subsequently used. After fixation, two PBS washes were performed and 0,5 % v/v of non-ionic detergentTriton X-100 in PBS was applied to the cells for 30 minutes at RT for permeabilisation, followed by three PBS washes. This step was necessary to check for potential Ag-Ab reaction inside the cells. The specimens were then blocked with BlockAid™ Blocking Solution for 1 hour at RT to avoid nonspecific binding of the mAb (Hoff, 2022). The dilution of the primary Ab (mAb 4A5) was assessed, and mAb 4A5 was used neat and diluted 1:100, 1:500 and 1:1000 in PBS; with the neat mAb giving the best signal. The secondary Ab used was a Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 at the recommended dilution of 1:1000. NucBlue™ Live ReadyProbes™ Reagent was applied for 10 minutes. While the results obtained were satisfactory however, for further improvement it was decided to check if the addition of Image-iT™ FX Signal Enhancer after the cell permeabilisation step was tested to assessed its impact on image quality. This compound was then added for 30 minutes. The protocol was kept the same thereafter. Coverslips were carefully mounted using ProLong™ Gold Antifade Mountant. Negative controls using Sp2/0-Ag14 cells conditioned medium instead of primary antibody, and PBS in place of the secondary mAb were set up using the same protocol and tested in parallel in all experiment.

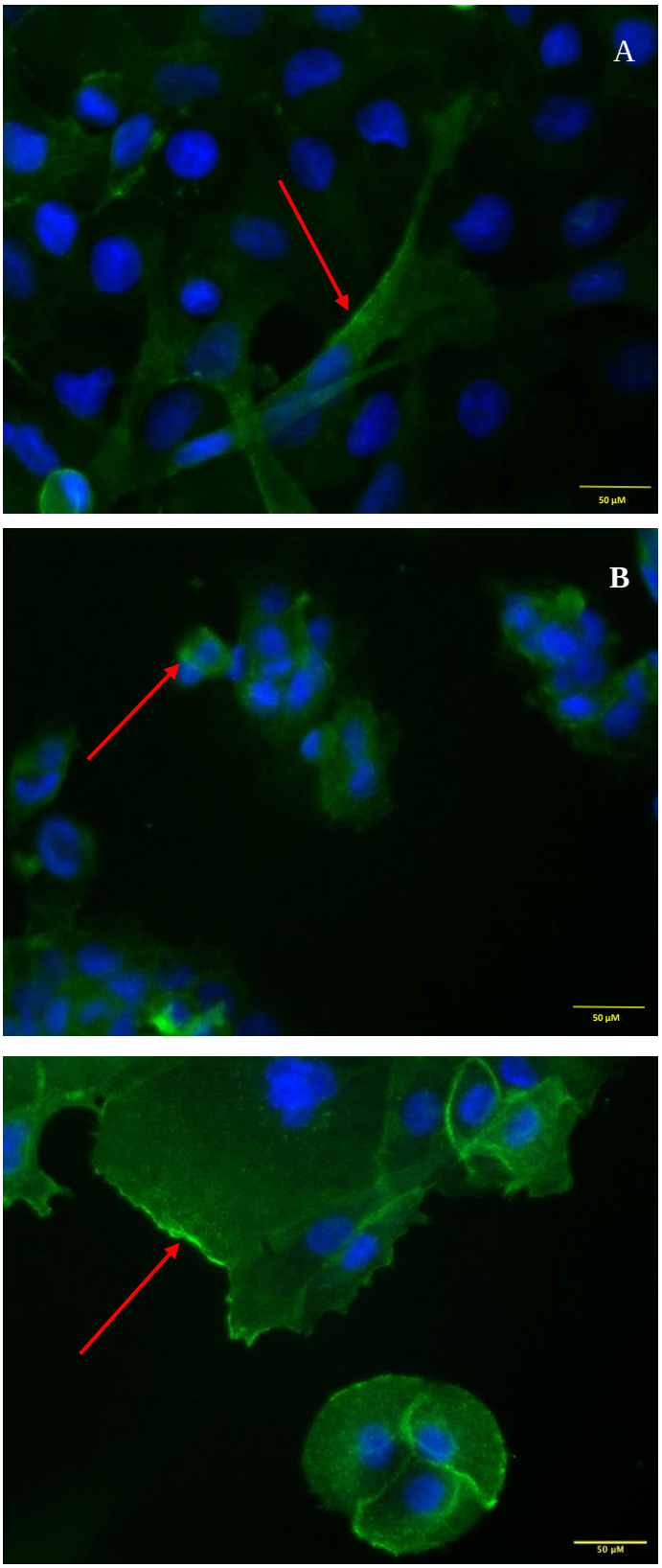


Figure 3-5: Representative microscopic images of immunofluorescent staining of mAb 4A5 on fixed A) PANC-1 cells, B) PIN 127 cells, C) SW1990 cells. The immunoreactivity appears to be membrane localised, as shown by the red arrows. Magnification 40x.

The images in Figure 3-5 show representative microscopic images of the signal obtained when mAb 4A5 was applied on fixed preparation of three PDAC cell lines PANC-1 (A), PIN 127 (B) and SW1990 (C). The three cell lines allowed visualisation of a membrane (red arrows) and cytoplasmic signal, which reflects that observed in unfixed cells. Punctate like immunoreactivity is observed in PANC-1 (A) and SW1990 (C) cells which appeared to exhibit a stronger membrane signal than PIN 127 cells (B).

3.2.4 Immunocytochemical studies of mAb 4A5

Immunohistochemistry (ICC) refers to the method that uses antibodies to check for certain antigens in a sample of cells. There are no extracellular matrix or stromal elements. Unlike IF, the secondary Ab is not coupled to a fluorophore but to a chromogen, in this instance Horse Radish Peroxidase (HRP), which produces a brown colour when in contact with an enzyme. The colouring can be visualised microscopically.

The prepared cells were stored at -80°C (section 2.4.1) until use. On the day of ICC, the cells were fixed with 4% formaldehyde for 5 minutes at RT, then rinsed 3 three times with PBS. The staining was carried out as described in section 2.4. The slides were exposed for 60 minutes to either mAb 4A5 neat or diluted 1:2 in the antibody diluent. A negative control consisting of the CM of Sp2-0/Ag14 cells was also analysed. Along with the immunogen PANC-1 and PIN 127 cells, the PDAC cell lines SW1990 and MiaPACA-2 were also studied.

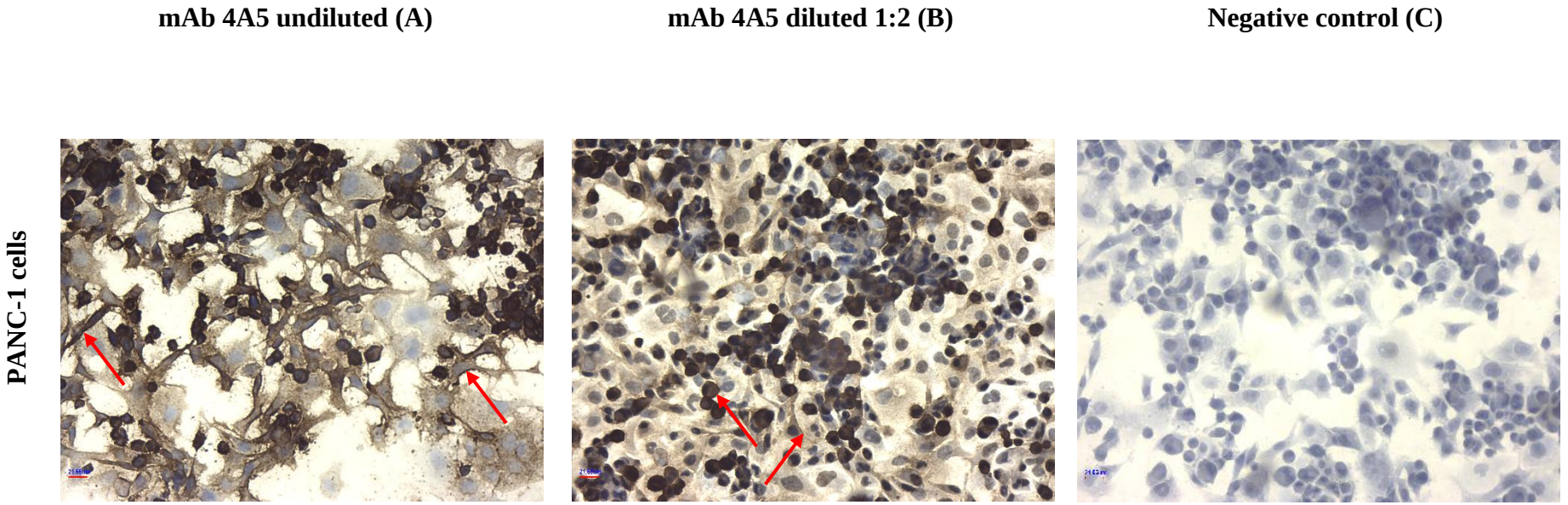


Figure 3-6: Representative microscopic images of immunocytochemical staining of mAb 4A5 A) undiluted, B) diluted 1:2 on PANC-1 cells and the corresponding negative control C). Membrane straining is observed, as indicated by the red arrows. Magnification 20x. The scale bar represents 21.66µm.

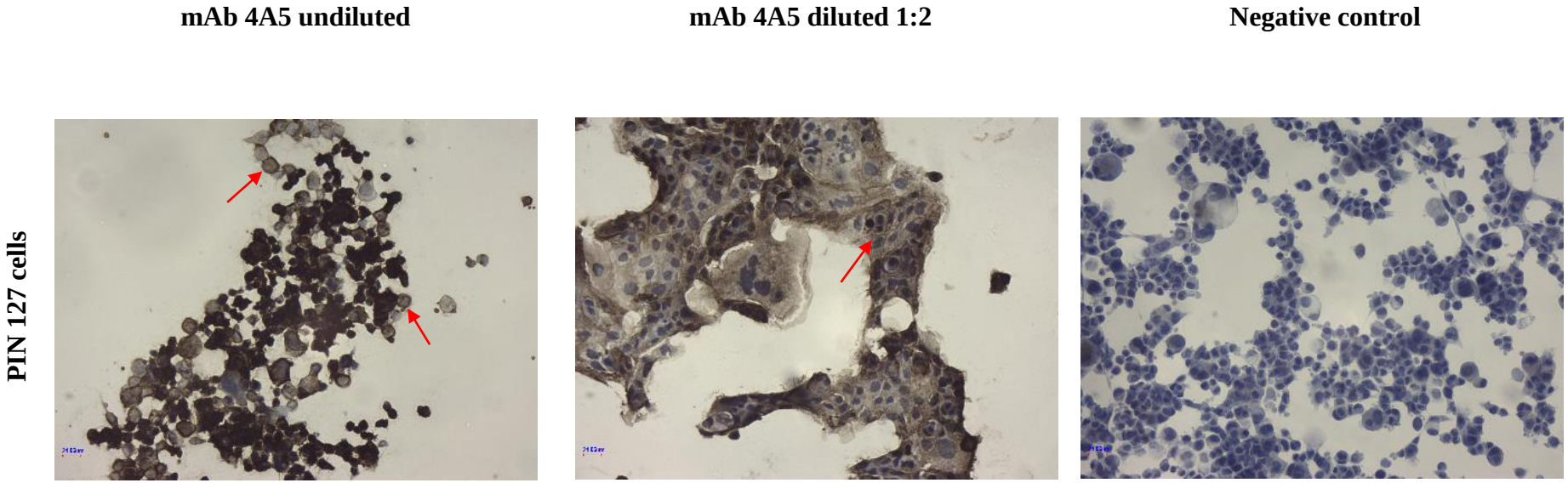


Figure 3-7: Representative microscopic images of immunocytochemical staining of mAb 4A5 A) undiluted, B) diluted 1:2 on PIN 127 cells and the corresponding negative control C). Membrane straining is observed as indicated by the red arrows. Magnification 20x. The scale bar represents 21.66µm.

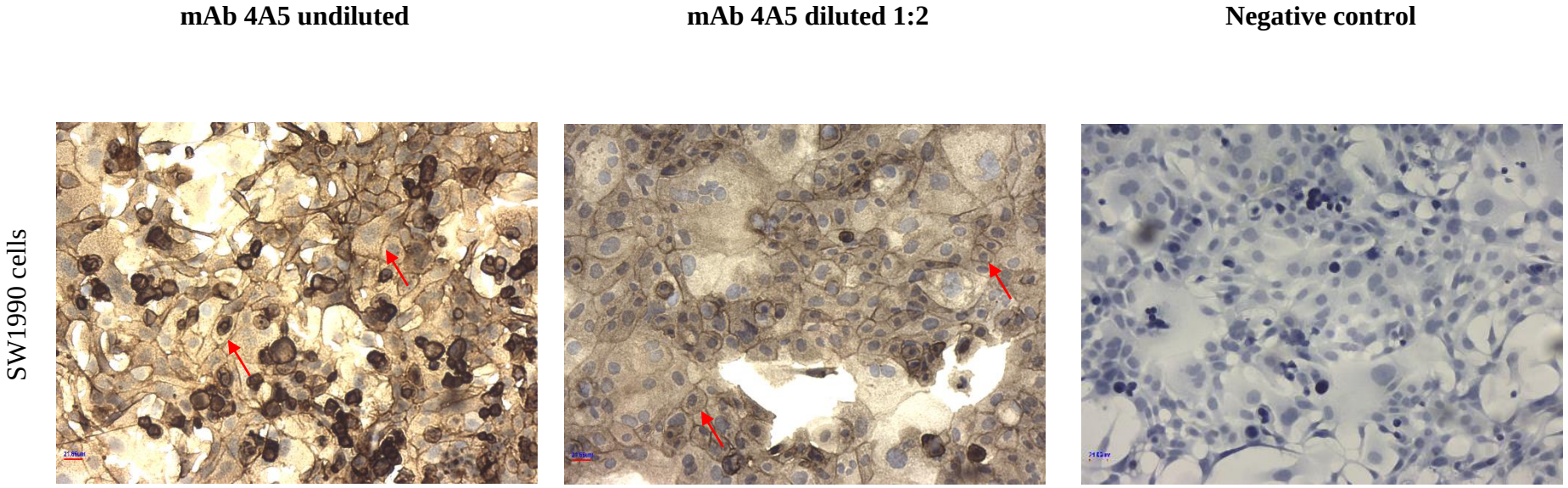


Figure 3-8: Representative microscopic images of immunocytochemical staining of mAb 4A5 A) undiluted, B) diluted 1:2 on SW1990 cells and the corresponding negative control C). Membrane straining is observed as indicated by the red arrows. Magnification 20x. The scale bar represents 21.66µm.

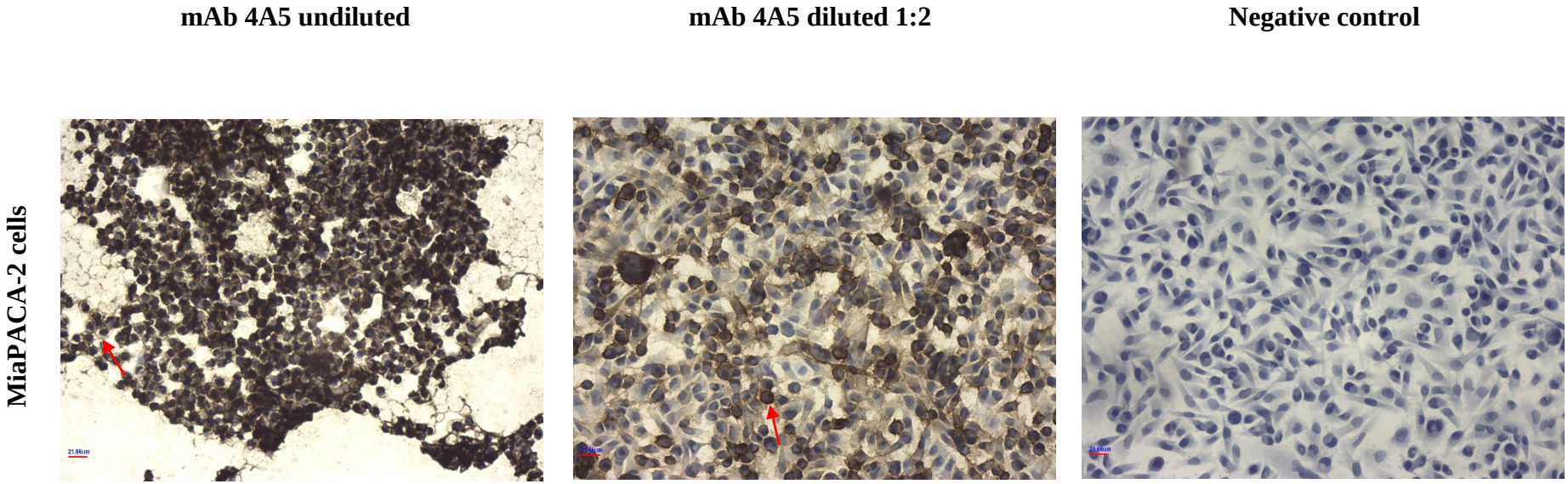


Figure 3-9: Representative microscopic images of immunocytochemical staining of mAb 4A5 A) undiluted, B) diluted 1:2 on MiaPACA-2 cells and the corresponding negative control C). Membrane straining is observed as indicated by the red arrows. Magnification 20x. The scale bar represents 21.66µm.

Figures 3-6, 3-7, 3-8 and 3-9 demonstrate the immunocytochemical staining profile of PANC-1, PIN 127, SW1990 and MiaPACA-2 cells respectively, when exposed to mAb 4A5 undiluted (A) and diluted 1:2 (B). An intense colouration can be observed with each dilution on all the cell lines. The mAb-antigen reaction appeared to be cytoplasmic and located on the cell-surface, as indicated by the red arrows. Each of the negative controls (C) showed no staining, indicating a specific reaction of mAb 4A5 to an antigen.

From these immunocytochemical observations, the undiluted mAb was used in the following immunohistochemical analysis of the mAb 4A5 antigen.

3.2.5 Immunohistochemical studies of mAb 4A5

Immunohistochemistry (IHC) differs from ICC in that the biological samples are cells or tissues fixed and embedded in a medium, like paraffin, and cut into thin sections. The samples reflect the morphology of the originating tissue. In this setting, the optimisation was carried out using sections of PIN 127 PDX tumour from which the cell line was established.

Pieces of tumours were fixed in formalin, paraffin embedded, and 5µm tissue sections were cut. Since antigens can be masked during the fixation, it is necessary to retrieve these to avoid false negative results and for such, different methods of epitope retrieval were tested.

3.2.5.1 Paraffin dewaxing

Antigen demasking was trialled through paraffin removal. For such, dewaxing was performed as per section 2.4.2

mAb 4A5 (A) **Negative control (B)**

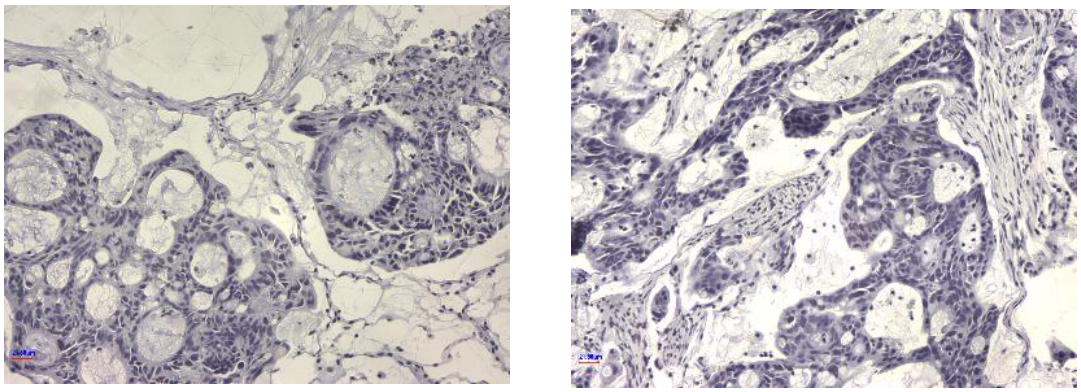


Figure 3-10: Representative microscopic images of immunohistochemical staining with A) mAb 4A5, B) negative control on PIN 127 tumour slides subjected to paraffin dewaxing. Magnification 20x. The scale bar represents 21.66µm.

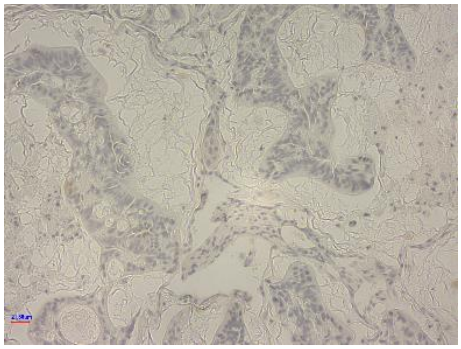
In Figure 3-10, the pictures of paraffin dewaxed PIN 127 tumour slides show no staining of mAb 4A5 (A), with the profile comparable to the staining of the negative control B), indicating that mAb 4A5 has not bound to an antigen.

3.2.5.2 Heat Induced Epitope Retrieval

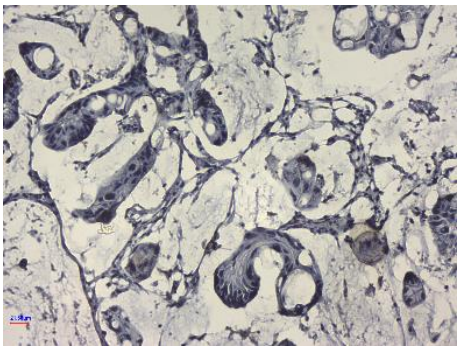
In order to unmask the mAb 4A5 antigens potentially present in PIN 127 tumours, another methodology was tested. Tissue sections were subjected to heat induced epitope retrieval (HIER) using either an acidic citrate buffer (pH 6) or a basic EDTA buffer (pH 9). Multiple incubation times of HIER were tested: 20 minutes, 30 minutes and 40 minutes.

Citrate Buffer (pH 6) HIER

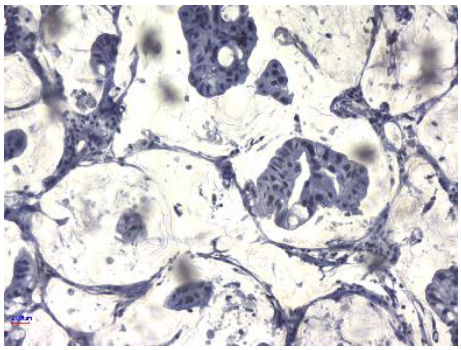
20 minutes (A)



30 minutes (B)



40 minutes (C)



Negative control

(D)

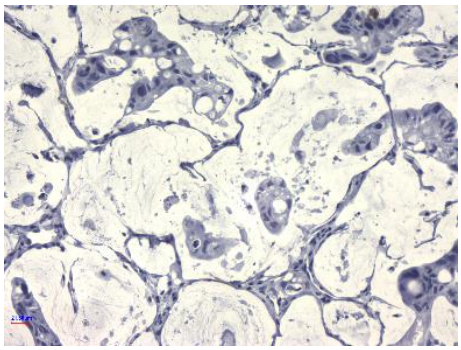
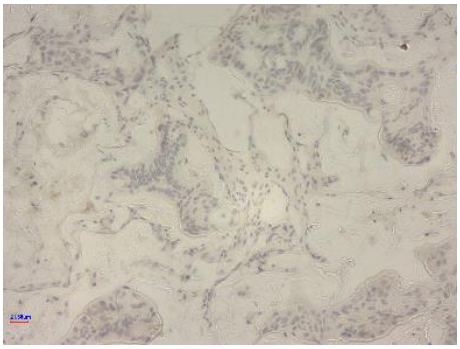


Figure 3-11: Representative microscopic images of immunohistochemical staining with mAb 4A5 on PIN 127 tumour slides subjected to pH6 HIER for A) 20 minutes, B) 30 minutes, C) 40 minutes. The negative control is represented in D). Magnification 20x. The scale bar represents 21.66µm.

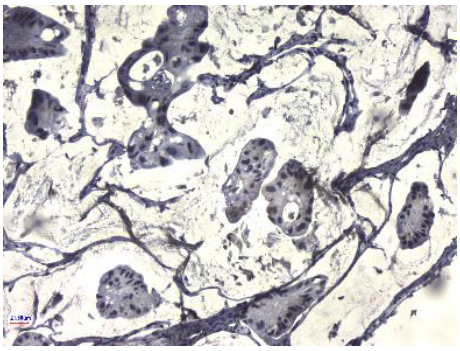
The images in Figure 3-11 show the results of ICC staining of mAb 4A5 after HIER at pH 6 for 20 minutes (A), 30 minutes, (B) and 40 minutes (C) and a negative control (D). No brown staining was observed on any of the specimens, suggesting that mAb 4A5 did not bind to an antigen on PIN127 PDX tumour tissue sections which were subjected to pH 6 HIER.

EDTA buffer (pH 9) HIER

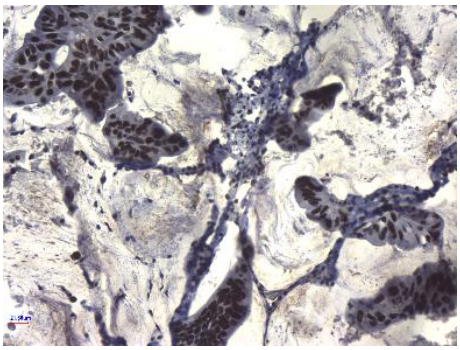
20 minutes (A)



30 minutes (B)



40 minutes (C)



Negative control (D)

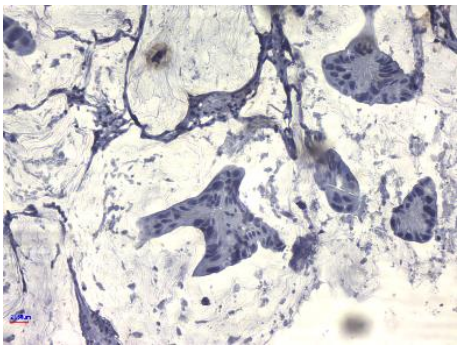


Figure 3-12: Representative microscopic images of immunohistochemical staining with mAb 4A5 on PIN 127 tumour slides subjected to pH9 HIER for A) 20 minutes, B) 30 minutes, C) 40 minutes. The negative control is represented in D). Magnification 20x. The scale bar represents 21.66µm.

Figure 3-12 demonstrates the staining of mAb 4A5 on PIN 127 PDX tumour tissue slides following HIER at pH 9. A 20 minutes HIER (A) compared to the negative control (D) where no staining was observed. After 30 minutes (B) and 40 minutes (C) HIER at pH 9, a brown colouration was observed within the nuclei of the cells. This could suggest that there is non-specific binding of the mAb (Wieczorek *et al*., 2013).

3.2.5.3 Paraffin-embedded cell blocks

To ensure the staining observed was non specific, paraffin embedded cell pellets of PIN 127 cells were generated to conserve the tissue sections of PIN 127 PDX tumour. We also examined PANC-1 paraffin embedded cells. The methodology for creating paraffin embedded blocks and sections is described in 2.4.2.1.

The slides generated from these cell blocks were submitted to 30 minutes HIER using either pH 6 citrate buffer or pH 9 EDTA-buffer. Staining was performed as previously described.

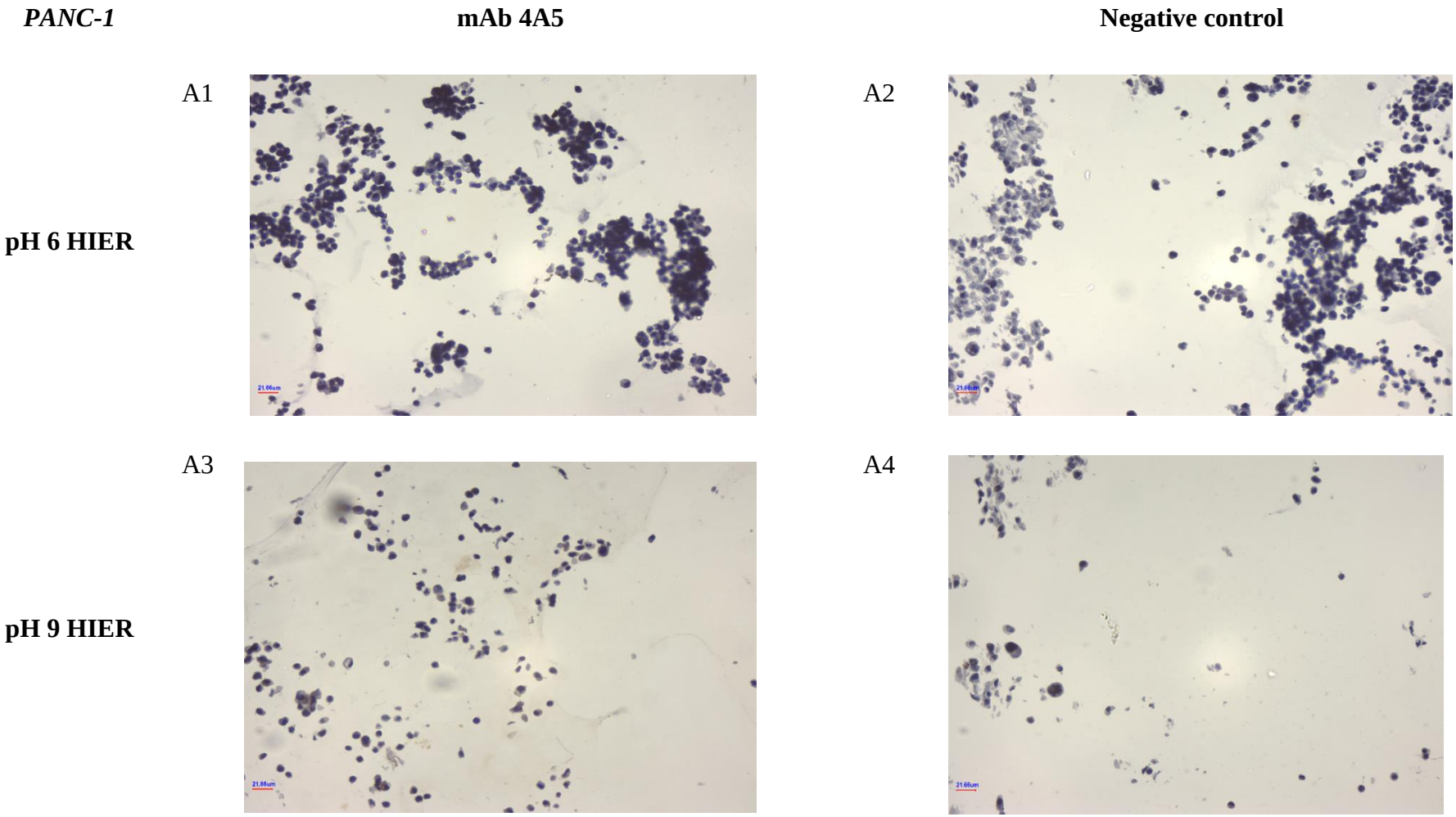
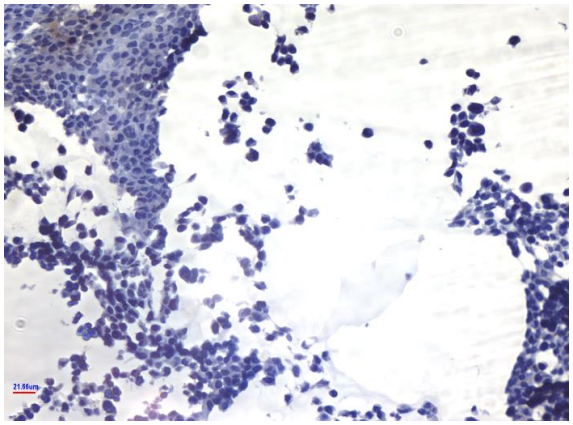


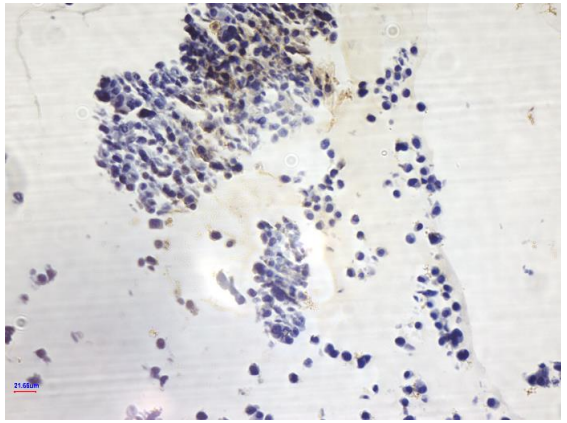
Figure 3-13: Microscopic observations representative of mAb 4A5 staining on PANC-1 paraffin embedded cells after HIER at pH 6 (A1) and pH 9 (A3). The corresponding negative controls are presented in (A2) and (A4) Magnification 20x. The scale bar represents 21.66µm.

PIN 127 **mAb 4A5** **Negative control**

B1

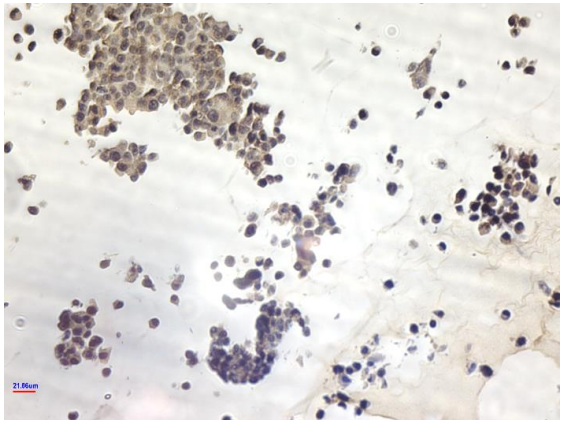


B2

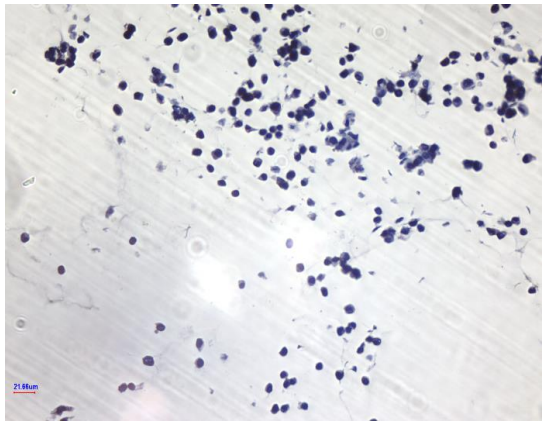


pH 6 HIER

B3



B4



pH 9 HIER

Figure 3-14: microscopic observations representative of mAb 4A5 staining on PANC-1 paraffin embedded cells after HIER at pH 6 (B1) and pH 9 (B3). The corresponding negative controls are presented in (B2) and (B4) Magnification 20x. The scale bar represents 21.66µm.

In Figure 3-13, no staining was observed on PANC-1 paraffin embedded cells exposed to mAb 4A5 following 30 minutes HIER at either pH 6 (A1) or pH 9 (A3). The pattern was similar to the corresponding negative control A2 and A4. This indicates that the immunostaining of mAb 4A5 on PANC-1 paraffin embedded cells was ineffective.

In Figure 3-14, both mAb incubated (B1) PIN 127 cells and its corresponding negative control (B2) exposed to an acidic pH 6 HIER developed the same pattern, with no brown colouration observed. Cytoplasmic staining was seen on the slides of PIN 127 paraffin embedded cells that were treated with pH 9 HIER and exposed to mAb 4A5 (B3), in comparison to the corresponding negative control (B4) that showed no staining. This mean that the epitope for mAb 4A5 seemed to be accessible by mAb 4A5, through HIER at pH9 for 30 minutes.

3.2.6 Immunoblotting studies

To prepare for a potential identification of the target of mAb 4A5 by immunoprecipitation, it was necessary to analyse the size of proteins from the cell lines used to immunise the animals for the hybridoma generation that reacted with mAb 4A5. For such, the Western blot methodology was used to detect the target protein of an antibody within a pool of proteins from cell lysates (Mahmood & Yang, 2012). In this technique, the proteins are discriminated based on their sizes and the use of immunoblotting helps assessing the antigen molecular weight of the given antibody (Bordeaux *et al*., 2010).

Whole cell lysates were used in all instances. The protein quantity was optimum at 30 µg. As per section 2.5.1, the protein content was denatured and ran through a 4-12% Bis-Tris gel. The optimum running times and voltage were determined to be 1h30 minutes and 150V. The transfer to a nitrocellulose membrane using the iBlot system was compared between 7 and 10 minutes, and the latter time was selected as being the most efficient.

To achieve a satisfactory immunoblotting profile, some conditions were optimised as seen in Table 3-5.

Table 3-5: Parameters to be optimised for the characterisation of mAb 4A5 through immunoblotting

Parameters to optimise
Method of detection
Primary antibody concentration
Secondary antibody dilution

3.2.6.1 Detection system

The enhanced chemiluminescence (ECL) detection methodology is based on an enzymatic reaction that produces light. In immunoblotting application, a secondary Ab is coupled with a substrate, such as horseradish peroxidase (HRP) substrate. An enzyme is then added and the substrate-enzyme chemical reaction generates light (Signore *et al*., 2017). Automated or manual detection system were used for the characterisation of mAb 4A5.

Li-Cor Odyssey detection system

Initially the immunoblotting results were analysed through the automated Li-Cor Odyssey imaging system, mainly for its ease of access and practicality. All reagents were used as per section 2.5.2. The mAb was applied crude on the blotting membranes. A negative control with the CM of Sp-2/0-Ag14 was performed in parallel.

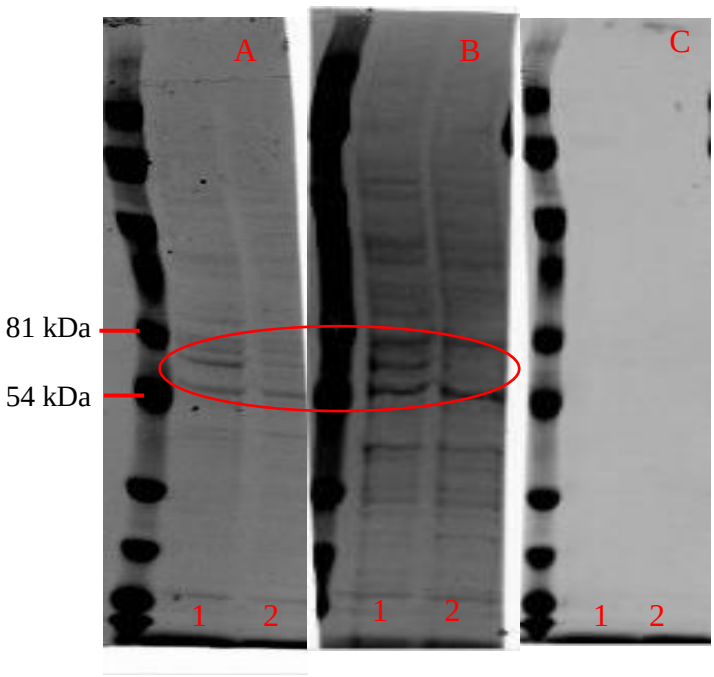


Figure 3-15: Western blotting images of mAb 4A5 generated by exposure of the membrane to the Li-Cor Odyssey platform to an intensity of 7,5 (B) or 8.5 (B) of whole cells lysates of 1) PANC-1 and 2) PIN 127 cells. The potential bands of interest are circled in red. Blot C represents the negative control.

Figure 3-15 shows the profiles of the Western blots obtained after exposure of the membranes at intensities 7.5 and 8.5 on the Li-Cor Odyssey apparatus. In the negative control, (C) no bands are observed, meaning the mAb is specific to a target. MAb 4A5 analysed at an intensity of 8.5 (B) resulted in overexposure of the membrane, rendering the protein ladder unreadable. MAb 4A5 analysed at an intensity of 7.5 (A) resulted in multiple bands observed. Stronger bands of possible interest developed between 54 and 81 kDa, as circled in red.

Exposure to X-Ray film

The Li-Cor Odyssey system is robust and fast for characterised or commercially available antibodies. For the characterisation of a novel antibody, more control of exposure intensities was required. Exposures on X-ray films (section 2.5.2) is a highly sensitive and widely accepted method that permits the detection of minimal amount of an antigen within a sample

1X NET buffer was used as blocking buffer and dilution buffer. The negative control was undiluted Sp2/0-Ag14 conditioned medium.

Two dilutions of mAb 4A5were compared, of 1:4 and 1:8.

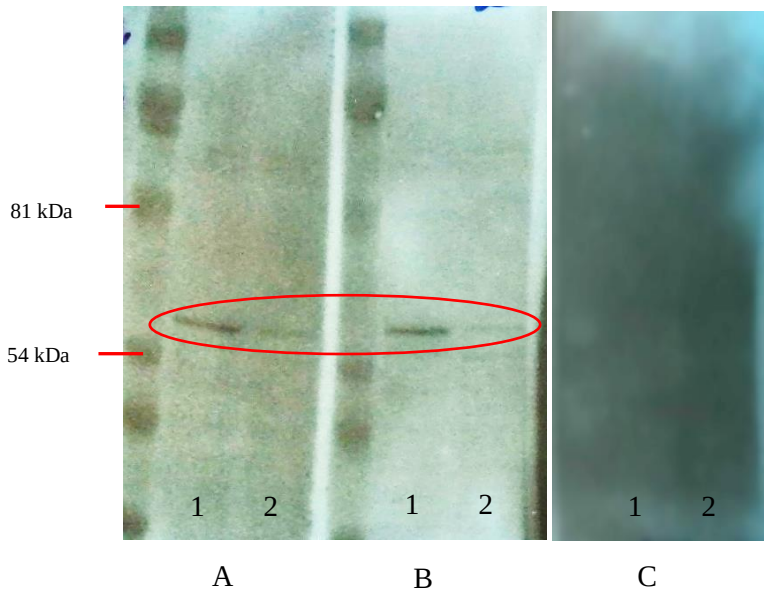


Figure 3-16: Immunoblotting images comparing two dilutions of mAb 4A5, 1:4 (A) and 1:8 (B). The blots were performed on PANC-1 (lane 1) and PIN 127 (lane 2) whole cell lysates. Blot C represents the negative control. The bands of interest are circled in red.

As seen in Figure 3-16, the two dilutions of the mAb achieved similar blotting profiles with a stronger band detected at around 60 kDa. This band was visualy less intense in the PIN 127 cell line, but no quantification was performed on these preliminary findings. The negative control (C) reaffirmed that mAb 4A5 bound specifically to an antigen.

In order to spare our stock of mAb 4A5, the 1:8 dilution of the primary Ab was subsequently preferred. The optimal concentration of the secondary goat anti-mouse Ab was then investigated. Three dilutions were tested and compared: 1:5,000, 1:10,000, and 1:15,000.

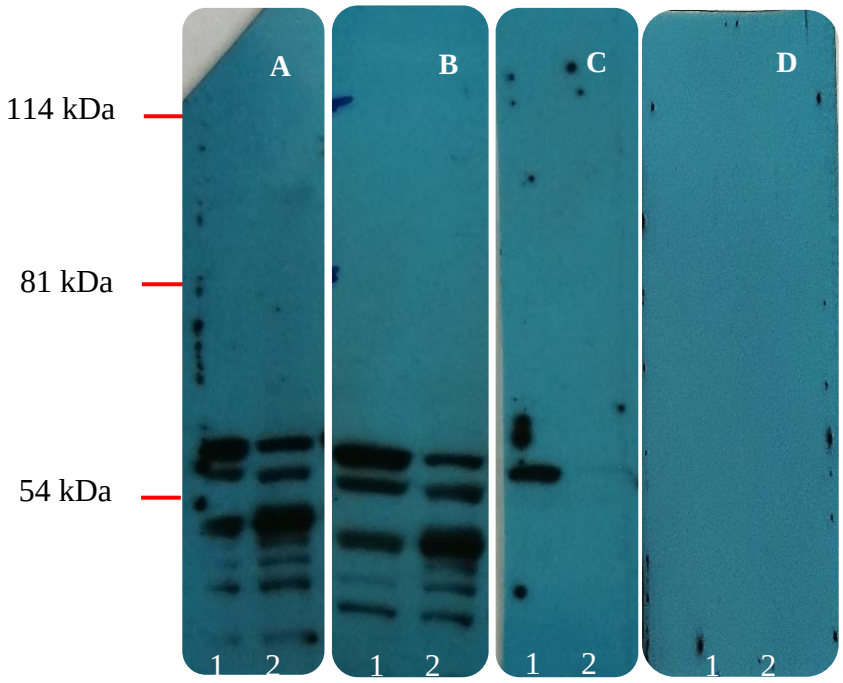


Figure 3-17: Immunoblotting profiles of mAb 4A5 exposed to PANC-1 cells (lane 1) and PIN 127 (lane 2) with different concentrations of a secondary antibody. The secondary Ab was diluted at 1:5,000 (A), 1:10,000(B) and 1:15,000 (C). The negative control is represented in (D).

As seen in Figure 3-17, the secondary Ab diluted at 1:5,000 (A) and 1:10,000 (B) enhances the detection of many bands including the 60Kda band previously observed. At a higher dilution of 1:15,000 (C), this band of interest is detected only in PANC-1 whole cell lysates.

This investigation demonstrates that a dilution of the secondary Ab between 1:10,000 and 1:15,000 would achieve optimum detection. Hence, a 1:12,500 dilution was further used.

3.2.6.2 Summary of immunoblotting optimisation

The detection of a band of interest reacting against mAb 4A5 was accomplished. The optimum conditions for the immunoblotting of mAb 4A5 on whole cell lysates of PANC-1 and PIN 127 cells are described in Table 3-6.

Table 3-6: Optimised parameters for the detection of a band of interest targeted by mAb 4A5 in PANC-1 and PIN 127 whole cell lysates

Parameters	Optimised conditions
Quantity of loaded proteins	30µg
Gel type	4-12% Bis-Tris Bolt gel
Running of the gel	150V for 90 minutes
Transfer	10 minutes transfer onto nitrocellulose membrane using iBlot
Blocking	1hour in NET
Primary antibody	mAb 4A5 diluted 1:8 in NET buffer overnight at +4°C
Washes in between antibodies	Three washes in PBS 0.1% tween-20
Secondary antibody	secondary goat anti-mouse Ab 1:12,500 in NET buffer for 1 hour at RT
Detection	enhanced chemiluminescence (ECL) followed by exposure on X-Ray film

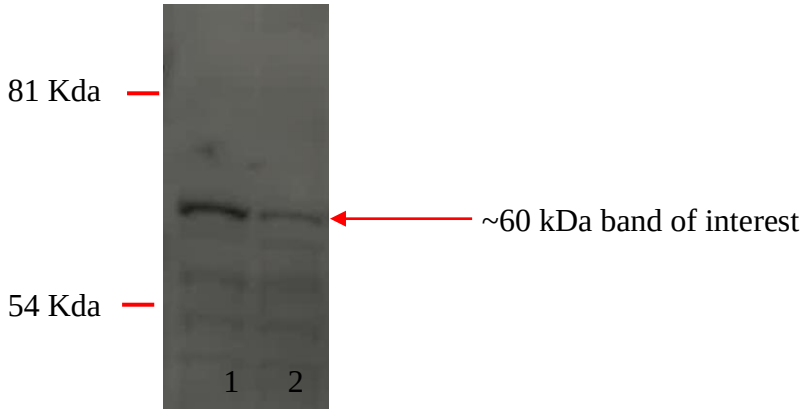


Figure 3-18: Representative image of optimised western blot of whole cell lysates of PANC-1 (lane 1) and PIN 127 (lane 2) whole cell lysates against mAb 4A5.

The protein target of mAb appears to have a molecular weight of approximately 60 kDa, as seen in Figure 3-18 (red arrow).

3.2.7 Immunoprecipitation studies

Following on from Western blot analysis, results indicated that the candidate reactive band to mAb 4A5 was the observed band at approximately 60 kDa. We proceeded to apply a direct immunoprecipitation (IP) approach as detailed in section 2.6, which is a modification of the method previously used to determine the target antigens of mAbs 7B7 and 9E1 (O'Sullivan et al., 2014, 2017). This IP method is used to isolate a protein, interacting with an antibody, from a complex of proteins in a given sample. The technique involves the anchoring of an antibody onto an immobilised support; in this work protein G agarose was used. The immobilised mAb was mixed with a protein sample, allowing for the formation of antibody-antigen complexes. These complexes were eluted, separated and analysed (Bonifacino *et al*., 2006).

The direct IP was carried out on PANC-1 and PIN 127 whole cell lysates. The controls included were Sp2/0-Ag14 CM and a mouse IgG in place of mAb 4A5, to ensure of the specificity of the mAb to its antigen. Three IP products per cell line were obtained and these were denatured and separated on SDS-PAGE. Two gels per cell line were performed, one was stained with GelCode Blue™ Coomassie staining and the other was used for immunoblotting.

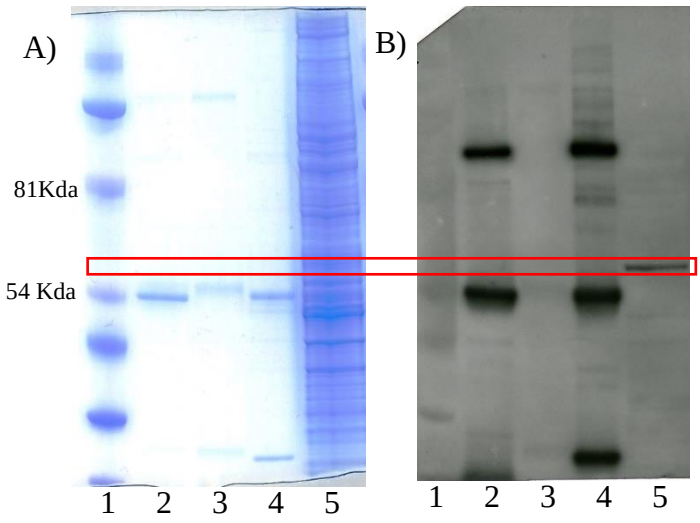


Figure 3-19: Direct immunoprecipitation analysis of mAb 4A5 against PANC-1 cells and corresponding controls by A) Coomassie staining, B) immunoblotting. Lane 1: protein marker, Lane 2: mAb 4A5 IP, Lane 3: Sp2/0-Ag14 IP, Lane 4: mouse IgG IP, Lane 5: PANC-1 whole cell lysate. The protein of interest band is circled in red.

Figure 3-19 presents images of the Coomassie stained gel (A) and immunoblot (B) of mAb 4A5 direct immunoprecipitates. The candidate band of interest can be observed in the immunoblot of PANC-1 whole cell lysate (B, lane 5), however it did not appear in any of 4A5 immunoprecipitates (either by Coomassie staining or immunoblotting). There might be other bands present in the immunoprecipitates with mAb 4A5 comparing to the mouse IgG control but it is difficult to observe. These visual observations suggest that the direct immunoprecipitation of mAb 4A5 against PANC-1 cells was inconclusive.

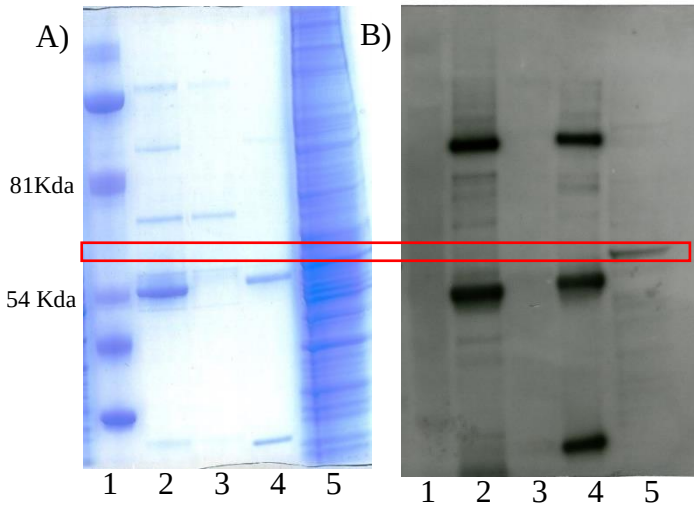


Figure 3-20 : Direct immunoprecipitation analysis of mAb 4A5 against PIN 127 cells and corresponding controls by A) Coomassie staining, B) immunoblotting. Lane 1: protein marker, Lane 2: mAb 4A5 IP, Lane 3: Sp2/0-Ag14 IP, Lane 4: mouse IgG IP, Lane 5: PANC-1 whole cell lysate. The protein of interest band is circled in red.

The profile of direct IP products of mAb 4A5 and corresponding controls against PIN 127 cells are seen in Figure 3-20. The Coomassie (A) and immunoblotting (B) analysis reflects the results obtained from the PANC-1 immunoprecipitates as seen in Figure 3-19. The protein band of interest observed in the whole cell lysates immunoblot (B, lane 5), is not detected in the immunoprecipitates (either by Coomassie or immunoblotting). Other bands, observed in the immunoblot, seemed pullet out with mAb 4A5 (B, lane 2) but not with the mouse IgG control (B, lane 4).

The direct immunoprecipitation studies were inconclusive as no proteins bound to mAb 4A5. No target of mAb 4A5 was isolated.

3.2.8 *In vitro* functional assays

While a target has not been identified to date, the impact of mAb 4A5 on the proliferative activity of its two immunogenic cell lines PANC-1 and PIN 127 was studied. Proliferation assays were set up to assessed the biological impact on the cell growth and analysed using an acid phosphatase assay as described in section 2.7.1.

3.2.8.1 Effect of purified mAb 4A5 on PANC-1 and PIN 127 cells

Preliminary experiments compared the effect of purified mAb 4A5 on its immunogen cell lines PANC-1 and PIN 127 after a three days exposure to the antibody. NAb™ Protein L spin columns were used to purify mAb containing CM as per section 2.2.7. These columns allow for a quick, small-scale affinity purification of human and mouse samples containing immunoglobulins with kappa light chains. The purity of the eluates was verified through GelCode™ Blue safe Coomassie staining (section 2.6.4).

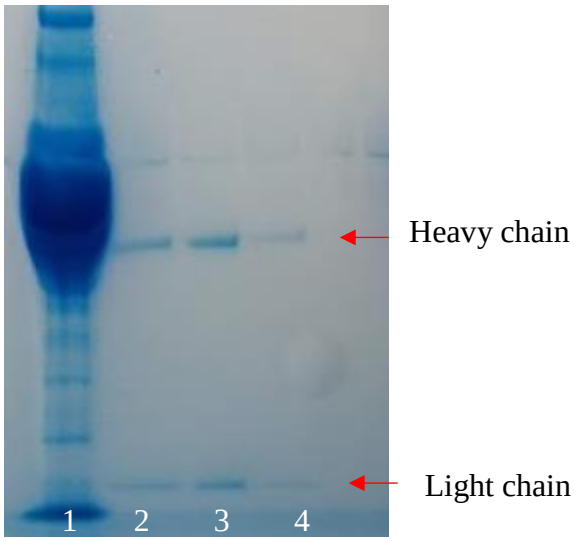
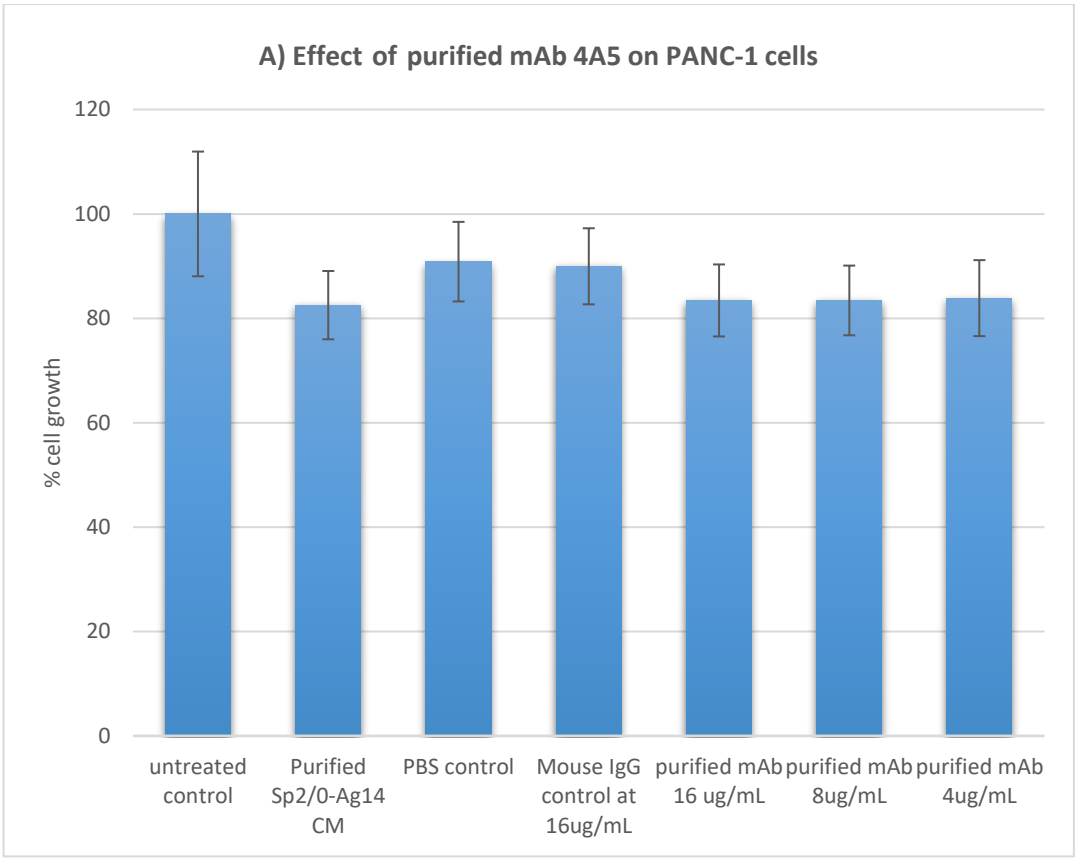


Figure 3-21: Coomassie staining profile of mAb 4A5 after purification through an Nab spin column. Lane 1 shows the unbound fraction. Lane 2, 3 and 4 represent the eluates 1, 2 and 3 respectively, containing the purified antibody. The denatured form of the antibody allows for the detection of the heavy chain and the light chain, as shown by the red arrows.

The Coomassie staining profile of purified mAb 4A5 is observed in Figure 3-21. The unbound fraction (1) shows multiple proteins detected, whereas in lanes 2 to 4, only the heavy chain and light chain bands can be observed. This means the mAb was purified.

Eluates 1, 2 and 3 were combined to increase the quantity of the mAb. The amount of IgG in the combined samples was quantified at 32 µg/mL by BCA assay. (section 2.5.1).

The purified mAb 4A5 was applied at a concentration of 16 µg/mL, 8 µg/mL and 4 µg/mL onto the cells. The conditioned medium of Sp2/0-Ag 14 was also processed through a Nab™ protein L spin column to serve as a negative control. Coomassie staining resulted in no bands detected (not shown). This negative control was added to the cells at a 1:2 v/v dilution. Along with this negative control, an untreated control representing 100% cell growth, a purified mouse IgG control and its corresponding diluting buffer (PBS) were also incorporated.



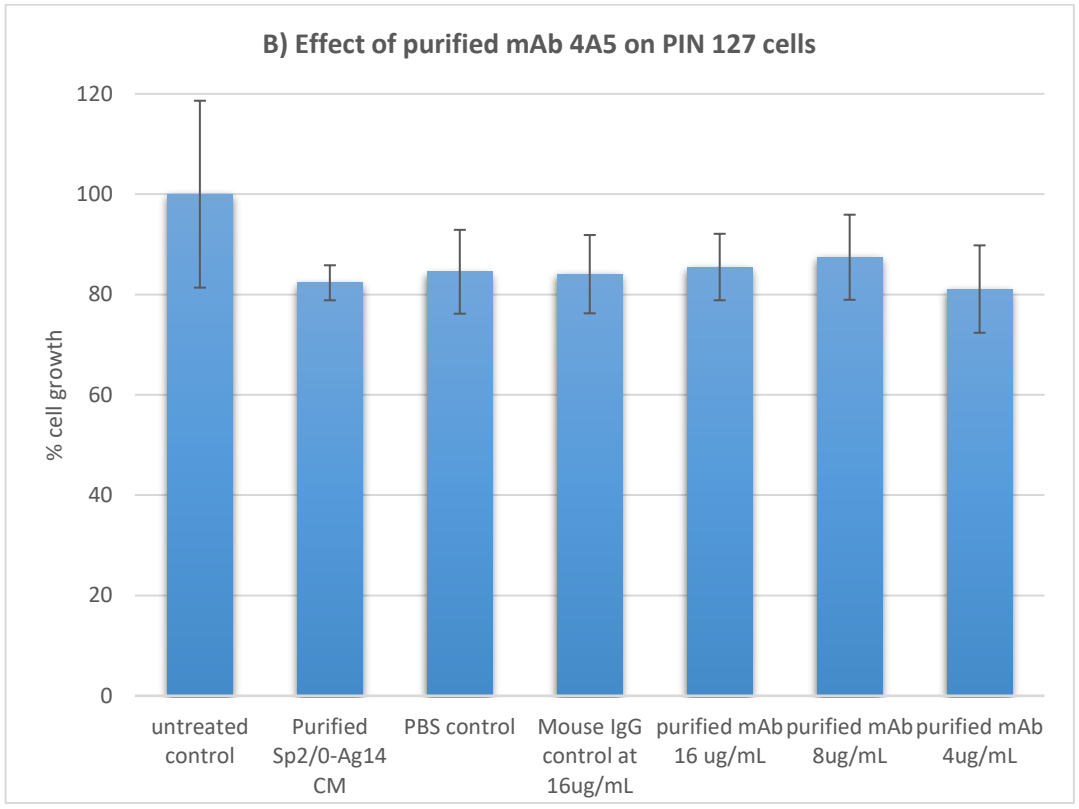
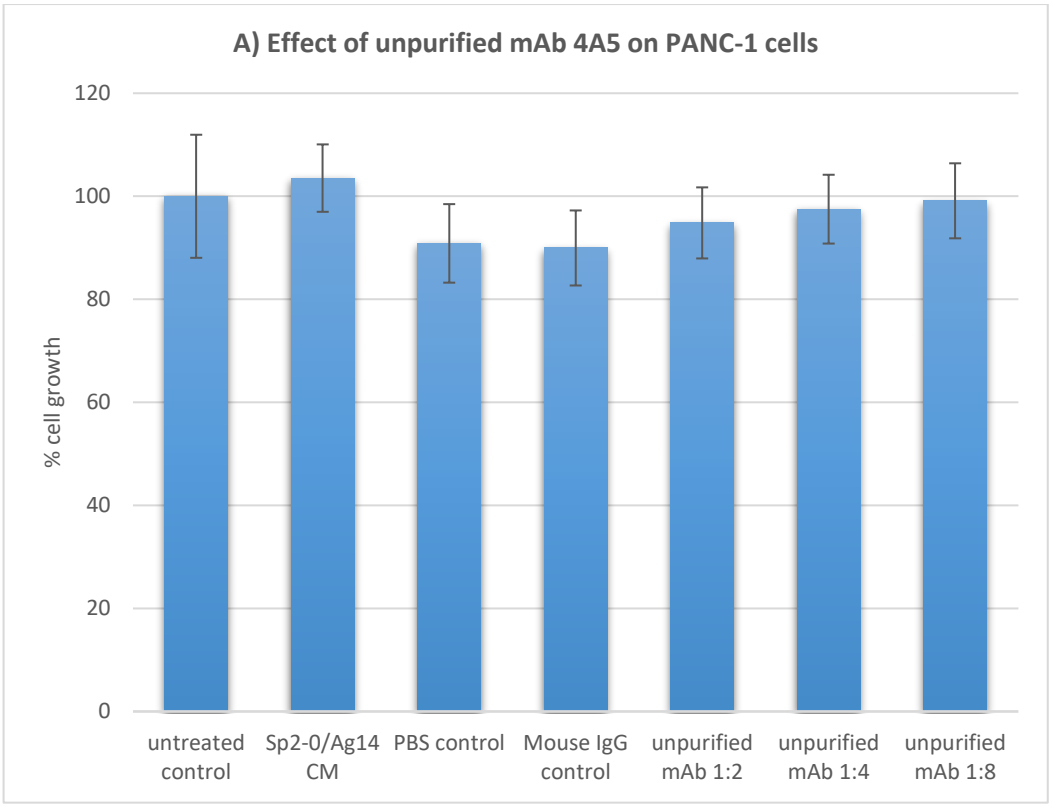


Figure 3-22: Growth profiles of PANC-1 (A) and PIN 127 (B) cells after 3 days exposure to purified mAb 4A5. The proliferation was analysed with an acid phosphatase assay. n=1. The error bars represent the variations between technical replicates.

Figure 3-22 shows that the growth of PANC-1 (A) and PIN 127 (B) cells was neither negatively nor positively affected by the addition of a purified form of mAb 4A5. The percentage of cell proliferation was similar in the purified Sp2/0-Ag14 CM control, the mouse IgG control, PBS control and each dilution of mAb 4A5; all resulting in 80 to 85% cell growth when compared to an untreated control (100%).

3.2.8.2 Effect of unpurified mAb 4A5 on PANC-1 and PIN 127 cells

The ineffectiveness of mAb 4A5 observed in 3.2.8.1 on the growth of PANC-1 and PIN 127 cells could be due from the low amount of IgG recovered after purification. To test that hypothesis, the incubation of mAb 4A5 in an impure state from the hybridoma CM was tested on PANC-1 and PIN 127 cells. In this experiment, the quantity of mAb 4A5 in the CM was not quantified. The mAb was added at 1:2, 1:4 and 1:8 dilutions. An unpurified Sp2/0-Ag14 CM control was tested at a 1:2 dilution. A mouse IgG control and its corresponding PBS buffer control were also analysed.



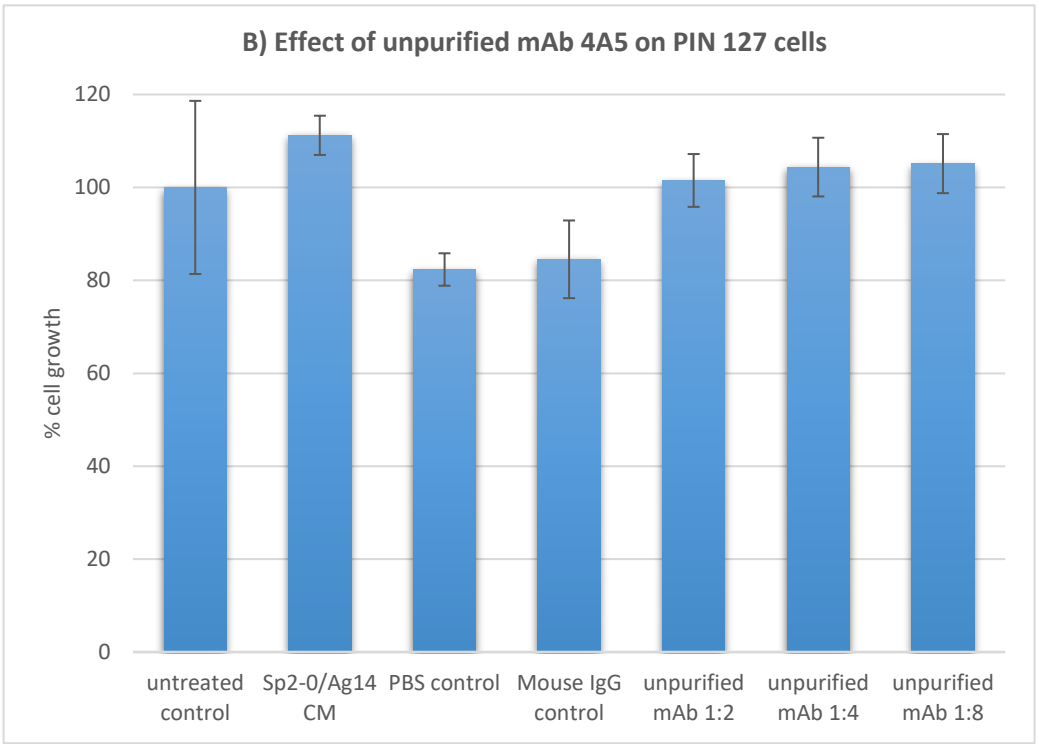
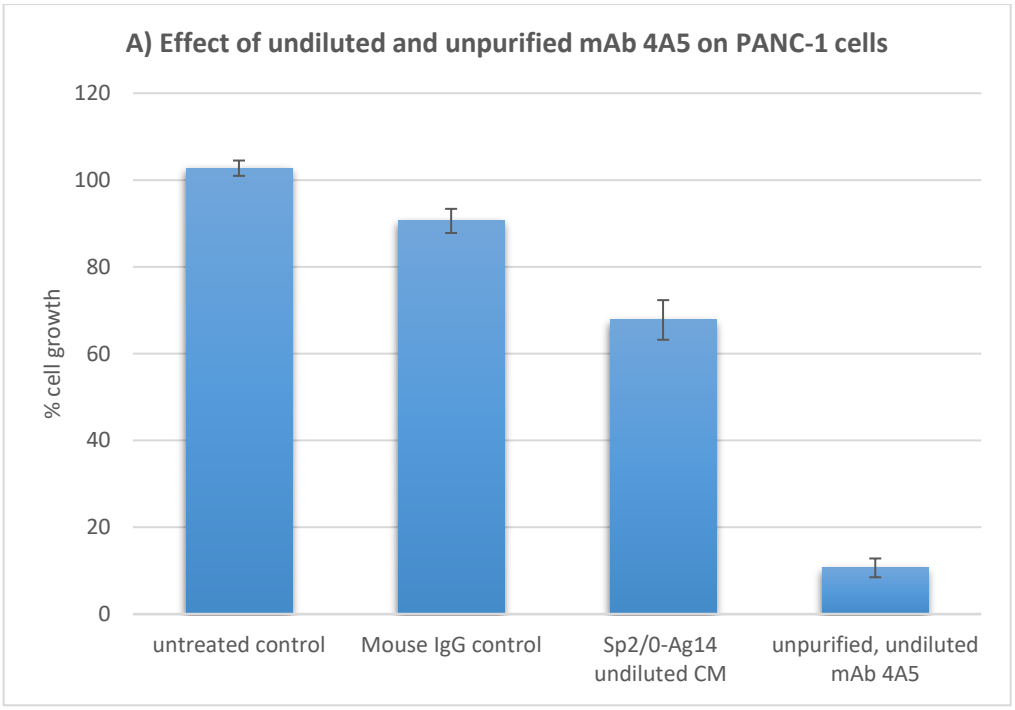


Figure 3-23: Growth profiles of A) PANC-1 and B) PIN 127 cells after 3 days incubation with the unpurified mAb 4A5 and controls. The proliferation was analysed using an acid phosphatase assay (n=1). The error bars represent the variations between technical replicates

The results displayed in Figure 3-23 show that the addition of unpurified mAb 4A5 at 1:2, 1:4 and 1:8 dilution, on PANC-1 (A) and PIN 127 (B) had no positive or negative effects on their proliferation compared to an untreated control.

3.2.8.3 Effect of undiluted and unpurified mAb 4A5 on PANC-1 and PIN 127 cells

In order to ascertain the ineffectiveness of mAb 4A5 on its immunogenic material, undiluted, and thus more concentrated, unpurified mAb 4A5 was applied on the cells. Therefore PANC-1 and PIN 127 cells were left to attach overnight and the following day, all growing medium was removed and replaced by either the undiluted mAb or the undiluted Sp2/0-Ag14 CM control. A mouse IgG control was also studied. The corresponding PBS buffer control was not included, as it was shown to have a similar effect than the mouse IgG control (see 3.2.8.1 and 3.2.8.2).



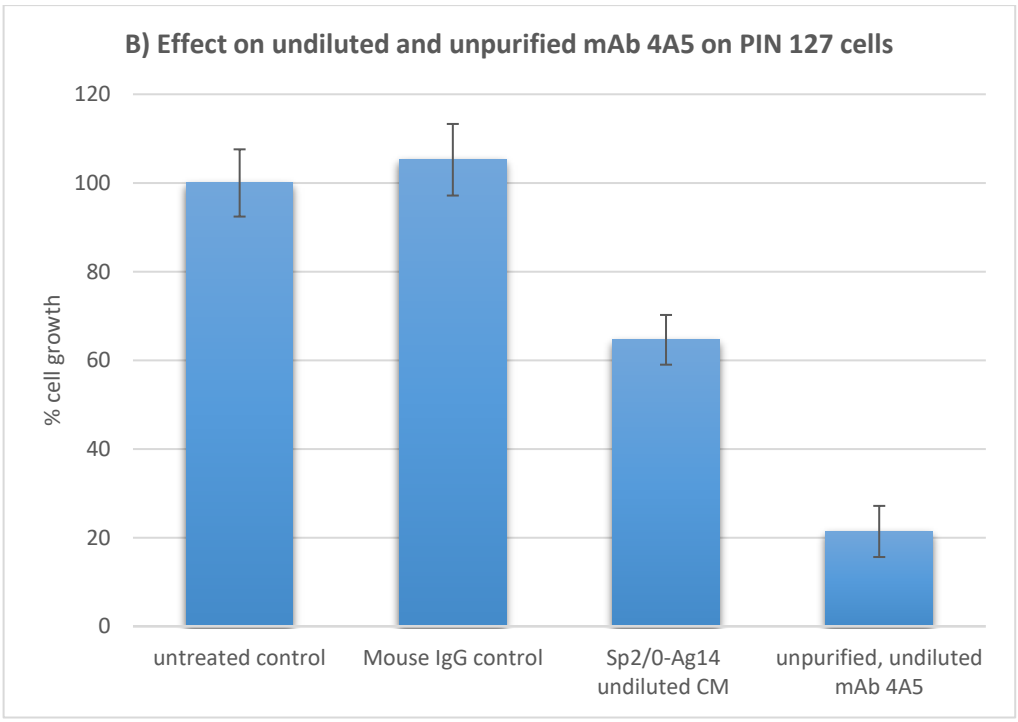


Figure 3-24: Growth profiles of A) PANC-1 and B) PIN 127 cells after 3 days incubation with unpurified and undiluted mAb 4A5 and controls (n=1). The proliferation was analysed using an acid phosphatase assay. The error bars represent the variations between the technical replicates.

Figure 3-24 represents the proliferation profiles obtained after exposing PANC-1 (A) and PIN 127 (B) cells to mAb 4A5 in an unpurified and undiluted form. The mAb appears to have a growth reducing impact on both cell lines when compared to the untreated control. However, the Sp2/0-Ag14 undiluted CM also led to a decrease in cell proliferation.

This could be explained by the fact that CMs are harvested when the cells are in a dying state and thus released toxins and waste products into the medium. CMs also contain reduced quantitites of nutrients such as L-Glutamine and glucose. When applied onto cells, all these metabolic wastes and reduced sources of nourishment could have a detrimental influence on the cell proliferation.

3.2.8.4 Effect of dialysed mAb 4A5 on PANC-1 cells

To establish if the growth reduction observed in PANC-1 and PIN 127 cells was due to the CM composition or the mAb, a proliferation assay was set up a dialysed mAb 4A5. Dialysis consists of the separation of molecules within a sample through passive diffusion against a buffer solution. The pore size of the dialysis membrane dictates what molecules are retained or passed through. This allows for removal of toxic metabolites, such as lactic acid and ammonium (Nath *et al.*, 2017). Twelve mL of PANC-1 complete growth medium, mouse IgG at 100µg/mL, unpurified Sp2/0-Ag14 CM and unpurified mAb 4A5 were dialysed against DMEM basal medium, (section 2.2.8). Four mL of each dialysed products were ultra-centrifuged at +4°C for 30 minutes at 3,000 g, on 50 kDa molecular weight cut off (MWCO) filters, and diluted back to the original sample volume (12 mL) in DMEM basal medium. All conditions were incubated for 3 days along with an untreated control.

Unlike PANC-1 cells, for which DMEM medium is used, the PIN 127 cells are routinely grown with DMEM/F-12 medium. As such, growth proliferation assays of PIN 127 cells could not be set up with the dialysates performed against DMEM. Due to time limitations, we were not in a position to perform the dialysis of mAb 4A5 and controls against DMEM/F-12.

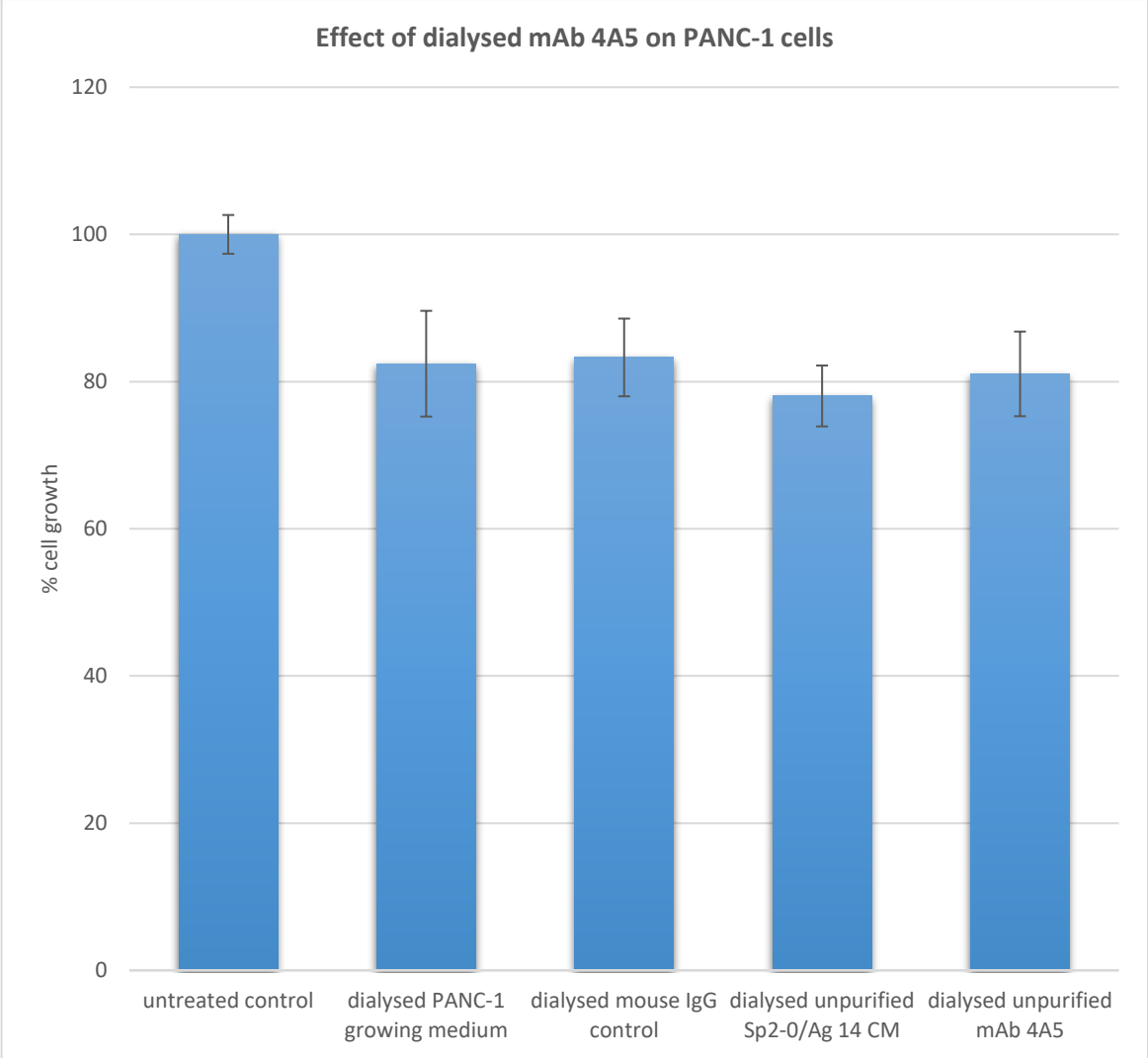


Figure 3-25: Growth profile of PANC-1 cells after a 3 days incubation with dialysed mAb 4A5 and corresponding controls (n=1). The proliferation was analysed using an acid phosphatase assay. The error bars represent the variations between the technical replicates.

The data in Figure 3-25 suggest that mAb 4A5 has no effect on the growth of PANC-1 cells. The proliferation profile of the PANC-1 cells with the dialysed mAb compares with all the dialysed controls , i.e the PANC-1 complete growth medium, mouse IgG at 100 µg/mL, unpurified Sp2/0-Ag14 CM. Therefore, it can be concluded that mAb 4A5 does not show any effect on the proliferation of one of its immunogenic cell line, the PANC-1 cells.

3.2.9 Summary of the characterisation of mAb 4A5

- MAb 4A5 was isolated following the successful generation of hybridomas directed against PANC-1 cells and PIN 127 PDX derived cells.
- MAb 4A5 was initially characterised as IgG1-κ and cryopreserved
- Immunofluorescence and immunocytochemical studies located the target of mAb 4A5 on the membrane and cytoplasm of PANC-1, PIN 127, SW1990, MiaPACA-2 and PIN 99 PDX derived cell lines.
- The target antigen appears to be highly expressed in PDAC cell lines comparing to some cell lines established from other neoplasm types.
- mAb 4A5 target antigen was not detected in paraffin embedded tissue sections of PIN 127 PDX tumour.
- The molecular size of the protein binding to mAb 4A5 was determined to be of approximately 60 kDa by immunoblotting on PANC-1 and PIN 127 cell lines.
- A direct immunoprecipitation approach was not successful in isolating this 60 kDa molecule from PANC-1 and PIN 127 whole cell lysates.
- MAb 4A5, tested in different forms (purified, unpurified, dialysed) demonstrated no positive of negative effects on the proliferation of PANC-1 and PIN 127 cells.

3.3 Development of a serum-free hybridoma cloning medium supplement

BriClone™, a well-established medium supplement supporting the growth of hybridomas in the post fusion period, is made from the conditioned medium of a human cancer cell line growing in complete media containing 5% fetal bovine serum (FBS). For confidential reasons, the origin of the cell line, named BRI-6 for the purpose of this work, cannot be disclosed.

Fetal bovine serum (FBS) is a rich and complex source of growth factors, hormones, lipids, enzymes and others unknown components commonly used *in vitro* for the expansion of cell lines. Due to the heterogeneity of each batch of serum, variations can occur leading to unreproducible data. Because it originates from live animals, FBS can be contaminated with different kind of elements such a viruses, bacteria or prion proteins that can be interfering with the outcome of experiments (Chelladurai *et al*., 2021). While FBS is harvested in highly regulated manners to avoid pain to the animals (Versteegen *et al*., 2021), ethical concerns associated with the use of live animals are always food for controversy. William Russell and Rex Burch ('The Principles of Humane Experimental Technique', 1960), have initiated the concept of the 3Rs (Replacement, Reduction and Refinement) which describes the importance of improving the way laboratory animals are treated. In line with this concept, it is necessary to find alternatives to the use of FBS and other animal derived products incorporated in the manufacturing of life science products.

Taking account of all the reasons above, the objective of this body of work was to develop a similar product to BriClone™ that does not contain serum or other animal derived products.

Although BriClone™ has never been fully characterised, it is known that the producer cells BRI-6 secrete interleukin-6 (IL-6). This cytokine has been reported to support the growth of B cells and by such the growth of hybridomas (Tosato *et al*., 1988) (Kishimoto Yoshizaki *et al*., 1984). Therefore, it was necessary to include this component in the development of a serum-free BriClone™ supplement. A recombinant human IL-6, containing no animal-derived compounds and no carrier-protein was purchased.

In order to remove the serum, there is a need to find replacements to mimic its benefits. FBS contains an abundance of growth factors and hormones, all necessary to support cell

proliferation and survival. As such, two components X and Y described as alternatives to FBS in mammalian cell cultures were included in the formulations to test.

The shelf life of the final product is of great importance, and to achieve stability, it is important to include a stabiliser in the formulation. Component Z was chosen as it was previously described as a good agent for protein conservation.

Since BriClone™ is sold to customers as a 20X concentrated product, it was agreed that the serum-free version would also be made at a similar concentration, to be diluted at a working concentration of 5% in the medium used post-fusion.

For the following sets of experiments, hybridoma fusion assays were carried out to observe the effect of the new serum-free product on freshly fused hybridoma cells (section 2.2.3). Due to ethical considerations and in order to minimise the number of mice (a maximum of 10 animals are allowed per annum for in-house use), only one 48-well-plate per condition was set up. A negative control plate (growing media not supplemented with 5% BriClone™) and a positive control plate (growing media supplemented with 5% BriClone™) were included in all early experiments. Hybridomas were left to grow undisturbed at 37°C with 5% CO2 for 12 days. Fusion efficiencies were determined by counting the number of wells containing hybridoma colonies and translating it to a percentage of fusion efficiency.

3.3.1 Basis of the formulation

All components used in the formulation of the basis of serum-free BriClone were diluted in DMEM containing 5% w/v component Z. All combinations were prepared at 20X concentration for a 1X (5% dilution) final and prepared fresh on the day of the hybridoma cell fusion assay. The concentrations of the compounds in the 20X solutions were:

- Interleukin-6: 50 ng/mL
- Component X: 200 µg/mL
- Component Y: 30 mg/L
- Mix of growth enhancing component : 1:5 v/v dilution (from the 100X solution)

The combinations tested were as seen in Table 3-7.

Table 3-7: Composition of formulations A to I tested for the basis of a serum free hybridoma cloning medium supplement.

	IL-6	Component X	Component Z	Mix of growth enhancing components
Formulation A	✓			
Formulation B		✓		
Formulation C			✓	
Formulation D				✓
Formulation E	✓	✓		
Formulation F		✓	✓	
Formulation G	✓	✓	✓	
Formulation H	✓			✓
Formulation I	✓		✓	✓

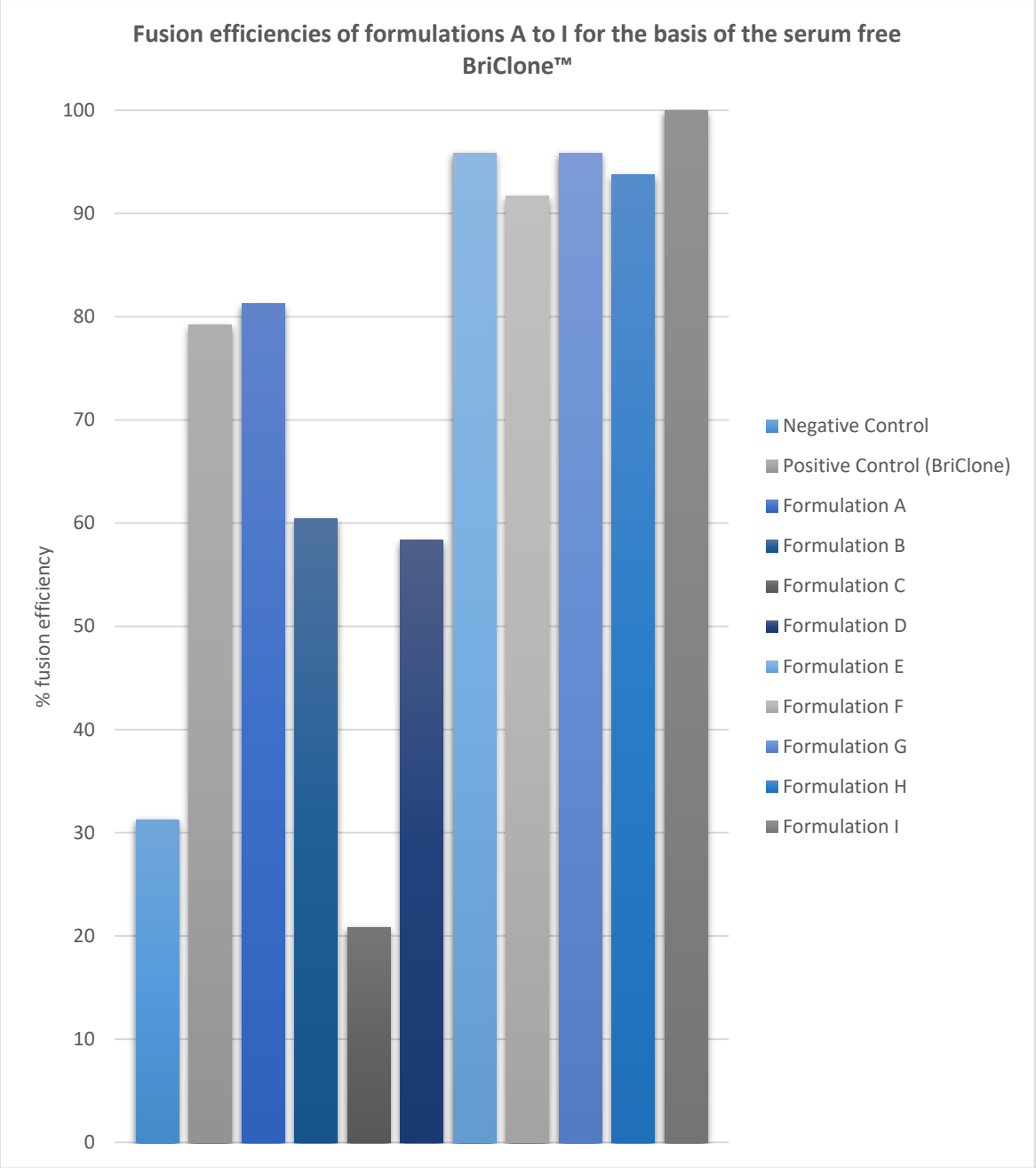


Figure 3-26: Fusion efficiencies generated from freshly fused cells grown with the addition of formulation A to I of a serum-free hybridoma cloning supplement (n=1)

Figure 3-26 represents the percentage of fusion efficiencies for each formulation tested. The negative control gave 31% colony formation and BriClone™ lead to 79%. With lower results than the parent product at 60%, 21% and 58%, formulations B (component X only), C (component Y only) and D (mix of components) will not be further analysed. Formulation A (IL-6 supplemented only) had a similar profile than BriClone™ and since formulations E, F, G. H and I all demonstrated high efficiency ratio of 92% to 100%, only these were deemed of interest. The best fusion efficiency (100%) was obtained from formulation I, made up of a cocktail of growth enhancing component combined with IL-6 and component Y. The subsequent two best formulations were E (IL-6 + component X) and G (IL-6 + component X + component Y) both giving 96% efficiency. F and G resulted in 92% and 94% colony formation respectively.

While formulation I gave the maximum percentage, it was decided that too many compounds were involved and that the cost of making this recipe would not be advantageous. Subsequently, and from the results generated in Figure 3-26, it was agreed to pursue the investigation of **formulations E and G.**

3.3.2 Stability and storage temperature studies

To verify how stable the formulations are, aliquots were prepared for analysis at 6 week and 4 month. Three different temperature storage were simultaneously tested: +4°C, -20°C and -80°C.

3.3.2.1 Six week stability study

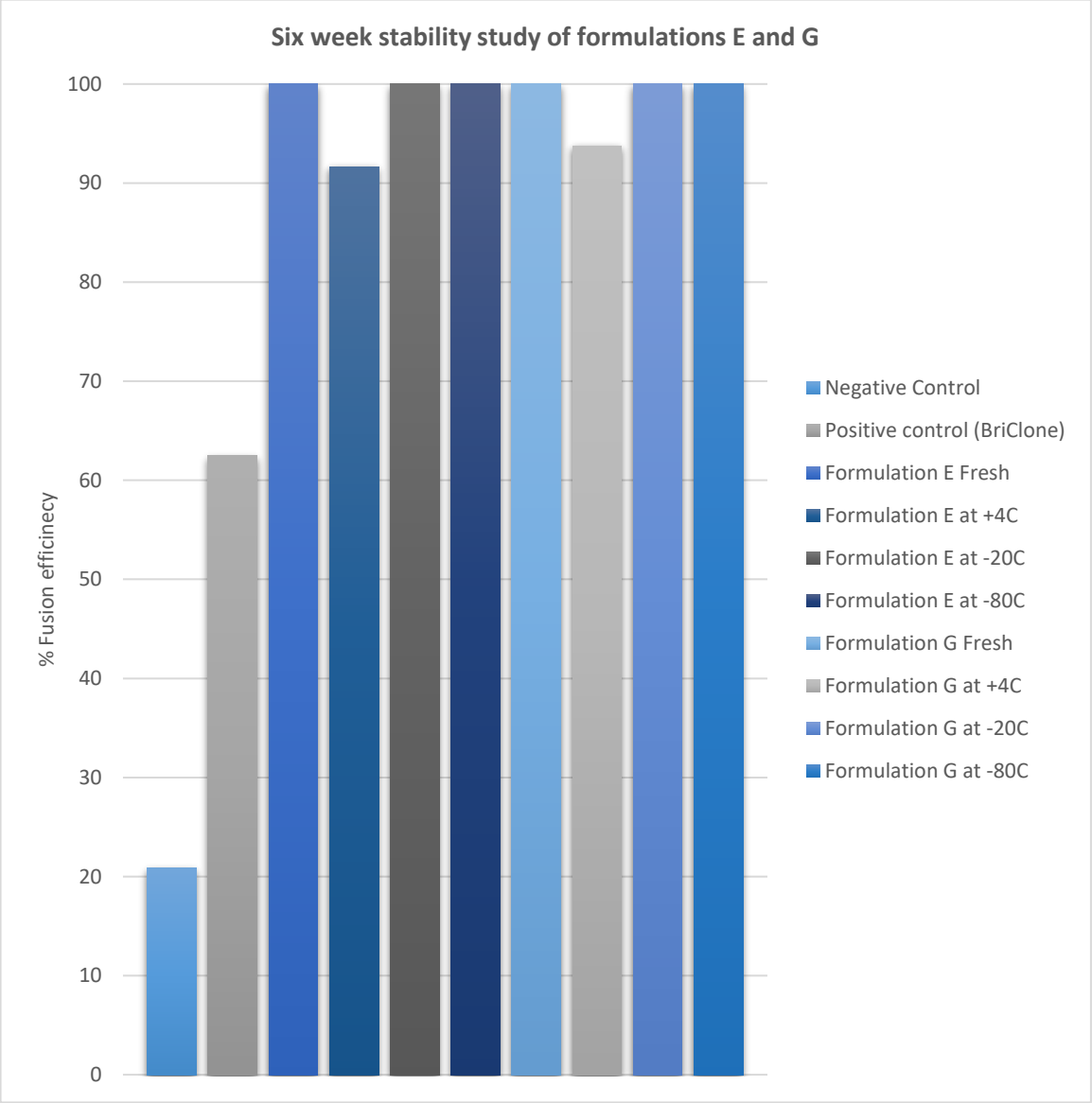


Figure 3-27: Fusion efficiencies generated from freshly fused cells grown with the addition of formulations E and G stored for 6 weeks at +4°C, –20°C and -80°C of serum free hybridoma cloning supplement. (n=1)

This assay (Figure 3-27) shows that freshly made up formulations E and G lead to 100% fusion efficiencies, and same data are observed with the products stored at -20° and -80°C for 6 weeks. Both formulations kept at +4°C demonstrated efficiencies of 92% and 94%. The parent product BriClone™ resulted in 63% of colony formation throughout the plate and the negative control efficiency was at 21%.

From these observations, it was agreed to set the temperature storage at either -20°C or -80°C.

3.3.2.2 Four month stability study

In the next fusion results, the +4°C temperature storage has been eliminated as it was guessed that the stability of each compound would lessen overtime, making this temperature not suitable for long term storage.

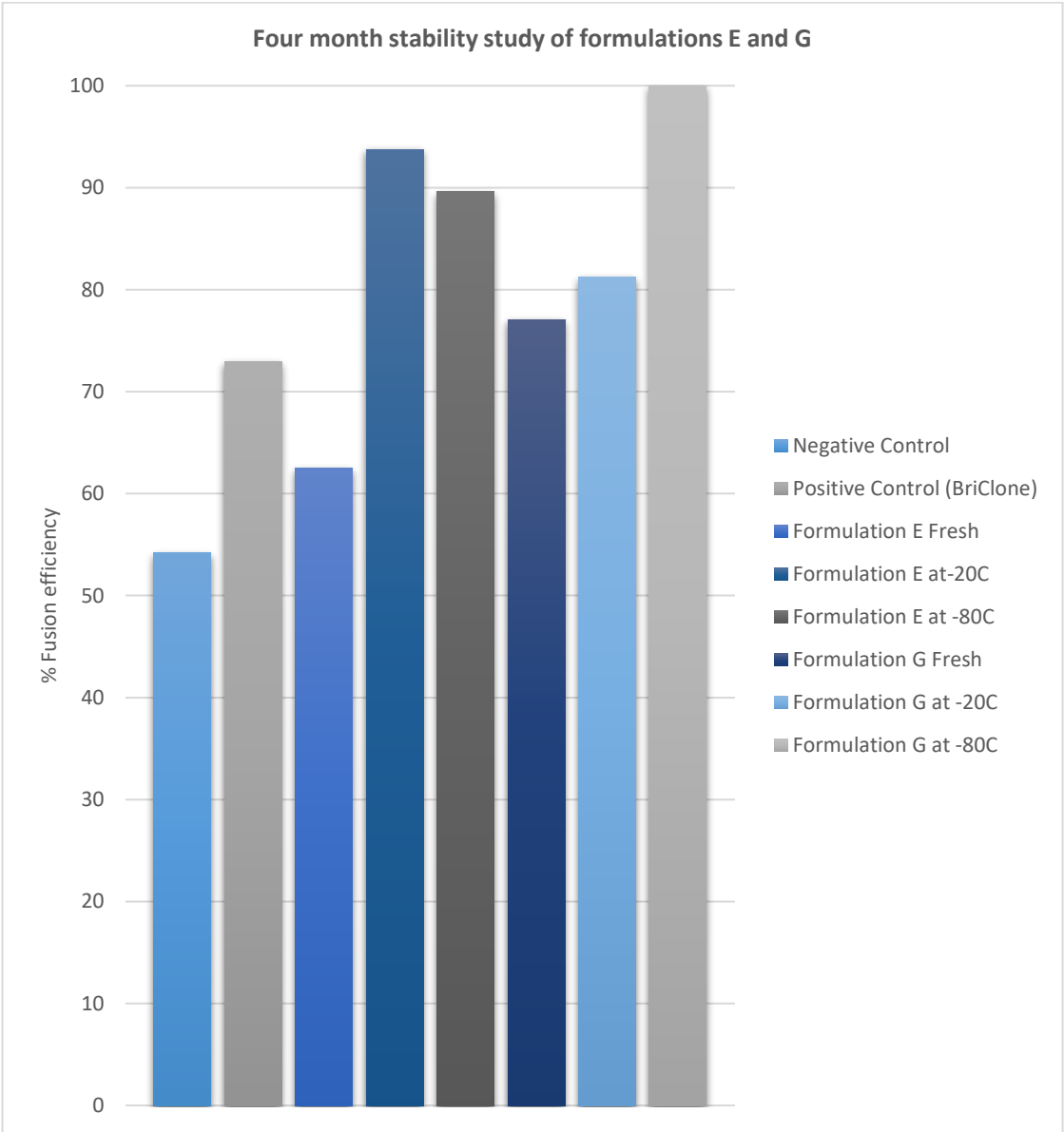


Figure 3-28: Fusion efficiencies generated from freshly fused cells grown with the addition of formulations E and G stored for four months at –20°C and -80°C (n=1)

As shown in Figure 3-28, the 4 months stability study fusion experiment demonstrated the best result (100% efficiency) is observed with the combination of IL-6, component X and Y (formulation G) kept at -80°C. The next highly satisfactory outcome were seen in formulation E stored at -20°C and -80°C, with respective efficiencies of 94% and 90%. In comparison, BriClone™ helped in the generation of 73% of hyridoma colonies. Formulations E fresh and G fresh and stored at -20 resulted in 63%, 77% and 81% respectively.

It is worth noting that ultra-low temperature freezers (-80°C) are not available in every laboratory. Subsequently, it was decided that this temperature would not be further assessed. Instead, the focus was set on the conditions that were kept at -20°C. With that in mind and with a fusion efficiency of 94%, formulation E was selected for further work.

3.3.3 Assessment of a final formulation

For budgeting reasons, it was suggested that more research could be done with the mixing and concentrations of the compounds. From this point, it was decided to reduce the concentration of IL-6 at 1ng/mL final (20ng/mL in the concentrated product) was agreed. The concentrations of component X and component Z could also be reduced and as such new formulations were looked into as per Table 3-8.

Table 3-8: Formulations J1 to L3 for the development of a serum-free hybridoma growth supplement

	% component Z	Component X (µg/mL)	IL-6 (ng/mL)
J1	5	200	20
J2	2.5	200	20
J3	1.25	200	20
K1	5	100	20
K2	2.5	100	20
K3	1.25	100	20
L1	5	50	20
L2	2.5	50	20
L3	1.25	50	20

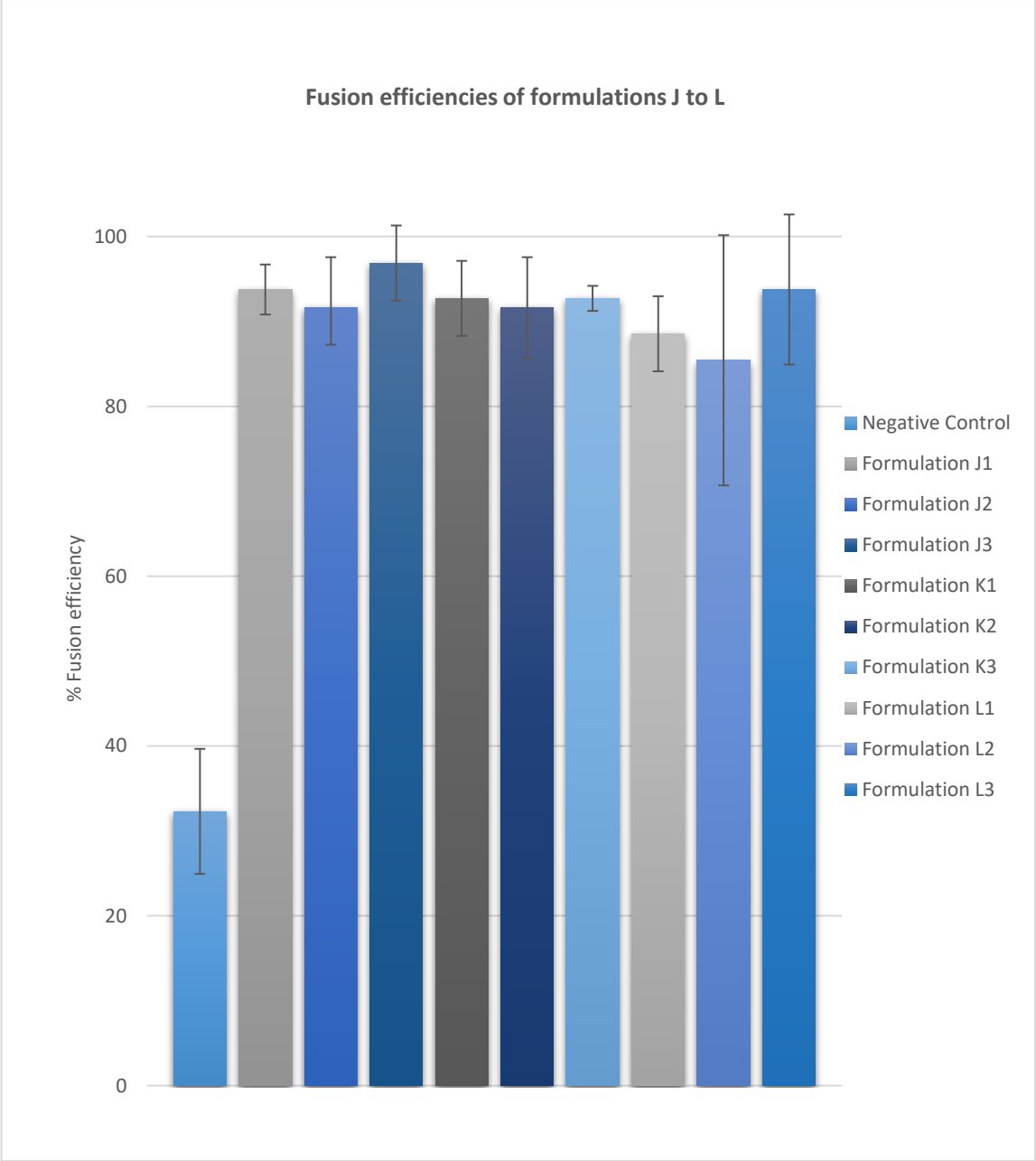


Figure 3-29: Fusion efficiencies generated from freshly fused cells grown with the addition of formulations J to L (n=2)

Looking across the results in Figure 3-29, all formulations lead to high fusion efficiencies compared to the negative control (32%). Blend J3 gives the best result at 97%, and L2 has the lowest score at 85%. These data were produced from materials prepared fresh on the day of the fusion assay. The formulations with a lower amount of stabilising agent component Z demonstrated effectiveness in generating colonies of hybridomas, however these low concentrations could affect the long term stability of the product. With that in mind , J1 was chosen as the final formulation for the preparation of a serum free hybridoma cloning supplement.

The components of this final formualtion are:

- **IL-6 at 20 ng/mL,**
- **Component X at 50 µg/mL**
- **all mixed in DMEM containing 5% w/v of component Z.**

Our newly developed product will be named BriClone-SF.

3.3.4 Stability study of BriClone-SF

The newly developed BriClone-SF cloning medium supplement was tested for its stability when stored at -20°C for a duration of 6 months and 9 months. These timepoints were compared to the product freshly made and a negative control.

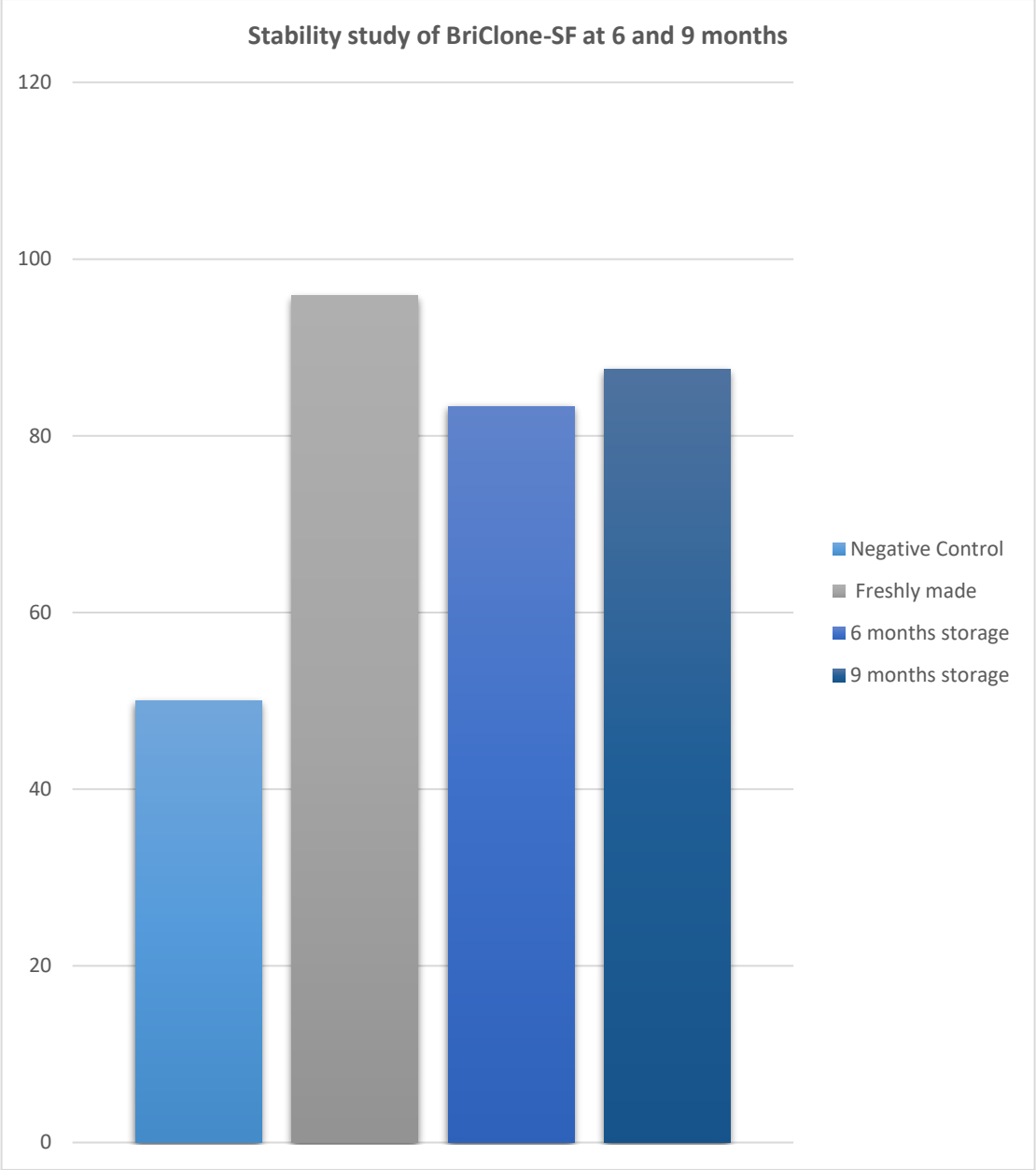


Figure 3-30: Fusion efficiciencies generated from freshly fused cells grown in medium supplemented with BriClone-SF stored for 6 and 9 months at -20°C (n=1)

The results in Figure 3-30 show the percentage of hybridoma colonies formed with each condition tested. The negative control lead to 50% of colony formation. With 96% fusion efficiency, the freshly made product surpassed the samples stored for 6 months and 9 months, which gave 83% and 88% respectively. These data are nevertheless satisfactory, meaning our supplement has an acceptable shelf life of at least 9 months without loss of potency.

3.3.5 Competitor analysis

There are commercially available chemically defined hybridoma cloning supplement. Our newly developed product was compared to some already-established products.

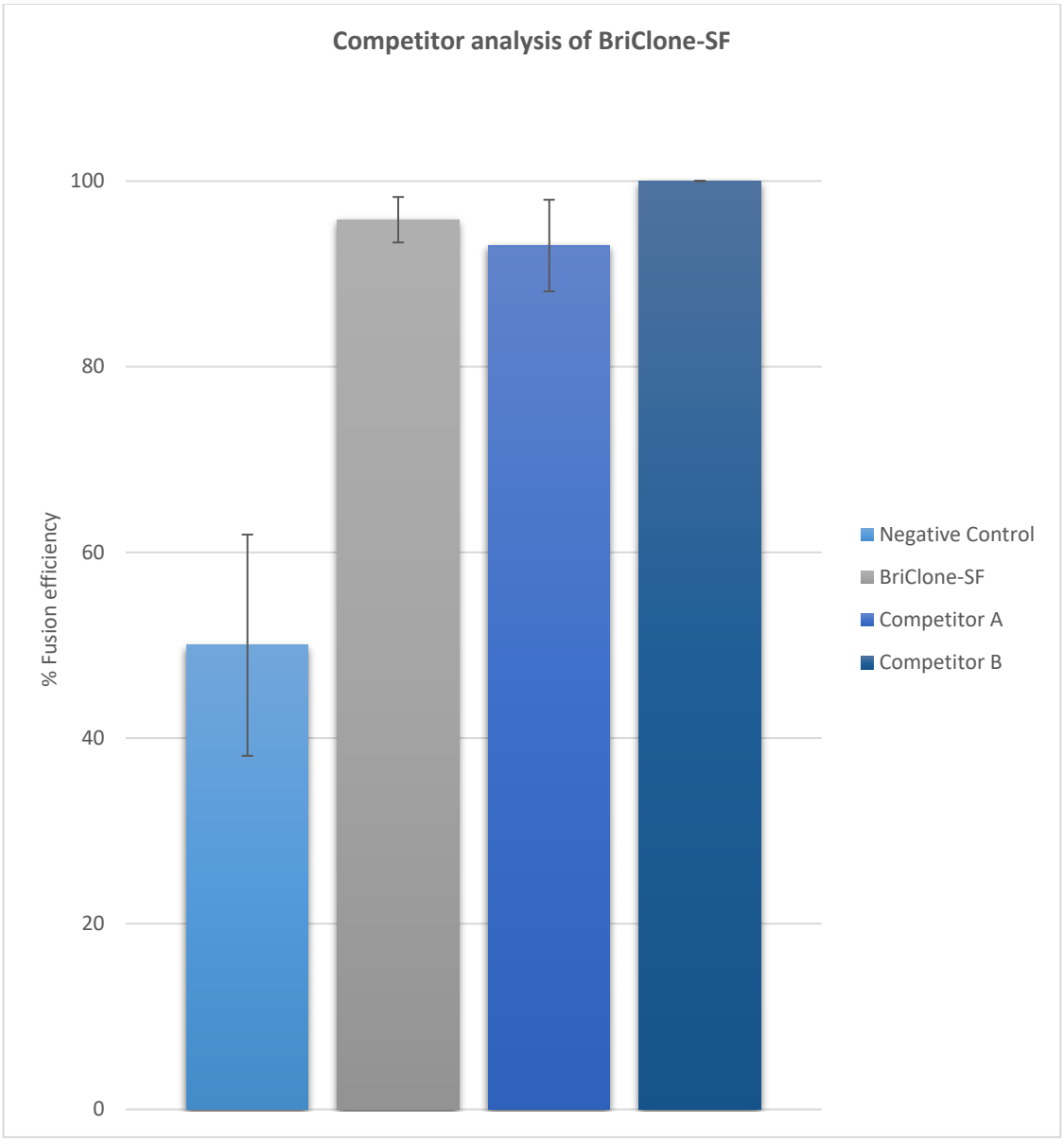


Figure 3-31: Fusion efficiencies generated from freshly fused cells grown with the addition of the newly developed BriClone-SF and commercialy available competitors A and B (n=2)

Figure 3-31 compares the results of fusion efficiencies of freshly prepared BriClone-SF against two competitors. The negative control resulted in 50% of hybridoma colonies formed. BriClone-SF lead to 96% efficiency, surpassing competitor A which produced 93% of colonies. Competitor B excelled with 100% efficiency.

These results indicates our newly formulated product BriClone-SF is a robust and reliable hybridoma growing supplement which notably improves the generation of freshly fused hybridoma.

3.3.6 Summary of the findings on the development of a serum-free, chemically defined hybridoma growing supplement

Animal derived products in scientific research may pose a problem in terms of product variability due their unknown components content, and for ethical concerns. Many formulations were tested for the creation of a serum free hybridoma cloning medium supplement that could compare to its long established parent product BriClone™. The efficiency in hybridoma colony formation after cell fusion was at the centre of these findings. The main components, interleukin-6 and component X, were determined to be necessary to achieve the best performances on the formation of hybridoma colonies 12 days post fusion. The concentrations of 20 ng/mL of recombinant human (animal free) IL-6 and 50µg/mL of component X showed the highest capacities. The necessity of the stabilising agent component Z at 5% w/v in the final product was deemed essential. This final formulation, named BriClone-SF was tested for its stability, with results indicating that a shelf life of at least nine months showed a high efficiency in hybridoma colonies generation. The optimum temperature for storage was set at -20°C. Data also showed that our newly developed serum free hybridoma cloning supplement is comparable to some commercially available products.

All the data suggest BriClone-SF is a robust, reliable and stable product. This could give the NICB the potential to manufacture and sell BriClone-SF along with its parent product BriClone™.

3.4 Development of a bioassay for use in the Quality Control of BriClone™

The production of BriClone™ in the NICB requires quality control testing to measure its efficacy. BriClone™ is sold globally as a hybridoma-growing supplement and is intended to be used for the generation of hybridomas, as it can significantly contribute to improved establishment of freshly fused hybridomas. The production of BriClone™ requires quality control (QC) testing for batch release. One of the QC tests performed to measure the potency of BriClone™ is the cell fusion assay, which is considered the reference in the NICB for hybridoma efficacy testing. However, it requires the sacrificing of BALB/c mice. In this QC test, the animals are naïve, meaning they have not been exposed to any antigen, therefore will not produce any specific mAb. A core principle of the emerging EU Directive 2010/63 emphasises a move towards removing the requirement for the use of animals is scientific research. This is in line with the principle of the 3Rs (Reduction, Refinement, Replacement) (*The 3Rs | NC3Rs*, no date-b), and therefore it would be fitting to develop an *in vitro* bioassay that would replace the need of these animals.

In this context, this results section focused on the development of an *in vitro* bioassay using established cell lines to test for the potency of BriClone™.

3.4.1 Development of a cell-based assay using IL-6 dependent cell lines

BriClone™ is produced from the conditioned medium (CM) of a proprietary human cancer cell-line, named BRI-6 for the purpose of this thesis. While fetal bovine serum (FBS) is used in the making of BriClone™ it is assumed that the main element having an effect on hybridoma development is the cytokine Interleukin-6 (IL-6), which is secreted by the producer cells. For that reason, it was decided to purchase and grow IL-6 dependent cell lines, with two myeloma cell lines, the human LOPRA-1 and the mouse 7-TD-1, having previously been described as being IL-6 independent (Lohmeyer *et al*., 1988) (Van Snick *et al*., 1986). A concentration of 10ng/mL was used in the following assays, based on the supplier's recommendations (*Leibniz Institute | LOPRA-1 | DSMZ*, no date), (*Leibniz Institute | 7-TD-1 | DSMZ*, no date)

Cells were seeded as per section 2.7.2 in complete growing media without IL-6. The conditions tested included a positive control, i.e. representing 100% growth (complete

growing medium with IL-6), a negative control (complete growing medium without IL-6) and serial dilutions of BriClone™ (complete growing medium with 5%, 2.5%, 1.25% and 0.6% BriClone™). The cells were incubated for 7 days and analysed by CyQUANT™ XTT Cell Viability Assay, as described in section 2.7.2. In the presence of actively respiring viable cells, the cellular redox sensitive XTT reagent change to formazan, forming an orange colour. The colorimetry is quantified through a spectrophotometer and converted to a percentage of growth.

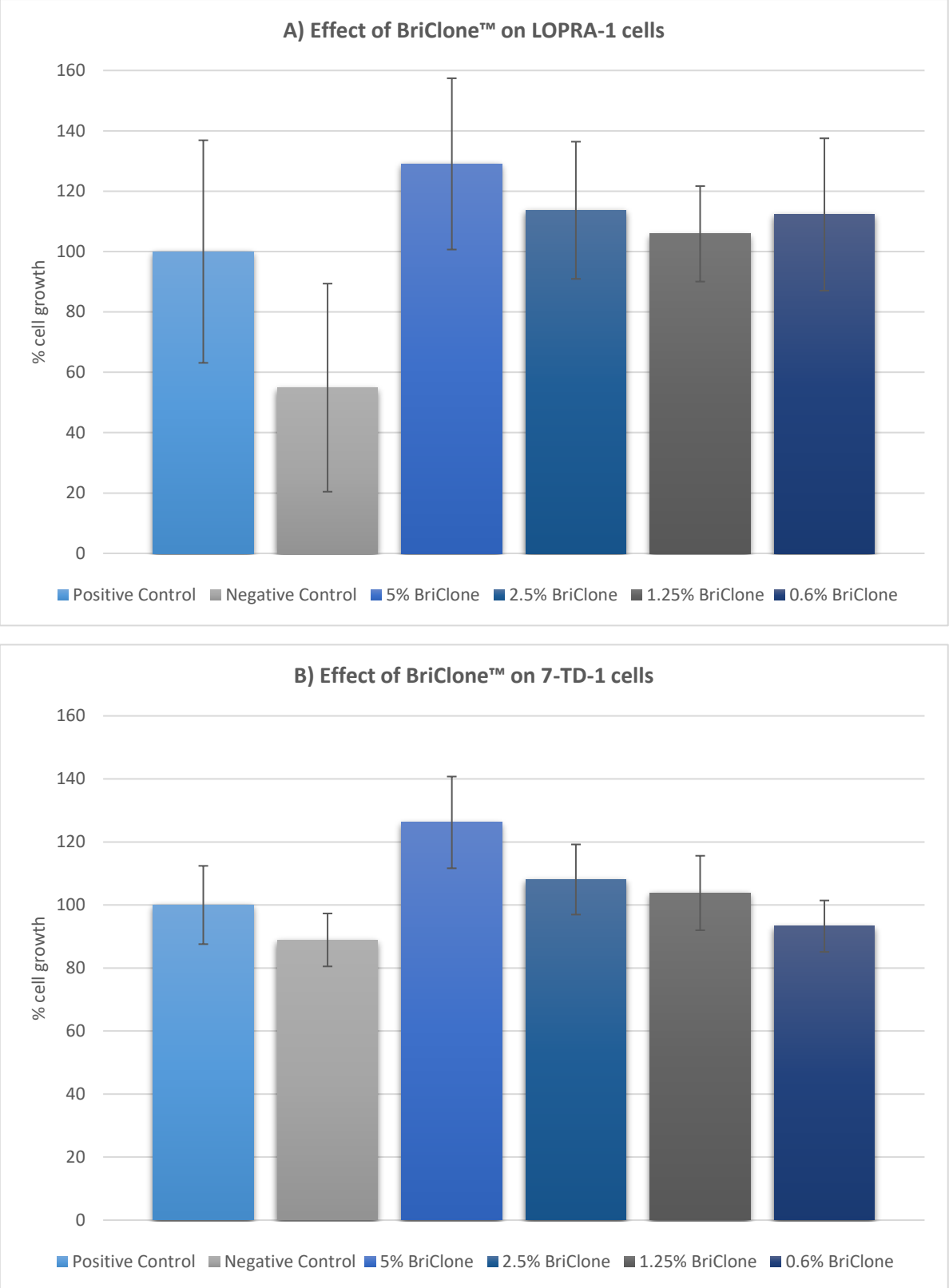


Figure 3-32: XTT proliferation assay of A) LOPRA-1 cells and B) 7-TD-1 cells after 7 days incubation with different concentrations of BriClone™ and respective controls (n=3).

The results in Figure 3-32 indicate that BriClone™ at 5% v/v seemed to have a positive effect on the growth of LOPRA-1 (A) and 7-TD-1 (B) cells, with a percentage cell growth of 130 % and 126% respectively compared to the positive controls (i.e. cells growing in their regular medium). The data in B) suggested that the 7-TD-1 cells might not be IL-6 dependent, as the percentage of cell growth of the positive control (containing 10mg/mL IL-6) and the negative control (without IL-6) looked similar. However, none of the data obtained were statically relevant. It is seen in Figure 3-32 that the variations are high and this is possibly caused by the difficulty encountered to maintain these two cell lines in healthy cultures in our laboratory. The viabilities were low throughout and long doubling times were observed. The three replicates were subsequently set up using cells with different states of health leading to different results, leading to biological variations. Due to the difficulties in maintaining the LOPRA-1 and 7-TD-1 cells in a vigorous form in our laboratories, it was concluded that they do not represent a suitable model for establishing a reliable cell-based assay.

3.4.2 Development of a cell-based assay using Sp2/0-Ag14 cells

It was consequently proposed to study the effect of BriClone™ on the cell line used as the fusion partner, the Sp2/0-Ag14 cells.

Sp2/0-Ag14 are commercially available cells used as fusion partner in the generation of hybridomas (Shulman *et al*., 1978), and routinely grown in our laboratories with DMEM Glutamax supplemented with 10% heat inactivated FBS. It was reported that a supplementation of IL-6 (1ng/mL) in the growing medium of Sp2/0-Ag14 cells containing 1% FBS had a similar effect than a regular medium containing 10% FBS (Chung *et al*., 1997). As such, it was suggested to adapt the growth of Sp2/0-Ag14 to low concentration of FBS in their growing medium. The Sp2/0-Ag14 cells were gradually adapted to reduced concentrations of FBS over a few weeks in culture, with the final concentration being 1%. The adapted cells showed phenotypical resemblance to their parents, but needed more frequent feeds. These cells hence represented a good candidate cell line model for testing the potential of the addition BriClone™ on their proliferation.

For the purpose of the following assays, these cells will be named Sp2-1%. XTT proliferation assays (section 2.7.2) were performed after three days incubation of the cells with the conditions to test.

3.4.2.1 Seeding of Sp2 1% cells in growing medium containing 1% FBS

Sp2-1% cells were seeded at in their regular growing medium (section 2.7.2). A positive control (regular growing medium) and an IL-6 control (10ng/mL IL-6) were included. Serial dilutions of BriClone™ starting at 10% v/v and ending at 1.25% v/v were analysed. All controls and BriClone™ dilutions were prepared in growing medium containing 1% FBS.

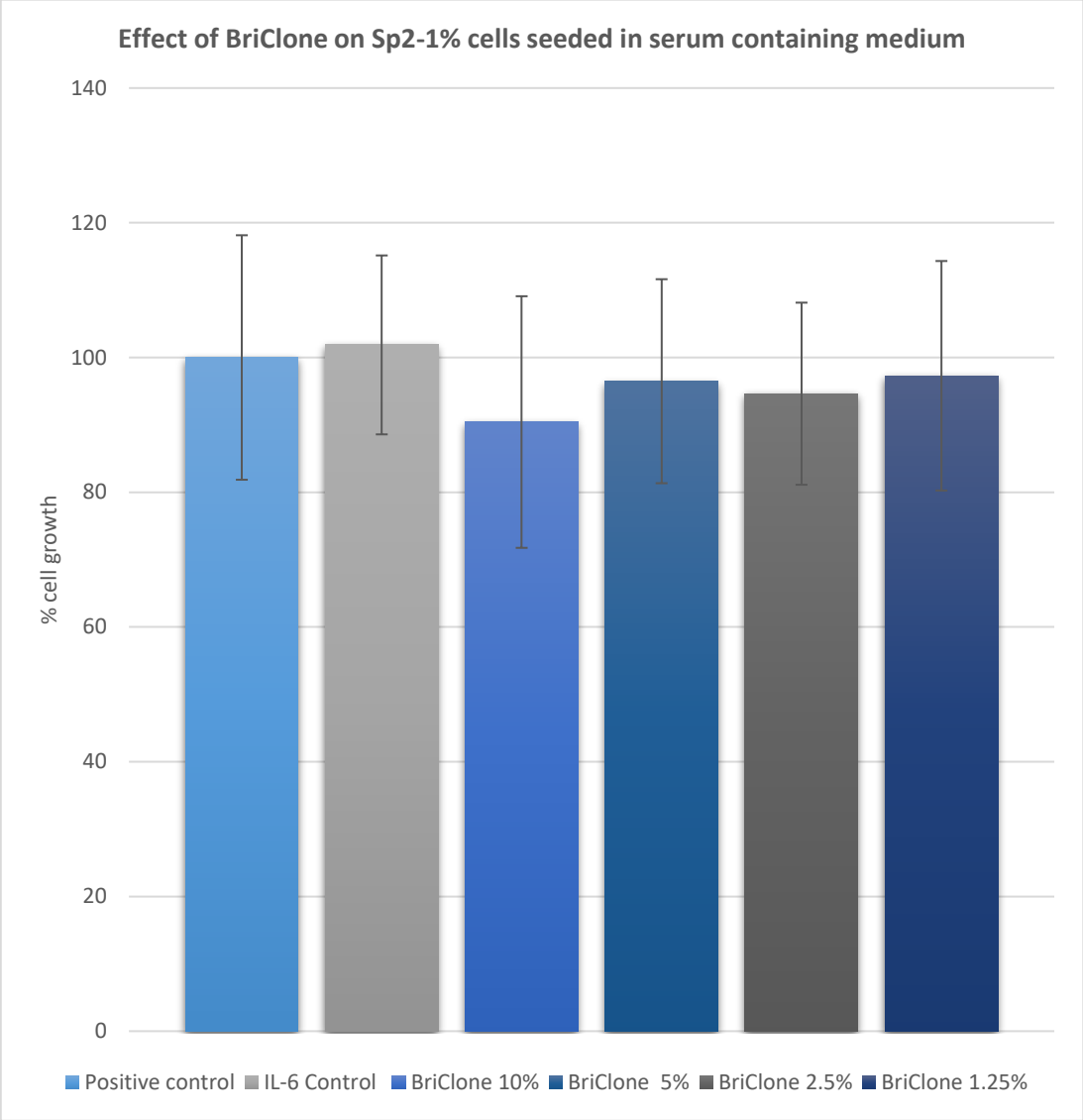


Figure 3-33 XTT proliferation assay of Sp2-1% cells seeded in medium containing 1% FBS and incubated 3 days with different concentrations of BriClone™ and controls. (n=2)

As seen in Figure 3-33, BriClone™ did not seem to improve the growth of the Sp2-1% cells after a 3 day incubation. All conditions resulted in a similar trend, with percentage of growth comparable to the positive control. The data analysed were not statistically significant.

It is noted that the cells were seeded with growing medium containing 1% of FBS and this minimal concentration of serum may have masked the effect of BriClone™. To test this hypothesis, the assay was then performed with cells seeded in basal medium only, containing no FBS.

3.4.2.2 Seeding of Sp2-1% in serum free medium

The Sp2-1% were prepared and seeded in basal serum free (SFM) medium. A growth control (no additives added) and two IL-6 controls (1ng/mL and 10 ng/mL) were included in the test. Three concentrations of v/v of BriClone™ were analysed: 5%, 10% and 20%.

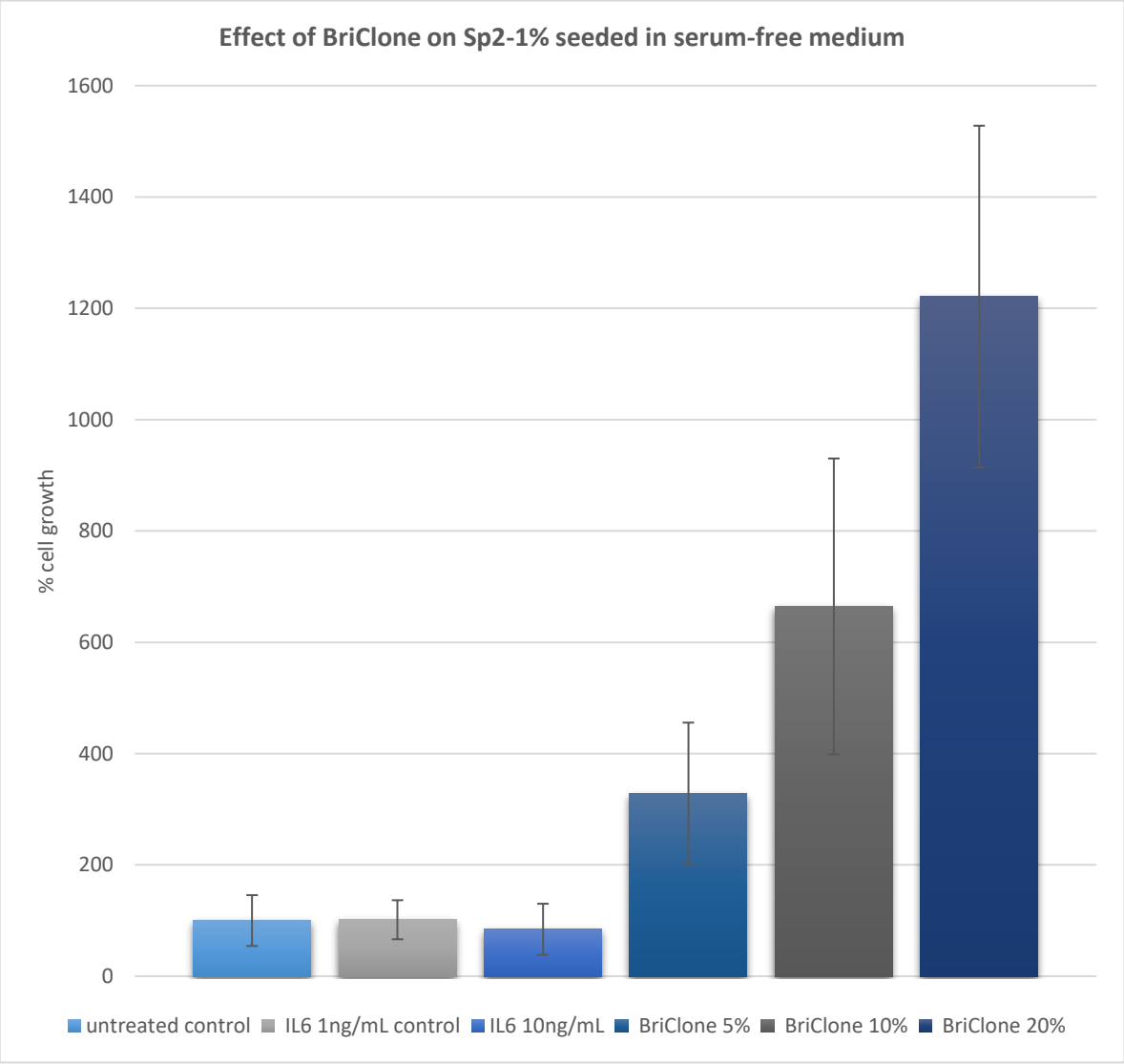


Figure 3-34: XTT proliferation assay of Sp2-1% cells seeded without serum and exposed for 3 days to different concentrations of BriClone™ and controls (n=3).

The analysis in Figure 3-34 presented that the two IL-6 controls showed a comparable trend to the untreated control, which would mean that IL-6 alone is not enough to enhance the growth of Sp2-1% cells. BriClone™ seemed to have a positive effect on the Sp2-1% cells proliferation comparing to an untreated control. The highest concentration of BriClone™ looked to have the best percentage of cell growth, followed by 10% and 5% concentrations.

BriClone derives from the conditioned medium (CM) of a cell line. During production, the complete growing medium used contains 5% FBS. Downstream, this CM is filtered through polyethersulfone membrane filters in order to remove complexes contained in the serum. It was observed through Coomassie staining (data not shown), that the profile of BriClone™ ressembles one from FBS, with multiple band of proteins stained. As such, it was speculated that the FBS remaining in BriClone™ could be the reason why Sp2-1% cells proliferated as well as in Figure 3-34.

3.4.3 Effect of a mimic of BriClone™ on Sp2-1% cell proliferation

To ensure of the hypothesis of section 3.4.2, it was decided to imitate BriClone™ but without the producer cells. For such, 250 mL of the complete medium used to make BriClone™ was incubated in a similar way as when BriClone™ is produced. This mimic medium was filtered through a 0.45 µm and a 0.2 µm to mirror the final filtration processes in the making of BriClone™.

BriClone™ and the mimic were compared on their effect on Sp2-1% cells proliferation. Concentrations 0f %%, 10% and 20% v/v were tested. As per section 3.4.2.2, the cells were seeded in SFM. An untreated SFM control was included.

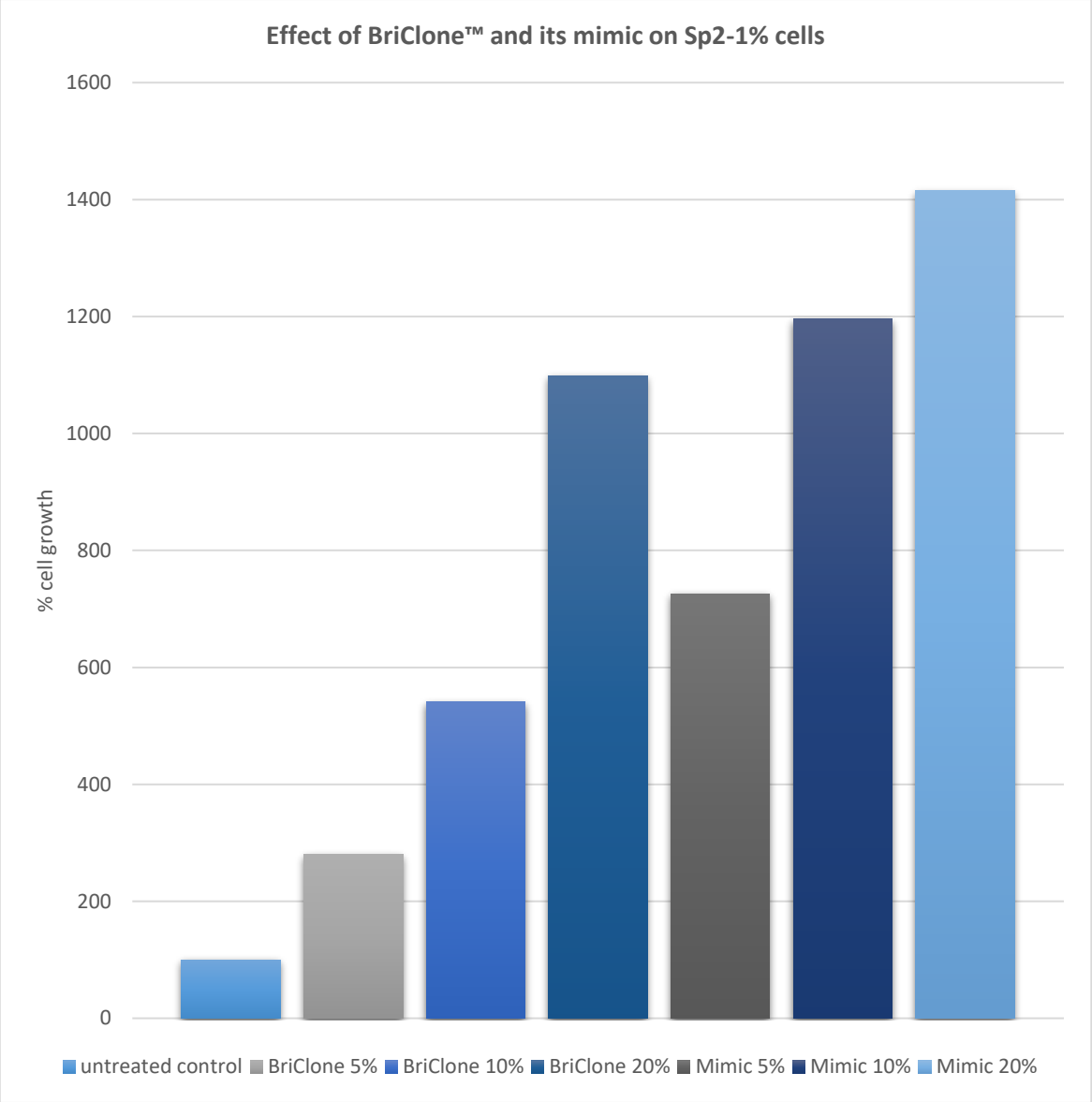


Figure 3-35: XTT proliferation assay comparing the effect of BriClone™ and its mimic on the proliferation of Sp2-1% cells after 3 days incubation (n=1).

As seen in Figure 3-35, both BriClone™ and its mimic product seemed to boost the Sp2-1% cells in a comparable ways. Both products showed similar trends, with the 20% v/v seemingly enabling the best cell growth.

These results would suggest that the traces of serum contained in BriClone™ are enough to support the Sp2-1% cell proliferation. This assay invalidates any benefits of BriClone™ on the Sp2-1% cells proliferation.

3.4.4 Summary of the findings in the development of a bioassay for the QC testing of BriClone

Quality control of any product is a critical step in batch release. One of the quality control tests required for BriClone™ production for potency testing is to generate hybridoma colonies through a fusion assay using BALB/c mice. In line with EU directives on the replacement of animal use when possible, we sought to create an *in vitro* cell based assay to assess the efficacy of BriClone™.

Commercially available cell lines were employed to create a suitable cell-based assay. Two IL-6 dependent cell lines, LOPRA-1 and 7-TD-1 were analysed to start with. Due to the issues observed with handling these two cell lines, the focus was shifted onto the Sp2/0-Ag-14 cells adapted to grow in a minimal amount of FBS of 1%, and these were exposed with various concentrations of BriClone™ or a mimic product. Both products has a similar effect on the cell proliferation Attempts to generate a dependable assay were unproductive, as it was observed that traces of fetal bovine serum present in BriClone™ seemed to induce cell proliferation.

To date, there has been no valid bioassay developed for replacing the use of live animals in the quality control of BriClone™, hence further research needs to be looked into for achieving the development of a reliable cell-based assay.

4. Discussion

4.1 Generation and characterisation of monoclonal antibodies against pancreatic adenocarcinoma

Due to the late appearance of symptoms and late stage diagnosis, pancreatic cancer adenocarcinoma (PDAC) remains a malignancy with a dismal prognosis, the overall survival (OS) at 5 years standing at less than 10 %. The success of conventional therapies in treating PDAC is limited, with severe side effects and development of chemotherapies resistance commonly experienced by the patients. The development of new effective treatments at molecular levels is therefore a priority. Immunotherapies, in combination with other drugs, may offer a hope (Turpin *et al*., 2022). In regards to pancreatic ductal adenocarcinoma, several monoclonal antibodies are currently under pre-clinical assessments. Recently, the antibody hRabMab1 directed against alternatively spliced tissue factor was shown to supress growth of human PDAC cells *in vivo* (Lewis *et al*., 2021). An anti-Glypican-1 antibody combined with an immunotoxin (GPC1) demonstrated inhibition in tumour growth in a PDAC xenograft model (Pan *et al*., 2022). Cell surface proteins, due to their accessibility, represent attractive targets. Recently it was reported that several transmembrane proteins overexpressed in PDAC could serve as promising targets for the treatment or (diagnosis) of this cancer (Meng *et al*., 2020) (Mashayekhi *et al*., 2021). Exploitation of candidate targets exhibiting high differential cancer specific expression holds the potential to develop targeted antibodies such as ADCs which are capable of selective drug delivery to cancer cells without adversely affecting patients (Parslow *et al*., 2016). ADCs are called biological bullets, due their selectivity towards their antigen and the fact that they are linked to a highly cytotoxic compound, leading to the killing of the targeted cells. Antibody drug conjugates (ADCs) along with other new formats of antibodies such as antibody-oligonucleotide conjugates (AOCs), and bispecific/multispecific antibodies are also currently undergoing clinical trials, but no biologics has been approved for clinical use to date. Sucessful target selection remains a key factor for the development of new effective antibody based therapies. There remains an urgent need to find novel targets that can be exploited using antibody based therapeutics to address the unmet needs associated with PDAC.

The successful generation of novel function blocking monoclonal antibodies targeting pancreatic cancer through the use of established cell lines was previously described (O'Sullivan *et al*., 2014) (O'Sullivan *et al*., 2017) (Arias-Pinilla *et al*., 2018). Patient derived tumour xenografts (PDXs) may offer the potential to induce mAb generation. PDX tumours

simulate the human tumour microenvironment structure, and replicate the heterogeneous aspect of the cancerous mass (Abdolahi *et al*., 2022). These models maintain essential histopathological features and genetic profiles of the original patient tumours including genomic mutational status, biochemical signalling, and response to tumour cell autonomously targeted therapeutics. Tumours have the potential to facilitate the isolation of mAbs recognising novel and clinically relevant target proteins involved in PDAC disease. A team of researchers at the NICB has successfully established a PDX biobank. Multiple patient tumours and PDX derived tumours make up this highly valuable biological sample collection. PDX derived cell lines were also derived from two tumours, the PIN 127 and PIN 99 cell lines, both cell lines were demonstrated to have conserved their human features by immunohistochemical analysis using the human-specific antibody Anti-Mitochondria antibody 113–1 (Roche *et al*., 2020). Previous research studies undertaken by Coleman *et al*, identified several proteins differentially expressed in PDAC cell lines, PDAC patient tumours and PDX tumours, and some of these candidates may have potential as therapeutic targets or biomarkers of PDAC (Coleman *et al*., 2018) (Coleman *et al*., 2020).

By applying hybridoma technology and using a combination of established PDAC cell lines, patient derived xenograft (PDX) cell lines, PDX first generation tumours and patient tumours (as described above), we have successfully developed a panel of mAbs that may have the potential to represent valuable PDAC associated molecular targets. Although recombinant technologies have changed the way monoclonal antibodies are produced, the generation of hybridomas as described by César Milstein and Georges Köhler in the early 1980s remains the popular and traditional method. Most recently, this key methodology has permitted the discovery of some of the most successful and FDA approved mAbs, such as nivolumab (anti-programmed cell death protein 1; anti-PD-1) and atezolizumab (anti-programmed cell death protein ligand 1; PD-L1) (Moraes *et al*., 2021).

In our study, three groups of BALB/c mice (n=2) were inoculated with mixture of immunogenic material, and their spleens were harvested post immunisation schedule for hybridoma generation. A combination of two immunogens, different for each group, was used to achieve a wider the immune response. We successfully generated positive hybridoma candidates from the three groups. Multiple hybridomas were selected and screened by immunofluorescence (IF) on unfixed cells to check for immunoreactivity. Any detected IF signals resulted in the hybridomas being single cloned to achieve monoclonality. Each clone

was further re-assessed by IF and all remaining reacting hybridomas were cryopreserved, forming a bank of clones of interest.

In group 1, the mice were immunised with PANC-1 cells and PIN 127 PDX cells. Post-fusion, hybridoma colonies were selected; and these were further re-screened for reactivity; six monoclonal candidates were chosen for further expansion and characterisation. Five of these are yet to be characterised but the lead hybridoma candidate, secreting an IgG1-κ antibody, was selected for further study. Most ADCs are based on the IgG1 isotype, since they possess greater immunogenic capacities, and can support antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Criscitiello *et al*., 2021). The selected clone was designated mAb 4A5 and comprehensively analysed. Due to time constraints, only this one lead mAb was characterised and was the focus of this thesis.

Multiple immunological based experimental methodologies were employed to assess the reaction of mAb 4A5 to its target antigen. Through the initial screenings, it was demonstrated that mAb 4A5 had a strong affinity to its two immunogens, PANC-1 and PIN 127 PDX cells. Apart from these two cell lines, the newly generated antibody was also applied to a panel of other cancer cell lines, from PDAC to other representative malignancies. IF on unfixed cells was carried out on SW1990, MiaPACA-2 and PIN 99 PDX cells representing pancreatic adenocarcinoma, and Caco-2 (Colon cancer), IGROV-1 and OAW42 (ovarian cancer), and MCF-7, HCC 1419, and ZR-75-1 (breast) to represent other neoplasms.

It was observed that mAb 4A5 showed strong membrane and cytoplasmic reactivity across the PDAC panel, whereas the signal on the other cancer cell lines was weak or almost undetectable. These results suggested that mAb 4A5 target antigen may potentially represent a membrane and cytoplasmic protein, which might be present in higher abundance in PDAC. However the specific localisation would have to be confirmed through detailed antibody localisation studies using confocal microscopy. In the context of an ADC development target, this would represent an ideal antigen, with higher expression by the cancer cells, and low to no expression in the surrounding normal cells, and localised on the surface of the cells (Khongorzul *et al*., 2020).

To confirm the localisation and study the expression of the target protein, immunofluorescence on fixed PANC-1, PIN 127 and SW1990 cells was performed. The images obtained from this experiment showed a strong “punctate type” of staining on the

surface of the cells, and the signal was also observed in the cytoplasm. Further studies were carried out, this time by immunocytochemistry (ICC). In this setting, only cells from PDAC were analysed ; the two immunogens PANC-1 and PIN 127, along with the SW1990 and MiaPACA-2 cells. The conditioned medium of the hybridoma expressing mAb 4A5 was directly applied onto the fixed cells leading to a strong staining observed on all specimens. Membrane and cytoplasmic reactivity was observed, and this corroborated the findings observed on IF. An ideal therapeutic/ADC should target a membrane protein only expressed in the tumour targeted (Fu, 2022). Since the reactive antigen for mAb 4A5 looked localised on the membrane of mainly PDAC cell lines, it could thus represent a potential therapeutic antibody target. This would need to be further studied across other cancer types.

Using tissue sections of PIN 127 PDX tumours, immunohistochemical analyses were performed. The resulting images showed that no or unspecific staining was achieved, suggesting that the tumour might not possess the target to mAb 4A5, which would invalidate the previous findings. However, there is an hypothesis that formalin fixation, followed by paraffin embedding, could degrade some antigenic sites in specimens (Xie *et al*., 2011) and lead to false positive or negative readings. Immunohistochemistry plays a huge role in clinical laboratories for diagnosis purposes (Duraiyan *et al*., 2012),and mAbs can be used as diagnostic tools to potentially recognise markers specific to a disease. Due to the absence of reaction of mAb 4A5 to its protein target antigen on tissue, we cannot state it is not expressed in PIN 127 PDX tumours.

Ahead of a potential identification of the target protein by immunoprecipitation, it was necessary to determine its molecular weight. Through optimisation of immunoblotting conditions, we discovered that mAb 4A5 reacted with a band of interest of around 60 kDa. Although no loading control was performed during analysis, it seemed that this ~60 kDa protein was present at higher levels in the PANC-1 whole cell lysates than in the PIN 127. The bands observed were not intense, meaning that the protein might be quite low abundant in the whole cell lysates. The optimised immunoblots were tested only with the two immunogenic cell lines, therefore it is not known yet if the other PDAC cell lines used in IF, ICC would display the same profile.

The detection of the band of interest prompted to perform preliminary direct immunoprecipitation (IP) studies using protein G agarose beads. A similar approach was successfully previously employed by our group to identify the target protein of two novel

antibodies (O'Sullivan *et al*., 2014) (O'Sullivan *et al*., 2017). IP enriches proteins, even in low quantities from complex samples. PANC-1 and PIN 127 whole cell lysates were subjected to IP in presence of mAb 4A5. The potentially pulled down proteins were analysed and compared by Coomassie staining and corresponding immunoblots. The target protein did not seem present in the immunoprecipitates of either PANC-1 or PIN 127 cells. Some potential bands observed in the immunnoprecipitates that were not present in the control mouse IgG. As observed with the immunocytochemical studies, the target of mAb 4A5 is potentially cell membrane localised. Membrane proteins are difficult to study due to their low level presence and instability outside the lipid layers, thus the handling and detection of this protein in complex samples is challenging (Kongpracha *et al*., 2022). All these findings require further study to identify the target antigen by LC/MS spectrometry.

While other technologies exist for the characterisation of mAbs not all of them were suitable in our case. For example the use of Biacore™ technology to study real-time analysis of biomolecular interactions based on surface plasma resonance, relies on the requirement to know what antigen binds the mAb, which has yet to be identified.

Our group has previously identified novel function blocking mAbs that were demonstrated to show significant effects on cancer invasion in PDAC *in vitro* models (O'Sullivan *et al*., 2014), (O'Sullivan *et al*., 2017). To investigate the potential anti-cancer activity of mab 4A5 we studied its effect on the proliferation of PANC-1 and PIN 127 cells. For such, growth assays were performed with different forms of the antibody, i.e. purified, unpurified, dialysed, and at several dilutions on the cells, and left to incubate for 72 hours. No cell growth reduction or increase was detected in the presence of mAb 4A5, this could suggest that the target of the antibody might not be involved in cell growth.

Other mechanisms can be modulated by monoclonal antibodies It was reported that a newly developed mAb N2E4 targeting the Neuropilin-2 (NRP2) transmembrane non-tyrosine-kinase glycoprotein, had the potency to inhibit the proliferation, migration, and invasion of PDAC cell lines *in vitro,* and to inhibit tumour growth and metastasis *in vivo* (Wang *et al*., 2021). An anti-human TM4SF5 antibody showed to reduce cell viability and to decrease the motility of human pancreatic cancer cells with a high expression of TM4SF5 (Park *et al*., 2020). By targeting the glycolytic enzyme alpha-enolase (ENO1) protein, researchers demonstrated that an anti-ENO1 inhibited invasion of human PDAC cell lines *in vitro* and that metastasis in immunodepressed mice was also prevented (Principe *et al*., 2015). No

effect on the PANC-1 and PIN 127 cell growth was observed with our mAb 4A5. Due to time constraints, we did not study other aspects such as invasion and migration that could in further studies.

4.2 Alternative to the use of animals and animal-derived containing products

4.2.1 Development of a chemically defined, serum free hybridoma growing supplement

The use of animal-derived product in life science is changing, with many alternatives being available for growing even notably difficult cell types (Pilgrim *et al*., 2022). In light of data reproducibility and ethical concerns, the employment of fetal bovine serum (FBS) for in vitro purposes has shifted towards more respectably considered animal-free products (Chelladurai *et al*., 2021). An overview of serum-free medium can be accessed freely at https://fcs-free.org/fcs-database (*Fetal Calf Free Serum Database - Fetal Calf Serum Free Database*, no date).

Hydridoma technology remains the traditional way for discovering and producing monoclonal antibodies (Zaroff & Tan, 2019), but the hybrid cells produced are fragile and unstable. Conditioned media (CM) contain biologically active factors that are released by previously cultured cells. Although undefined, they have proven to enhance the proliferation of freshly fused hybridomas *in vitro* and expression of their monoclonal antibody (Okuda, 2018; Zhu et al., 1993). While efficacious, CM are the results of harvested cell line growing media commonly containing at least 5% of FBS. Developed by a group of researchers at Dublin City University for in-house use BriClone™ is the result CM of IL-6 secreting producer cells grown in a complete medium containing 5% FBS. For over thirty years, BriClone™ has been commercialised and has a continuing worldwide success when used as a supplement for the formation of freshly fused hybridoma colonies (Okazaki *et al*., 2001) (Hinali *et al*., 2013). Although the downstream process in BriClone™ production aim to remove the serum particles in the CM, traces may remain in the final product. To fall in line with the 3Rs, we proposed to develop a serum-free alternative to BriClone™, which would show similar performances. Based on our knowledge that BriClone™ contains IL-6, in undefined quantities, we incorporated this cytokine in the formulation of the new serum-free substitute. Interleukin-6 (IL-6) is a potent soluble cytokine involved in many physiological

functions (Bazin & Lemieux, 1989); it does help in the maturation of B cells into antibody secreting plasma cells (Yoshizaki *et al*., 1984). This ingredient was tested alongside other compounds that are commonly used for replacing FBS, such as component X. To achieve stability of the newly developed product, component Y was also incorporated in the formulation. After eliminating many combinations with less potency for the generation of hybridoma colonies post-fusion, one final chemically defined and free of animal content blend surpassed the long established effectiveness of BriClone™. When added at the same recommended concentration of BriClone, i.e 5 % v/v in the medium used for seeding hybridoma, the efficiencies observed most often neared the 100% efficacy. This new product, named BriClone-SF was produced in DMEM containing 5% v/v of component Y, 20 ng/mL animal free recombinant human IL-6 and 50 µg/mL of component X. When stored at -20°C, this alternative for BriClone™ was proven to be stable for at least nine months without any decrease in hybridoma colony formation. Two competitor products, with a mentioned shelf-life of 1.5 years on their datasheet, were compared to BriClone-SF. Resulting percentages of successful hydridoma generation with BriClone-SF were similar to these two commercially available products. Our data also supports the findings that BriClone-SF is stable when stored at -20°C at least nine months.

4.2.2 Development of an *in vitro* cell-based assay for the testing of BriClone™

In line with the 3RS principles, the replacement of animals in product testing and scientific research is to be considered wherever possible (Tannenbaum & Bennett, 2015). *In vitro* bioassays for testing the potency of a product need to be relevant, reliable and represent accurately the biological activity of the compound to test (Moreira *et al*., 2020). The production of BriClone™ requires the use of one or two BALB/c mice for each quality control (QC) testing for batch release. The spleen of the naïve mice is used to check for expected high percentage of efficiencies in the formation of freshly fused hybridoma colonies.

To move away from the need of live animals in this QC setting, it was proposed to create an *in vitro* bioassay that would have the ability to prove the potency of BriClone™ in similar way than the fusion assay. Since one of the known active component of BriClone™ is interleukin-6 (IL-6), the first models tested were two IL-6 dependent described established cell line. The cells were exposed to various concentrations of BriClone ™ in microplates

and their cell proliferation analysed by XTT assay after a week in culture. The LOPRA-1 and 7-TD-1 cell lines were found to be difficult to handle in our laboratories. Their growth was never exponential, their doubling time was almost a week and their viabilities were constantly low, sometimes reaching 50% of viable cells only. These observations are not described in the literature, and could be related to the stocks from the NICB, but since the data obtained from the growth assays of these two cell lines showed high variations, the LOPRA-1 and 7-TD-1 cells were deemed unreliable. Moving away from these two myeloma cell lines, it was suggested to look at another myeloma established cell line for further assessment. The Sp2/0-Ag14 is a common cell line used as fusion partner for the generation of hybridomas. IL-6 supplementation was reported to enhance the growth of Sp2/0-Ag14 cells cultivated with low amounts of FBS (Chung *et al*., 1997). Through gradual reductions in the concentration of FBS added to the growing medium of these cells, we obtained a healthy culture capable of proliferation displaying phenotypic resemblance to their parents. A minimal final concentration of 1% FBS was kept in the growing medium. Named Sp2-1%, the new cell line underwent several assays in the presence of BriClone™. Preliminary results suggested that the addition of BriClone™ at a concentration of at least 5% v/v led to a boost in the Sp2-1% growth by comparison to a untreated control. BriClone™ is a conditioned medium produced with a medium containing 10% FBS, and while the downstream process involves two steps of filtration, there is a possibility that not all the serum is removed from the final product, To ensure the traces of serum are not responsible for the boost in proliferation of the Sp2-1% cells, it was necessary to investigate this hypothesis. For this purpose, a mimic of BriClone™ was generated. For such, the growing medium used in the production of BriClone™ underwent a similar process than the regular production process, but with no producer cells cultivated. This mimic was added to Sp2-1% cells and compared to BriClone™. It was observed that the proliferation of the Sp2-1% cells was exacerbated similarly with BriClone™ and its mimic. This concluded that the traces of serum, seemed to be responsible for the cell proliferation of the Sp2-1% cell line. The depletion of high-abundance proteins in FBS such as bovine albumin, could be necessary for observing the dynamic of other proteins in the conditioned medium (Nakamura *et al*., 2021). Commercially available ligand-based affinity columns proved to be efficient in removing up to 80% of bovine albumin (Stastna & Van Eyk, 2012) hence, this methodology could prove useful in enriching BriClone™ with less abundant proteins for setting up the QC cell-based assay with Sp2-1% cells.

While we found the handling of the two IL-6 dependent cell lines LOPRA-1 and 7-TD-1 challenging, it could be of interest to study other IL-6 dependent cell lines. The INA-6 human myeloma cell line (Burger *et a*l., 1998) and B9 hybridoma cells, both commercially available, are described as needing 10 ng/mL and 50 pg/ml recombinant human IL-6 respectively for their proliferation. They both require exogenous addition of IL-6 and the B9 hybridoma cells are used for simple sensitive assays for human, rat, rabbit, pig and mouse IL-6 (Nordan *et a*l., 1996).

As such, although progresses have been made towards the development of an *in vitro* cell based assay for the quality control of BriClone™, It is hoped that in follow on studies a reliable system will be successfully developed.

5. Conclusions and Future perspectives

Pancreatic adenocarcinoma (PDAC) remains a virtually incurable cancer with current therapies. Immuno-oncology, an approach successful on other types of neoplasm, offers the possibility for better treatment of PDAC. While several monoclonal antibodies (mAbs) are undergoing pre-clinical studies and clinical trials, to date, none have been approved to treat patients with PDAC. As such, there is a need for discovering new targets of PDAC which could be targeted by mAbs. The use of established cell lines and patient derived xenograft (PDX) material was used as an approach to generate novel mAbs against PDAC through the traditional hybridoma technology method. Following the sucessfull generation of mAbs directed against three groups of different immunogenic material, a number of hybridomas of interest secreting mAbs of different classes were generated, screened and cryopreserved. These mAbs have the potential be investigated further to establish if they are targeting cell surface proteins and also if their respective target antigens can be identified using an IP approach coupled with LC-MS spectrometry there may also be potential for identification of novel targets associated with PDAC.. Although, these hybridomas need to be reassessed for the immunoreactivity against their immunogens, they could be selected as interesting candidates and characterised through functional studies (potential anti-cancer activity) and immune-based methodologies (target expression in PDAC *in vitro* and *in vivo*).

In this thesis, one lead newly developed mAb 4A5 was successfully generated and comprehensively characterised. It was found that mAb 4A5 reacted with a seemingly cytoplasmic and membrane localised protein, potentially present in higher abundance on PDAC cells representing established cell lines but exhibiting none/very little abundance in some cell lines representing other cancer. A larger cohort of cancer cell lines, PDAC cells representing all PDAC classification types (Deer, *et al*., 2010) and other cancer types and non-cancer types could be analysed to confirm or not the presence of the target protein of mAb 4A5. The National Institute for Cellular Biotechnology possesses an extensive bank of cryopreserved established cell lines from many cancer types, and selected representative cells could be used to analyse the expression of mAb 4A5 target by immunofluorescence, immunocytochemical and immunoblotting studies.

Immunohistochemical studies were inconclusive in detecting the antigen of mAb 4A5 on PIN 127 PDX tumour sections, with no to seemingly unspecific nuclear staining observed. There is a possibility that the antigen was destroyed or degraded during the formalin fixation process. More research could be conducted into why this monoclonal antibody is ineffective

on tissue sections of PDAC tumour. The optimal cutting temperature compound (OCT) used for tissue embedding prior to frozen sectioning could offer an alternative. If the antigen is unmasked on PIN 127 PDX tissue sections, OCT could then be applied to many more patient tumours and PDX derived tumours that have been generated by the PDX team and currently banked at the NICB (Roche *et al*., 2020). The expression of the target antigen of mAb 4A5 on tissue cannot be made at this stage.

Following Western blotting, the target protein was discovered to be of an approximate molecular weight of 60 kDa. However, since Western blots were carried out with whole cell lysates, our current immunoblotting settings cannot confirm if this target is a membrane protein. As such, we could generate enriched membrane proteins lysates and conducted more immunoblotting studies to check the target on the cell surface of the immunogen cell lines. We could also apply both whole cell and membrane enriched lysates to other cancer cell types to analyse the target potential abundance in PDAC.

In order to identify the protein target, the first approach used was a direct immunoprecipitation, which had already used by our group in the discovery of mAbs 9E1 and 7B7 directed against Annexin A6 and the KU70/80 heterodimer respectively (O'Sullivan *et al*., 2014) (O'Sullivan *et al*., 2017). Although it proved inconclusive for the 60 kDa target of mAb, some other bands were possibly pulled out on interpretation of the gels, however these observations would need to be confirmed in further IP studies. A second interesting approach to help identify the antigen would be to conduct a 2D gel electrophoresis followed by both Western blot and Coomassie staining. Instead of a protein band, a protein spot is obtained as the proteins from the starting material have been separated in two-dimensions. The Coomassie stained gel can be matched to the Western blot, and the protein stained spot of interest can be excised, trypsin digested, and the protein identified by Liquid chromatography–mass spectrometry (LC–MS).

While we demonstrated that mAb 4A5 has no effect on the proliferation of PANC-1 and PIN 127 cells, further functional studies can be conducted. The effect of mAb 4A5 could be studied in the mechanisms of cell invasion, migration or anoikis. These mechanisms have been looked into in many other cancers by our group, and the methodologies for such are well established within the NICB. Other PDAC cell lines and a wider selection of other cancer types could also be included. If any effects were observed, it would also be interesting to check if mAb 4A5 is internalised by the cells. If successful, internalisation of the 4A5

mAb antibody-antigen complex could be shown (and if the 4A5 target antigen can be revealed) the potential target antigen would represent a target that could be potentially targeted using an ADC.

To conclude this body of work, there is an urgent need for more PDAC targets to be identified. The hybridoma generation approach we used has the potential to be exploited for novel cancer target discovery.

Replacing animals and animal derived products in life science is a constant necessity, both in terms of data reliability and ethical concerns. The hybridoma technology remains the traditional way of discovering and producing monoclonal antibodies. Hybridomas are fragile cells and their formation is enhanced by the addition of growth supplement in their media. Our well-established supplement, BriClone™ has a great track record and has been steadily used by researchers worldwide for the last thirty years. Since BriClone™ contains traces of fetal bovine serum we proposed to design a serum-free, chemically defined hybridoma growing supplement. The resulting formulation, BriClone-SF, which is intended to help with the formation of freshly fused hybridoma colonies showed to be a robust and reliable product. It showed comparable to better performances in establishing colonies of hybridoma post-fusion than its parent product BriClone™ and two market available similar products. The stability of BriClone-SF has been demonstrated to be nine months without loss of performance. To make BriClone-SF even more attractive to the market, we would need to ensure this product is stable for a longer period. As such more stability timepoints, for example up to 18 months, which is the shelf-life of BriClone™ would need to be studied.

For its development, BriClone-SF was added at 5% v/v in selective HAT containing medium containing 10% FBS. To fully implement the replacement part of the 3Rs, it could be of interest to check if BriClone-SF supplement still performs as well in serum-free conditions, where the freshly-fused hybridomas are seeded in their selective medium containing 0% of FBS.

Thirdly, we looked at developing a quality control cell based assay for BriClone™ that would replace the current need to harvest the spleen of mice. Interleukin-6 being one of the active component of BriClone™, it was suggested that IL-6 independent cell lines would be a good model. Two myeloma cell lines, LOPRA-1 and 7-TD-1 were used for that purpose but were found to be too tricky to handle. Through the use of a mimic, it was observed that

the traces of FBS still present in BriClone™ were responsible for the growth of Sp2-1% cells. To conduct more research into establishing a reliable assay, we could look at using other IL-6 dependent cell lines. The INA-6 cells is another human myeloma established cell line described as being IL-6 dependent, and therefore could be purchased from the DSMZ and used for growth assays using BriClone™. Another cell line found to be requiring IL-6 for survival and proliferation is the mouse hybridoma cell line B9, which as per the supplier (ECACC) needs a constant 10 ng/mL intake of the cytokine and serves as a model for IL-6 based assay. In conclusion, no *in vitro* cell based assay has been developed to replace the fusion assay used as a standard in the NICB for the QC testing of BriClone™ to date. There is however, is plenty of scope for more research to find an adequate test.

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Annex I

NET buffer recipe

- To make 2L of 10X NET

Compound	Stock concentration	Volume (mL)	Final concentration
NaCl	5M	600	1.5M
EDTA	0.5M	200	0.05
Trs pH 7.8	1M	1000	0.5M
Triton X-100	100 %	10	0.5 %
UHP	100 %	190	
Total volume		2000	

5M NaCl: add 750 mL UHP to 219.15 g NaCl

0.5M EDTA: add 900 mL UHP to 186.12g EDTA (MW: 372.24 g/L) on stirrin hot plate. Add pellets of NaOH until EDTA dissolution and pH 8 reaches 8. Make up to 1000 mL.

1M Tris pH 7.8: weigh 127g Tris-HCl and 23.6g Tris Base and make up to 1000 mL with UHP. Ensure pH is 7.8.

- To make 20 L of 1X NET: add in order

1. 17.5 L of UHP in a suitable container
2. 2L of 10X NET
3. Dissolve 50g of gelatin in 0.5 mL of UHP in a 1L glass beaker in microwave. The gelatin should be clear and free of lumps or strands.
4. Add gelatin to 10X NET/ UHP