**Characterisation and Development of 2448 and C51: Novel Monoclonal
Antibodies Targeting Ovarian and Breast Cancer**

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Abbreviations

ANXA2	Annexin A2
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
Asn	Asparagine
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CA125	Cancer Antigen 125
Ca ²⁺	Calcium ion
CDC	Complement-dependent cytotoxicity
CDR	Complementary determining region
C _H	Constant domain, heavy chain
CHO	Chinese hamster ovary
C _L	Constant domain, light chain
CO ₂	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
DM1	Mertansine/emtansine
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EMT	Epithelial-Mesenchymal Transition
EOC	Epithelial ovarian carcinoma
EpCAM	Epithelial cell adhesion molecule
Fab	Fragment, antigen binding
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment, crystallisable
FcγIIIa	Fc gamma receptor IIIa
FcRn	Neonatal Fc receptor
FcγR	Fragment crystallized gamma receptor
FDA	(U.S.) Food and Drug Administration
FFPE	Formalin fixed paraffin embedded
FITC	Fluorescein isothiocyanate
Fuc	Fucose
g	Gravitational force
GlcNAC	<i>N</i> -Acetyl glucosamine
HER2	Human epidermal growth factor receptor-2
hESC	Human embryonic stem cell
HRP	Horseradish peroxidase
i.p.	Intraperitoneal

i.v.	Intravenous
IB	Immunoblot
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IP	Immunoprecipitation
K_d	Dissociation constant
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
M	Mutant
mAb	Monoclonal antibody
MMAE	Monomethyl auristatin E
MS	Mass spectrometry
NFAT	Nuclear factor of activated T cells
NaN_3	Sodium azide
NK	Natural killer
OS	Overall survival
pAb	Polyclonal antibody
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PD	Pharmacodynamics
PI	Propidium iodide
PK	Pharmacokinetics
PNGase F	Peptide-N-glycosidase F
PODXL	Podocalyxin
PVDF	Polyvinylidene fluoride
RIP	Ribosome inactivating protein
RNAi	RNA interference
RT	Room temperature
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
t	Time
THPA	The Human Protein Atlas
TGF- β 1	Transforming growth factor beta-1
TMA	Tissue microarray
TN	Tunicamycin
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
V_H	Variable domain, heavy chain
V_L	Variable domain, light chain
WT	Wildtype

Summary

Monoclonal antibodies (mAbs) represent an attractive approach in treating cancer. Even so, a limited number of antigens are available for targeted therapy and new targets are needed to address the diverse clinical spectrum of ovarian and breast cancer.

Oncofetal antigens are one category of tumour antigens. In order to discover cell surface oncofetal proteins, our group generated a panel of novel mAbs by immunising mice with live human embryonic stem cells (hESCs). From this panel, two mAbs, 2448 (IgG) and C51 (IgM) were found to not only recognize the surface of undifferentiated hESC but also a wide selection of human carcinomas.

The research presented in this thesis aims to demonstrate how these mAbs could form the basis of mAb or ADC therapy in ovarian and breast cancer. Antibodies were screened against a wide range of cancer cells and normal cell lines using flow cytometry. The screening data showed specific binding to various subsets of ovarian and breast cancer cell lines. Moreover, both demonstrated stronger reactivity to cells expressing a more epithelial phenotype along the epithelial-to-mesenchymal transition (EMT), a critical process in cancer progression.

Interestingly, both mAbs were also shown to react with a 38 kDa protein band by western blot analysis. Immunoprecipitation (IP) followed by mass spectrometry analysis, revealed the target antigen to be annexin A2 (ANXA2), a calcium-dependent phospholipid binding protein. Target identification was confirmed by cross-IP with a commercial anti-ANXA2 antibody and ANXA2-siRNA knockdown. Subsequently, enzymatic and chemical digestion of glycans revealed that binding of mAbs was N-glycosylation dependent

Functional assays also demonstrated that 2448 and C51 as good candidates for antibody or ADC cancer therapy. Antibody C51 but not 2448, exhibited direct cytotoxicity via oncosis and complement dependent cytotoxicity (CDC). As both 2448 and C51 were of murine origin, chimeric variants, ch2448 and chC51, were also evaluated and shown to elicit potent antibody-dependent cell-mediated cytotoxicity (ADCC) activity. Antibodies also internalized into

cancer cells and could potentially be used to deliver cytotoxic moieties into cancer cells as ADCs. *In vivo* activity of ch2448 was demonstrated using a mouse xenograft model. The ability of ch2448 to target subcutaneous ovarian cancer tumours was demonstrated by *in vivo* and *ex vivo* imaging. Fc-mediated anti-tumour activity was delayed tumour proliferation.

This study characterises and develops 2448 and C51 as novel and functional anti-tumour mAbs. Overall, the findings of this thesis can potentially form the basis of a new antibody or ADC therapy in ovarian and breast cancer.

Chapter 1 Introduction

1.1 Background

Ovarian and breast cancer remain two of the most widespread and deadly diseases afflicting women around the world. Conventional treatments do not take into account the wide molecular heterogeneity found in both cancers. As targeted therapies, monoclonal antibodies (mAbs) and small molecules take advantage of differences between cancer and normal cells. Both have been approved in the treatment of haematological cancers and solid tumours. Unlike small molecule inhibitors, mAbs exhibit important pharmacological properties as drugs. These include the ability to induce several clinically-validated mechanisms of action: direct tumour cell killing, targeting of the tumour microenvironment, Fc-mediated modulation of the host immune system and delivery of a cytotoxic payload as an antibody-drug conjugate (ADC) [1–8].

The safety and efficacy of mAb therapy greatly depends on its target antigen. Multiple types of antigens exist for cancer treatment. These include different transmembrane signalling receptors, soluble growth factors, cell surface glycoproteins, glycolipids and glycans. Critical considerations for the target antigen are the specificity and level of expression on cancer cells and absence or minimal expression on normal cells.

Various approaches are available to address both the discovery of new targets and generate mAbs against them [9]. One antibody-based discovery approach uses live cells as the immunogen [10]. This approach allows for the discovery of cell surface antigens and can be done without bias towards a particular antigen target. In contrast to using peptides or recombinant proteins, this strategy also preserves native non-linear and conformational epitopes. These include epitopes that are exposed on the cell surface or depend on post-translational modifications, in particular glycosylation, which is significantly present on a number of proteins [11, 12] and is closely associated with cancer progression [13–15].

In order to generate novel mAbs against oncofetal antigens, live human embryonic stem cells (hESCs) were previously used to immunize BALB/c mice and establish a panel of mAbs using hybridoma technology [16, 17]. Clones

were first selected based on a primary screen of hybridoma supernatant against hESC (HES-3) and a negative screen against fibroblasts (HFF and IMR90). Using this strategy, mAbs that targeted known oncofetal antigens such as epithelial cell adhesion molecule (EpCAM, CD326) and podocalyxin-like protein-1 (PODXL-1) were identified and reported [18, 19]. To identify new mAbs for potential use in cancer therapy, a secondary screen was then carried out with a panel of different cancer cell types.

Two mAbs, 2448 and C51 (an IgG₁ and an IgM respectively), were identified as having high-binding against a wide range of cancer indications, including ovarian and breast cancer. Minimal binding was observed on primary normal cells such as hepatocytes, red blood cells and peripheral mononuclear cells. Subsequently, 2448 and C51 were shortlisted for further *in vitro* and *in vivo* characterisation and development.

1.2 Thesis objective

The main objective of this thesis is to establish the therapeutic potential of novel mAbs, 2448 and C51, for the treatment of ovarian and breast cancers. This study can be subdivided into two parts: (1) Antibody characterisation and (2) Functional evaluation.

- (1) The first part will focus on the characterisation of the antigen targets. This involves screening of the mAbs against different cell lines to establish specificity, immunoprecipitation/mass spectrometry to identify the antigen target and siRNA knockdown to validate the antigen ID. The nature of the epitopes will then be investigated via a series of glycosylation assays. To evaluate more clinically-relevant mAbs, chimeric variants of 2448 and C51 were also characterised.
- (2) Next, a functional evaluation of the mAbs will be done *in vitro*, using cell-based assays and *in vivo*, using mouse xenograft models. Based on these studies, possible mechanisms of action for the mAbs are elucidated. A glycoengineered mAb variant and ADC will also be assessed for enhanced therapeutic efficacy.

1.3 Thesis organization

The thesis comprises of seven chapters:

Chapter 1 describes the background and objectives of the research.

Chapter 2 reviews the published literature, giving an overview of currently available antibody-based therapies for ovarian and breast cancers. Overall emphasis is given to the different antibody mechanisms of action, focusing mainly on ADCC activity and the delivery of cytotoxic payloads as ADCs. Sections on the role of the Epithelial to Mesenchymal Transition (EMT) in cancer and oncofetal antigens are also included because of their relevance to the study.

Chapter 3 details the materials and methods used in this research.

Chapter 4 describes the characterisation of (mouse) 2448, C51 and the respective chimeric variants ch2448 and chC51. Binding profiles to the surface target on various ovarian and breast cancer cell lines and the selectivity of mAbs to an EMT-status are detailed. Next, the identification of the antigen target is carried out. Both mAbs are determined to bind to annexin A2 (ANXA2). Subsequent investigation reveals that 2448 and C51 bind to unique N-glycosylation epitopes. Evaluation of chimeric (human/mouse) 2448 (ch2448) and C51 (chC51) was also done.

Chapter 5 is a functional study of the mAbs *in vitro*. C51 is shown to induce direct tumour-cell killing via oncosis and CDC activity. ADCC activity is described for ch2448 and chC51. A glycoengineered variant of ch2448, aF-ch2448, with enhanced ADCC activity is also reported. Finally, the use of the mAbs as ADCs is explored. Results from Chapter 5 suggest that ch2448 as an unconjugated mAb or as an ADC, is a promising candidate for therapy.

Chapter 6 documents the functional study of ch2448 *in vivo* using tumour xenograft mouse models. Data from human tumour xenografts demonstrates preclinical efficacy of ch2448 via an Fc-mediated MOA.

Chapter 7 summarizes the results and discusses possible future directions.

Chapter 2 Literature Review

2.1 Cancer

Cancer is the neoplastic transformation of cells to a state of abnormal (or uncontrolled) growth with potential to spread to surrounding tissue away from its site of origin. Intense research efforts have been devoted to the study of cancer which encompasses a wide spectrum of diseases, generally defined by the (tissue or organ) site of initiation. Although certain hallmarks of cancer have been identified (as reviewed in [20]), cancer is a heterogeneous disease, comprising of clinically and molecularly diverse subtypes, various genetic alterations, different morphological characteristics and differences in drug sensitivity and clinical outcome, extending not only between different tumours, but even within tumours of the same origin (intra-tumour heterogeneity) [21–25].

Cancer is a leading cause of death worldwide and is expected to increase in the future [26–28]. An estimated 14.1 million new cases are diagnosed every year with an annual estimate of 8.2 million deaths worldwide [27]. Ovarian and breast cancer are two of the most frequently diagnosed cancers in women (Table 2.1).

Table 2.1 Top cancers among women

Rank	Origin	Estimated number of cases
1	Breast	1,671,149
2	Colorectum	614,304
3	Lung, bronchus and trachea	583,100
4	Cervix uteri	527,624
5	Stomach	320,301
6	Corpus uteri**	319,605
7	Ovary	238,714
8	Thyroid	229,923
9	Liver	228,082
10	Non-Hodgkin lymphoma	168,098
11	Pancreas	159,711
12	Leukemia	151,289

**endometrial cancer

Data from: <http://globocan.iarc.fr> [27]

2.1.2 Ovarian cancer

Ovarian cancer is the 7th most common (239,000 estimated cases, 3.6% of female cancer cases) and the 5th most deadly cancer among women (152,000 estimated deaths, 4.3% female cancer deaths), making it the most deadly gynaecological malignancy [27, 29–31]. Epithelial ovarian carcinoma (EOC), which accounts for 90% of all malignant ovarian tumour neoplasms, can be further divided into several histological subtypes (serous, endometrioid, clear cell, mucinous and undifferentiated). Each subtype has a unique gene expression profile and responsiveness to therapy. High-grade serous carcinoma is the most common subtype. It accounts for an estimated 70% of all ovarian carcinomas and a majority of all ovarian cancer deaths [32].

The poor mortality rates of ovarian cancer can be partially attributed to late presentation of the disease. Only 15% of all cases are diagnosed at an early stage [30, 33]. The five-year survival rates of EOC patients dramatically fall from 90% for localized disease, to < 30% after metastasis has occurred [30]. EOC presents vague symptoms (e.g. abdominal or pelvic pain, persistent bloating, abnormal satiety) that are not typically evident in early stages of the disease [34]. It is often overlooked due to the similarities in symptoms with other gynaecological diseases, leading to late stage diagnosis for a majority of cases. Additionally, there is an incomplete understanding of the origin and pathogenesis of ovarian cancers [35]. The most commonly-used biomarker for ovarian cancer is CA-125 or cancer antigen 125 [36], a membrane associated mucin that is elevated in ovarian cancer patients. Due to its lack of specificity and sensitivity, CA-125 is used more frequently to monitor cancer progression than as a reliable diagnostic marker of EOC [36].

The standard treatment of care for EOC is maximal cytoreductive surgery followed by taxane or platinum-based chemotherapy. Ovarian cancer patients typically respond to initial treatment (70% overall response rate and 50% after 5 months) [37, 38] however, relapse with progressive or recurrent disease within 3 years is common (75%). Advanced stages of EOC (stage III or IV) typically involve the deadly spread of cancer to the peritoneal cavity and the production of malignant ascites, eventually leading to death [33].

Given the poor mortality rates of ovarian cancer, translational research is needed for the development of better diagnostic tests and treatments [39, 40].

2.1.3 Breast cancer

Breast cancer is the most frequently diagnosed cancer among women (1.7 million estimated cases, 25.2% of female cancer cases) and remains a deadly disease (522,000 estimated deaths, 14.7% of all female cancer deaths) [27]. Like ovarian cancer, breast cancer is a heterogeneous diseases that encompasses different molecular subtypes and responsiveness to therapies [41]. Most breast cancers (95%) are adenocarcinomas, arising from cells lining the ducts or lobes of the mammary gland. In addition to morphological studies, classifications of breast cancers combine molecular profiling and gene expression patterns [42–44]. For clinical treatment, tumours are often defined by the expression of the oestrogen receptor (ER-positive), the progesterone receptor (PR-positive), and the overexpression of the epidermal-growth-factor receptor (HER2-positive). When tumours express none of these, the breast cancer is classified as triple-negative breast cancer (TNBC). Additional gene studies have further delineated breast cancers as luminal A, luminal B, basal and normal-like.

Various treatment options exist for breast cancer. Surgery (such as mastectomy, lumpectomy or quadrantectomy) is often combined with chemotherapy and radiotherapy. Other treatments depend on the molecular subtype being treated. For example, hormone-antagonist therapies are used to treat ER-positive and/or progesterone-positive subtypes. Moreover, HER2-positive breast cancers are treated by targeted approaches (which will be further discussed later in the chapter) [45].

The general prognosis of breast cancer has greatly improved in the past decades. The current overall five-year survival rate is approximately 90% [30]. Notably, increased awareness and technological advances like mammography have resulted in diagnoses at an earlier stage, when treatment interventions are more successful [46, 47].

Nevertheless, limited treatment options exist for certain subtypes and metastatic disease. TNBC has been a particular focus for drug discovery

research [48, 49]. TNBC accounts for an estimated 20% of all breast cancers and has a five-year survival rate of 77%. Recurrent disease (locally, regionally and/or at distant sites) also remains difficult to treat. Cancer cells often become multi-resistant towards different cytotoxic agents and an estimated 20% of TNBC patients relapse with distant metastasis. Reported five-year survival rates for distant metastatic disease is approximately 25% [30].

2.1.4 Role of the epithelial-mesenchymal transition in cancer

The majority of cancer-related death arises from complications during tumour progression and metastasis [50–52]. During these processes, phenotypic changes allow cancer to spread away from the primary site of origin and into surrounding or distant tissues. Therefore, cancer involves not only cell growth (in terms of numbers), but also the conversion of cells into different shapes, sizes and arrangements (i.e. cell organization). The epithelial-mesenchymal transition (EMT) has been proposed as a critical cellular program in this process.

EMT was first studied in embryonic development (Type 1 EMT) but similar processes were later identified in wound healing, tissue regeneration, and organ fibrosis (Type 2 EMT). In cancer, (Type 3) EMT and MET are associated with invasion and metastasis. Studies show that EMT may also confer cancer cells with stem cell characteristics [53, 54] and promote immune invasion and drug resistance [55, 56].

EMT and its reverse process, the mesenchymal to epithelial transition (MET), allow cells to partially or fully gain or lose epithelial and mesenchymal characteristics. This dynamic process involves drastic morphological and phenotypic changes during which epithelial cells lose their apical basal polarity and cell-cell adhesion, gaining a more migratory phenotype [57, 58] (Figure 2.1). Cancer cells undergoing EMT change from having polygonal columnar morphology to more spindle-shaped morphology with greater migratory potential. Hallmarks include the loss of functional E-cadherin, loss of expression of adhesion proteins (such as claudins and occludins) and the upregulation of fibronectin and vimentin. Other notable changes include the expression of specific transcription factors and microRNAs, the reorganization

of cytoskeletal proteins and the production of enzymes that can degrade the ECM. Moreover, EMT involves common cancer-signalling pathways such as the TGF- β , Notch and Wnt signalling pathways and has been extensively studied at the transcriptional, post-transcriptional, translational and post-translational levels [59, 60].

Although EMT has typically been observed using *in vitro* cell models, increasing evidence from *in vivo* data and clinical samples suggest its relevance in treating cancer [61–63]. Multiple reviews have described the distinct and important roles of EMT (and MET) in ovarian [64–66] and breast cancer [65, 67–69] (Figure 2.1).

Figure 2.1 The Epithelial-Mesenchymal Transition in ovarian and breast cancer. (A) The Epithelial-Mesenchymal Transition (EMT) and its reverse process, the mesenchymal to epithelial transition (MET), enable cells to express different properties with epithelial characteristics on one end of the spectrum and mesenchymal characteristics on the other. Epithelial cells have apical basal polarity and express proteins such as E-cadherin and other adhesion and junction proteins like claudin, zonula occludens-1, cytokeratin and desmoplakin. After undergoing EMT, cells have a more mesenchymal phenotype with elongated and fibroblastic-like morphology, making the cells more motile and invasive. Mesenchymal markers include N-cadherin, vimentin and fibronectin and FSP-1 (fibroblast specific protein 1). The EMT process is regulated by several transcriptional factors including SNAIL 1 and 2, ZEB 1 and 2 and Twist 1. (B,C) EMT and MET play distinct roles in cancer progression and metastasis. (B) In ovarian cancer, EMT and MET are involved in the dissemination of tumour cells in the peritoneal cavity, where multiple rounds of partial or full EMT and MET lead to the formation of spheroids and the establishment of secondary tumours. (C) In breast cancer, EMT and MET are closely associated with metastasis. After undergoing EMT, cells extravasate into the lymphatic or circulatory system. MET then allows cells to establish macrometastasis at distant sites. Image was licensed from Elsevier (reference number #4100690589127) and modified from [70].

New cancer strategies are incorporating knowledge about EMT to better target cancer according to its different EMT phenotypes. Recent studies suggest the possibility of using a universal EMT-signature to characterize carcinomas [56, 71]. Appropriate EMT-staging can also provide insights into the prognosis and

possibility of cancer recurrence. Attempts are being made to stratify patients according to EMT status [72] and various clinical markers of EMT have been studied, including adhesion proteins (e.g. E-cadherin, CD44), regulatory miRNAs (e.g. miR-200, miR-34) and transcription factors (e.g. Twist, Snail and Slug) [73]. These markers of EMT are also being explored as therapeutic targets in cancer (as reviewed in [65, 70, 74]).

2.1.5 Treatment options

2.1.5.1 Conventional Therapy

Conventional cancer therapeutics heavily relies on radiotherapy, which treats localized regions of cancer, and chemotherapy, which is administered systemically throughout the body. Both are often used in parallel and have harmful side effects to patients. Traditional chemotherapies are cytotoxic compounds mainly targeting DNA synthesis and regulatory events related to cell division. These drugs target and kill rapidly dividing cells such as cancer cells, however, they also have dose-limiting toxicities to various organs, killing normal cells especially those that also rapidly divide, including hair follicles, bone marrow cells and cells found in the intestinal epithelium, mouth and reproductive system [75].

2.1.5.2 Targeted Therapy

In hopes of improving the therapeutic index of cancer drugs and the overall survival of cancer patients, targeted therapy incorporates more molecular and genetic information of a disease into treatment strategies [76]. In oncology, two main types of drugs are used in targeted cancer therapy: small molecule inhibitors and monoclonal antibodies (mAbs) [77]. To specifically target and kill cancer, these drugs take advantage of differences found between cells in a normal and disease state.

Small molecule inhibitors are low molecular weight organic compounds (< 1 kDa) that can specifically regulate biological processes [78, 79]. The most common types of small molecules are proteasome inhibitors or kinase inhibitors. The classic example is Imatinib, commonly known as Gleevec, an

inhibitor of BCR-ABL tyrosine kinases in multiple cancers, most notably chronic myelogenous leukaemia [80–83]. Tyrosine kinase inhibitors like Imatinib typically compete with adenosine triphosphate (ATP) binding in order to inhibit enzymatic activity [79]. Another kinase inhibitor, Lapatinib (Tyverb), targets EGFR and HER2 and is used in combination with chemotherapy or hormone therapy for breast cancer treatment. More recently, poly-ADP ribose polymerase (PARP) inhibitors are now emerging as important small molecule inhibitor for BRCA1 and BRCA2 –related ovarian and breast cancers. Olaparib (Lynparza) is the first PARP inhibitor that has been approved for use as a single agent in the treatment of ovarian cancer. Other small molecules are currently being investigated in clinical trials.

In contrast to small molecules, antibodies are relatively larger biomolecules (> 100 kDa) and biologically more complex. Antibodies, or immunoglobulins (Ig), are glycoproteins that are generally composed of 82–96% protein and 4–18% carbohydrate [84]. These biomolecules are normally produced by B-cells, forming part of the adaptive immune response against foreign substances (antigens). Monoclonal antibodies (mAbs) are antibodies typically from a single clone or cell line [85]. Unlike polyclonal antibodies (pAbs), mAbs are homogeneous and specifically recognize a single epitope on an antigen target. This specificity makes mAbs attractive for use in research and medical applications including autoimmune diseases and heart disease. As cancer therapeutics, mAbs can specifically bind to tumour antigens and exert various mechanisms of action (as discussed later in section 2.2.4).

Both small molecules and mAbs are promising types of targeted therapies with their own advantages [77]. For example, most small molecules are taken orally unlike mAbs, mAbs which are generally administered intravenously. Small molecules are also able to target binding pockets inaccessible to mAbs. These include those outside the blood stream. In particular, small molecules can more easily interact with intracellular targets, including the cytosolic domain of transmembrane receptors. Conversely, mAbs generally act on molecules that are expressed on the cell surface or are secreted.

Antibodies have their own advantages as drugs. Due in part to its larger size and interactions with Fc receptors (e.g. FcRn), mAbs have longer circulation

half-lives in the body (days to weeks) [86]. Small molecules tend to be less specific than mAbs with a greater range of side effects [87]. Both small molecules and mAbs can target haematological cancers and solid tumours, sometimes even targeting the same growth factor or receptor (e.g. EGFR, HER2 and VEGF). Better clinical outcomes were observed in treatments with mAbs compared to those with only small molecule inhibitors [87]. Traditionally, (chimeric and humanized) mAbs have also had better approval rates than small molecules [88, 89]. The conserved structure of a mAb as well as other various factors may contribute to their overall more favourable safety and efficacy profiles.

Commercial considerations also play a significant role in the attractiveness of mAbs. Therapeutic mAbs can be favoured by biotechnology and pharmaceutical companies because it remains the top-selling biologic in a billion dollar industry [90, 91]. Production of mAbs can entail significantly high economic cost but, large-scale commercial production processes have been established and costs are expected to go down in the future [92]. High barriers to entry and intellectual property further contribute to the marketability of mAbs.

2.2 Antibody-based Cancer Therapy

2.2.1 Historical development of therapeutic mAbs

In the past thirty years, mAbs have become an increasingly important part of targeted cancer treatment [1–8]. The shift in interest and funding towards antibody-based cancer therapy is correlated with a dramatic increase in the number of publications related to the field (Figure 2.2).

Figure 2.2 The growth of antibody-based cancer therapy research. Antibody-based cancer therapy is a growing field of interest. In the past thirty years, research publications in the field have dramatically increased. The graph was made based on search results in PubMed using the keywords “antibody”, “cancer” and “therapy”.

The evolution of antibody therapeutics (Figure 2.3) can be traced back to serum therapy of the 1890s, which was used to treat diphtheria and tetanus [93–95]. These therapies however suffered greatly due to the lack of efficacy and consistent production. Around the same time, the idea of targeted treatment was first conceptualized in Paul Ehrlich’s “magic bullet” hypothesis, which suggested that drugs such as “antibodies” could be used to selectively target diseases with high potency and minimal off-target toxicity [96]. The concept was later expanded to include targeted cancer therapy.

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Figure 2.3 Milestones in antibody-based cancer therapy. Technological advances and important discoveries are depicted (in shaded boxes) on a timeline, highlighting the development of therapeutic antibodies (Abs). The major advance of hybridoma technology in 1975 resulted in the use of monoclonal antibodies (mAbs) as drugs. Humanisation technologies in the 1980s and 1990s eventually led to the clinical use of chimeric, humanized and fully-human antibodies. In the past 20 years, various mAbs against cancer have been approved. Below the timeline, selected mAbs and antibody drug conjugates (ADCs) against cancer are also shown according to the year of marketing approval in the US or EU or Japan. Recently approved mAbs include ADCs, a glycoengineered mAb and a bispecific mAb, representing the diversity of available antibody-based cancer therapies. Note: *, Fab fragment with IgG₁ isotype; **, Approval by the European Medicines Agency (EMA); ***, Approval by the Ministry of Health, Labour and Welfare (MHLW) of Japan.

The feasibility of antibody-based therapy greatly improved with hybridoma technology [85, 96]. The ability to fuse a single B-cell clone and a multiple myeloma cell allowed for the isolation and sufficient production of mAbs (from a single clonal pool) for use in laboratory research and clinical therapy. Muromonab-CD3 (OKT3), a mouse IgG_{2a}, was the first mAb to be approved for clinical use as an immunosuppressant for transplant recipients [97]. Early mAbs, like OKT3, were of rodent origin and suffered from low efficacy due partly to immunogenicity and the rapid clearance from the body when given as a drug [98].

Next, humanisation technologies further improved the prospects of mAb-therapy. Advances in antibody engineering allowed for the incorporation of human sequence elements into mouse-generated mAbs [99, 100], leading to the creation of chimeric or humanized mAbs with reduced immunogenicity [101, 102]. Fully-human mAbs were also developed by two different technologies: phage display [103] and transgenic mice [104–106]. Significant resources are currently being devoted to advancing new antibody technologies [88, 90, 107–109].

In general, antibody-based therapies are well-tolerated in cancer patients today. They have less serious side effects compared to murine mAbs of the past and compared to systemic chemotherapy [110, 111]. Common side effects include less severe infusion reactions or allergic reactions or side effects that are closely associated with the specific antigen target (e.g. cardiotoxicity with anti-HER mAbs [112] and dermatitis with anti-EGFR mAbs [113])

As of today, there are over twenty mAbs approved by the FDA for the treatment of haematological or solid tumour malignancies (Table 2.2) [6, 114, 115]. These mAbs are used as single agents or in combination with other conventional drugs. Examples include rituximab and trastuzumab, two blockbuster drugs which have been integrated into the modality of care for non-Hodgkin lymphoma and HER2-positive breast cancer. Continued growth is expected in the near future. In 2015, fifty-three mAbs were evaluated in Phase III clinical trials [116]. Seventeen of which were in the cancer field.

Table 2.2 FDA-approved monoclonal antibodies for cancer therapy

Year †	Antibody (Trade Name)	Subclass	Indication	Target
1997	Rituximab (Rituxan/Mabthera)	IgG ₁	CLL, NHL	CD20
1998	Trastuzumab (Herceptin)	IgG ₁	breast, gastric	ERBB-2
2000	Gemtuzumab ozogamicin (Mylotarg)*	IgG ₄	AML*	CD30
2001	Alemtuzumab (Campath)	IgG ₁	CLL	CD52
2002	Ibritumomab tiuxetan (Zevalin)	IgG ₁	NHL	CD20
2003	Tositumomab (Bexxar)*	IgG _{2a}	NHL*	CD20
2004	Bevacizumab (Avastin)	IgG ₁	breast*, colorectal, cervical, glioblastoma, kidney, lung, ovarian	VEGF
2004	Cetuximab (Erbix)	IgG ₁	colorectal, head, neck	EGFR
2006	Panitumumab (Vectibix)	IgG ₂	colorectal	EGFR
2009	Ofatumumab (Arzerra)	IgG ₁	CLL	CD20
2011	Brentuximab vedotin (Adcetris)	IgG ₁	HL	CD30
2011	Ipilimumab (Yervoy)	IgG ₁	melanoma	CTLA4
2012	Pertuzumab (Perjeta/Omnitarg)	IgG ₁	breast	ERBB-2
2013	Ado-trastuzumab emtansine (Kadcyla)	IgG ₁	breast	ERBB-2
2013	Obinutuzumab (Gazyva)	IgG ₁	CLL, NHL	CD20
2014	Ramucirumab (Cyramza)	IgG ₁	colorectal, gastric, lung	VEGFR2
2014	Pembrolizumab (Keytruda)	IgG ₄	head and neck, melanoma, lung	PD-1
2014	Nivolumab (Opdivo)	IgG ₄	kidney, HL, melanoma, lung	PD-1
2014	Blinatumomab (Blinicyto)	NA [#]	ALL	CD3, CD19 [#]
2015	Dinutuximab (Unituxin)	IgG ₁	neuroblastoma	GD2
2015	Daratumumab (Darzalex)	IgG ₁	multiple myeloma	CD38
2015	Necitumumab (Portrazza)	IgG ₁	lung	EGFR
2015	Elotuzumab (Empliciti)	IgG ₁	multiple myeloma	SLAMF7
2016	Atezolizumab (Tecentriq)	IgG ₁	bladder, lung	PD-L1
2016	Olaratumab (Lartruvo)	IgG ₁	STS	PDGFR α

†year of approval for first indication, *withdrawal from market, [#]bispecific T-cell engager (BiTE) composed of two scFvs. NA = not applicable, FDA = US Food and Drug Administration, AML = acute myeloid leukaemia, CLL = chronic lymphocytic leukaemia, HL = Hodgkin's lymphoma, NHL = Non-Hodgkin's lymphoma, STS = soft tissue sarcoma.

2.2.2 Antibody structure

2.2.2.1 Immunoglobulin G

The IgG (Immunoglobulin G) is the predominant antibody isotype for cancer therapies (Table 2.2). As the most abundant isotype found in normal human plasma, IgGs, (excluding IgG3s [117, 118]) tend to have longer serum half-lives (days to weeks) than other human antibody isotypes (IgA, IgD, IgE, IgM [84]). In addition, the IgG₁ subclass has other therapeutically-relevant features like higher affinity, better specificity and more favourable interactions with Fc receptors. These include interactions with FcRn for improved pharmacokinetic properties and interactions with Fc gamma-receptor IIIa, for the ability to induce more potent effector functions [119]. Other subclasses, however, have also been specifically used to purposefully eliminate these interactions. For example, two approved mAbs targeting antigens on normal T-cells are IgG₄s, which do not elicit ADCC or CDC activity [120]. Furthermore, isotypes such as IgM, IgA [121, 122] as well as non-human isotypes are being explored for unique properties. So far, none has been commercially approved due in part to issues with low affinity, poor tissue penetration and manufacturing challenges. At least for the near future, IgG₁ is likely to remain the preferred format for cancer therapy,

The therapeutic advantages of a mAb can be directly linked to its modular domain structure. The general chemical structure of IgGs was first elucidated in structural and functional studies in the early 1960s (Figure 2.3). As depicted in Figure 2.4, a human IgG comprises of two heavy (H) chains (γ : 1, 2, 3 or 4) and two light (L) chains (either κ or λ : 1, 2, 3 or 4). Each polypeptide chain contains an NH₂-terminal variable (V) domain and one or more COOH-terminal constant (C) domains. Each domain is approximately 110-130 amino acids (12-13 kDa). The two H chains connect at the hinge region (between CH₁ and CH₂) by a variable number of disulphide bonds depending on its subclass: 2 for IgG₁ and IgG₄, 4 for IgG₂ and 11 for IgG₃. The H and L chains are approximately 50 kDa and 25 kDa, respectively. An IgG can also be broken down into fragments based on their functional properties: the antigen binding fragment (Fab) and the constant, crystallisable fragment (Fc). Binding

of the antibody to its antigen is determined by the complementary determining regions (CDRs) located in hypervariable loops in the Fab-region.

Figure 2.4 Schematic representation of an IgG1 structure. An IgG is a glycoprotein composed of 2 heavy (H) chains and 2 light (L) polypeptide chain which are held together by disulphide bonds. For an IgG₁, two disulphide bonds link the two H chains at the hinge region and another disulphide bond (between the last cysteine residue of the L chain and the fifth cysteine residue of the H chain) links H and L chain. An IgG is often described as a “Y”-shaped structure of two Fab fragments, the “arms” and an Fc fragment, the “trunk”. The Fab fragments contain the variable (V) regions which bind to an epitope on a target antigen. The 6 non-consecutively arranged CDRs of each Fab determine the binding specificity. The Fc fragment is made up of constant (C) regions where the binding sites for Fc receptors are located. Each Fab fragment consists of four domains (C_{H1}, C_L, V_H, and V_L). The Fc region consists of two constant (C) domains (C_{H2}, C_{H3}). Note: CDR, Complementarity determining region; C_H, Constant heavy chain; C_L, Constant light chain; Fab, fragment antigen-binding; Fc, fragment crystallisable region; V_H, Constant light chain; V_L, Variable light chain.

2.2.2.2 Other antibody formats

Although not the focus of this review, antibody-based formats, such as fragments and non-conventional constructs, are also currently being developed in hopes of improving therapeutic efficacy [123–126]. Alternative

formats can provide better tumour penetration or confer additional multi-specific or multivalent targeting properties.

An important type of antibody-based format is the bispecific mAb [127–131]. A typical therapeutic IgG₁ is bivalent but monospecific, with binding of each Fab arm to the same epitope. Bispecific mAbs bind two different epitopes of the same antigen or of two different antigens. This ability can efficiently inhibit downstream signalling process of targets on the same tumour cell or help redirect immune effector cells to tumour cells. The recent approvals of two bispecific mAbs, blinatumomab and catumaxomab, [116, 132–134] highlights the success and ongoing development of bispecific mAbs (as extensively reviewed in [127, 135–137]).

2.2.3 Tumour antigens

2.2.3.1 Finding the ideal antigen target

In general, the ideal tumour antigen is easily accessible, abundant and homogenously expressed over time on tumours with minimal expression on normal tissues, particularly those of vital organs. Most tumour antigens are membrane-bound and found on the cell surface. For these antigens, a secondary consideration is the expression of soluble antigen in the blood stream, which could function as a sink, diverting a mAb from its intended tumour target.

Antibodies targeting CD20, EGFR or HER2 account for eleven of the FDA-approved mAbs used in cancer therapy. Whilst a number of more recently-approved mAbs target checkpoint inhibitors that are widely expressed on T-cells, most mAbs targeting tumour antigens are only therapeutically relevant for a certain subset of cancers. The discovery of new antigen targets remains an important part of antibody drug discovery and development.

2.2.3.2 Oncofetal antigens

Some tumour-associated antigens are also expressed in non-cancerous cells during particular stages of differentiation and development [138–140]. For example, early stem cell surface markers like TRA-1-60 and the stage specific

embryonic antigen SSEA-3 were originally raised against embryonal carcinoma cells [141]. Antigens associated with fetal development and tumour tissue are classified as oncofetal antigens.

The first oncofetal antigens were discovered in the 1960s, including alpha-fetoprotein (AFP) which is found in hepatocellular cancer and germ cell tumours [142] and carcinoembryonic antigen (CEA), which is primarily associated with colorectal cancer [143]. Examples that were later discovered include sialylated Lewis A antigen (CA19-9) for pancreatic cancer and Mucin 16 (CA-125) for ovarian cancer [144, 145].

Oncofetal antigens are closely associated with cancers of epithelial cells (carcinomas). These antigens can appear in multiple cancer indications. For example, CEA is not only associated with colorectal cancer but also breast, gastric, lung and pancreatic cancers [146, 147]. Other oncofetal antigens, however, are more closely associated with a few cancer indications. For example, glypican-3 is upregulated in liver cancer but downregulated in lung and gastric cancer [148, 149].

Furthermore, post-translational modifications (PTMs) such as glycosylation have been reported as important features of oncofetal antigens (Table 2.3). As an additional layer of detail, glycosylation can be exploited to potentially improve the specificity of an antigen as a biomarker. For example, one of the four glycoforms of alpha-fetoprotein (AFP), can be specifically targeted as a more relevant biomarker [150–152]. In the case of the oncofetal Thomsen-Friedenreich (TF) tumour antigen, the antigen itself is defined as a glycan epitope that is present on multiple proteins [153]. Examples of oncofetal antigens and their post-translational features are shown below in Table 2.3.

Oncofetal antigens encompass many of the commonly-used serum biomarkers for cancer and are frequently used to monitor disease progression and to a lesser extent, as a diagnostic or prognostic marker. More recently, oncofetal antigens have also been proposed as promising targets for cancer vaccines [139] and therapies. Oncofetal antigens that have recently been explored as targets for mAb-based therapy include CEA [154, 155], CA125 [156], EpCAM [157], iLRP [158] and ROR1 [159, 160].

Table 2.3 Examples of oncofetal antigens and post-translational properties.

Oncofetal antigen	Interesting post-translational properties	References
Alpha-1-fetoprotein (AFP)	Tumour-specific glycoforms of AFP (fucosylated AFP-L3) may improve specificity of AFP as a diagnostic biomarker for hepatocellular cancer.	[151]
Carcinoembryonic antigen (CEA)	CEA attaches to the tumour cell-surface by a glycosylphosphatidylinositol (GPI) anchor and is highly glycosylated, with N-glycans making up 50-60% of the molecular mass.	[146]
Glypican 3 (GPC3)	GPC3 attaches to the tumour cell-surface by a glycosylphosphatidylinositol (GPI) anchor and belongs to the heparin sulfate proteoglycan family.	[149]
Immature Laminin receptor protein (iLRP)	After acetylation, iLRP (37 kDa) dimerizes to generate the mature 67 kDa form.	[158]
Oncofetal fibronectin (onfFN)	The addition of an O-glycan (GalNAc α 1-O-Ser/Thr or Gal β 1-3GalNAc α 1-O-Ser/Thr) on normal fibronectin defines onfFN.	[161]
Podocalyxin (PODXL)	PODXL is a heavily glycosylated type-1 transmembrane sialomucin.	[162, 163]
Receptor tyrosine kinase-like orphan receptor 1 (ROR1)	ROR1 localization and signalling is regulated by N-glycosylation and mono-ubiquitination.	[164]
Trophoblast glycoprotein (TPBG/5T4)	The conformational epitope of 5T4 depends on N-glycosylation (and the presence of intact intramolecular disulphide bonds).	[165]
Thomsen–Friedenreich disaccharide (T or TF antigen)	The TF antigen is a glycan (Gal β 1–3GalNAc) antigen that is expressed on splice variants of the cell-surface adhesion molecule, CD44 and the transmembrane mucin protein, MUC1.	[153]

2.2.4 Antibody mechanisms of action

Antibodies have several clinically-validated mechanisms of action (MOA) for tumour cell killing: direct tumour cell killing, modulation of the immune system, targeting of the tumour microenvironment and delivery of a cytotoxic agent (Figure 2.5). Since most mAbs combine different mechanisms of action, determining the relative contribution of each mechanism can be challenging, especially in a clinical setting, where mAbs are administered in combination with chemotherapy and radiotherapy. A complete understanding of their mechanisms can be difficult to obtain even for mAbs that have successfully been used in the clinic for years. This review will focus on ADCC and ADC because of their particular relevance to this study. Table 2.4 summarizes

reported mechanisms of action for various clinically approved mAbs and the primary literature supporting these findings.

Figure 2.5 Antibody mechanisms of action. There are several known mechanisms of action that have been validated in preclinical and clinical studies. These include direct tumour cell-killing, immune-mediated tumour cell killing, alteration of the tumour microenvironment and the (payload) delivery of a drug or cytotoxic agent. NK = natural killer ADCC = antibody-dependent cell-mediated cytotoxicity, CDC = complement dependent cytotoxicity, ADCP = antibody-dependent cellular phagocytosis, VEGF = vascular endothelial growth factor, ADC = antibody drug conjugate.

2.2.4.1 Direct tumour cell killing

A mAb can directly induce cell killing by acting as a receptor agonist or antagonist of downstream signalling pathways. Abrogation of signalling can involve directly blocking ligand-receptor interaction or the down-modulation of receptors on the cell surface. Disruption of growth factor receptor signalling has been validated for various mAbs targeting different antigen targets for example HER2, EGFR and more recently in preclinical development, the insulin-like growth factor receptor, IGF-2R.

Another example of direct tumour cell killing is oncosis. This mode of action uses a non-apoptotic pathway to induce homotypic cell-adhesion and pore formation upon antibody binding [166, 167]. Antibody-mediated oncosis has been studied in our group [17] and has been described for various mAbs, particularly mAbs targeting glycan antigens [168–170]. Oncosis has not been identified as the main mechanisms of action for clinical mAbs. However, a similar ROS-mediated cell –death has been described for second generation anti-CD20 antibodies, including the recently approved obinituzumab [171]. The epitope and orientation of antibody-antigen interaction was shown as critical for this mechanism of action [171].

2.2.4.2 Targeting the tumour microenvironment

Antibodies can also be used to target antigens in the stroma and vasculature of the tumour microenvironment [172–175]. These mAbs can involve the inhibition of angiogenesis or vascularization, two important processes for tumour growth and metastasis [176, 177]. Angiogenesis is a process in which tumours develop new vasculature by mediation of pro-angiogenic and anti-angiogenic factors (e.g. vascular endothelial growth factor (VEGF)[172], fibroblast growth factor [178], and the platelet-derived growth factor [179]). The inhibition of angiogenesis can deprive tumours from essential nutrients and oxygen, thus inhibiting cell growth beyond a certain size (typically, 1-2 mm³) [180, 181].

Indirectly targeting tumour cells allow mAbs to take advantage of the more genetically stable tumour vasculature and cancer-related stroma surrounding the tumour. This approach increases the possibility of targets that are commonly found in multiple cancer indications. For example, the anti-VEGF mAb, bevacizumab, was approved in combination with chemotherapy for a wide variety of cancers including colorectal, kidney, breast, and ovarian cancer. Bevacizumab targets and sequesters the soluble growth factor VEGF, blocking interaction with its receptors, VEGFR-1 and VEGFR-2 [172, 177, 182–184]. Recent attempts have been made to target other cancer-related proteins in the extracellular matrix [185].

2.2.4.3 Modulation of the host immune system

Antibody therapy can also modulate the host innate or adaptive immune system, often through Fc-mediated interactions. Preclinical and clinical studies have demonstrated the importance of complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and the regulation of T-cell activity for approved mAbs in cancer therapy

CDC results from activation of a complement cascade, comprising of proteolytic enzymes, cofactors and regulatory proteins [186–188]. Antibodies involve the classical pathway for complement activation. Here, the IgM or IgG form immune complexes with its target antigen, allowing it to act as a scaffold for various complement proteins. The first step involves C1q binding to the Fc region. This interaction triggers a complement cascade leading first to the formation of C4b2a (C3 convertase) and the Membrane Attack Complex (MAC) which can lyse cell membranes. Mutagenesis and structural studies have revealed important binding sites for C1q and CDC activity [189–192]. In the field of antibody-based cancer therapy, the role of CDC has been extensively studied with anti-CD20 mAbs (eg. rituximab and ofatumumab), however, the extent of its role in overall clinical efficacy remains controversial [193, 194].

Alternatively, ADCC activity has been recognized as the primary mechanism of action of mAbs. ADCC (and antibody-dependent cellular phagocytosis, ADCP) activity involves a mAb acting as a bridge between the target tumour cells and various immune effector cells. The Fc region of a mAb recognises activating and inhibitory Fc-gamma receptors that are expressed on variety of natural killer cells, granulocytes and macrophages. Crosslinking of activating Fc-gamma receptors triggers the release of cytotoxic granules such as perforin and granzymes [195], leading to tumour cell lysis. These activities are heavily influenced by the interactions between the Fc and the Fc receptors, depending on antibody isotype, Fc sequence and Fc glycosylation [196].

The significance of ADCC activity has been demonstrated in several clinically approved mAbs, including rituximab and trastuzumab. Studies have shown that immunostimulatory agents (e.g. IL-2) increase the formation of ADCC

synapses present in trastuzumab-treated tumours [197, 198]. Furthermore, mice lacking activating (or inhibitory) Fc receptors showed less (or more) effective ADCC-induced lysis of tumour cells upon treatment with trastuzumab or rituximab [199].

Clinical evidence involves correlative studies in patients. Natural polymorphisms on the activating receptor Fc gamma-receptor IIIA (CD16) were found to correlate with clinical response of mAb treatment [200]. Effector cells carrying the homozygous V/V 158 polymorphisms had higher affinity to mAbs than those with the heterozygous V/F or homozygous F/F 158 polymorphisms. Patients who were homozygous for the higher affinity alleles had more favourable clinical outcomes, suggesting that ADCC activity was an important mechanism of action [201–204].

More recently, mAbs have also shown promise in modulating the adaptive immune system [205]. The clinical success of mAbs in activating T-cells was shown by targeting immune checkpoint inhibitors such as the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and Programmed cell death protein 1 (PD-1) (Khalil et al., 2016). These new cancer immunotherapies are not covered here but have been extensively described in a number of recent review articles ([206–210]).

2.2.4.4 Delivery of a drug or cytotoxic agent

Antibodies can also be used as a delivery system for cytotoxic agents [211–215]. For example, mAbs have been conjugated to radioisotopes (radio immunotherapies, RITs), toxins (immunotoxins), enzymes (antibody-directed enzyme prodrug therapy, ADEPT) and small-molecule cytotoxic drugs (antibody drug conjugates, ADC). In these cases, antibodies are used to specifically target tumour cells and in some cases, facilitate internalization for cytosolic delivery. Furthermore, mAbs confer useful pharmacokinetics (PK) and biodistribution properties, increasing the half-life of a cytotoxic agent of low molecular weight.

Typically, an ADC is made up of three important components: the targeting mAb, the cytotoxic agent and the linker between them (Figure 2.6). The first

specifically targets and binds to the cell surface. The second confers a cytotoxic effect. The last component links the targeting component with the cytotoxic drug.

Figure 2.6 Components of an antibody drug conjugate (ADC). A typical ADC is made up of three components: antibody, linker and cytotoxic drug.

Most ADCs require internalization and degradation of the mAb portion in order to release their cytotoxic payload. Following selective antigen binding, an ADC is typically internalized and processed by the endocytic pathway, leading to cleavage of the linker or degradation of the mAb. This results in the release of the payload inside the cell where the drug can induce cytotoxicity.

Although early ADCs used drugs (e.g. doxorubicin) that were already in clinical use, many were found not to be substantially better than the drug alone in terms of efficacy. Consequently, this led to a search for more potent cytotoxic agents including highly cytotoxic plant and bacterial toxins such as saporin and ricin. Today, most ADCs in clinical development consist of IgG₁ mAbs conjugated with anti-mitotic tubulin inhibitors such as auristatins or derivatives of maytansines which bind to the vinca-binding domain of tubulin. Both agents interfere with microtubule dynamics and cause cell cycle arrest in the G2/M phase [216, 217]. The compounds are highly toxic, exhibiting 100- to 1000-fold

higher potency than standard chemotherapeutic drugs [218]. The dose limiting toxicities of ADCs can be vastly different than unconjugated mAbs with off-target toxicity of the drug usually determining maximal drug tolerance. Conjugates of auristatins and maytansanoids exhibit IC₅₀ values at 10 to 40 picomolar concentrations.

The complexity of an ADC raises special considerations in drug development. Off target toxicity of the drug and potential bystander effects are two major concerns. Development can involve an empirical (experimental) approach of optimizing the three components of an ADC: the mAb, the drug and the linker. Parameters such as the rate intracellular trafficking, the drug to antibody ratio (DAR) and type of linker (e.g. cleavable versus non-cleavable linker for drug-release) can have a dramatic effect on the overall efficacy of an ADC [219].

The demonstration of clinical efficacy of ADCs has led to a growing interest in the ADC field and advances in ADC linker/drug chemistry [220]. Recent reviews have summarize these preclinical and clinical advances, highlighting challenges in the design of new ADCs [5, 212, 214, 221, 222]. New areas of research and development include platforms for site-specific conjugation of the linker [223, 224]. The two most recently approved ADCs for cancer are the anti-CD30, brentuximab vedotin [225–227] and the anti-HER2, ado-trastuzumab emtansine (T-DM1) [228, 229]. In the case of T-DM1, the ADC was developed based on a mAb that was already shown to possess endogenous anti-tumour effects. Approaches similar to ADC are also being explored with other bio macromolecules (e.g. siRNA, DNA, peptides) that may require cytosolic delivery to exert potent cytotoxicity.

2.2.5 Improving therapeutic activity via antibody engineering

New antibody engineering strategies are currently in development to enhance the safety and efficacy of therapeutic mAbs. Fc engineering has emerged as an important component of antibody development with demonstrated clinical success. Both amino acid substitutions or glycoengineering in the Fc region has led to the development of mAbs with better immunogenicity properties and therapeutic efficacy [230, 231].

Different approaches have successfully altered the Fc interaction with C1q for enhanced CDC activity [232, 233] and Fc interaction with the neonatal FcRn receptor in order to prolong antibody clearance rate [234, 235]. The most established studies involve Fc-engineering to alter interactions with Fc gamma receptors, in particular the Fc-gamma receptor IIIA for ADCC activity [236, 237].

With the approval of glycoengineered mAbs, mogamulizumab (Poteligeo) and obinutuzumab (Gazyva), glycoengineering has emerged as one of the most clinically successful approaches [238–240]. Biochemical and structural studies have extensively characterized the glycosylation of Fc receptors (as reviewed in [196]). Antibodies lacking the core (alpha-1,6-linked) fucose show enhanced binding of Fc to Fc receptors by greater than 1-10 fold difference and improved ADCC target cell lysis by greater than 2 to 50- fold [241–243].

Table 2.4 Reported mechanisms of action of anti-tumour mAbs

Antibody (Trade Name)	Main mechanisms of actions	Reference
Rituximab (Rituxan/Mabthera)	<ul style="list-style-type: none"> • Direct cell-killing (via apoptosis) • Immune-mediated (ADCC and CDC) 	[244–247]
Trastuzumab (Herceptin)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) • Immune-mediated (ADCC and CDC) 	Reviewed in [248]
Gemtuzumab ozogamicin (Mylotarg)*	<ul style="list-style-type: none"> • Delivery of drug (calicheamicin) 	Reviewed in [249]
Alemtuzumab (Campath)	<ul style="list-style-type: none"> • Direct cell-killing (via apoptosis) • Immune-mediated (ADCC and CDC) 	Reviewed in [250].
Ibritumomab tiuxetan (Zevalin)	<ul style="list-style-type: none"> • Delivery of radioisotope (^{90}Y) 	[251]
Tositumomab (Bexxar)*	<ul style="list-style-type: none"> • Direct cell-killing (via apoptosis) • Delivery of radioisotope (^{131}I) • Immune-mediated (ADCC and CDC) 	Reviewed in [252], [253]
Bevacizumab (Avastin)	<ul style="list-style-type: none"> • Targeting the tumour microenvironment 	Reviewed in [254]
Cetuximab (Erbix)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) • Immune-mediated (ADCC and CDC) 	[255]
Panitumumab (Vectibix)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) • Immune-mediated (ADCC) 	[256, 257]
Ofatumumab (Arzerra)	<ul style="list-style-type: none"> • Immune-mediated (ADCC and CDC) 	Reviewed in [258]
Brentuximab vedotin (Adcetris)	<ul style="list-style-type: none"> • Delivery of drug (MMAE) 	[259–262]
Ipilimumab (Yervoy)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) 	Reviewed in [263]
Pertuzumab (Perjeta/Omnitarg)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) • Immune-mediated (ADCC) 	[264, 265]
Ado-trastuzumab emtansine (Kadcyla)	<ul style="list-style-type: none"> • Direct cell-killing (via signal inhibition) • Immune-mediated (ADCC) • Delivery of cytotoxic drug (DM1) 	[242]
Obinutuzumab (Gazyva)	<ul style="list-style-type: none"> • Direct cell-killing (via apoptosis and non-apoptosis mechanism) • Immune-mediated (ADCC/ADCP and CDC) 	[266–268]
Ramucirumab (Cyramza)	<ul style="list-style-type: none"> • Targeting the tumour microenvironment 	[269–271] and reviewed in [254]
Pembrolizumab (Keytruda)	<ul style="list-style-type: none"> • Immune-mediated (Checkpoint inhibition) 	[272]
Nivolumab (Opdivo)	<ul style="list-style-type: none"> • Immune-mediated (Checkpoint inhibition) 	[273]
Blinatumomab (Blincyto)	<ul style="list-style-type: none"> • Immune-mediated (T-cell recognition) 	[274]
Daratumumab (Darzalex)	<ul style="list-style-type: none"> • Direct cell-killing (via non-apoptosis mechanism) • Immune-mediated (ADCC/ADCP, CDC and other) 	[275–277]
Elotuzumab (Empliciti)	<ul style="list-style-type: none"> • Immune-mediated (ADCC, NK-cell activation) 	[278–280]
Atezolizumab (Tecentriq)	<ul style="list-style-type: none"> • Immune-mediated (Checkpoint inhibition) 	[281–283]

2.3 Status of current antibody-based therapeutics for ovarian and breast cancer

Antibody-based therapies are generally well-tolerated in cancer patients with less serious side effects compared to chemotherapy [110]. Side-effects are often associated with specific antigen targets. For example, trastuzumab which targets HER2 is sometimes linked to cardiotoxicity [112]. Lack of therapeutic efficacy is driven more often by resistance than by a limiting drug side effect of mAbs.

Antibody-based therapies for solid tumours have been most successful in breast and colon cancer. For breast cancer patients, the use of mAbs targeting the ErbB family (which includes HER2) has improved overall survival and time to disease progression of breast cancer patients. Nevertheless, clinical benefits of these mAbs are only applicable for patients with tumours that overexpress ERBB2 [284–286], which is estimated to be 15–20% of all primary breast tumours [287–289]. In addition resistance (both intrinsic and acquired) remains a persistent issue in anti-HER2-treatments [290]. The use of bevacizumab in breast cancer is more controversial, as is no longer approved by the FDA for treating breast cancer [291, 292].

Fewer antibody therapies exist for ovarian cancer. Notably, bevacizumab was approved by the FDA for treatment of platinum-resistant recurrent epithelial ovarian cancer. It has been shown to increase progression free survival but not overall survival for ovarian cancer patients [293–295]. The EMA has also approved a bispecific hybrid (mouse IgG_{2a} and rat IgG₂) mAb that targets EpCAM and CD3 [132, 134]. This mAb is used for intraperitoneal treatment of malignant ascites, an accumulation of fluid in the peritoneal cavity which can be found in patients with advanced ovarian cancer [296, 297].

Excluding checkpoint inhibition targets (such as PD-1, PD-L1/PD-L2, CTLA-4, NKG2A), the most widely investigated tumour antigen targets for treatment of ovarian cancer include VEGF, members of the epidermal growth factor receptor (EGFR) family (which includes HER2 and HER3), CA125, Folate receptor-alpha, MUC1 and EpCAM [298–300]. Examples of mAbs undergoing phase 3 clinical trials (www.ClinicalTrials.gov) include catumaxomab (which

targets EpCAM and CD3), farletuzumab (which targets folate receptor-alpha), and trastuzumab and pertuzumab (which both target HER2) [301].

2.4 Summary

Ovarian and breast cancer are deadly diseases characterized by molecular heterogeneity. Development of new treatment options is needed so as to target different cancer subtypes and more effectively treat them. Monoclonal antibody (mAb) and antibody drug conjugate (ADC) therapies represent an attractive targeted approach. Taking advantage of differences between cancer cells and normal cells, mAbs preferentially bind cell-surface antigens and exert clinical efficacy through various mechanisms of action. These include direct tumour cell-killing, immune-mediated killing (via ADCC and CDC activity) and the delivery of a cytotoxic payload. Despite the current availability of mAbs, there remains a need to develop new mAb-based therapies for ovarian and breast cancer. An important area of research is the identification of new antigen targets and generation of functional mAbs against them.

Chapter 3 Materials and Methods

3.1 Cell Culture

Cells were stored in freezing medium of 90% FBS (Hyclone; #SV30160.03) and 10% DMSO (Sigma Aldrich; #D2660) and cultured at low passage numbers (< 40 for *in vitro* studies) and (< 20 for *in vivo* studies). Cells were cultured at 37°C in a humidified incubator with 5% CO₂. Serous ovarian carcinoma cell lines used in this study include A2780, HEY, IGROV1, OVCAR3, OVCAR8, SKOV3(D10), SKOV3. Breast cancer cell lines include BT549, MCF7, MCF7-D10, MCF-2101, MCF10A and T47D. Cell counting was done based on the Trypan Blue (Sigma; #T8154) dye exclusion method or by use of a NucleoCounter (ChemoMetec) as per the manufacturer's instructions.

(see Table 3.1 for list of cell lines and media formulations)

Table 3.1 Cell lines

Name	Description*	Media
A2780	Ovarian serous carcinoma-	RPMI, 10% FBS
BT549	Breast ductal carcinoma	RPMI, 10% FBS
HEY	Ovarian serous carcinoma-	RPMI, 10% FBS
IGROV1	Ovarian serous carcinoma-	RPMI, 10% FBS
IOSE523	Immortalized ovarian surface epithelium	MCDB105, M199 (1:1 mix), 10% FBS
MCF7	Breast adenocarcinoma derived from metastatic site: pleural effusion	RPMI, 10% FBS
MCF7-2101	TNF- α resistant MCF7-D10 cells	DMEM, 10% FBS
MCF7-D10	Breast adenocarcinoma derived from metastatic site: pleural effusion	DMEM, 10% FBS
OVCAR3	(Poorly differentiated papillary)Ovarian serous carcinoma-	RPMI, 20% FBS, Insulin
OVCAR8	Ovarian serous carcinoma-	DMEM, 10% FBS
SKOV3(D10)	Ovarian serous carcinoma-	DMEM, 10% FBS
SKOV3	Ovarian serous carcinoma-	RPMI, 10% FBS
T47D	Breast ductal carcinoma derived from metastatic site: pleural effusion	RPMI, 10% FBS

All cell culture media was from Invitrogen and fetal bovine serum (FBS) was purchased from Gibco. ATCC, American Type Culture Collection

3.2 Antibodies and antibody labelling kits

The following commercial antibodies were used in the study: anti-beta-actin (Cell Signaling Technology; #4967), GAPDH (Cell Signaling Technology; #14C10), human IgG₁ kappa light chain isotype control (Southern Biotech; #0151K), mouse IgG₁ isotype control (Invitrogen; #M100), mouse anti-human Fc-specific antibody (Sigma; #B3773), anti-annexin A2 monoclonal antibody (BD Transduction laboratories; #61006), anti-annexin A2 monoclonal antibody (Invitrogen; #ZO16), anti-annexin A2 polyclonal antibody (Santa Cruz; #SC-9061).

(see Table 3.2 and 3.3 for list of secondary conjugates and dye labelling kits)

Table 3.2 Secondary conjugates

Name	Species	Conjugate	Dilution	Commercial source (cat #)
Anti-mouse pAb	Goat	FITC	1:500	DAKO (F0479)
Anti-mouse pAb	Goat	HRP	1:10000	DAKO (P0447)
Anti-human pAb	Goat	HRP	1:10000	DAKO (P0214)
Anti-human pAb	Rabbit	FITC	1:500	DAKO (F0200)
Anti-human pAb	Goat	Alexa Fluor 488	1:300	Invitrogen (A11013)
Anti-mouse IgM pAb	Goat	Alexa Fluor 488	1:300	Invitrogen (A21042)
Anti-human pAb	Goat	Alexa Fluor 647	1:300	Invitrogen (A21445)
Anti-mouse pAb	Rabbit	Alexa Fluor 488	1:300	Invitrogen (A11059)
Anti-mouse pAb	Goat	Alexa Fluor 647	1:300	Invitrogen (A32728)
Anti-human kappa light chain mAb	Goat	FITC	1:80	Sigma (F3761)
Streptavidin	NA	HRP	1:20	DAKO (P0397)
Streptavidin	NA	FITC	1:100	DAKO (F0422)

HRP (horseradish peroxidase), FITC (fluorescein isothiocyanate), WB (Western blotting), ICC (Immunocytochemistry), F (Flow cytometry), cat # - product category number

Table 3.3 Fluorescent dyes

Name	Excitation/Emission	Commercial source	Description
CypHER5e™	644/655 nm	GE Healthcare	Amine-reactive, pH sensitive cyanine dye with minimum/maximum fluorescence at pH 7.4 and pH
LYNX Dylight®488	493/598 nm	Bio-Rad	Amine-reactive dye with high fluorescence intensity over a broad pH range (pH 4-9)
XenoLight®CF750	755/775 nm	Caliper Life Sciences	Amine-reactive near infrared (NIR) fluorescent dye

3.3 Antibody generation and purification

Monoclonals 2448 and C51 were generated by mouse hybridoma technology as previously reported [17, 302]. Hybridomas were maintained in ClonaCell™-HY Medium E (Stem Cells Technologies; #03805) at 37°C in a humidified incubator with 5% CO₂. Purification of mouse antibodies was done by the Downstream Processing Group at the Bioprocessing Technology Institute using affinity chromatography or steric exclusion chromatography, respectively [303].

Chimeric (including afucosylated) antibodies were expressed in DG44-CHO cells and maintained in BTI's proprietary serum-free media. Chimerisation was done by the Animal Cell Technology group at the Bioprocessing Technology Institute [304].

Purification was done on using the ÄKTA Explorer 100 (GE Healthcare). Supernatants from both transient and stable transfections were subjected to Protein A chromatography (Tosoh; Toyopearl AF-rProtein A-650F) and ion exchange chromatography (Biorad; UNOsphere™ Q). Purified products were evaluated on a Superdex200 PC 3.2/30 column (GE Healthcare) using a high performance liquid chromatography system (Shimadzu). Antibodies were additionally analysed by SDS-PAGE, and protein concentrations were determined by absorbance at A₂₈₀ using Nanodrop 1000 (Thermo scientific).

For *in vivo* studies, endotoxin was measured to ensure levels were below 0.1 EU/mL using the Endosafe® Endotoxin Testing System (Charles River) [305, 306]. Samples of purified protein product are shown in Figure

Figure 3.1 Chromatogram of purified ch2448 after UNOsphere QTM chromatography. A sample of purified ch2448 was evaluated using HPLC.

The following in-house antibodies were also used as isotype controls: mouse mAb 8 [307]; mouse mAb 63 (mouse IgM isotype control) and chimeric mAb chTNA2 (chimeric IgG isotype control).

3.4 Antibody fragmentation

The IdeS protease (Genovis; FabRICATOR™, #A0-FR8) was used to generate F(ab')₂ fragments from whole IgG. IdeS was originally discovered as streptococcal Mac-1 from *Streptococcus pyogenes* and cleaves at a specific site after amino acid residue glycine 236 in human IgG₁ [308]. Digestion with IdeS protease produces three products: one F(ab')₂ fragment and two half-Fc fragments (Figure 3.2).

F(ab')₂ fragmentation was done per manufacturers' instructions. Briefly, IdeS enzyme was added to purified antibody at 1 unit of enzyme per 1 µg of antibody in 50 mM HEPES/ 150 mM NaCl buffer solution. The mixture incubated at 37°C for 1 h. Histadine-tagged IdeS enzyme was removed by Ni-NTA spin columns (Qiagen; #31014).

The lysine specific gingipain cysteine protease, KGP (Genovis; GingisKHAN™, #B0-GKH) was used to generate Fab fragments from whole IgG. KGP was originally derived as gingipain K of *Porphyromonas gingivalis*. It specifically cleaves at a specific site after amino acid residue lysine 223 in human IgG₁ [309]. Digestion with the KGP protease produces one Fc fragment and two Fab fragments (Figure 3.2).

Briefly, KGP protease was incubated with purified antibody at 1 unit of enzyme per 1 µg of antibody in PBS/2 mM cysteine reaction buffer. After incubation at 37°C for 2 h, cysteine was removed by centrifugal ultrafiltration (GE Healthcare; VIVASPIN® 500, # VS0101). Digestion products were analysed by gel electrophoresis and western blot analysis, size exclusion chromatography and flow cytometry.

Figure 3.2 Generation of F(ab')₂ and Fab fragments. (A) Schematic representation of antibody digestion to create F(ab')₂ and Fab fragments. After reduction of disulphide bonds, heavy and light chains are separated. (B) The Coomassie-blue-stained SDS-PAGE gel of digestion products are shown in non-reducing (NR) and reducing (R) conditions.

3.5 Flow Cytometry and Analysis

Cells were harvested as single-cell suspensions using trypsin (Thermo Fisher Scientific; #25200). Each sample of $1-2 \times 10^5$ cells was thoroughly washed in 1% bovine serum albumin (BSA; Sigma Aldrich; #A9647) in phosphate buffered saline (PBS) buffer (Thermo Fisher Scientific; #14190). Samples were incubated with primary antibody at 4°C for 45 min, washed and then incubated with the appropriate fluorophore-conjugated secondary antibody for 15 min at 4°C. After incubation, cells were washed and sample data acquisition was done by on a BD FACSCalibur™ (BD Biosciences) or Guava® easyCyte (Millipore). Data analysis was done using FlowJo™ software v7.6.3 (Tree Star). Percentage of binding was determined from M gating set at the 97th-98th-percentile based on the negative control.

3.5.1 High-throughput screening:

Cells were incubated in 100 µl of hybridoma supernatant at 4°C for 30 min, followed by appropriate secondary antibody incubation for 15 min at 4°C. Binding was analysed on BD FACSCalibur™ flow cytometer (BD Biosciences). Screening was done using cultured supernatant from hybridoma clones of 2448 and C51.

3.5.2 Binding saturation curves:

Saturating levels of 2448 and C51 were determined using flow cytometry. Cells were harvested as single-cell suspensions using trypsin (Invitrogen) and washed in 1% BSA/PBS buffer. Cells were then diluted to a final density of 1×10^5 cells per 100 µl of cold buffer and incubated with serial dilutions of primary antibody (1 to 300 µg/mL) conjugated to FITC (fluorescein isothiocyanate) for 30 min on ice. After a final wash, cells were resuspended in buffer and analysed on BD FACSCalibur flow cytometer (BD Biosciences) as described in Section 3.5. Data analysis was done using FlowJo™ software v7.6.3 (Tree Star). Representative binding curves from three independent experiments are shown. Fitting of binding data to a one to one site specific binding model (GraphPad; GraphPad Prism 6) was done to generate estimated K_d values.

3.5.3 Competitive inhibition assay:

Cells were incubated at saturating concentrations of mAb with single mAb alone, both mAbs added simultaneously or one after the other. Incubations were done for at least 15 min each at 4°C, washed in 1% BSA/PBS and subsequently followed by incubation of appropriate secondary antibodies. Binding was analysed on a BD FACSCalibur™ flow cytometer (BD Biosciences) or Guava® easyCyte (Millipore) as described in Section 3.5.

3.6 Immunocytochemistry

Cell cultures were fixed with 4% paraformaldehyde for 20 min. For permeabilized cells, 0.1% Triton X-100/PBS (Bio-Rad) was added for 3×5 min, and finally blocked with either 10% goat serum/PBS (DAKO) for 1 h at room temperature. Overnight incubation with primary antibodies was done followed

by incubation with appropriate Alexafluor-conjugated secondary antibodies (see Table 3.2). DNA was counterstained with DAPI (1:1000, Invitrogen; #D1306). All incubations were done at 4°C. Images were taken using a Zeiss Axiovert 200 inverted microscope.

3.7 Gel electrophoresis and western blot analysis;

Cancer cells were harvested by manual scraping and washed in PBS. Membrane protein enrichment was done via the Membrane Protein Extraction Kit (BioVision; # K268-50) as per the manufacturer's instructions. Lysis buffer contained 2% Triton X-100 (Bio-Rad; #1610407) in PBS. Lysate was clarified by centrifugation at 10,000 g for 1-2 min at 4°C. If not used on the same day as harvest, lysate was supplemented with protease inhibitors (Calbiochem; #539131). Total protein concentration was determined using the DC™ Protein Assay (Bio-Rad; #5000116).

Protein samples of 30 µg (or as amount indicated) were prepared with loading dye for a final concentration of 50 mM Tris-HCl, 2% SDS, 10% glycerol and 0.02% bromophenol blue. For reducing samples, β-mercaptoethanol was added to a final concentration of 2-5%. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 – 12% Bis-Tris gradient gel (Invitrogen; #NP0321 or #NP0323) with 1X MOPS buffer (Invitrogen; #NP001). SeeBlue Plus2® protein standard (Invitrogen; #LC5925) was also run on the gel alongside protein samples. Proteins were visualized by Coomassie Blue R-250 staining (Sigma Aldrich; #B7920) and/or analysed by western blotting.

Western Blot transfer was done onto PVDF membranes (Bio-Rad; #1620115) that were subsequently blocked with either 5% non-fat milk or Odyssey® Blocking Buffer (LI-COR; #92740000) for 1 h. Incubation with primary mAb (hybridoma supernatant at 1:1 dilution in blocking buffer or affinity-purified antibody 10 µg/mL of blocking buffer) was done overnight at 4°C. Appropriate secondary antibody was incubated for 1-2 h, and proteins were detected by chemiluminescence using ECL substrate (GE Healthcare; #RPN2232). Image capture was done on film or by the ChemiDoc™ Imaging System (Bio-Rad).

3.8 Immunoprecipitation

Immunoprecipitation was done to enrich for the antigen target of 2448. Briefly, membrane-enriched lysate (as described in Section 3.7) was precleared with Protein G-Sepharose resin (GE Healthcare; # 17-0618-01) and clarified by centrifugation. Samples at 1-5 mg/mL were then incubated overnight on a rotator with 2448-coated Protein G-Sepharose beads. After washing at least five times with PBS buffer, a final wash was done with saline solution containing 150 mM NaCl at pH 7.4, immunoprecipitation samples were eluted in gel electrophoresis sample loading buffer. All steps were done at 4°C or on ice.

For C51, immunoprecipitation was done using the automated MEA 2 system (PhyNexus) with immobilised streptavidin tips. Biotin-conjugated anti-mouse IgM antibody (Sigma) was used for indirect C51 capture. Cell lysates at a protein concentration of 1-5 mg/mL were used. Washing between capture steps was done using PBS (pH 7.4). The last wash before elution was with saline solution containing 150 mM NaCl. Immunoprecipitation samples were eluted in 200 mM NaH₂PO₄, 140 mM NaCl buffer at pH 2.5. Immunoprecipitation of C51 was done by Melissa Wirawan and Ms. Fong Wey Jia.

Immunoprecipitation products of each mAb were separated on multiple gels run in parallel (as described in Section 3.7). Replicate samples were either transferred by western blot (as described in Section 3.7) or silver stained. Silver staining was done using the SilverQuest silver staining kit (Invitrogen; #LC6070) as per the manufacturer's protocol. Silver stained bands corresponding to the antigen of interest (as visualized on western blot) were manually excised. The excised band was soaked overnight in 2.5 mM ammonium bicarbonate in 50% acetonitrile solution at 4°C. The band was subjected to in-gel proteolysis with trypsin and sent for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were processed by the Proteomics Group at the Bioprocessing Technology Institute."

3.9 Transient siRNA-mediated knockdown

IGROV1 cells were plated on 6-well plates (at 3×10^5 cells) or T175 flasks (at 1×10^6 cells). Transfection was done at 30 to 50% confluency using Lipofectamine RNAiMAX (Invitrogen; #13778030) in serum-free media. Cell cultures were incubated with 30 pM of a validated set of human annexin A2-specific siRNA (Invitrogen; #S1384) or scramble siRNA control (Invitrogen; #4390846). Transfection media was removed and replaced with fresh media containing serum after 5 h. Cells were harvested within 72 h for analysis.

3.10 Periodate treatment

In order to determine if antibodies recognized glycan targets [310], periodate treatment was done on cell lysate after western blotting. Sodium *meta*-periodate (Sigma; #S1878) was used as a mild oxidative, opening the saccharide rings between vicinal diols and thereby leaving two aldehyde groups. Firstly, cell lysate was run on SDS-PAGE and transferred to PVDF membranes. Subsequently, membranes were washed twice with sodium acetate buffer (100 mM at pH 4.5) (Merck Millipore; #1.06268.0250), which was followed by two incubations with sodium *meta*-periodate (100 mM) in acetate buffer. Each wash was done for 30 min at room temperature in the dark. Membranes were then washed four times with buffer and quenched with 0.5 M of sodium borohydride (Sigma; #21346-2) in PBS for 30 min at room temperature. Membranes were washed and blocked with Odyssey® Blocking Buffer (LI-COR; #92740000) for another 30 min prior to addition of primary mAbs. For the control blot, all incubations were done as previously described but without the addition of sodium *meta*-periodate. Samples were analysed with primary antibodies 2448, C51 and actin as a negative control.

3.11 PNGase F treatment

Peptide -N-Glycosidase F (PNGase F) is commonly used for the efficient release of N-linked glycans on (glyco) proteins. The PNGase F treatment was optimized per the manufactures' instructions (New England Biolabs; #P0704S/L). PNGase F cleaves the β -glycosidic amide bond between Asn residue and the first GlcNAc of the N-glycan (ie GlcNAc-Asn), releasing nearly

all N-glycans on glycoproteins excluding alpha-(1,3)-linked core-fucose [311, 312]).

3.11.1 Release of N-glycans from membrane glycoproteins

Briefly, membrane protein enriched cell lysate (10 µg) was first denatured using 1 µl of 10X glycoprotein denaturing buffer (5% SDS without DTT) and water to make up a 10µl reaction volume. Glycoproteins were heated to 100°C for 5 min. A total reaction volume of 20 µl was prepared with the denatured glycoproteins by adding 2 µl of 10× G7 reaction buffer, 2 µl of 10% NP40, and 1 µl of PNGase F, and 5µl of water. Samples were incubated in the reaction buffer for 1 h at 37°C. After incubation, samples were boiled in 5X loading dye and separated on a 4-12% gradient gel followed by Western blot analysis (see 3.7). Samples were probed with primary antibody 2448 and C51 followed by appropriate secondary antibodies.

3.11.2 Release of N-glycans from antibody ch2448

The removal of N-glycans from purified ch2448 was similarly done using PNGase F as described in Section 3.11.1. As a control, ch2448 was buffer-treated without PNGase F.

3.12 Tunicamycin treatment

Tunicamycin is a nucleoside antibiotic that targets GlcNAc transferase [313, 314]. At high concentrations, tunicamycin can induce apoptosis in cancer cells [315] however at lower concentrations, it can be used to inhibit N-glycan synthesis of intact (eukaryotic) cells [316–318]. IGROV1 cells at 60-70% confluency were treated with tunicamycin (Sigma; #T7765) at a final concentration of 0.25 µg/mL. After incubation of 24 h, cells harvested and analysed by flow cytometry and western blot analysis.

3.13 Direct tumour cell-killing assay

Cells were harvested as single cell suspension using trypsin and resuspended at a 100 µl volume in FACS buffer (1% BSA/PBS). Samples were incubated at saturating concentrations of primary mAbs (from hybridoma supernatant or purified mAb) at a total volume of 200 µl at 4°C for 45 min. Cells were washed

and resuspended in 1% BSA/PBS containing propidium iodide (PI) at 2 mg/mL. Analysis was done by flow cytometry (as described in Section 3.5). IgG or IgM antibodies were used as isotype controls. Live cells were measured based on PI exclusion of live cell populations [319].

3.14 Complement-dependent cytotoxicity assay

Normal human serum (Quidel; # A112) was used as a source of complement proteins (CH50, Bb, C4d, C3a, iC3b, and the *terminal complement complex*). 10,000 cells were incubated with primary mAb and either NHS or heat-inactivated NHS. (Complement activity was inactivated by heating NHS for 30 minutes at 56°C.) After 6 hours at 37°C, 5% CO₂, plates were spun down and cytotoxicity was measured by the release of lactate dehydrogenase (LDH) using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega; G1780) according to the manufacturer's instructions. Maximal lysis was determined by lysis in 0.4% Triton X-100. Percent Cytotoxicity was measured as: (*Experimental-Control (no Ab)*)/ *Maximal lysis-Control (no Ab)*).

3.15 Antibody-dependent cell-mediated cytotoxicity assay

ADCC activity was measured using a reporter bioassay (Promega; ADCC Reporter Bioassay, #G7010). Compared to assays which use peripheral blood mononuclear cells (PBMC) or purified NK cell populations, this assays used a reporter gene readout for activation of the nuclear factor of activated T-cells (NFAT) signalling pathway (Figure 3.3). Jurkat cells were engineered to express human Fc-gamma IIIa receptors via the Fc region. Antibodies that bound to target antigens could also to bind to these engineered effector cells. Upon sufficient binding, antibodies activated an NFAT response element upstream of the luciferase gene, leading to production of the luciferase enzyme. Effector cells were then lysed and interaction of luciferase with luciferin and ATP in solution produce a luminescent signal readout [320, 321].

The ADCC bioassay was carried out according to the manufacturer's protocol Briefly, cells were seeded at 5,000 cells per well in a 96-well clear bottom black tissue culture plates (Corning; #3904) in low 4% IgG-serum (Promega; #G711A) media. Serial dilutions of primary antibody were incubated in triplicate wells for approximately 15 min at 37°C, 5% CO₂. Following

incubation, engineered effector cells were added to the wells at approximately 150,000 cells per well. After more than 5 h (or as indicated in results), BioGlo™ Luciferase Assay Substrate (Promega; #G719A and #G720A) was added to the wells and luminescence was measured using the Infinite® 200 microplate reader (Tecan). Estimated EC₅₀ values were calculated by non-linear regression on log-transformed data using GraphPad Prism 6 (GraphPad).

Figure 3.3 Schematic diagram of ADCC assays.

3.16 Internalization Assay

Antibodies were lysine conjugated to the CypHER5E™ mono NHS ester dye (GE Healthcare), a pH-sensitive dye that maximally fluoresces in low pH environments such as intracellular endosomal and lysosomal compartments [322, 323]. Conjugation was done according to the manufacturer's protocol. Briefly, purified antibody 2448 was incubated with a 2-3 molar excess of dye for 2 h on ice. The reaction mixture was then buffer exchanged and concentrated (VIVASPIN™ 500, Sartorius Stedim Biotech). The dye load of the antibody was determined by spectrophotometry (NanoDrop™, Thermo Fisher Scientific). The dye to protein ratio (D/P) was calculated by the formula: $D/P = A_{\text{dye}} \epsilon_{\text{protein}} / (A_{280\text{nm}} - A_{\text{dye}}) \epsilon_{\text{dye}}$, where A is the absorbance and ϵ is the molar extinction coefficient. Dye excitation and emission wavelengths were 644nm and 655nm respectively.

IGROV1 cells were harvested and incubated in the presence of sodium azide (Sigma #S8032) at 0.1% w/v in 1% BSA/PBS) or buffer alone for 30 min at 4°C. CypHER5e™ dye-conjugated mAb or free-dye control was then added to cells with or without sodium azide. Cells were transferred to 37°C to induce internalization, and fluorescence was measured at different time points (0.5 h, 0.45 h, 1 h) on the BD FACS Aria™ II (BD Biosciences) flow cytometer. The mean fluorescence intensity (MFI) of internalized CypHer5E™ was analysed using FlowJo™ software v7.6.3 (Tree Star). Internalization was measured as an increase in dye fluorescence intensity.

3.17 Cell Proliferation Assays

Cells were seeded in 96-well plates (Corning; #3904), and allowed to grow overnight. Metabolically active cells were measured based on the presence of ATP, using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega; #G7570). Data are expressed as a percentage of control, measuring the viability of treated cells against that of untreated cells. The linear range of the assay was determined for each cell line.

3.18 Antibody drug conjugate assays using secondary conjugates

3.18.1 Saporin conjugates

The potential of mAbs to be used as ADCs were evaluated using antibody conjugates of saporin: MAb-ZAP, Anti-M-ZAP, HUM-ZAP (Advanced Targeting Systems; #IT-04, #IT-30, #IT-22) [309].

Cells were seeded in 96-well plates (Corning; 3904) at 1000-2000 cells per well in 90 µl of media. After 24 h, the primary mAbs (2448, C51, ch2448 or chC51) at 10 µg/ml were complexed with appropriate secondary antibody conjugates: mAb-ZAP, Anti-M-ZAP, HUM-ZAP (Advanced Targeting Systems), at 1:3 molar ratio for 15 min at room temperature. Primary mAb alone, secondary conjugate or mixtures at 10 µl volume were added to wells. Buffer alone was added to control wells. After 72 h post treatment, live (metabolically active) cells were measured based on the presence of ATP, using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega; #G7570). Data

are expressed as the % control, measuring the viability of treated cells with that of untreated (buffer only) control cells.

3.18.2 Monomethyl auristatin E conjugates

The Fab and F(ab')₂ fragments of ch2448 were evaluated using a similar assay as described in the prior section (Section 3.18.1) but with anti-human IgG Fab-specific secondary antibody conjugate (Moradec; #AH-122AE-20). OVCAR3 cells were seeded at 1000 cells per well. Primary Fab and F(ab')₂ fragments were tested at two concentrations, 20 mM and 40 mM.

The secondary antibody targets the Fab region of the antibody and is conjugated to monomethyl auristatin E (MMAE) via a cleavable linker. The cleavable linker is stable in extracellular fluids but cleaved by cathepsin in endosomes.

3.19 Antibody drug conjugate assays with ch2448-saporin conjugate

Direct conjugation of saporin to antibodies was done by Advanced Targeting Systems (ATS). Drug to antibody molar ratios of ch2448-saporin, chTNA2-saporin and human Ig-saporin were 2.5, 2.9 and 3.1, respectively. Evaluation of binding specificity was carried out by flow cytometry analysis (as described in Section 3.5).

Cytotoxicity of ch2448-saporin was evaluated on IGROV1, SKOV3 and IOSE523 cell lines. Cells were seeded in 96-well plates (Corning; #3904) at 1000 cells per well in 90 µl of media. The following day, ch2448-saporin and chTNA2-saporin were serially diluted and 10 µl of each dilution was added to wells. As a control, free saporin and ch2448 were added in a separate set of plates. After 72 h of incubation, the cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega) according to the manufacturer's instructions. Data are expressed as the % control, measuring the viability of treated cells with that of untreated cells. Dose response curves and IC₅₀ values were calculated using GraphPad Prism 6 (GraphPad).

3.20 Real-time monitoring of cell growth

Cell growth was continuously monitored over time using the xCelligence® real-time cell analyzer (Roche). Viability of adherent cell cultures was monitored

based on cell impedance [324]. Cell culture media was first loaded onto the 96-well E-plate to measure background impedance. Cells were then plated at either 1,000 cells per well for BT549, IGROV1, IOSE523, OVCAR3, SKOV3 or 2,000 cells per well for MCF7, T47D and allowed to grow overnight in normal cell culture conditions. Cells were treated with antibody (ch2448) or antibody conjugates (ch2448-saporin or human IgG-saporin) at the beginning of the log phase of cell growth. Control wells were treated with buffer alone. All experiments were done in at least 3 wells per treatment condition. The outermost wells of the plate were not used.

For dose response curves, IGROV1 and MCF7 cells were treated with dilutions of ch2448-saporin. The cells were monitored for growth until cells reached a death phase. Cell indices of wells were normalized after antibody treatment. The IC₅₀ value on IGROV1 cells was determined using the accompanying real-time cell analysis (RTCA) software (Roche).

3.21 Tumour xenograft

Four- to six-week-old female BALB/c nude mice (InVivos) were used in this study. On day 0, mice were inoculated with a total of 5×10^6 IGROV1 cells in 100 μ l of cell media and high concentration matrigel (BD biosciences; #354248) at a 1:1 dilution volume. On the following day, antibodies or buffer saline control (50 mM HEPES, 150 mM NaCl) was injected intraperitoneally. Subsequent administration was done on a weekly basis as indicated. Antibodies, ch2448 IgG or F(ab')₂ (or vehicle control) was administered by intraperitoneal injection at 100 μ l volumes at indicated doses. Size of the primary tumour was measured weekly by using digital calipers. Mice were euthanized when tumour size was $> 2000 \text{ mm}^3$ or when persistent side effects (e.g. swollen lymph nodes or drastic body weight loss) were observed over a period of more than two weeks. Euthanasia was done by CO₂ inhalation followed by cervical dislocation.

Tumour volumes (TV) were calculated based on the formula: $TV = ((L \times L \times W)/2)$, where W (width) and L (length) are the short and long diameter, respectively. A student's t test was used to assess the statistical significance treated and untreated animals. Tumours were also harvested to test for

antibody binding or for immunohistochemistry. For collection of tumours, animals were euthanized and immediately harvested. For flow cytometry, tumours were cut-up and washed in PBS and gentamicin (Life technologies; #15750060). The samples were then re-cultured for 1 to 2 days and then analysed by flow cytometry. For immunohistochemistry, samples were washed with PBS and either snap-frozen in liquid nitrogen or placed in paraformaldehyde (Merk; #1.04003.2500) for 24 h which was subsequently replaced with 100% ethanol (Merk; #1.00983.2500). Animals were handled according to Biopolis IACUC Protocol No.: 151001 in accordance with the National Advisory Committee For Laboratory Animal Research (NACLAR) Guidelines. Studies were done using The Animal Study Services at the Biological Resource Center was used in subcutaneous xenograft studies.

3.22 Enzyme-linked immunosorbent assay (ELISA)

In order to confirm the presence of ch2448 in mice, blood samples were collected in heparin-containing tubes by tail vein bleed or cheek vein bleed. Human IgG levels in were measured using ELISA. Maxisorb 96-well plates (Nunc) were coated with anti-human IgG in coating buffer (0.05M NaHCO₃, pH 9.6) overnight. The following day, plates were washed with PBST (0.05% Tween (Bio-Rad) in phosphate-buffered saline) and blocked with 3% BSA/PBST for 1 h at 37°C. Mouse serum was pooled from each treatment group and diluted 10X and 100X with 1% BSA/PBS. Samples and a serially diluted ch2448 antibody standard were added into coated wells. Samples were allowed to incubate for 1 h at 37°C. After incubation wells were washed with PBST and dried. Secondary HRP-conjugate (see Table 3.2) was added and incubated for 1 h at 37°C., o-Phenylenediamine dihydrochloride substrate solution (Sigma; #P1526) was prepared per manufacturer's protocol. Substrate solution was added and allowed to develop. Reaction was suspended by the addition of 3M hydrochloric acid (Sigma; #258148). Optical density values at 420 nm (OD₄₂₀) were measured on the Infinite® 200 microplate reader (Tecan).

3.23 Biodistribution study

Human IgG mAb (Southern Biotech), chTNB1 and ch2448 were labelled with a near infrared fluorescent (NIR) dye CF750 using the XenoLight CF750 rapid antibody-labelling kit (Caliper Life Sciences) according to the manufacturer's instructions.

Female BALB/c nude mice were inoculated with a total of 5×10^6 IGROV1 cells in 100 μ l of cell media and high concentration matrigel (BD biosciences; #354248) at a 1:1 dilution volume. When tumours reached 300-400 mm³ (on Week 10), dye-conjugated ch2448 and control conjugate chTNB1 were administered by i.p. injection (or tail-vein intravenous (i.v.) injection) at 100 μ g per 100 μ l of buffer (50mM HEPES, 150mM NaCl) per mouse. At 72 h and 94 h post injection, mice were anesthetized with 2-3% isoflurane and imaged using the IVIS® Spectrum imaging system (Caliper Life Sciences). Data was analysed using the Living Image software 3.2 (Caliper Life Sciences).

Thereafter, mice were euthanized by CO₂ inhalation followed by cervical dislocation. Tumours and organs such as were harvested. The organs were thoroughly washed, placed side by side and imaged using the IVIS® Spectrum imaging system (Caliper Life Sciences). The level of ch2448-NIR present in mice was measured by photon radiance of selected regions of interest that were drawn around each tumour or organ.

Chapter 4 Characterisation of novel monoclonal antibodies against ovarian and breast cancer

4.1 Introduction

Monoclonal antibodies (mAbs) can be used to target antigens that are differentially expressed on cancer cells compared to normal cells. This approach requires the identification of tumour antigens and the generation of mAbs against them. So far, only a limited number of antigen targets have been exploited for therapies in solid tumours (see Chapter 2, Table 2.2). The most clinically successful have been against VEGF, EGFR and HER2. Notably, the anti-HER2 mAbs are only applicable for a subset of breast cancers overexpressing the HER2 gene. To target the spectrum of malignancies, there remains an important need for the discovery of novel mAbs against new antigen targets.

Oncofetal antigens represent one type of tumour antigens that is present during embryonic development while also being upregulated during cancer progression. Human embryonic stem cells (hESC) represent an important source for the discovery of these antigens [139, 141, 325–327]. Interestingly, many of these antigens contain unique glycan epitopes [328].

In the past several decades, there has been renewed interest and developments in the field of cancer glycobiology [329, 330]. Aberrant cell surface glycosylation is a prevalent feature in cancer. Different levels of glycosyltransferases and glycosidases have been reported in cancer cells and these have been linked to overexpression, truncation of glycan structures, or tumour-specific glycan epitopes [331]. Glycosylation may contribute to protein stability, surface translocation or enhance signal transduction. Glycoproteins, glycolipids [332] and glycans themselves have been explored as targets for antibody therapy [333–337].

In order to generate novel mAbs against cell-surface oncofetal antigens, live human embryonic stem cells (hESCs) were used to immunize BALB/c mice and establish a panel of mAbs by hybridoma technology [16, 17]. The mAbs bound strongly on hESC and minimally on human fibroblast and other normal cells. As the antibodies are generated against the entire repertoire of cell

surface proteins, the antigen target is also not directly known and requires additional effort to isolate and characterise.

In this thesis, 2448 and C51 were investigated for their potential as targeted treatment for ovarian and breast cancers. This chapter reports on the *in vitro* characterisation of the mAbs, focusing on identifying the antigen targets and specific epitopes.

4.2 Binding profile of 2448 and C51 on ovarian and breast cancer cells

4.2.1 Antibodies 2448 and C51 bind to the cell-surface

Antibodies 2448 and C51 were previously found to bind on the surface of hESC and a range of cancers. To further investigate the selectivity within ovarian and breast cancers, high-throughput screening was done on a panel of eighteen ovarian and sixteen breast (cancer and normal) cell lines. All ovarian cancer cells belonged to the serous ovarian carcinoma subtype. Breast cancers cells included including HER2 positive cell populations e.g. HCC2218, HCC1954, SKBr3, MDA453 and BT474 and HER2 negative populations e.g. HCC1937, BT20, and HS578T. Surface binding on live cells was measured by flow cytometry. Strong binding of either 2448 or C51 was observed in ten of the ovarian cancer cell lines and fourteen breast cancer cell lines (Figure 4.1).

Figure 4.1 Binding of 2448 and C51 on live ovarian and breast cancer cells. High-throughput fluorescence-activated cell sorting (FACS) screening was done using supernatant from hybridoma clones (8, C51 and 2448) against (A) ovarian cancer cell lines and an immortalized ovarian surface epithelium cell line, IOSE523 and (B) breast cancer cell lines and an immortalized mammary epithelial cell line, MCF10A. Cell lines were classified according to an EMT spectrum: Epithelial (E), Intermediate Epithelial, Intermediate Mesenchymal and Mesenchymal (M). The anti-EpCAM mAb (Clone 8) was used as a control to screen for cell lines with an epithelial phenotype. Binding intensity was measured on a scale of 0 to 4: 0 < 10% binding, 1 = 10-40% binding, 2 = 40-60%, 3 = 60-80% and 4 > 80%. HCC1395 was not classified (NC).

Representative histogram data of six cell lines that are commonly used throughout this thesis are shown in Figure 4.2. The percentage of binding was analysed by shifts in the mean fluorescence intensity (MFI). For ovarian cancer cell lines, IGROV1 and OVCAR3, and breast cancer cell lines, MCF7 and T47D, complete rightward shifts along the x-axis of the binding histograms was observed. These indicated positives binding and minimal overlap with the negative-binding cell populations. No binding was observed on the normal immortalized ovarian cell line, IOSE523 and the triple-negative breast cancer cell line, BT549. Similar to flow cytometry results, immunocytochemistry revealed surface binding of 2448 on cells cultured on two-dimensional surfaces (Figure 4.2). Homogenous staining was confirmed by membrane staining of cells.

Figure 4.2 Representative images of 2448 and C51 on ovarian cancer cells. IGROV1 cells were stained with FITC-conjugated antibodies. Membrane staining was observed by wide-field epifluorescence microscopy. DAPI was used as a counterstain for the cell nuclei.

Figure 4.3 Representative histograms of binding by flow cytometry. Cell suspensions of various ovarian and breast cells were incubated with 2448 or C51 and secondary antibodies conjugated to FITC. Binding (dashed blue lined tinted histogram) was graded based on binding percentages after a marker (M) gating with a 2-2.5% cut-off from the negative control (solid red lined empty histogram).

4.2.2 Antibodies 2448 and C51 bind to cells with an epithelial phenotype

The epithelial to mesenchymal transition (EMT) describes a process in which epithelial cells transform into cells with more mesenchymal characteristics (see Chapter 2, Section 2.1.4). Although EMT is more closely related to cancer progression, it has also been associated with the differentiation of hESC. [338, 339]. For example, the oncofetal antigen, 5T4, is transiently expressed during hESC differentiation, but it is also a mesenchymal marker for cancer-related EMT [340, 341]. Epithelial markers are also selectively expressed in undifferentiated hESC. Examples include EpCAM [19, 342–345] and E-cadherin [346–348].

To determine if 2448 and C51 were targeting cancer subpopulations of an epithelial or mesenchymal phenotype, cell lines were organized into four categories based on an Epithelial to Mesenchymal Transition (EMT) spectrum: Epithelial (E), Intermediate Epithelial (IE), Intermediate Mesenchymal (IM) and Mesenchymal (M) [72, 67]. Results from high-throughput screening revealed that 2448 and C51 bound to a subset of ovarian and breast cancer cells (Figure 4.1). Interestingly, both mAbs had similar binding to twenty of the same cell lines. Positive binding was observed with percentages ranging from 40% to over 80%. Strong to moderate binding was observed on cells classified as “E” and moderate to weak binding was observed on those classified as “IE”. Moreover, binding of mAbs was significantly weaker or non-existent on cell lines classified as “IM” or “M”. Notable exceptions include SKOV3, CH1 and HS578T cell lines. The results suggested that 2448 and C51 preferentially bind to cells with having an epithelial phenotype. Due to the limited number of breast cancer cell lines, this trend was more strongly observed on ovarian cancer cell lines.

To extend these observations, 2448 was also screened against two isogenic breast cancer cell lines, MCF7-D10 and MCF7-2101. These cell lines represented different states of EMT. MCF7-2101 was derived from the parental MCF7-D10 line after treatment with TNF-alpha, a known inducer of EMT [67, 349, 350]. Therefore, MCF7-D10 represented an epithelial state and

MCF7-2101 represented a mesenchymal state, after cells have undergone EMT.

Cell surface binding of 2448 was first evaluated on live MCF7-D10 and MCF7-2101 cell lines by flow cytometry (Figure 4.4). Strong binding was observed on MCF7-D10 as indicated by a shift in the mean fluorescence intensity. Conversely, binding was not observed on MCF7-2101. Only after cells were fixed and permeabilised was binding observed on MCF7-2101, suggesting recognition of intracellular antigens.

Figure 4.4 Binding of 2448 on MCF-D10 and MCF7-2101 isogenic cell lines. Antibody binding (blue tinted) compared to the negative control (red) was positive for live MCF7-D10 cells, but not MCF7-2101 cells. Positive binding was observed for fixed and permeabilised MCF7-D10 and MCF7-2101 cells. Binding was graded based off binding percentages after a marker (M) gating with a 2-2.5% cut-off from the negative control (solid red lined empty histogram).

To further investigate, immunocytochemistry was also done. As expected, MCF7-2101 cells were morphologically characterised by a more mesenchymal phenotype compared to the MCF7-D10 cells. Differences include increased cell scattering and more elongated fibroblastic-like cells (Figure 4.5). Immunostaining revealed strong binding of 2448 on the membranes and tight junctions of live MCF7-D10 cells but not MCF7-2101 cells (Figure 4.5A). When cells were fixed and permeabilised, punctuated staining patterns of 2448 were observed, confirming the presence of intracellular antigens (Figure 4.5B). Overall, 2448 specifically recognised antigens that were exposed on the surface of cells with an epithelial phenotype.

Figure 4.5 Microscopy images of 2448 on MCF-D10 and MCF7-2101 isogenic cell lines. Cells were stained with 2448 directly conjugated with FITC (2448-FITC). Phase contrast images revealed EMT-like morphological changes. MCF7-D10 cells were more epithelial with cuboidal or “cobblestone-like” cells compared to MCF7-2101 cells which were more isolated and elongated. (A) Binding of 2448 was observed on the membrane of MCF7-D10 cells. No binding was observed on live MCF7-2101 cells. (B) Binding of 2448 was observed on both fixed and permeabilised MCF7-D10 and MCF7-2101 cells.

4.3 Characterisation of chimeric variants, ch2448 and chC51

To date, most cancer therapeutic mAbs are IgGs that are at least chimerical, if not humanised. Therefore, in order to develop 2448 and C51 as therapeutics, chimeric variants were generated. The variable genes from the mouse hybridomas of 2448 and C51 were isolated and cloned into multi-promoter single expression vectors containing human IgG₁ constant genes [304]. These vectors were then transfected into DG44 Chinese hamster ovary (CHO) cell lines which enabled high expression of chimeric 2448 (ch2448) and C51 (chC51). Peptide sequencing by mass spectrometry confirmed matching identity of the variable regions of ch2448 and chC51 with the parental 2448 and C51 respectively (data not shown).

To confirm no loss of binding specificity after chimerisation, ch2448 and chC51 were then tested on IGROV1, a positive antibody-binding cell line and IOSE523, a non-binding cell line. Both ch2448 and chC51 retained binding on IGROV1 cells and no binding was observed on IOSE523 (Figure 4.6, *left and right panels*). A flow cytometry-based competitive inhibition assay was done to confirm no loss of epitope specificity. When 2448 and C51 were pre-incubated with IGROV1 cells, binding inhibition of the respective chimeric variants was observed as shown by a (leftward) shift of fluorescence intensity (Figure 4.6, *middle panels*). Taken together the experiments showed no loss epitope specificity for the chimeric mAbs.

Figure 4.6 Comparison of binding between mouse and chimeric variants. Cells were pre-incubated with mouse mAb (or negative binding isotype control) followed by chimeric mAbs. Binding of chimeric mAbs was inhibited by mouse mAbs (*top middle and bottom middle panels*) compared with isotype controls (*top left and bottom left panels*). Binding of the chimeric mAbs was not observed on the IOSE523 cell line.

4.4 Binding isotherms of 2448 and C51

Next, binding isotherms (i.e. binding curves) of 2448 and C51 were generated by flow cytometry (Figure 4.7A). Live IGROV1 cells were incubated with various concentrations of mAb. Saturation of the cell surface was observed at 10^{-4} M. To eliminate possible overlapping of mAbs, Fab fragments were also generated and tested. For both 2448 and C51, saturation occurred at micromolar concentrations with a linear concentration range between 10^{-8} M and 10^{-6} M. At saturating conditions, higher maximum fluorescence of chC51 suggested more antigen binding sites per cell for chC51 compared to ch2448 (Figure 4.7A).

The relative affinities were then estimated based on the binding isotherms. Some indication of the antibody binding kinetics can be extrapolated from flow

cytometry-based measurements [351–354]. A simplified method using linear transformation of the saturation binding curves was used to compare apparent affinities of 2448 and C51 [355]. Based on the model, the dissociation constant (K_d) of the mAbs were found to be close in range with one another (at 0.27 μM for ch2448 and at 0.22 μM for chC51) (Figure 4.7).

These values closely correspond with affinity measurements of 2448 done on hESC [16]. In that more extensive study, binding isotherms and batch uptake kinetics were used to calculate a binding affinity (K_d) of 1.5 μM . Characterisation of the binding kinetics (e.g. by Surface Plasmon Resonance or other methods) are ongoing and will be discussed in Chapter 7.

Figure 4.7 Binding isotherms of 2448 and C51 on live IGROV1 cells. (A) Various concentrations of 2448, C51, and Fab fragments ch2448 and chC51 were tested for binding on IGROV1 cells. Estimated linear ranges of binding (LR) were observed by. Binding was measured as the normalized geometric mean fluorescence (nGMFI) using fluorescence activated cell sorting (FACS). (B) Saturation binding curves of ch2448 and chC51 Fab fragments were generated by the receptor binding model equation: $Y = B_{max} * X / (K_d + X) + NS * X + B$; B_{max} is the maximum specific binding in Y units (GMFI, geometric mean fluorescence intensity); K_d is the equilibrium binding constant or the concentration to achieve half-maximal binding at equilibrium in X units (μM); NS is the slope of nonspecific binding; and B is the background amount of nonspecific binding with no added Fab fragments. The estimated K_d values of ch2448 and chC51 were relatively close to one another.

4.5 Antigen target identification of 2448 and C51

4.5.1 Antibodies 2448 and C51 recognize similar antigen bands

In order to characterise the antigen targets of 2448 and C51, antibody detection on western blot was done against three cell lines that are commonly used in ovarian cancer research: IGROV1, OVCAR3 and SKOV3 (Figure 4.8). Membrane protein enrichment of lysate was required to visualize antigen bands of 2448. Protein smears with a range of molecular weights from around 33 kDa to 40 kDa were observed on the IGROV1, OVCAR3 and SKOV3 cell lines. Similar to results from flow cytometry (Figure 4.1), 2448 and C51 shared similar binding patterns on western blot (Figure 4.8)

Figure 4.8 Detection of antigen targets on western blot. Membranes were immunoblotted (IB) with 2448 and C51. Both antibodies recognised western blot protein bands around 39 kDa on sample preparations of IGROV1, OVCAR3 and SKOV3 cancer cells. Bands appeared as smears ranging in molecular weights from around 33 kDa to over 40 kDa.

4.5.2 Identification of annexin A2 (ANXA2) as the antigen target

Next, antigen identification was carried out by immunoprecipitation (IP) followed by mass spectrometry (MS) analysis. IP with 2448 and C51 were done to enrich for their respective antigen of interest (Figure 4.8). On a parallel silver stained gel, protein band corresponding to those of the western blot

were then excised for mass spectrometry analysis. Interestingly, the top hit for both 2448 and C51 was identified as human annexin A2 (ANXA2 Accession No. P07355). The finding was consistent in multiple rounds of independent experiments and peptide coverage > 50% was found to span across the amino acid sequence of ANXA2.

Figure 4.9 Identification of annexin A2 as the antigen target. Immunoprecipitation (IP) with 2448 and C51 enriched for unique antigen bands around 39 kDa (←). Bands were not present on the respective antibody alone control lanes. Target identification was carried out by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for multiple rounds of IP. A search against protein databases yielded a match to annexin A2 (ANXA2), as shown by the sample description. Peptide coverage (as highlighted) spanned across the amino acid sequence of ANXA2 (> 50%). Ab = antibody AA = amino acid, MW = molecular weight.

To confirm ANXA2 as the antigen target, a cross-immunoprecipitation was also carried out using a commercial anti-ANXA2 mAb (Figure 4.9). Samples from the IP with 2448 were immunoblotted against 2448 and the commercial antibody. The reverse procedure was also done using the commercial anti-ANXA2 mAb. 2448 and the commercial mAb were able to recognise similar antigen bands on both IP products (Figure 4.9A). These results showed that 2448 recognised and was able to immunoprecipitate ANXA2. Similar findings were observed for C51 (Figure 4.9B).

A

B

Figure 4.10 Confirmation of annexin A2 as the antigen target. A cross-immunoprecipitation (IP) was done with a commercial anti-annexin A2 mAb (α -ANXA2) was carried out. When the IP product of (A) 2448 and (B) C51 was probed with the commercial mAb, an antigen band of similar size was observed. Cross-IP with the commercial mAb pulled-down a band of similar size, which was reactive to 2448 and C51. (C51 data generated by research student Ms. Melissa Wirawan.)

To validate the IP/mass spectrometry study [356], a knockdown study of ANXA2 in was carried out. IGROV1 Cells were transfected with a validated pool of ANXA2-specific siRNAs. Partial knockdown (40 to 60% decrease) of ANXA2 was observed using a commercial anti-ANXA2 mAb on western blot (Figure 4.11). As 2448 required membrane-protein enrichment for visualisation of antigen bands Figure 4.8, knockdown was done on both membrane protein-enriched lysate (Figure 4.11A) and whole cell lysate (Figure 4.11B). Knockdown of ANXA2 corresponded to a (70-80%) loss of antigen recognition by 2448 and C51. (As C51 did not required membrane-protein enrichment, western blotting was done on whole cell lysate.) Overall, recognition of 2448 and C51 (Figure 4.11B) was markedly decreased in the ANXA2-knockdown-lysate versus the control, confirming ANXA2 as the antigen target of 2448 and C51.

Figure 4.11 Validation of antigen target by siRNA-mediated knockdown of annexin A2. IGROV1 cells were treated with ANXA2 siRNA. A decrease in ANXA2 expression on cell lysate corresponded to a loss in antigen recognition for (A) 2448 and (B) C51. As a control, a commercial anti-ANXA2 mAb was used to demonstrate downregulation of ANXA2 expression. Results were normalised against beta-actin.

4.6 Elucidating the epitopes of 2448 and C51

4.6.1 Antibodies 2448 and C51 bind to N-glycan-dependent epitopes

Unlike the commercial anti-ANXA2 mAb, 2448 and C51 recognised antigen bands that remained smeared even after forward or reverse immunoprecipitation (Figure 4.10). The results suggested that the smeared bands were not due to protein degradation or overloading but rather differences in epitopes, possibly glycosylation. The ANXA2 amino acid

sequence contains potential glycosylation sites for both N and O glycosylation [357]. Although not studied in detail, glycoforms of annexin A2 have been identified in different cell types [358–362]. For example, in ovarian cancer the existence of multiple isoforms of ANXA2 was identified by 2D gel electrophoresis followed by mass spectrometry [363] and in another study, a positive correlation between histological expression patterns of ANXA2 and the Lewis Y antigen was reported in ovarian cancer tissues [364]. Therefore, to investigate if 2448 and C51 were binding to a glycosylated motif, a series of glycan experiments was conducted.

Periodate treatment of lysate creates a mild oxidative reaction, cleaving the cis-diols of glycans and creating amine-reactive aldehydes. After treatment, antibody recognition of glycans is abolished. First, periodate treatment of IGROV1 lysate was done after SDS-PAGE and western blotting (Figure 4.12). Binding of 2448 and C51 was completely abolished suggesting that antibody binding to its antigen was dependent on glycosylation.

Glycans on membrane proteins are generally classified as N-linked glycans or O-linked glycans. N-linked glycans attach to an asparagine or arginine side chain residue proteins via a glycosidic amide bond. O-linked glycans attach to protein via a hydroxyl oxygen of a serine or threonine residue [365]. To determine if mAb binding required N-glycosylation, lysate was digested with the enzyme peptide N-glycosidase (PNGase) F and probed with 2448 (Figure 4.12A) and C51 (Figure 4.12B). From the western blot analysis, the original antigen bands of 2448 and C51 were lost after the removal of N-linked glycans with PNGase F. To confirm the results, another experiment was done using tunicamycin, an inhibitor of N-glycoprotein synthesis. Cells were incubated with tunicamycin in culture for 48 h. Cells were then lysed and resolved on by SDS-PAGE. From western blot analysis, binding was similarly loss on tunicamycin-treated cells. Unlike 2448 and C51, the commercial anti-ANXA2 mAb was not sensitive to any of the de-glycosylation treatments. Interestingly, no drop in molecular weight was observed after PNGase F treatment. Therefore, ANXA2 was suspected to not be highly glycosylated. Taken together, it was concluded that the binding of 2448 and C51 is associated with N-glycosylation.

Figure 4.12 N-glycosylation epitopes of 2448 and C51. Binding of (A) 2448 and (B) C51 after chemical degradation of glycans and inhibition of glycan synthesis. IGROV1 lysate was resolved on SDS-PAGE and transferred to PVDF membrane. The membrane was treated with and without periodate followed by borohydride and then subjected to immunoblot with 2448. Antigen bands were completely abolished after treatment with periodate. Removal of N-glycans was done using two methods. Lysate was first treated with PNGase F then resolved on SDS-PAGE and subjected to western Blot. Cells were cultured in the presence of tunicamycin then lysed and subjected to western Blot. Both treatments resulted in the loss of antibody recognition of 2448 and C51. (C) Alternatively, the commercial anti-ANXA2 mAb was not sensitive to any of these treatments.

4.6.2 Antibodies 2448 and C51 bind to different epitopes

As both 2448 and C51 bound to N-glycan epitopes of ANXA2, a series of flow cytometry-based competitive inhibition assays were subsequently done to determine if the antibodies were binding to the same epitope. Two secondary antibodies with different fluorophores were used to independently evaluate the binding of 2448 and C51. The secondary antibodies were specific for either the heavy and light chains of gamma immunoglobulins or the mouse mu

immunoglobulin, which was confirmed by single staining (Figure 3.13A, *top panels*).

Partial binding inhibition of 2448 was observed when cells were pre-incubated with C51 (Figure 3.13A, *bottom right panel*) compared to when mAbs were incubated simultaneously (Figure 3.13A, *bottom left panel*). A negative control experiment with appropriate isotypes was also carried out (data not shown). The partial inhibition of 2448 suggested that the epitopes of the mAbs were either the same or in close proximity to one another, with the possibility of steric hindrance. Interestingly, no inhibition of C51 was observed when the experiment was reversed. Binding of C51 did not decrease when cells were pre-incubated with 2448 (Figure 4.13A, *bottom middle panel*).

The difference might be due in part to differences in isotypes, as 2448 is an IgG and C51 is an IgM. The multivalency of C51 may confer stronger avidity, allowing C51 to outcompete 2448. Alternatively, C51 may also elicit dramatic changes in antigen expression by its increased ability to crosslink the antigen target.

To mitigate these scenarios and investigate if C51 was inhibiting 2448 by steric hindrance, 2448 was next compared with the IgG variant of C51, chC51. No significant difference in binding was observed when cells were incubated in the presence of 2448 and chC51 regardless of the order in which the mAbs were added (Figure 4.13B, *bottom three panels*). Therefore 2448 and ch51 did not competitively inhibit one another, indicating both had distinct and non-competing epitopes. Taken together, these results suggest that 2448 and C51 bind in close proximity to one another, but do not share the same epitope. Furthermore, the apparent inhibition of 2448 by C51 was due to steric hindrance.

Figure 4.13. Competitive binding inhibition of 2448 and C51. Binding inhibition was measured by flow cytometry. (A) Binding of 2448 was significantly inhibited by C51 as indicated by a downward shift (↓) in fluorescence (*bottom right panel*). (B) No competitive inhibition was observed between 2448 and chC51. No significant shifts in fluorescence was observed when mAbs were incubated together (*bottom panels*) compared to the single stain controls (*top middle and right dot plots*). Note: The top panels of (A) and (B) depict negative staining and single stains of antibodies. The bottom panels of (A) and (B) depict double stains with mAbs incubated at the same time (*bottom left panel*) or one after the other (*bottom middle and right*)

4.7 Comparison study with a commercial anti-ANXA2 mAb

Previous results showed that 2448 and C51 target N-glycan epitopes of ANXA2. In order to determine if the ANXA2 epitopes bound by 2448 and C51

are unique, the mAbs were then compared with a commercial anti-ANXA2 mAb targeting amino acids residues 123- 328. This mAb was used in multiple ANXA2-related publications [366, 367]. A polyclonal antibody (pAb) targeting human ANXA2 at amino acids 1-50 was also evaluated.

By flow cytometry, only 2448 and C51 bound on the cell surface of live IGROV1 ovarian cancer cells. In contrast, the commercial antibodies were unable to bind to the cell surface on live cells but showed significant binding after the cells were permeabilised. The data suggest that 2448 and C51 are binding to unique epitopes on the extracellular surface of the cancer cells whilst the commercial antibodies were detecting intracellular ANXA2. Similar binding properties were found on other cancer cell lines (data not shown).

Figure 4.14 Cell surface binding of 2448, C51 and commercial anti-ANXA2 antibodies. IGROV1 cells were stained and measured by flow cytometry. Both 2448 and C51 showed binding to live cells compared to commercial anti-ANXA2 antibodies (as indicated by a shifts in the red-lined histograms). Binding of commercial anti-ANXA2 antibodies was only observed when cells were fixed and permeabilised.

To further investigate, ANXA2 expression was also analysed by western blot Using lysate from different ovarian and breast cancer cell lines (Figure 4.15) parallel blots with same amounts of lysate for each cell line was probed with either the commercial mAb or 2448. Protein expression was then compared.

Antibody recognition with the commercial anti-ANXA2 mAb (Figure 4.15A) was dramatically different compared to 2448 (Figure 4.15B). Antigen bands also appeared sharp and more distinct with most of them detected as single bands.

Two exceptions were MCF7 and OVCAR3 which had multiple bands, possibly indicating fragmentation. Similar glycosylation experiments (data not shown) as described in Section 4.6.1 also revealed that binding of the commercial mAb was not sensitive to periodate treatment. Therefore unlike 2448, binding was not to a glycan epitope. The commercial mAb also detected ANXA2 on all seven cell lines regardless of their EMT classification. Conversely, 2448 was only able to recognise a subset of cancer cell lines by western blot analysis (as shown on western blot run in parallel (Figure 4.15)). The data corresponded to binding based on flow cytometry as previously described (Figure 4.1). Overall, the results provided supporting evidence that the hESC-generated mAbs targeted unique epitopes of cell surface ANXA2.

A

B

Figure 4.15 Expression of annexin A2 on membrane-protein (MP) enriched lysate. Antibody recognition of ANXA2 was compared (A) The commercial mAb recognised ANXA2 in all cancer cell lines (as shown by western blot). (B) Antibody 2448 only recognised ANXA2 on a subset of cells that were classified as having an epithelial phenotype of EMT.

4.8 Summary and Discussion

4.8.1 Summary

2448 and C51 were identified as potential therapeutic candidates for ovarian and breast cancer therapy. These mAbs were generated by whole cell-immunisation of hESC in mice and subsequently found to bind on the surface of various cancer cells. Minimal binding was found on normal cell lines. This chapter reports the *in vitro* characterisation of 2448 and C51.

The binding selectivity of 2448 and C51 was first studied within ovarian and breast cancer. Screening against live cells was done and the mAbs were found to preferentially bind on a subset of cancer cell lines. Based on an EMT spectrum (from epithelial to mesenchymal), these cells expressed an epithelial phenotype. The respective chimeric variants ch2448 and ch51 were also characterized. The chimeric mAbs retained binding specificity of the parental mAbs and had similar saturation binding curves to one another.

IP followed by LC-MS/MS analysis identified annexin A2 (ANXA2) as the antigen target for both 2448 and C51. Moreover, treatments with glycosylation inhibitors or enzymatic digest of glycans showed that antibody binding was via N-glycosylation. As a comparison, a study with a commercial mAb was done. Competition experiments showed that the epitopes of 2448 and C51 were unique and non-overlapping. Based on western blot analysis, both mAbs bound a subset of ANXA2 present in cancer cells.

4.8.2 Discussion

4.8.2.1 Background of annexin A2 in cancer

Annexin A2 is a member of the annexin family of Ca^{2+} -dependent phospholipid binding proteins [357, 368]. ANXA2 can be found in the nucleus, but is predominantly localized in the cytoplasm and membrane surfaces of cells. It also exist in multiple forms, both as a monomer and as a heterotetramer in complex with the plasminogen receptor protein S100A(p11) [368–371].

As a multifunctional protein, ANXA2 is found in various normal cells including endothelial cells [372–375] and skin keratinocytes [376]. ANXA2 was also reported on various normal tissue such as the lung and female reproductive tract (see The Human Protein Atlas (THPA) [377]). ANXA2 has not been extensively studied in hESC nor been identified as an oncofetal protein. Based on proteomics data from our institute [378], ANXA2 was shown to be present in hESCs, induced pluripotent stem cells (iPSCs) and foreskin fibroblast.

Multiple studies have described ANXA2 as a potential biomarker or therapeutic target in cancer (as reviewed in [379–382]). In contrast to tumour suppressors (e.g. p53), the ANXA2 gene (Gene ID: 302) is not commonly mutated in cancer. Aberrant mRNA and protein expression have been identified in a variety of cancers (as summarized in Table 3.1). In addition, high ANXA2 expression was also associated with poorer clinical outcomes [382]. These include lower overall survival (OS), lower disease-free survival (DFS) and an increased likelihood of tumour invasion, lymph node metastasis and chemoresistance.

To date, no clinical trials using mAbs targeting ANXA2 have been published. However, ANXA2 has been clinically tested as a serum diagnostic marker for hepatocellular cancer [383]. Additionally, ANXA2 was identified as a molecular target for chlorotoxin, a cancer-targeting peptide which is currently being investigated as a drug and as an optical imaging agent for gliomas [384–386] .

Recently, ANXA2 was identified as a potential therapeutic target for epithelial ovarian cancer [366, 387]. High levels of ANXA2 in the membrane and cytoplasm was reported in serous ovarian cancer tissue and its surrounding stroma [388]. Analysis of publicly available microarray data sets also revealed a negative association between high ANXA2 mRNA and IHC expression with ovarian cancer patient survival [388].

Table 4.1 Expression of annexin A2 in cancer

	Cancer	Expression	Reference
1	ALL	↑ (gene) prednisolone-resistant infant ALL	[389]
2	APL	↑ (mRNA) levels in promyelocytic cell line vs early myeloid cell line	[390]
3	Angiosarcoma	↑ in benign and malignant vascular tumour tissue	[391]
4	Breast	↑ in invasive cell lines and cancer tissue	[392, 393]
5	Cervical	↑ in cancer tissue	[394–396]
6	Colorectal	↑ in cancer tissue and correlation with histopathological grade	[397, 398]
7	Head and neck	↓ poorly differentiated tumours and dysplastic tissue	[399]
8	ISA	↓ cancer tissue	[400]
9	ESCC	↓ in cancer cell lines and tissue	[401, 402]
10	Endometrial	↑ in cancer cell lines and tissue	[387, 403]
11	Gastric	↑ in cancer cell lines and tissue	[404–406]
12	Glioma	↑ in high grade cancer cell lines and tumour tissue	[407]
13	Lung	↑ in cancer cell lines and tissue	[393, 408]
14	MM	↑ cancer cell lines	[409]
15	OSCC	↑ in cell lines and correlation with histopathological grade of tumour tissue	[410, 411]
16	Osteosarcoma	↓ in metastatic cell line and (mRNA) levels in metastatic tissue vs. primary tumour	[412]
17	Ovarian	↑ in cancer cell lines and tissue	[364, 366, 387, 413]
18	Pancreatic	↑ in gemcitabine-resistant cell lines and invasive cell line	[414–416]
19	Prostate	↑ and ↓ expression (mRNA and protein) in cancer cell lines and tissue.	[417–422]
20	Renal clear cell	↑ in metastatic tumour tissue and correlates with differentiation and clinical outcome	[423, 424]

↑ = Increased expression, ↓ = Decreased expression, ALL - Acute lymphoblastic leukaemia, APL - Acute promyelocytic leukaemia, ISA - Intestinal-type sinonasal adenocarcinoma, ESCC - Oesophageal squamous cell, MM - Multiple myeloma, OSCC - Oral squamous cell carcinoma.

The role of ANXA2 in cancer progression has not yet been fully elucidated. Various studies have shown that ANXA2 can play a role in tumour cell adhesion [366, 425–427], migration and invasion [366, 367, 407, 428–430], proliferation [431], neovascularization and angiogenesis [375, 432] and membrane trafficking [433–436].

Figure 4.16 Functional roles of annexin A2 in cancer. Annexin A2 is a multifunctional protein with various reported roles in cancer. The glycoprotein has multiple interacting partners.

4.8.2.2 Targeting unique cell-surface, N-glycosylated epitopes

Antibodies 2448 and C51 were shown to target unique non-competing oncofetal epitopes of ANXA2. These epitopes were found to share three important traits: cell surface recognition, N-linked glycosylation and restricted expression on cells with an epithelial phenotype of EMT. Importantly, conventional antibody generation techniques may not be able to replicate these unique properties of 2448 and C51.

First, surface epitopes of ANXA2 may be challenging to recapitulate in recombinant peptides. ANXA2 has been reported at the plasma membrane both in the inner leaflet and at the cell surface for multiple cell types including cancer [361, 367, 371, 425, 437–441]. However, ANXA2 lacks a transmembrane domain and a classical signal peptide sequence. Instead, various mechanisms have been proposed for its translocation to the cell

surface. For example, ANXA2 may be anchored by an ANXA2-receptor [442] or shed in association with other proteins [435, 443, 444]. Post-translational mechanisms have also been proposed as a mechanism of surface expression. ANXA2 was shown to translocate from the cytosol to the cell surface by a tyrosine 23 (Tyr23) phosphorylation-dependent manner in breast cancer [367, 445] and by glycosylation in various cell-types [440, 361]. ANXA2 at the cell surface has been associated with fibrinolysis, exocytosis and endocytosis, cell-cell adhesion, and membrane-cytoskeletal interactions. For example, knockdown of ANXA2 in ovarian cancer cells resulted in the loss of the ability to bind to peritoneal cells in a co-culture system [366]. In breast and pancreatic cancer, ANXA2 was reported to promote cancer progression via binding strongly to proteins like the p11, cathepsin B, tenascin C and t-PA. These facilitate plasmin generation and a proteolytic cascade. This activation of pro-enzymes leads to selective degradation of the ECM and thus, enhances cancer cell migration and invasion.

Besides targeting the cell surface, 2448 and C51 also bind preferentially on cells that express an epithelial phenotype. Identification of cell populations expressing an epithelial phenotype could potentially be used to monitor EMT progression as well as aid in the development of anti-EMT therapies [65, 72, 74].

The role that ANXA2 plays during the EMT process has not been fully elucidated. Several other studies also suggest that ANXA2 is linked to cancer-related EMT [367, 405, 446–449]. Interestingly, downregulation of a phosphorylated form of ANXA2 correlated with the loss of ability of pancreatic cancer cells to undergo TGF β -Rho mediated EMT [367]. Knockdown of ANXA2 in breast cancer cells also resulted in a more epithelial phenotype and the loss of its ability to undergo EGF-induced EMT [432, 447]. Therefore, ANXA2 was proposed as a promoter of EMT by activation of the EGFR pathway, a critical pathway in EMT. Interestingly, a negative correlation was observed between the expression of ANXA2 and E-cadherin, an epithelial cell marker of EMT [405, 447, 450]. These studies suggest that ANXA2 could be a better mesenchymal marker in gastric and breast cancer. Alternatively, ANXA2

is known to complex with an S100A10 protein involved in the formation of E-cadherin-based adherens junctions in Madin-Darby Canine Kidney Cells [451].

Overall, the detailed role(s) of ANXA2 during EMT has not been elucidated. From results of this thesis, antibody recognition of 2448 and C51 were lost in cells expressing a more mesenchymal phenotype. The differences expression is most likely due to the unique epitopes of 2448 and C51. Overall, the findings suggest that the role of ANXA2 may be complex.

As mentioned earlier, ANXA2 is subjected to various post-translational modifications, mostly in its amino terminal (Figure 4.17). Different isoforms of ANXA2 have been identified in two-dimensional gel electrophoresis studies. Truncated forms of ANXA2 due to proteases such as MMPs, glycogen synthase and plasmin were reported in human monocytes, endothelial and cancer cells [425, 431]. Various phosphorylated forms of ANXA2 have also been reported [367, 389, 429, 438, 452].

2448 and C51 were shown to bind N-glycosylated ANXA2 (Figure 4.12). Furthermore, these were shown to be non-competing epitopes suggesting that the branching of glycans was sufficiently extensive so that neither (as IgGs) sterically interfered with the others binding. The unique glycosylation of ANXA2 may explain why 2448 and C51 target epithelial cells and not mesenchymal cells, which also express ANXA2 according to a commercial anti-ANXA2 mAb (Figure 3.15).

Oncofetal antigens have been associated with changes in glycosylation (see Chapter 1, Table 2.2). Furthermore, changes in cell surface glycosylation has also been implicated in tumour progression and EMT (as reviewed in [453, 454]). For example, oncofetal fibronectin [455, 456] is defined by site-specific O-glycosylation that occurs during EMT [161, 457, 458].

As mentioned earlier, glycoforms of ANXA2 have yet to be studied in detail, but were identified in numerous cell types [361, 358, 360, 359]. For example, N-glycosylation of ANXA2 was shown to be critical for surface translocation in human airway epithelial cells [361]. Interestingly, glycosylation was also proposed as an alternative way for ANXA2 translocation in HER2-negative breast cancer cells [440]. Furthermore, cell surface ANXA2 was reported to

interact with galectin-3, a highly specific plant lectin that binds to *N*-acetyl-D-galactosamine [440]. Findings of this chapter have yet to determine the exact epitope of 2448 and C51. Based only on the results presented here alone, it is difficult to determine the extent of antibody-binding to the glycan portion or the peptide portion of annexin A2. Due to the difficulty of generating soluble ANXA2 with the appropriate glycosylation, no epitope mapping was done. Based on potential glycosylation sites as shown in Figure 4.17, binding is likely around extensive glycans at asp-52 [357], This site is located close to the variable N-terminus of the ANXA2 amino acid sequence (Figure 4.17A and B). Further investigations into the glycan-epitope of 2448 and C51 are ongoing.

Figure 4.17 Annexin A2 schematic with glycosylation sites.(A) Representation of annexin A2 (ANXA2) domains and reported glycosylation sites, cleavage sites, phosphorylation site, and hydrophobic residues. (B) Full amino acid sequence of ANXA2 with consensus N-glycosylation sequence at asparagine (N) 52. (C) The crystal structure of ANXA2 (Protein Data Bank (PDB) ID: 1XJ; 10.2210/pdb1xjl/pdb) with a highlighted potential N-glycosylation site (↑).Note: ^a, ^b and ^c denote that sites were determined by web-based tools (https://www.expasy.org/proteomics/post-translational_modification)

Overall, the ability of 2448, ch2448, C51 and chC51 to bind cell surface and glycosylated epitopes of ANXA2 may confer added binding specificity compared to other anti-ANXA2 mAbs (Table 3.2). These would be important properties for drug targeting of ANXA2, which is not only upregulated in cancer but also ubiquitously present in many different cell types. In the next chapter, the epitopes of 2448 and C51 are functionally validated. Various potential therapeutic mechanisms of actions are explored.

Table 4.2 Epitopes of 2448, C51 and other anti-ANXA2 antibodies

Ab-binding property	2448	C51	Commercially-available		Examples from literature	
			mAb (#5)	pAb (#9061)	mAb ^a	mAb ^b
EMT marker	✓	✓	-	-	NR ^c	NR ^d
Cell surface	✓ (F, IB, IF)	✓ (F, IB, IF)	-	-	(IF/IB/IHC)	(IF/IB/IHC)
Anti-glycan	✓	✓	-	-	NR	NR
Immunogen	hESC	hESC	aa.123-328	aa.1-50	Fragments f/ BAE using angiostatin aa. 314-324, 234-245, 330-339	AG1523 fibroblasts of human origin

✓ = Yes, - = No, NR = not reported, Ab = antibody, mAb = monoclonal antibody, pAb = polyclonal antibody, ANXA2 = Annexin A2, EMT = Epithelial to Mesenchymal Transition, F = flow cytometry, IB = immunoblotting, IF = immunofluorescence (microscopy), IHC = Immunohistochemistry, aa. = amino acids, hESC = human embryonic stem cells, BAE = bovine aortic endothelial cells

^a[459], ^b[460]

Chapter 5 Functional studies of 2448 and C51, *in vitro*

5.1 Introduction

The previous chapter detailed results on the characterisation of monoclonal antibodies (mAbs) 2448, C51 and their respective chimeric variants, ch2448 and chC51. These mAbs were shown to bind on the surface of ovarian and breast cancer cells, making them potential candidates for mAb-based therapy

Therapeutic mAbs can exert various mechanisms of action (MOA) for mediating anti-tumour activity. Important mechanisms include direct tumour cell-killing e.g. antagonism or receptor signalling pathways, immune modulation e.g. Fc-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity, and delivery of a cytotoxic payload as an antibody drug conjugate (ADC) (see Chapter 2, Table 2.4). Determining the relative contribution of each MOA can be challenging even when mAbs are used as a single agent. Most mAbs elicit more than one mechanism with additive, overlapping or synergistic effects impacting overall clinical benefit.

Although a complete understanding of an antibody's MOA is not a requirement for clinical success, functional evaluation of a mAb is important in providing insight into whether a mAb and its epitope is suitable for therapeutic development. In this chapter, an *in vitro* functional study of the mAbs was done. In order to demonstrate the potential of these mAbs to be used as therapies, three MOA of 2448, ch2448, C51 and chC51 were investigated: direct tumour cell killing via oncosis, immune-mediated tumour cell killing via ADCC or CDC activity and the intracellular delivery of cytotoxic agents.

5.2 Results

5.2.1 Direct tumour cell-killing

Direct tumour cell-killing involves an antibody binding to a tumour cell and endogenously inducing cytotoxicity. For example, a mAb can act as a receptor antagonist, inhibiting signalling pathways by blocking ligand-receptor interactions and downregulating receptors (see Chapter 2, Section 2.2.4.1)

Many of these mechanisms involve the cell-programmed death program, apoptosis [461]. Alternatively, direct cell-killing can be via non-apoptotic pathways. For example our group previously reported on the ability of an anti-PODXL mAb to induce a dose-dependent oncotic death mechanism in human embryonic stem cells (hESC) [17]. This mechanism involves rapid homotypic cell aggregation and pore formation on the plasma membrane. Other groups have also observed this death mechanism with other mAbs and cell types. These include anti-glycan mAbs against cancer [168–170]. Most notably, type 2 anti-CD20 mAbs for the treatment of B-cell lymphoma [462, 463] were functionally-defined based partly on its ability to induce such a death mechanism [464, 465].

Antibodies 2448 and C51 were first assessed for the ability to directly kill cells via oncosis. Single cell suspensions were incubated with supernatant from the hybridoma clones of 2448 and C51 or media-only controls. To arrest apoptosis, studies were conducted at low temperature [166, 466, 467]. Cells treated with 2448 had no significant decrease in cell viability (even at saturating conditions). In contrast, cells treated with C51 had a significant and rapid increase in PI uptake, indicating cell-death associated with a loss of membrane integrity, a hallmark of oncosis (Figure 5.1). Less than 50% relative viability was observed in IGROV1 and MCF7 cells treated with C51 compared to media-only control cells. 60% and 90% relative viability was observed for T47D and SKOV3 respectively. Furthermore, no cytotoxicity was observed in cell lines that did not bind to C51 (MCF7-2101 and IOSE523). Minimal cytotoxicity was observed in media-only controls.

The chimeric version of C51, chC51 was then investigated. Interestingly, even with increasing concentrations of up to 1 mg/mL and doubling the length of incubation time, minimal increases in direct cytotoxicity was observed in multiple cell lines (data not shown). Overall, results indicated that pentameric C51 (and not monomeric 2448, ch2448 and chC51) was capable of endogenously producing observable oncotic cell death. The lack of direct killing for chC51 suggested that hyper-cross-linking of the antigen target, possibly due to the valence of an IgM might be critical for direct cell-killing via oncosis. Similar studies with mAb 84 showed that oncotic cell death was

associated with the ability of an IgM (versus a bivalent, IgG) to cross-link multiple antigens across the cell surface [17, 468]. A similar mechanism might be occurring for C51.

A

B

Figure 5.1 Direct cell-killing of C51. (A) Representative binding profiles and PI uptake on MCF7-10 and MCF7-2101 after cells were treated with supernatant from hybridoma clones or media only control. (B) Summary of C51 direct cell-killing on IGROV1, SKOV3, IOSE523, MCF7 and T47D cell lines. Cytotoxicity was observed as a decrease in viability normalized against no treatment control. C51 was cytotoxic to IGROV1, SKOV3, MCF7 and T47D cancer cells but not cytotoxic to the immortalized ovarian cell line IOSE523. Results are represented as mean $\pm$ standard deviation of at least 2 independent experiments. (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, unpaired t-test).

5.2.2 Immune-mediated cell killing

Since limited direct-tumour cell killing was observed for 2448 and ch2448, the mAbs were then tested for other functional activity. Immune-mediated tumour cell killing is associated with interaction of the antibody Fc region with different components of the innate immune system. Two immune-mediated mechanisms were tested: Antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Both have been reported as important mechanisms of action for mAbs such as trastuzumab and rituximab.

5.2.2.1 Investigation of CDC activity

First, to test for CDC activity (see Chapter 2, Section 2.2.4.3), mouse mAbs (2448, C51) and chimeric mAbs (ch2448, chC51) were co-incubated with complement proteins from normal human serum (Figure 4.2A). C51 demonstrated detectable levels of cell lysis in the presence of human complement. Up to 30% lysis was observed at high binding saturation concentrations of C51. For controls, cells were incubated with mAb and heat-inactivated complement. To validate these results, conserved sites that are important for C1q binding were also identified on the peptide sequence of mouse C51. Important residues for human IgG₁ include D270, K322, P329 and P331 of the C_H2 domain [190–192, 469–471]. Surprising though, C1q binding sites were also identified on 2448, chC51 and ch2448 but these mAbs were not able to elicit CDC activity.

Figure 5.2 Immune-mediated mechanisms of action (A) CDC activity was measured after plated IGROV1 cells were incubated in the presence of antibody and serum containing complement activation fragments. Cytotoxicity was measured by level of LDH present in the cell culture wells. (B) Plated IGROV1 cells were incubated with ADCC reporter cell lines and various concentrations of antibodies. Reporter cell lines were engineered to express human Fc γ IIIa. After 6 h, ADCC activity was measured based on NFAT-mediated expression of a luciferase reporter element. $n = 2$ independent experiments

5.2.2.2 Investigation of ADCC activity

ADCC activity (see Chapter 2, Section 2.2.4.3) was measured by expression of a luciferase reporter element attached to the NFAT (nuclear factor of activated T-cells) ADCC pathway. Engineered effector cells expressed the human (high) variant Fc-gamma IIIA receptors with the V/V polymorphism. The mAbs were evaluated against two negative controls: mAbs with engineered effector cells alone and mAbs with target cancer cells alone. When target cancer cells were incubated with primary mAb and engineered effector cells, strong ADCC activity observed on chimeric mAbs. In contrast, 2448, a mouse IgG₁ with low affinity to human Fc-gamma receptors [472], showed no ADCC activity. Similarly, C51 showed no ADCC activity. Results corresponded with a lack of activity observed among other IgMs [122, 473].

To extend these observations, ch2448 (a chimeric IgG₁) was subsequently selected for a more in-depth study. ADCC activity of ch2448 was evaluated at various concentrations and across different cancer cell lines. Different levels of

ADCC activity were observed depending on the cell line. Potent ADCC activity on IGROV1 cells was exhibited with an EC₅₀ value in the micromolar (10⁻⁶ M) range. Saturation was reached at 10⁻⁵ M (Figure 5.3A). Next, three other ovarian cancer cell lines were assessed. Positive ADCC activity corresponded to the-binding of ch2448 on cells (as described in the previous chapter, see 3.5.1). OVCAR3 which had high binding of ch2448 also had greater ADCC activity. Moderate binding on the SKOV3(D10) cell line and the non-binding IOSE523 cell line (Figure 5.3A) corresponded with less potent ADCC activity. Observations were also extended to breast cancer cell lines (Figure 5.3C). ADCC activity was detected on the MCF7-D10, MCF7, T47D and HCC18937 cell lines but not the MCF7-2101 cell line.

Figure 5.3 Antibody-dependent cell-mediated cytotoxicity (ADCC) activity of ch2448. ADCC activity was measured against (A and B) ovarian and (C and D) breast cell lines. Cell lines which bound to ch2448 (IGROV1, OVCAR3, SKOV3(D10), MCF7-D10, MCF7, T47D, HCC1937) had concentration-dependent ADCC activity. Cell lines that did not bind to ch2448 (MCF7-2101, IOSE523 and BT549) had no ADCC activity. Activity was measured as fold increase against control wells without ch2448. Values are means $\pm$ standard deviations of triplicates.

5.2.2.2.1 De-glycosylation of N-glycans of ch2448

Several groups have investigated different ways to enhance ADCC activity. One approach is to increase the affinity between the Fc portion of a mAb and the human Fc-gamma IIIA receptor [474], either through amino acid engineering or glycoengineering. Glycoengineering (as reviewed in [196, 475, 476]) has successfully been used to modify Fc regions of mAbs targeting a variety of antigens [243, 477–479]. Recently, glycoengineered mAbs with enhanced ADCC activity were approved against haematological cancers expressing CD20 and CCR4 (CC chemokine receptor 4). Although no glycoengineered mAbs have so far been approved against solid tumours,

preclinical studies have shown promising results. Examples against solid tumours include anti-EGFR [241, 463] and anti-HER2 mAbs [480, 481].

A large amount of glycan heterogeneity can be found in the Fc region of a therapeutic mAbs [196, 476]. This includes different combinations of high mannose, complex and hybrid N-glycans, with or without core fucosylation (i.e. fucosylation in the innermost *N*-acetyl glucosamine). Although complete loss of these glycans can lead to protein instability or aggregation of mAbs, glycoengineering techniques can specifically add or remove glycan residues to influence Fc receptor interaction.

One well-established strategy is the removal of core (alpha 1, 6)-linked fucose at the conserved asn-297 site [239]. Loss of a core fucose can change the conformation of the IgG₁ and improve interaction between glycans on the Fc-gamma IIIA receptor and Fc regions of IgG₁ [482, 483]. This increase in affinity was shown to be specific for Fc-gamma IIIA compared to other Fc receptors, FcRn or C1q [243].

In order to test if ch2448 could be glycoengineered for enhanced ADCC activity, the N-glycosylation of ch2448 was first evaluated. *N*-glycans of ch2448 were first removed using PNGase F treatment as indicated by a lower shift in the antibody heavy chain (Figure 5.4A). After de-glycosylation, binding to its antigen target was conserved but was also accompanied by loss of ADCC activity (Figure 5.4B and C). These results corresponded to the literature [484, 485] and suggested that afucosylation of ch2448 could increase ADCC activity of non-glycoengineered ch2448 without compromising its antigen binding.

Figure 5.4 Evaluation of N-glycosylation of ch2448. PNGase F was used to remove N-glycans on ch2448. (A) The reduction in band size (*) on a silver stained gel was observed after treatment with PNGase F, indicating a large presence of N-glycans on the heavy chain of ch2448. (B) No difference in binding of the antibody to antigen was observed on IGROV1 cells by flow cytometry. (C) The enzymatic digest of IgG resulted in a loss of ADCC activity. Values are means $\pm$ standard deviations of triplicates.

5.2.2.2.2 Afucosylation of ch2448 (aF-ch2448)

Next, an afucosylated variant was generated in order to enhance ADCC activity. Several ways to produce afucosylated mAbs have been reported. These include the use of different engineered cell lines [479, 486, 487] or the use of glycosylation inhibitors [488].

The Expression Engineering Group at The Bioprocessing Technology Institute has generated novel CHO mutants with a knockout of the GDP-fucose transporter gene (Slc35c1). The GDP-fucose transporter plays an essential role in the translocation of GDP-fucose from the cytosol to the Golgi, where a fucose molecule can be added on the first *N*-acetyl glucosamine (GlcNAc) moiety of the glycan [489].

An afucosylated version of ch2448 was first generated utilizing this mutant DG44-CHO cell line. To confirm the loss of the core fucose, chimeric 2448 and aF-ch2448 were resolved on SDS-PAGE and stained with the *Aleuria Aurantia* Lectin (AAL) on western blot. The WT but not the MT was visible when stained with AAL, confirming the loss of core fucose (Figure 5.5). A drop in the percentage of fucosylation was also confirmed from 100% to < 1.5%, based on glyco-analysis studies (data not shown). To determine that the antibody-antigen binding was not affected, a binding titration curve was analysed by flow cytometry on IGROV1 cells. Both ch2448 and aF-ch2448 showed similar binding on cells, indicating no loss of specificity (Figure 5.5).

Figure 5.5 Characterisation of afucosylated ch2448 (aF-ch2448). (A) Confirmation that aF-ch2448 (Mutant) lacks the core fucose but retains binding specificity of ch2448 to IGROV1 cells. Silver stain shows antibodies at ~150 kDa in non-reducing conditions. Samples that were run in parallel show that the *Aleuria Aurantia* lectin was not able to recognize aF-ch2448, demonstrating loss of core fucose. (B) The binding specificity of aF-ch2448 (Mutant) retains similar specificity of ch2448 on live IGROV1 cells by flow cytometry. nMFI = normalized mean fluorescence intensity. Results are representative of 3 independent experiments.

The aF-ch2448 mutant (MT) was then tested for ADCC activity against different ovarian and breast cancer cell lines. Increased ADCC activity was observed with aF-ch2448 compared to ch2448 for OVCAR3, IGROV1, SKOV3

and MCF7D10 cells. No ADCC activity for either ch2448 or aF-ch2448 was observed on the MCF72101 cell line.

The efficacy as measured by the change in fold induction or the difference between maximum and minimum fold induction, was greater for aF-ch2448 than ch2448. Increases in the maximal levels of ADCC activity were observed on OVCAR3, IGROV1 and MCF7-D10 cell lines (as indicated by the increase of the upper asymptote of dose-response curves) (Figure 5.). Maximal ADCC activity was not reached for SKOV3 at the experimental range of concentrations tested. An induction increase of 20-fold was observed at the highest tested concentration.

Figure 5.6 ADCC activity of ch2448 versus aF-ch2448 on ovarian and breast cancer cells. The ADCC activity was measured on OVCAR3, IGROV1, SKOV3, MC7-D10 and MCF7-2101. ADCC activity was measured as fold induction of the NFAT pathway. Values are means $\pm$ standard deviations of triplicates. Similar results were obtained in 3 independent experiments.

The potency of aFch2448 was also greater than ch2448. Among the different cell lines, OVCAR3 had the lowest EC_{50} and SKOV3 had the highest. Therefore, ch2448 elicited the most potent ADCC activity against OVCAR3.

Correspondingly, OVCAR3 had high binding of ch2448 and SKOV3 had low binding based on flow cytometry (see Chapter 4, Figure 4.1). The results suggested a possible correlation between potency and cell-surface binding. A range of fold differences of 19.3 to 26.4 were observed in the EC₅₀ values of ch2448 compared to aF-ch2448 for all cancer cell lines that bound to ch2448. Minimal ADCC activity was observed in the MCF7-2101 cell line for both ch2448 and aF-ch2448.

Table 5.1 ADCC activity of ch2448 and aF-ch2448

Cell Type	Average EC ₅₀ * (mg/mL)		Fold Difference ^a
	ch2448 (WT)	aF-ch2448 (MT)	
IGROV1	0.034965	0.001325	26.39864
OVCAR3	0.010483	0.000446	23.50273
SKOV3	0.06616	0.003407	19.41884
MCF7-D10	0.03069	0.001366	22.46706
MCF7-2101	nd	0.0008857	ND

* Average of two independent experiments

^a EC₅₀ of ch2448 / EC₅₀ of aF-ch2448

WT = wildtype, MT = mutant, ND = not determined

Next, to confirm the difference in drug efficacy, incubation times were increased for OVCAR3, a cell line with high ADCC activity (Figure 5.A) and SKOV3, a cell line with low ADCC activity (Figure 5.B). For OVCAR3, maximal ADCC activity of ch2448 was not able to reach that of aF-ch2448 (as indicated by a lower asymptote for the dose response curve of ch2448). Furthermore, aF-ch2448 activity against SKOV3 increased with time. Minimal increase in fold induction was observed for ch2448. As antibody-antigen binding did not differ between ch2448 and aF-ch2448, the increase in maximal ADCC activity was likely due to Fc-FcR binding. This hypothesis was proven in the following chapter.

Figure 5.7 ADCC activity of ch2448 versus aF-ch2448 over time. ADCC activity of aF-ch2448 and ch2448 was measured at three different time points on OVCAR3 and SKOV3. Afucosylation of ch2448 was able to increase maximal fold induction on both OVCAR3 and SKOV3. Greater differences in fold induction were observed as incubation times increased. Values are means $\pm$ standard deviations of triplicates. Similar results were obtained in 2 independent experiments.

5.2.3 Delivery of cytotoxic payloads

5.2.3.1 Antibody 2448 internalization

To induce drug-mediated cytotoxicity, most ADCs rely on the ability of a mAb to efficiently internalize into cells. To the best of my knowledge, ANXA2 has not been explored as a target for ADC therapy. However, ANXA2 has been reported to play a critical role in mediating internalization of cytotoxic peptides targeting cell surface ANXA2 [385, 490]. Furthermore, ANXA2 has been reported to interact with clathrin assembly proteins [491] and play a regulatory role in endocytosis, [492]. These data suggest that anti-ANXA2 mAbs may internalize, making them suitable candidates as ADCs.

To determine if 2448 can internalize upon cell surface binding, the mAb was first evaluated using a pH-sensitive dye. This dye would maximally fluoresce in acidic endosomal and lysosomal compartments of cells. An increase in fluorescence intensity was used as a measurement of internalization.

Cells were incubated with dye-conjugated 2448 and compared to free dye alone (Figure 5.8). Within 15 min of incubation, the mAb was internalized in the cells and exhibited high fluorescence (grey histogram) as opposed to cells incubated with the free dye (white histogram). In addition, a decrease in fluorescence intensity was observed when cells were depleted of ATP by the addition of sodium azide (dotted histogram). The data showed that 2448 internalizes into the cells upon binding to cell surface ANXA2 and the internalization is energy-dependent, suggesting that clathrin-mediated endocytosis is a likely mechanism of internalization.

Figure 5.8. Rapid internalization of fluorescently-labelled 2448. 2448 was labelled with a pH sensitive dye (Cypher5e) that maximally fluoresces at low pH levels found in endosomal and lysosomal cellular compartments. Cells were incubated in the presence of Cypher5e-conjugated 2448 (or Cypher5e dye alone as a control). 2448 was internalized into the cell as indicated by an increase in fluorescence. In addition, cells pre-treated with sodium azide exhibited lower fluorescence, indicating less internalization of Cypher5e-conjugated 2448.

5.2.3.2 Investigation of potential ADC activity

Next, chimeric and mouse variants of 2448 and C51 were evaluated for the ability to deliver and release a toxic payload after internalization. Conjugates of saporin were used to screen for the ability of the mAbs to be used as ADCs. Saporin is a ribosome inactivating protein (RIP) is derived from plant *Saponaria officinalis*. It can act as a rRNA *N*-glycosylase in cells, inactivating the large 60S ribosomal subunit, blocking translation and causing apoptosis

[493]. For preliminary experiments, instead of conjugating the toxin directly to the antibody, a secondary mAb (either anti-mouse Ig or anti-human Ig) conjugated to saporin was used [309]. The primary mAbs can complex with the secondary antibodies and internalize into target cells, releasing saporin into the intracellular space.

Cells were incubated in culture with primary mAb (2448, ch2448, C51 or chC51) and an appropriate secondary antibody conjugated to saporin (mAbZAP, MZAP or HZAP). After 72 h of incubation, relative cell viability (% Control) was measured by the percentage of live cells in the treatment group compared to the buffer-treated control (Figure 5.9).

Similar results were observed between the murine (2448, C51) antibodies and the respective chimeric forms (ch2448, chC51). Cytotoxicity was demonstrated by the ADCs on IGROV1 and MCF7 cell lines with a decrease in 20-60% cell viability compared to the buffer control, primary mAb alone or saporin secondary Ab alone. No cytotoxicity was observed for IOSE523 and BT549 cell lines which the primary mAbs do not bind to. No difference in cell viability was observed between cells incubated with primary mAb-alone and cells incubated with the buffer control. However, the secondary antibodies conjugated to saporin exhibited slight inhibition of cell growth in three cell lines BT549, IOSE523 and T47D (Figure 5.9A and C). Overall, primary antibodies were able to deliver saporin into various tumour cells that it binds to and induce potent cytotoxicity

Figure 5.9 Antibody-based delivery of a cytotoxic payload. Antibodies complexed with secondary saporin conjugates (ZAP) were tested for cytotoxicity. Live cells were measured as a percentage of control cells treated with buffer alone. (A, C) 2448 and C51 were complexed with anti-mouse IgG saporin conjugates (mAbZAP) and anti-mouse IgM saporin conjugates (MZAP), respectively. (B,D) Both ch2448 and chC51 were tested using anti-human IgG antibody conjugates (HZAP). Note: Results are represented as mean $\pm$ standard deviation of three independent experiments with triplicate wells (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, unpaired t-test). For (A), T47D cells were tested once in quadruplicate wells.

Extending these observations, the role of valency for antibody internalization was also evaluated. The bivalent F(ab')₂ of ch2448 was compared against the monovalent Fab fragment of ch2448. These antibody fragments were generated via enzymatic digestion and assessed using a similar ADC assay. Instead of using saporin, however, these mAbs were tested with secondary antibodies conjugated to monomethyl auristatin E (MMAE), a tubulin inhibitor that is used in brentuximab vedotin, a recently approved ADC in [225, 259]. Unlike the secondary antibodies used in the previous assay, the secondary MMAE conjugate specifically targets the Fab region of human IgG.

To ensure the same amount of Fab was available for both formats, the bivalent F(ab')₂ and the monovalent Fab were compared at a 2:1 molar ratio. The change in format from a bivalent ch2448 F(ab')₂ at 20 nM to a monovalent Fab at 10 nM resulted in an increase of 20% cell viability upon treatment (Figure 4.9). When fragments were compared at half the concentration, even less killing of 25% was observed in cells treated with the Fab fragment, indicating a dose-dependent effect. Alternatively, minimal loss of cytotoxicity was observed using the F(ab')₂ fragment of ch2448. The differences in killing were also observed by microscopy (Figure 5.10). Unlike Fab-treated cells, F(ab')₂-treated cells had unhealthy cell morphology, indicating a more potent cytotoxic effect of MMAE. Overall, the monovalent format did not internalize as readily as the bivalent format, suggesting internalization was antibody-mediated and not simply the result of endogenous rates of antigen turnover.

Figure 5.10 Comparison of F(ab')₂ and Fab internalization. Cells were incubated in the presence of F(ab')₂ or Fab fragments and evaluated for the ability to internalize antibody conjugates of MMAE. Results are represented as mean $\pm$ standard deviation of three independent experiments with at least triplicate wells. Note: ** and *** indicate $p < 0.01$ and 0.005 , respectively.

5.2.3.3 Antibody ch2448 as an ADC (ch2448-saporin)

As it had the greatest cytotoxic effect on IGROV1, ch2448 was selected for further studies as an ADC. The mAb was sent for direct conjugation to saporin (ch2448-saporin). This drug conjugate had an approximate drug to antibody (DAR) molar ratio of 2.5. In order to ensure that the conjugation of saporin to ch2448 did not affect cell-surface specificity, binding of unconjugated ch2448 was first compared to that of the conjugate (ch244-saporin). Similar binding profiles were observed against IGROV1, MCF7 and SKOV3 cells (Figure 5.11A). In addition, the ch2448-saporin conjugate did not bind to cell lines that ch2448 were not able to bind (data not shown). Therefore, the addition of saporin did not interfere with binding of ch2448 to its antigen. The human IgG conjugated to saporin (IgG-saporin) was also tested as a negative control.

ch2448 directly conjugated to saporin, induced greater cytotoxicity compared to ch2448 at the same molar concentration but complexed with secondary saporin conjugates. A range of 20-30% increase in cytotoxicity was observed in IGROV1, MCF7 and SKOV3 cells (Figure 5.11B). In addition, potent cytotoxicity of the conjugate was visually confirmed by the presence of cell-debris and unhealthy “shrivelled” morphology of remaining cells (Figure 5.11C).

Figure 5.11 Direct conjugation of ch2448 to saporin (ch2448-saporin). (A) Binding specificity of ch2448 was measured before and after direct conjugation to saporin (ch2448-saporin). Similar binding profiles on IGROV1, MCF7 and SKOV3 were observed by flow cytometry. In addition, the human IgG-saporin (IgG-saporin) control conjugate did not bind across all three cell lines. (B) IgG-saporin and ch2448-saporin were incubated in the presence of IGROV1, MCF7 and SKOV3 in cell culture. After three days, cell viability was measured as a percentage of buffer-treated vehicle control cells. Significant cytotoxicity was observed for ch2448 but not IgG-saporin, as indicated by decrease in viability. (C) Microscopy images were taken at 72 h after treatment. A significant reduction was observed after incubation with ch2448-saporin. Remaining cells displayed unhealthy cell morphology. Note: *, **, and *** indicate $p < 0.05$, 0.01 and 0.005, respectively.

Next, dose-dependent cytotoxicity was observed when IGROV1 and SKOV3 cells were treated with ch2448-saporin at various concentrations (Figure 5.12A). Another in-house mAb conjugated to saporin, chTNA2-saporin, was used as a negative isotype control (because it did not bind to either the IGROV1 or SKOV3 cell lines). Even at a higher molar ratio of saporin to mAb (2.9 versus 2.3-2.5), chTNA2-saporin required a 10-fold higher concentration to exhibit similar levels of cytotoxicity as compared to ch2448-saporin. As an additional control, IGROV1 and SKOV3 were also treated with free saporin (30 kDa) and ch2448. Minimal to no cytotoxicity was observed at the experimental concentrations that were used to test for cytotoxicity of ch2448-saporin. Non-specific toxicity of saporin was similar for both IGROV1 and SKOV3. Cytotoxicity was observed at concentrations greater than 10^{-8} M with slightly less potency against SKOV3 (Figure 5.12B). Based on multiple independent experiments, the IC_{50} values for ch2448-saporin were calculated and determined to be in the nanomolar range for IGROV1 and SKOV3.

A

B

Figure 5.12 Cytotoxicity of ch2448-saporin, chTNA2-saporin and free saporin. IGROV1 and SKOV3 cells were incubated with ch2448-saporin, chTNA2-saporin and free saporin. The cytotoxicity was evaluated by measuring the viability of treated-cells as a percentage of the cells treated with buffer alone. ATP levels were used as a measurement of metabolically active cells. (A) Dose dependent cytotoxicity was observed for both ch2448-saporin and chTNA2-saporin. A greater cytotoxic effect was observed with ch2448-saporin than the chTNA2-saporin control. IC_{50} values of 6.59 nM and 17.43 nM were calculated for IGROV1 and SKOV3 cells, respectively. (B) Cytotoxicity of free saporin was only observed at concentrations greater than $0.1 \mu\text{M}$. Values are means $\pm$ standard deviations of triplicates. Nonlinear regression was performed to determine the IC_{50} values using GraphPad Prism 6. Calculated IC_{50} values were similar in two independent experiments.

Previous analyses of ch2448 were based on end-point readouts of cell-based assays [319]. In order to demonstrate the sustained inhibition of cell growth, real-time monitoring of cells was also done via label-free, impedance-based cell growth analysis [324]. IGROV1, MCF7, SKOV3 and T47D cells were seeded and allowed to attach and spread overnight. Unconjugated ch2448 was then added in four ovarian and breast cancer cell lines with positive binding for ch2448. No biological effects were observed at antibody

concentrations as high as 100 µg/mL (Figure 5.13). To confirm the results on IGROV1 cells, ch2448 was also dose escalated up to 1 mg/mL with no observed differences compared to the buffer control (data not shown).

Figure 5.13 Real-time monitoring of cells treated with unconjugated ch2448. The effect of ch2448 on cell proliferation was monitored on IGROV1, MCF7, SKOV3 and T47D cell cultures. Cells were seeded and treated with either ch2448 or buffer as a vehicle control. Cell proliferation of cell cultures were monitored over time based on impedance. No significant differences in cell growth were observed between treated cells and the control. Cell indices were normalized just after antibody (Ab) treatment..

Next, the cytotoxic effect of ch2448-saporin was tested against IGROV1, MCF7 and IOSE523. The saporin conjugate induced potent cytotoxicity in IGROV1 cells, as indicated by the flat growth curve compared to the buffer-treated and IgG-saporin conjugate-treated cells (Figure 5.14). Similarly, ch2448-saporin also induced potent cytotoxicity in MCF7. However, some non-specific cytotoxicity was observed with the human IgG-saporin control. No difference between ch2448-saporin and the saporin-conjugate control was observed against the normal ovarian cell line, IOSE523. Overall, potent and specific cytotoxicity of ch2448-saporin was demonstrated over a period of 120 h.

The experiment was then repeated with various concentrations of ch2448-saporin. An IC_{50} value of ch244-saporin against IGROV1 cells was calculated at $t = 156$ h, when cells treated at the lowest concentration were reaching a stationary phase of cell growth (Figure 5.15). The IC_{50} value was consistent with IC_{50} values that were calculated in the prior section using a different assay (Figure 5.12).

Figure 5.14 Real-time monitoring of cells treated with ch2448-saporin. IGROV1, IOSE523 and MCF7 cells were seeded in 96-well plates. At 24 h for MCF7 and 48 h for IGROV1 and IOSE523, ch2448-saporin conjugate (ch2448-saporin), human Ig-saporin conjugate (hIgG-saporin) or buffer control were added. Growth was subsequently monitored for 72 h (for IGROV1 and IOSE523) and 96 h (for MCF7). IGROV1 cells treated with ch2448-saporin showed sustained inhibition of growth compared to hIg-saporin and buffer control. MCF7 cells treated with ch2448-saporin also showed growth inhibition however, non-targeted cytotoxicity was also demonstrated with hIgG-saporin. No significant differences were observed for the growth curve of IOSE523 which did not bind to ch2448-saporin.

Figure 5.15 Dose-response of ch2448-saporin activity over time. (A) IGROV1 and MCF7 were treated with different concentrations of ch2448-saporin at $T = 48 \text{ h}$. IGROV1 cells and MCF7 cells were monitored until untreated cells reached death phase (at $T = 250 \text{ h}$ for IGROV1 and $T = 130 \text{ h}$ for MCF7). A dose-dependent inhibition of cell growth was observed. Cell index readings were normalized at time of ch2448-saporin treatment. (B) A dose response curve was generated at $t = 156 \text{ h}$.

5.3 Summary and Discussion

5.3.1 Summary

Therapeutic mAbs have demonstrated various anti-tumour mechanisms of action in preclinical and clinical studies (see Chapter 2, Section 2.2.4). Many of these mechanisms are considered the major contributors to the efficacy of anti-tumour mAbs.

The novel mAbs 2448 and C51, and their chimeric variants ch2448 and chC51 bind to unique glycan epitopes of ANXA2. To test the functional relevance of these epitopes and develop the mAbs as therapeutics, this chapter investigated their potential mechanisms of action. Results of this chapter are summarized in Table 5.2.

Table 5.2 Results from *in vitro* functional studies

Mechanism of action	2448	ch2448	C51	chC51
Direct tumour cell-killing (via oncosis)	X	X	✓	X
Complement-dependent cytotoxicity (CDC)	X	X	✓	X
Antibody cell-mediated cytotoxicity (ADCC)	X	✓ ⁺	X	✓
Payload delivery as an antibody drug conjugate (ADC)	✓	✓ ⁺	✓	✓

✓ = demonstrated, X = not demonstrated, - = inconclusive, ✓⁺ = demonstrated with engineered variants for (enhanced) activity

5.3.2 Discussion

5.3.2.1 Functional activity

Unlike 2448, C51 elicits both direct tumour cell killing and CDC activity. These features of C51 can likely be attributed to its multivalent nature as an IgM. Despite sharing the same epitopes, C51 and its chimeric IgG variant, chC51, have strong differences in their ability to directly kill tumour cells via oncosis and elicit CDC activity.

As an IgM, C51 is able to crosslink multiple proteins on the cell surface as well as bind to multiple components in the complement pathway. In accordance with mAb 84 [17], the avidity of C51 seems to be critical for oncosis. Likewise, high avidity and multivalent IgM (as a hexamer but not as a pentamer) is also more efficient in complement binding and activation compared to IgG [189]. Multiple studies have shown that affinity between a single head of C1q and antibody Fc is low. (The differences in the hinge region flexibility or composition of glycans of the CH2 domains may also influence the difference in activities between IgM and IgG [494, 495].)

Most research efforts have focused on IgGs as therapeutic mAbs (as discussed in Chapter 2). Some groups have sought the use of the IgM as an alternative to IgG. Even so, most mAbs in development remain IgGs. Therefore, C51 would be more difficult to develop as a therapeutic mAb. Since the epitope of C51 could still be functionally interesting, novel antibody formats can be used to further explore these properties [127, 189]. One possible strategy would be to enhance cross-linking of the bivalent chC51. For

example, site-directed mutagenesis on the Fc region could be done to create multimeric chC51.

Two other possible mechanisms of action were observed for ch2448 and chC51: ADCC and the ability to deliver a toxin as an ADC. Both mechanisms were found to play primary roles in the efficacy of multiple mAbs. Results showed that ch2448 and chC51 elicited potent ADCC activity. ADCC activity depends on the isotype of the mAb and the surface expression (including the antigen density) of the mAb [496, 497]. Likewise, only the chimeric mAbs and the mouse parental mAbs were able to elicit potent ADCC activity. The ability of ch2448 and chC51 also corresponded to binding activity of mAbs, inducing cell death only when the antigen is sufficiently expressed.

Next, a glycoengineered variant of ch2448, aF-ch2448, was shown to exhibit superior anti-tumour efficacy. The loss of core fucose resulted in greater ADCC activity compared to the non-glycoengineered variant. These results corresponded to other afucosylated mAbs targeting known antigen targets [243, 477, 478]. ADCC assays using PBMCs or NK cell populations, showed a 3 to 10-fold increases in the EC_{50} value of an afucosylated anti-CD20 mAb. Comparable increases were found of up to 10-11 fold in afucosylated anti-EGFR and anti-HER mAbs [242]. The better efficacy of aF-ch2448 supports the possibility that anti-glycoprotein mAbs like ch2448 may also be good candidates this type of glycoengineering. .

Besides enhancing its ability to induce ADCC, the ability to act as an ADC was explored. The ability to internalize and deliver cytotoxic payloads was first investigated using a mAb-dye conjugate and then with secondary antibodies conjugated to a cytotoxic agent. Improved cytotoxicity was demonstrated after direct conjugation of saporin. Results opened up the possibility of further optimizing and enhancing drug conjugate toxicity based on variations of drug and linker combinations.

Results showed that internalization and the ability to deliver a cytotoxic agent was driven by antibody-antigen binding. Using an ADC assay, none of the mAbs were able to induce cytotoxicity on non-binding cell lines. All four mAbs had varying levels of cytotoxicity and could be used as ADCs. Differences

between the cytotoxicity of ch248 and chC51 could be attributed to differences in epitopes. For example, mAbs targeting different anti-HER2 domains have demonstrated different capacities to deliver cytotoxic agents into tumour cells [498–500]. Interestingly, minimal difference was observed for SKOV3, HCC1937 and T47D, which all have positive binding for the mAbs. In the case of SKOV3, the lower cytotoxic effect could be attributed to a lower binding percentage of ch2448 (as measured by flow cytometry, see Chapter 1, Figure 4.1). Results of HCC1937 and T47 however, suggested that the expression of the epitope alone is insufficient to produce a uniform cytotoxic effect. Besides the sensitivity of the cell line to the drug, other important considerations include the cell proliferation rate, time to confluency and the efficiency of antigen processing. The cell-surface binding of the mAb may also vary greatly depending on the different cell-type morphologies and how well cells adhere to the cell culture wells.

Furthermore, when the bivalent and monovalent formats were tested, there was decreased mAb internalization and a loss of cytotoxicity. As both the F(ab')₂ and Fab fragments bind to the same epitopes, this decrease may be due to the stronger affinity of F(ab')₂ or its ability to better crosslink antigen targets on the cell surface. Because Fab fragments could still induce some cytotoxicity, the bivalent binding likely enhances any regular turnover of antigen versus being its sole determinant.

Next, ch2448 was directly conjugated to saporin and evaluated. Here, ch2448-saporin is used as a proof of concept, however, more potent cytotoxicity could be achieved with optimization of linker and toxin combinations [390, 501, 502]. Clinical development of saporin conjugates are limited due in part to immunogenicity and hepatotoxicity in humans. Alternate drugs are currently the focus of intense development with the approvals of T-DM1 and brentuximab vedotin. For example MMAE-secondary conjugates have showed more potent cytotoxicity compared to saporin-secondary conjugates [503]. Potent cytotoxicity of ch2448-saporin was demonstrated with IC₅₀ values in the nanomolar range. Similar IC₅₀ values were observed with other mAb-saporin conjugates[493, 504–507]. As a comparison to approved ADCs, the IC₅₀s for

in vitro inhibition of cell growth by trastuzumab emtansine (T-DM1) is in the sub-nanomolar range (e.g. 0.04 nM for HER2-positive BT474 cells [229]).

5.3.2.2 Anti-ANXA2 mAbs

The use of mAbs to target ANXA2 has also been reported by other groups. However these studies have mainly focused on the ability of anti-ANXA2 mAbs to inhibit cancer cell motility and invasion [460, 366, 508, 459, 367]. Similar to knockdown studies of ANXA2, these mAbs were reported to inhibit the role of ANXA2 as a regulator of plasmin, a fibrinolytic protease involved in angiogenesis, tumour invasion and metastasis.

The functional activities of these mAbs are based on their neutralization activity and particularly on the ability to inhibit localized generation of plasmin. Neutralizing anti-ANXA2 mAbs have been tested in various cancers, including ovarian and breast cancer [460, 366, 508, 459, 367] and interestingly on macrophages and endothelial cells [509, 510]. Lokman et al. described the use of an anti-ANXA2 mAb to reduce ovarian cancer cell invasion using a chick chorioallantoic membrane assay and in a SKOV3 i.p tumour xenograft via apoptosis [381]. In another study, Sharma et al. described the use of anti-ANXA2 mAbs to inhibit neoangiogenesis in a mammary fat-pad xenograft model by reducing localised plasmin generation and activation of MMP-2/9 [459]. Anti-ANXA2 mAbs also significantly inhibited EGF-induced proliferation and metastasis in another breast cancer study [367, 459]. Here, results from cell-based assays showed that inhibition was via blocking of EGFR homo-dimerization, phosphorylation and internalization [460].

To the best of my knowledge, other publications have not demonstrated the ability of anti-ANXA2 mAbs to elicit ADCC activity nor have they been developed as ADCs. Furthermore, these mAbs were developed for enhanced ADCC activity (aF-ch2448) and ADC activity (ch2448-saporin). Overall, the functional study of this chapter highlights the uniqueness of the mAbs as potential candidates for unconjugated mAb or ADC therapy. In the following chapter, observations are extended to *in vivo* studies.

Chapter 6 Functional studies of ch2448, *in vivo*

6.1 Introduction

In vitro studies of 2448, C51, ch2448 and chC51 were reported in the previous chapters. These mAbs were functionally validated and could potentially be used as therapeutics in ovarian and breast cancer. In particular, ch2448 was able to exert potent antibody-dependent cell mediated cytotoxicity (ADCC) activity and induce cytotoxicity as an antibody drug conjugate (ADC). In order to extend these studies, this chapter reports on the *in vivo* functional characterisation of ch2448.

In vivo properties of unconjugated ch2448 were investigated using tumour xenograft mouse models [511–517]. In these models, human tumour cells are implanted into immune-compromised mice e.g. Nod/SCID or athymic nude mice [518]. Here, subcutaneous human tumour xenografts were first established in female BALB/c nude mice. In the first model, anti-tumour activity was observed after treatment with unconjugated ch2448. Based on the *in vitro* data reported in the previous chapter, it was postulated that *in vivo* activity of ch2448 could be attributed to ADCC activity. To investigate the role of this Fc-mediated mechanism of action, the F(ab')₂ fragment of ch2448 and the afucosylated ch2448 (aF-ch2448) were also assessed for reduced and enhanced activity, respectively. Biodistribution studies were also carried out with a conjugated near-infrared (NIR) dye.

6.2 Results

6.2.1 Treatment of ch2448 inhibits growth of tumour xenografts

A subcutaneous tumour xenograft mouse model was used to study the *in vivo* efficacy of ch2448. Mice were inoculated with IGROV1 tumour cells in order to establish a tumour xenograft. After 24 h, mice were pre-emptively administered purified ch2448. The tumour growth and changes in bodyweight were recorded by weekly measurements (Figure 6.1).

To observe any *in vivo* activity of ch2448, high dosage was used in the xenograft studies. Experiments followed a treatment regime similar to those used by a collaborator [519]. Initial experiments were done using 1 mg per

injection and given weekly for up to six weeks. The results of initial experiments indicated that after week 10, a rapid increase in tumour growth was observed (*data not shown*). Therefore, following experiments included a second round of antibody injections in attempts to further attenuate tumour growth.

Treatment of ch2448 significantly delayed tumour growth. As shown in Figure 6.1, an increase in tumour size in untreated (control) mice was observed after week 3. No increase was observed in mice treated with ch2448, which was only observed after week 10. A significant difference in tumour volumes was observed at week 14 ($p < 0.001$). Moreover, two mice from the control group were euthanized due to tumour volumes exceeding 1500 mm³ in the subsequent weeks. Alternatively, the average tumour volume of treated mice was below 500 mm³.

Antibody treated was also well-tolerated in mice. Animals weighed from 20-26 g at the start of treatment with minimal fluctuations in body weight throughout the study. No drastic loss in body weight ($> 10\%$), side effects or abnormal behaviours were observed in either the treatment group or untreated control group. A preliminary pharmacokinetic analysis in mice was also done in a repeated experimental study. Mouse serum was sampled on weeks 4, 8 and 12 during the treatment schedule. By capture ELISA using an anti-human secondary antibody, high levels $> 250 \mu\text{g/mL}$ of ch2448 were found in the serum of ch2448-treated mice as compared to those treated with buffer alone.

To check that the tumour xenografts maintained antigen expression of 2448, tumours at the end of the study were also harvested and processed to test for binding on flow cytometry. Preliminary results indicate 2448 was able to bind to at least some population of cells from the xenografts. Ongoing immunohistochemistry studies on these tumour xenografts are underway.

Figure 6.1 *In vivo* efficacy of ch2448. (A) On day 0, nude mice were implanted subcutaneously with IGROV1 cells. The following day mice were administered ch2448 (or buffer) by intraperitoneal (i.p.) injection and weekly thereafter for a total of 10 doses (↓) of 1 mg per mouse. Treatment of ch2448 delayed IGROV1 tumour growth. On week 14, tumour volume of a buffer control mice reached 1500 mm³ and was euthanized per protocol. (B) No adverse effects were observed between buffer and ch2448-treated mice as supported by minimal fluctuations in body weight. Tumour volume = $((L \times L \times W)/2)$, where W (width) and L (length) are the shortest diameter, respectively. Statistical significance is measured by a two-sided unpaired Student's t-test. *, **, and *** indicate $p < 0.05$, 0.01 and 0.005, respectively. All values and bars are represented as mean $\pm$ standard error for $n = 5$ mice, p value = 0.0037.

6.2.2 Fc-mediated activity of ch2448

Based on *in vitro* studies, ch2448 did not endogenously suppress growth as an unconjugated mAb (see Chapter 5, Figure 5.13). Therefore, indirect immune-mediated cell killing was investigated as a cause of *in vivo* biological activity. As an IgG₁, ch2448 can interact with the host immune system via Fc-Fc receptor interactions. In the mice immune system, the homolog receptor to the ADCC-related human Fc-gamma receptor IIIA is the murine Fc- gamma

receptor IV. This murine receptor is present on murine monocytes and macrophages but not on murine NK cells. Fc-mediated immune effector functions of anti-tumour mAbs that were humanised have been evaluated with mouse effector cells, *in vitro* and *in vivo* [199, 520, 521].

6.2.2.1 Generation and evaluation of ch2448 as a F(ab')₂

To assess its role in mediating anti-tumour activity of ch2448 *in vivo*, the Fc portion of ch2448 was first removed by enzymatic digestion. The remaining (separated) F(ab')₂ and Fc fragments of ch2448 were then evaluated for efficacy in another IGROV1 tumour xenograft study. The loss of the Fc region would eliminate any Fc-mediated activity, allowing the effect of antigen binding to be studied independently.

Approximately double the amount of F(ab')₂ (1 mg per dose) versus the IgG (500 µg per dose) was used. The 2 to 1 dose ratio was similar to another study that compared F(ab')₂ and IgG [505]. Since the previous study used a high dose of antibody (Figure 6.1), the lower dosing of IgG ch2448 served as a dose de-escalation study. Various considerations were taken into account in the comparison of ch2448 as a F(ab')₂ (100 kDa) versus a full-length IgG (150 kDa). Although above the renal clearance threshold, F(ab')₂ fragments can have significantly lower circulatory half-lives compared to an intact antibody [522]. This might be due to a loss of Fc and Fc-Rn interactions which are also present in mice [523, 524]. This property might reduce potential activity of the F(ab')₂ fragment compared to the IgG in mice [523, 525]. The difference may also be offset by the small size of a F(ab')₂ fragment, allowing for better penetration into a solid tumour, increasing the availability of fragments at the tumour site. Given the repeated administration of mAb in the treatment regime and the use of a high concentration of F(ab')₂ fragments, the concentrations should not be limiting.

The F(ab')₂ fragment of ch2448 was first evaluated *in vitro*. As shown by flow cytometry, antigen binding of the F(ab')₂ (and lack of Fc-binding) was detected on IGROV1 cells using an anti-human kappa light chain secondary antibody and an Fc-specific secondary antibody (Figure 6.A). The F(ab')₂ fragment was

also confirmed to not elicit ADCC activity, *in vitro* (Figure 6.B). The digestion products of F(ab')₂ and Fc fragments were then administered in a similar mouse xenograft as described in the previous section. In this case, only the full-length IgG exhibited anti-tumour activity. On week 12, significant difference between the tumour volumes of mice treated with full length ch2448 and mice treated with ch2448 F(ab')₂ and Fc fragments (Figure 6.C, $p < 0.05$). In accordance with results from the previous section, ch2448 also delayed IGROV1 tumour growth compared to the vehicle control. Interestingly, in this case, there was a significant drop in the tumour volume which may be due to slightly more rapid growth of the tumours. No significant difference was observed between mice treated with the buffer control and those treated with F(ab')₂ and Fc fragments of ch2448, suggesting that biological activity of ch2448 was at least due in part to Fc-mediated activity like ADCC.

Figure 6.2 Evaluation of ch2448 as a F(ab')₂ fragment (versus as an IgG). (A) The F(ab')₂ of ch2448 retains a similar binding profile as ch2448 on IGROV1 cells. A FITC-conjugated anti-human kappa light chain mAb was used as the secondary detection antibody. Binding to the Fc region is not present on F(ab')₂ fragments or on cells when using an anti-human Fc secondary detection antibody. (B) F(ab')₂ fragment of ch2448 does not elicit ADCC activity compared to (ch2448) IgG control. For combination of IgG and fragments of ch2448, the level of ADCC activity is determined by the ratio of IgG to F(ab')₂ + Fc fragments. (C) Treatment (↓) of either full-length IgG or F(ab')₂ and Fc was given intraperitoneally at 500 µg and 1 mg per injection, respectively. Results are the mean of n = 6 mice with the highest and lowest tumour volumes removed for analysis. Error bars represent standard error of the mean. Significance testing was done using one-way ANOVA test. A significant difference was observed in ch2448-treated mice and control group (p < .01). No significance was observed comparing F(ab')₂ + Fc group and control group.

6.2.2.2 Preliminary *in vivo* study of aF-ch2448

To confirm that ADCC activity was mediating anti-tumour activity *in vivo*, the afucosylated variant of ch2448 (aF-ch2448) was also tested in the established xenograft mouse model. Based on the superior ability of aF-ch2448 to induce potent ADCC activity *in vitro* (see Chapter 5, Figure 5.), a lower dose of 300 µg per injection was given to demonstrate its potential superior ability to elicit ADCC activity. Different concentrations of aF-ch2448 are currently being optimised for efficacy. Preliminary data showed that aF-ch2448 induced better anti-tumour activity than ch2448

Although not statistically significant, the development of tumours in mice treated with buffer control or ch2448 was greater than those treated with aF-ch2448 (Figure 6.3A). By week 5, mice in both the control group and ch2448-treatment group had tumours of up to 2000 mm³ in size (Figure 6.3B). Alternatively, no tumours formed in mice treated with aF-ch2448 until after week 10 (Figure 6.3B). After week 10, only two mice grew tumours of less than 700 mm³ in size. Treated mice exhibited no signs of systemic toxicity such as behavioural changes or weight loss. The results suggested that afucosylation of ch2448 enhanced the *in vivo* activity of ch2448, suggesting that *in vivo* activity was mediated via ADCC activity.

Figure 6.3 *In vivo* efficacy of aF-ch2448 (A) On day 0, nude mice were implanted subcutaneously with IGROV1 cells. The following day mice were administered ch2448 or aF-ch2448 (or buffer) intraperitoneally and weekly thereafter for a total of five treatments (↓). Treatment of ch2448 delayed IGROV1 tumour growth. On week 14, tumour volume of a buffer control mice reached 1500 mm³ and was euthanized per protocol. (B) No adverse effects were observed between buffer and ch2448-treated mice as supported by minimal fluctuations in body weight. Tumour volume = $((L \times L \times W)/2)$, where W (width) and L (length) are the shortest diameter, respectively. * indicates that mice were euthanized due to the tumour volume reaching 2000 mm³, ** indicated mouse had swollen lymph node and was euthanized. Results of this experiment were non-significant.

6.2.3 Biodistribution of ch2448

To visualize the ability of ch2448 to target cancer *in vivo*, biodistribution of the mAb was monitored using a near infrared dye (NIR) [130, 526–529]. The dye was directly conjugated to ch2448 via its amine reactive group. The chimeric IgG₁ (chTNB1), which had the same constant regions as ch2448, was used as a negative control. Similar degrees of labelling were measured by spectral absorbance for the dye conjugates, 1.24 for the ch2448-NIR dye conjugate and 1.45 for the chTNB1-NIR dye conjugate. Conjugation of the NIR dye was shown to not affect the binding specificity on IGROV1 cells (Figure 6.4A). Binding of ch2448 was observed before and after conjugation (ch2448-NIR) whereas the controls showed no binding. To confirm dye conjugation of

ch2448, the NIR fluorescence was also measured as a shift in NIR fluorescence compared to the negative conjugate control (Figure 6.4B).

Figure 6.4 Binding of a near infrared dye conjugate of ch2448 (A) Antibody binding of ch2448-NIR (or control conjugate, chTNB1) was measured using a secondary antibody conjugated to FITC. Binding specificity of ch2448-NIR was retained as indicated by a similar shift in fluorescence intensity as compared to that of unconjugated ch2448. No binding was observed for either chTNB1 or chTNB1-NIR. (B) Conjugation of the dye to ch2448 was confirmed by an increase in NIR fluorescence for ch2448-NIR as compared to the negative control, for chTNB1-NIR.

Antibody therapies in humans are usually administered by intravenous (i.v.) injection. Even so, intraperitoneal (i.p.) injection targeting malignant ascites in the intraperitoneal cavity, and subcutaneous injection involving antibody absorption by the lymphatic system, have also explored. As with human patients, most mouse models use i.v. injection for administration of mAb, allowing for direct access to the vasculature of the mouse.

To confirm that sufficient levels of i.p.-administered ch2448 reached the subcutaneous tumour site, administration of ch2448-NIR by i.p. injection was compared with i.v. tail injection. Subcutaneous IGROV1 tumours were first grown to 400-500 mm³ in size. Thereafter, ch2448 was administered either by

i.p. or i.v. injection. After 72 h, animals were imaged and comparable fluorescence levels for the mAb were observed regardless of the route of administration (Figure 6.5). Overall results showed that ch2448 had sustained localisation at the tumour site.

Figure 6.5 Comparison of i.p. injection versus i.v. tail injection of ch2448. The ability of ch2448 to reach the subcutaneous tumour site was shown regardless of an intraperitoneal (i.p.) or an intravenous (i.v.) route of administration. Mice with tumour volumes that range from 300 to 400 mm³ were injected with a ch2448-NIR (near-infrared) dye conjugate. After 72 h post injection, mice were imaged in dorsal and ventral positions. Strong uptake of ch2448-NIR was seen in the right flank of mice (↓), corresponding to the location of the subcutaneous tumours (○).

6.2.3.1 *In vivo* and *ex vivo* imaging of ch2448-NIR

Whole body imaging of mice was done to evaluate the ability of ch2448 to specifically target tumour xenografts in mice. Tumour-bearing mice were treated with either ch2448-NIR or chTNB1-NIR by i.p. injection. After 1 h post injection, mice were imaged in a dorsal position. Fluorescence intensity was detected throughout the bodies of all mice, especially in areas surrounding the kidneys (Figure 6.6A).

After 96 h post-injection of ch2448-NIR, mice treated with ch2448-NIR-had significantly higher levels of fluorescence compared to those of the non-targeting chTNB1-NIR control. Fluorescence was localised in the subcutaneous tumour regions. Tumour volumes for all four mice were roughly the same size (from 442.7 mm³ to 585.8 mm³). Among ch2448-NIR treated mice, higher fluorescence intensity correlated with a larger tumour volume (Figure 6.6B and C).

Figure 6.6 *In vivo* whole body imaging of ch2448-NIR-treated mice. Treatment of ch2448-NIR was given at $t = 0$ h. (A) At $t = 1$ h, ch2448-NIR was disseminated throughout the intraperitoneal cavities of mice. (B) Four days after treatment ($t = 94$ h), high levels of ch2448-NIR was observed at the subcutaneous tumour site. (C) Individual images of mice show that fluorescence levels at tumour site were at least four times greater in ch2448-NIR treated mice than in chTNB1-NIR-treated mice. The tumour volumes of all mice were around the same size (as measured by callipers and calculated by $\frac{1}{2} \times \text{Length} \times \text{Width}^2$ of tumour). Note: Fluorescence was measured by total flux with units p/sec/cm²/sr where p = photons, sr = steradian.

To further investigate, *ex vivo* imaging of animal organs was also done. Tumours and various organs were harvested from mice treated with ch2448-NIR and chTNB1-NIR. The tumours from ch2448-NIR treated mice had more than 6 times greater levels NIR-fluorescence than in tumours from control mice. Lower levels of NIR-fluorescence were detected in other organs such as the kidneys, liver, lungs, ovaries, spleen and stomach. As expected, no fluorescence was observed in the brain indicating that the mAb did not cross the blood/brain barrier. Overall, the control conjugate chTNB1-NIR had

detectable but relatively low uptake in the stomach, ovaries and spleen of the mice with minimal binding on the tumour.

Figure 6.7 *Ex vivo* imaging of tumour and major organs from ch2448-NIR-treated mice. Tumours and essential organs (brain, lungs, kidneys, stomach, heart, spleen, ovaries and uterine horns, liver) of antibody-treated mice were harvested for imaging. After a three times wash with PBS, the tumours and organs were imaged to detect NIR-fluorescence levels. Tumour fluorescence levels were significantly higher for the tumours of ch2448-NIR treated mice compared to other organs of the mice and compared to the chTNB1-NIR treated tumour. Results are representative from 2 mice per treatment group.

6.3 Summary and discussion

In this chapter, treatment of unconjugated ch2448 was shown to inhibit tumour cell growth in subcutaneous IGROV1 xenografts mouse models. Additionally, based on the failure of F(ab')₂ fragments to exhibit similar biological activity and the superior ability of aF-ch2448 to elicit ADCC, it was inferred that the *in vivo* activity of ch2448 involved ADCC activity.

Although anti-ANXA2 mAbs have demonstrated a reduced tumour burden in ovarian [366], breast [459, 530] and glioma cancer [407] mouse models, their activities were not attributed to ADCC. Instead, these studies focused on different roles of ANXA2, especially as an activator of plasmin generation [366, 459, 530]. In an orthopic xenograft breast cancer model, anti-ANXA2 inhibited neovascularization [459]. Anti-ANXA2 mAbs were also shown to induce apoptosis (based on the presence of caspase expression) in an i.p. ovarian cancer xenograft model [366]. Overall, these models speculated that anti-ANXA2 mAbs interacted with the tumour microenvironment in order to induce anti-tumour growth and metastasis.

As ch2448 was studied by a subcutaneous xenograft model, the relevance of anti-ANXA2 mAbs regulating plasmin activation and other angiogenic activities is limited. Plasmin activation likely has limited role in subcutaneous tumour growth [431, 531] and no metastasis was observed in the model. Furthermore, as shown in Chapter 4, ch2448 binds to a unique epitope of ANXA2 which may not be able to induce these mechanisms of action.

Instead, the *in vivo* efficacy of ch2448 relied on the capacity of ch2448 to interact with Fc receptors on mouse effector cells [532, 533]. Although nude mice do not have a thymus, they retain relatively intact innate immune systems, which include NK cells, macrophages and complement proteins [534]. Furthermore, even though the Fc-receptors comprise of different allelic variants, the homology of Fc receptors between species has been demonstrated [533, 535]. In both *in vitro* and *in vivo* studies, human IgG₁ was shown to interact with mouse Fc receptors and induce ADCC or ADCP activity with mouse NK cells or mouse macrophages [535, 536]. Previous studies with trastuzumab and rituximab have demonstrated the ability of mouse effector cell engagement with humanized mAbs [537].

Despite the ability of human IgG₁ to interact with activating mouse Fc receptors, the binding is not as strong as human Fc to human Fc receptors. Therefore, the host immune system of mice is not ideal for studying ADCC activity of humanized mAbs, suggesting that the effect of ch2448 might be even better in mouse models with humanised immune systems [533, 537, 538] and in human clinical trials. Groups have explored models that used transgenic expressing human Fc-gamma receptors, [538, 539] or the transfer of human cells (PBMCs, hematopoietic stem cells) into immunodeficient mice [537]. These models could potentially be used to further assess ch2448.

6.3.1 Biodistribution of ch2448

Beyond looking at the efficacy of ch2448, biodistribution studies were also done using an NIR dye conjugated to ch2448 (ch2448-NIR). Non-invasive imaging revealed strong accumulation of ch2448-NIR at the tumour site regardless of i.p. or i.v. route of administration (Figure 6.5). Similarly, when compared to an isotype control, significantly greater levels of ch2448-NIR were

detected at the tumour site compared to other organs, both *in vivo* and *ex vivo* (Figure 6.6, Figure 6.7). These data support the previous results showing that ch2448 inhibited growth of subcutaneously implanted tumours.

The exact and quantifiable amounts of ch2448 was also not determined in this study because imaging was done on a two dimensional surface, relying on limited cell-surface measurements. The studies therefore do not give any information on the penetration of ch2448 into the solid tumour. Ongoing work involves quantifying the level of ch2448 in the xenograft tumours. Samples can be homogenized and measured for accumulation of antibody (with NIR-dye or ELISA or immunohistochemistry).

6.3.2 Ongoing work

Apart from the subcutaneous xenograft model described in this thesis, our group is optimizing other mouse models that more closely mimic ovarian cancer progression in the clinical setting. This includes an intraperitoneal xenograft model using tumour cells overexpressing luciferase. These cells are injected in the intraperitoneal cavity of mice where they can proliferate and spread, more closely resembling the microenvironment and clinical events like the generation of ovarian cyst fluid and the formation of malignant ascites [540, 541]. Measurement of tumour cell growth is measured by injection of luciferin and imaging of tumour cells via bioluminescence.

In a clinical setting, mAbs would also likely require the ability to overcome high physiological barriers [542, 543]. To determine if antibody treatment is effective against a bulky tumour mass and not just tumour implantation, it would also be interesting to change the treatment regime schedule (from a pre-emptive model) to one that is initiated after the tumour has grown to a certain size. This model would be similar as the biodistribution model where antibodies were given after tumour volumes reached 400-500 mm³. It would also be beneficial to carry out dose response curves and increase the number of mice for statistical significance.

Overall, previous chapters showed that ch2448 targeted a unique epitope on ovarian cancer cells (Chapter 4) and could induce potent *in vitro* ADCC activity via an Fc-mediated mechanism of action (Chapter 5). Here, ch2448 was

evaluated for *in vivo* activity, demonstrating anti-tumour efficacy via a similar MOA.

Chapter 7 Concluding remarks and recommendations

7.1 Summary and translational relevance

In the past three decades, monoclonal antibodies (mAbs) have emerged as important drugs for the targeted treatment of cancer (Chapter 2, Section 2.2). To develop new therapies, an important focus of biomedical research is the discovery and exploitation of new antigen targets. This thesis reports on 2448 (IgG_{1K}) and C51 (IgM), two novel mAbs that were identified as targeting not only human embryonic stem cells (hESC) but also multiple cancers. The main objective of this thesis was to establish the potential therapeutic value of these candidate mAbs in targeting ovarian and breast cancer. This was accomplished by a systematic characterisation and functional study of 2448 and C51.

The first part of the thesis focused on the ability of 2448 and C51 to target surface antigens on ovarian and breast cancer cells. Interestingly, the mAbs had similar binding patterns. High binding was demonstrated on more than twenty different cancer cell lines (including both HER2-positive and HER2-negative breast cancer cell lines). Furthermore, results suggested that antibody recognition corresponded to a stage of EMT, a critical cellular program that has been studied in both ovarian and breast cancer progression. When cancer cell lines were classified based on an EMT spectrum, 2448 and C51 demonstrated preferential binding to a subset of cells. This subset was classified by an epithelial or intermediate epithelial phenotype. Concomitantly, minimal binding was observed on cell lines with mesenchymal or intermediate mesenchymal phenotypes.

Surprisingly, the antigen target for both mAbs was identified as annexin A2 (ANXA2). As described in Chapter 4 Section 4.8.2, ANXA2 has previously been reported as a promising target for cancer drug development. Recent studies have shown overexpression of ANXA2 in both ovarian and breast cancer. So far, however, no therapeutic anti-ANXA2 mAbs have been reported in clinical trials.

Changes in cell surface glycosylation are common features in both the differentiation of hESC [544] and cancer-related EMT [453, 454]. However, to date, glycosylation has not been the focus of studies surrounding the development of ANXA2 as a therapeutic target. Corresponding with studies showing glycoforms of ANXA2 (see Chapter 4, 4.8.2), further investigation revealed binding of 2448 and C51 via N-glycan epitopes on ANXA2. Compared to a commercial mAb of ANXA2, results showed that 2448 and C51 bound a subset of ANXA2 that was localised on the cell surface, possibly due to their unique glycan epitopes.

The ability to bind specific epitopes on tumour cells is one major advantage of therapeutic mAbs. Another is the ability to exert distinct mechanism(s) of action (MOA). Therefore, 2448, C51 and their chimeric variants ch2448 and chC51 were next functionally validated both *in vitro* and *in vivo*.

Although ANXA2 has been explored as a target for mAb therapy, most of the mAbs acted as neutralizing antibodies and in particular, focused on the role of ANXA2 in cell to cell adhesion and in promoting tumour cell motility invasion and angiogenesis. Interestingly, both C51 and ch2448 exerted different MOA against ovarian and breast cancer cells.

Antibody C51 demonstrated direct tumour cell killing via oncosis and complement dependent cytotoxicity (CDC) activity. Interestingly, after chimerisation of C51 to the IgG variant (chC51), both oncosis and CDC activities were lost, suggesting that the class of antibody (IgM) was important for the functionality of C51. Since IgMs are less favourable for development as a therapeutic, efforts were therefore focused on studying ch2448, the chimeric variant of 2448. Two potential MOA were demonstrated for ch2448: the ability to induce antibody-dependent cell-mediated cytotoxicity (ADCC) activity and the ability to internalize and deliver cytotoxic payloads as an antibody drug conjugate (ADC).

First, ch2448 demonstrated potent ADCC activity *in vitro*. ADCC activity is believed to be a critical MOA for some of the most clinically successful mAbs including those against solid tumours. To enhance ADCC activity, an

afucosylated variant (aF-ch2448) was also generated and when tested, demonstrated a > 10-fold improvement in ADCC activity compared to ch2448.

These *in vitro* results were also supported by *in vivo* studies. Unconjugated ch2448 inhibited IGROV1 tumour cell growth in subcutaneous mouse xenografts. As ch2448, by itself, was not able to induce direct-tumour cell killing *in vitro*, anti-tumour activity was hypothesized to be based on ADCC activity. The importance of Fc-mediated activity was shown by lack of *in vivo* anti-tumour activity using a ch2448 F(ab')₂ fragment. Moreover, preliminary results suggested that aF-ch2448 had superior anti-tumour activity compared to the non-glycoengineered ch2448.

Secondly, ch2448 was also tested as an ADC. A fluorescently-labelled 2448 first showed that ANXA2 could efficiently be internalized into cells after mAb binding, an important consideration for the development of an ADC. Next, as a proof of concept, ch2448 was used to deliver the saporin toxin in cell based assays *in vitro*. Here, ch2448 delivered secondary conjugates of saporin and induce cytotoxicity in some though not all cell lines that it bound to. More importantly, no cytotoxicity was observed on cell lines that ch2448 was not able to bind. To extend the studies, saporin was also directly conjugated to ch2448 (ch2448-saporin) and this yielded improved cytotoxicity with IC₅₀ values in the sub-nanomolar range for IGROV1 and SKOV3 cells.

Taken together, these studies show that 2448 and C51 bind to unique oncofetal epitopes of ANXA2 in ovarian and breast cancer. The ability to identify new functional targets validates our lab's antibody discovery approach of using whole cell immunisation and mouse hybridoma technology. Like antibodies previously generated in our lab [545, 546], 2448 and C51 target cell-surface and glycoprotein epitopes that are conserved across various carcinomas. These targets could be difficult to recapitulate using conventional recombinant protein systems. Therefore, these glycoprotein antigens are unique and can be used for therapeutic targeting.

To the best of our knowledge, 2448 and C51 are the first reported mAbs to target glycan-dependent epitopes of ANXA2. Anti-ANXA2 mAbs are typically generated from recombinant peptides (or proteins that do not include the

potential N-glycosylation site of ANXA2 [547]). These mAbs are not explicitly shown to target glycosylated epitopes. Furthermore, many have only demonstrated membrane staining of ANXA2 (by immunofluorescence or western blot) but not surface recognition on live cells (by flow cytometry [366, 548].) To support these observations, one commercial anti-ANXA2 mAb was compared against 2448 and C51. This mAb did not bind to surface ANXA2 on cancer cells (Figure 4.14) and did not bind to glycosylated epitopes (Figure 4.12).

Beyond targeting unique epitopes of ANXA2, mAbs 2448 and C51 were also functionally evaluated as therapeutic candidates. Four mechanisms of action were observed: direct tumour cell killing via oncosis, potent ADCC and ADC activity. Although neutralizing anti-ANXA2 mAbs have been reported by other groups, so far none has demonstrated ADC or ADCC activity. In addition, none of the mAbs reported from the literature were derived from hESC. Benchmarking of C51 and ch2448 with three commercially-available anti-ANXA2 mAbs from the literature are summarised in the table below (Table 7.1).

Table 7.1 Properties of ch2448, C51 and anti-ANXA2 mAbs reported in the literature

	Clone	Species	Class	Immunogen	Cell surface	Anti-Glycan	Proliferation or migration/invasion	ADCC/CDC	ADC	Ref.
1.	ch2448	Chimeric	IgG ₁	Human embryonic stem cells	Yes FACS, IB	Yes	Not observed (<i>in vitro</i>)	ADCC <i>in vitro</i> , <i>in vivo</i> (aF-ch2448)	Yes <i>in vitro</i> (ch2448-sap)	(Ch. 4-6)
2.	C51	Mouse	IgM	Human embryonic stem cells	Yes FACS, IB	Yes	Direct tumour cell killing via oncosis (<i>in vitro</i>)	CDC <i>in vitro</i>	NR	(Ch. 4-6)
3.	5	Mouse	IgG ₁	Human annexin II, aa. 123-328	No FACS* (IC/IB/IHC)	NR	Inhibits invasion, induces apoptosis (<i>in vitro</i> and <i>in vivo</i>)	NR	NR	[366]
4.	Multiple	NR	NR	Tryptic fragments purified f/ Bovine Aortic Endothelial (BAE) cells using angiotatin, aa. 314–324, 234–245, and 330–339	FACS NR (IC/IB/IHC)	NR	Inhibits neoangiogenesis (<i>in vitro</i> and <i>in vivo</i>)	NR	NR	[459, 508, 530, 549, 550]
5.	D1/274.5	Mouse	IgG _{2a}	AG1523 fibroblasts of human origin	FACS NR (IC/IB)	NR	Inhibits EGF-induced proliferation and migration (<i>in vitro</i>)	NR	NR	[460]

NR - not reported, IC - immunocytochemistry, IB - immunoblot, IHC - immunohistochemistry, ADC - antibody drug conjugate, ADCC – antibody-dependent cell-mediated cytotoxicity, CDC - complement dependent cytotoxicity, Ch. – chapter(s), *Based on own experiments.

7.2 Future directions and important considerations

7.2.1 Future directions of antibody characterisation

Future studies should continue to evaluate the therapeutic potential of C51 and ch2448. So far, this study has utilised various ovarian and breast cancer cell lines commonly reported in cancer research [551–554]. The next step would be to demonstrate equivalent specificity and efficacy on primary cell lines and tissues. As ANXA2 is expressed in normal tissue (see Chapter 4), differential epitope expression of 2448 and C51 in diseased (i.e. tumours) versus normal tissues would be a critical safety consideration for clinical use of the mAbs.

Immunohistochemistry (IHC) is one method that is commonly used to predict target specificity and therapeutic response of antibody treatments for cancer [555]. Tumour staining can provide information on the antigen density requirement for efficient ADCC activity, ADC activity or overall treatment efficacy. It may also yield insight into correlations between clinicopathological features and the epitope expression of 2448 and C51. Studies would be especially important because the epitope expression of 2448 and C51 involve glycosylation, an aspect not typically considered in all approved mAbs. Preliminary studies on in-house cell line and tissue arrays are currently underway. Variables that are being optimized include sample preparation (either formalin-fixed and paraffin-embedded or fresh frozen), antigen retrieval and appropriate antibody concentration.

In order to provide more complete knowledge on the binding specificities of 2448 and C51, mapping of the glycan epitopes can also be done. Potential findings may yield further insights into how glycan changes relate to EMT and the various glycoforms of ANXA2 which have yet to be studied in detail [361, 358, 360, 359]. In order to determine important glycan residues of 2448 and C51, glycan arrays using immobilized lectins can be done [556, 557]. Other approaches include site-directed mutagenesis on predicted glycosylation sites and glycomics studies by mass spectrometry techniques.

Next, it would also be important to determine accurate affinities of these mAbs. The affinity of mAbs is an important parameter because it determines how well

an antibody can overcome *in vivo* barriers to reach the tumour site and induce an anti-tumour effect. Due to difficulties in obtaining sufficient purified antigen (with the appropriate glycan structures on ANXA2) for surface plasmon resonance affinity measurements, a simplified flow-cytometry-based assay was used to estimate binding affinities of 2448 and C51. However these results do not give accurate estimates of absolute K_d values [352]. A more accurate measurement would be of high importance, especially since multiple anti-glycan mAbs have been reported to be of low affinities. Another method is currently being optimized to measure affinity on live cells [532]. Once affinity can be measured, further antibody engineering techniques such as affinity maturation can be applied to optimize affinities.

Antibodies 2448 and C51 could also be used as diagnostic mAbs. Like other oncofetal proteins such as AFP (alpha fetoprotein) and CEA (carcinoembryonic antigen), ANXA2 has been proposed as a serum diagnostic marker in hepatocellular cancer. Furthermore, cleaved and post translationally modified forms of ANXA2 have been reported in the secretome of serous ovarian cancer and identified in the tumour stroma [388]. Whether 2448 or C51 can distinguish between these isoforms is still unknown.

7.2.2 Future directions of functional studies

The scope of functional studies could also be widened to include other assays. It would be interesting to widen the possible functional activities of the mAbs, either through exploring different cancer models or by the creation of other antibody formats. In Chapter 6, some of these more clinically relevant mouse models were discussed in terms of different types of mice, different sites of inoculation and different sites of administration.

Functional studies may also be extended to primary cells and tissues. Patient-derived xenograft (PDX) mouse models for ovarian and breast cancer have successfully been reported in the literature [558–561]. These models would preserve more of the tumour heterogeneity found in patients.

Next, the ability of the mAbs to potentially exert other MOA (see Chapter 2) can be investigated. This thesis focused on a limited number of antibody MOA, particularly ADCC activity and delivery of cytotoxic agents (Table 7.1). ANXA2

is associated with adhesion as well as migration and invasion in cancer. Other groups have reported on anti-ANXA2 mAbs with different anti-tumour activity, including anti-migratory and anti-invasive activity. Therefore, it would be interesting to investigate the ability of 2448 and C51 to alter the tumour microenvironment.

Based on scratch migration assays and three-dimensional spheroid assays, 2448 and C51 have so far not demonstrated anti-migratory or anti-adhesive activity (data not shown). These assays, however, were not done in co-culture with other cell types that are critical for tumour cell adhesion. Further studies might include more extensive models, including *in vivo* models that have previously been used with anti-ANXA2 mAbs. These include the chick chorioallantoic membrane angiogenesis assay and intraperitoneal xenograft models [381, 530]. Another type of assay would involve studying the effect of 2448 and C51 on the EMT process. The ability of mAbs to monitor EMT progression can be further developed using *in vitro* or *in vivo* models of EMT [562]. These models could replicate the dynamic EMT phenotypic states in patients and extend the study of 2448 and C51 to other carcinomas including those of the colon, lung or stomach.

7.3 Future perspective of 2448 and C51

Since the start of this thesis project, there has been continued interest in targeting ANXA2 as a strategy for antibody-based cancer therapy. As discussed in Chapter 4 Section 4.8.2, no attempts to target glycosylated epitopes of ANXA2 have been published to date. In addition, cancer-associated glycosylation of ANXA2 has not been a focus of study. Instead, the overexpression of ANXA2 on normal versus tumour tissue is based on mRNA or protein expression alone (Chapter 4 Table 4.1). According to findings of this thesis, further studies on annexin glycosylation and the epitopes of 2448 and C51 are warranted. These would provide a better understanding on how 2448 and C51 are different than other anti-ANXA2 mAbs. As 2448 and C51 recognise cells with an epithelial phenotype, these studies might also provide insight into the relationship between glycosylation and the expression of ANXA2 during differentiation and cancer-related EMT.

From a translational perspective, 2448 and C51 can be further developed to maximise their clinical utility. From a drug development perspective, these mAbs have undergone target identification, functional *in vitro* validation and in the case of 2448, preliminary animal studies. Before 2448 and C51 can move closer to clinical trials, numerous challenges remain. These include validation on their specificity and selectivity on primary tissues or cells; more rigorous animal studies (including non-human primate toxicology studies) and further optimization (humanization and affinity maturation). The previous section addresses some of these issues. Additionally, results are being presented to drug development experts in both academia and industry for review.

Going forward, new strategies and technologies can be incorporated into the development of 2448 and C51. In the field of targeted therapy, these include dual or multi-specific targeting of antigens via antibody cocktails (eg. pertuzumab and trastuzumab [563, 564]) or bispecific mAbs (see Chapter 2, Section 2.2.2.2); combination of therapeutic mAbs with other anti-cancer drugs and conjugation of mAbs to different payloads [565, 566]. In the past few years, chimeric antigen receptor-modified T-cells have notably generated the most excitement in cancer research. In order to recognise tumour cells, these biologics express extracellular single-chain variable fragments (scFv) derived from an antibody [567]. Leveraging on these developments, 2448 and C51 are currently being used in two independent research projects in my lab. Based on the ability to monitor and target cancer-related EMT, these mAbs are being studied in combination with an EMT reversal agent. In addition, the CDRs of 2448 are being utilised to develop a new CAR-T therapy against ovarian cancer. These projects would build upon the work compiled in this thesis.

7.4 Conclusion

Taken together this thesis presents 2448 and C51 as novel therapeutic candidates for ovarian and breast cancer. The mAbs were first found to bind unique glycosylated epitopes of ANXA2 on hESC and epithelial ovarian and breast cancers. Furthermore, the mAbs were functionally validated and developed for better therapeutic efficacy. As an unconjugated mAb, ch2448 was enhanced via afucosylation. A proof of concept ADC was also tested showing that ch2448 could be optimised to deliver cytotoxic agents.

The results from this thesis have been filed as a patent (10201606178X) and are in review for scientific journal publication. Important findings are highlighted below:

- (1) Monoclonal antibodies (mAbs) 2448 and C51 bind a subset of ovarian and breast cancer cells that express an epithelial phenotype.
- (2) Chimeric mAbs ch2448 and chC51 retain binding specificity of 2448 and C51.
- (3) Based on immunoprecipitation and siRNA knockdown studies, 2448 and C51 target annexin A2 (ANXA2).
- (4) Binding of 2448 and C51 is N-linked glycan dependent.
- (5) Binding of 2448 and C51 target unique and non-overlapping epitopes.
- (6) mAb C51 but not chC51 exhibits complement dependent cytotoxicity (CDC) activity, *in vitro*
- (7) mAb ch2448 exhibits ADCC activity; The afucosylated variant (aF-ch2448) enhances ADCC activity, *in vitro and in vivo*.
- (8) mAb 2448, ch2448, C51 and chC51 can be used to deliver cytotoxic payloads into cells, *in vitro*.
- (9) ch2448 conjugated to saporin (ch2448-sap) exhibits potent cytotoxicity as an antibody drug conjugate (ADC), *in vitro*.
- (10) Unconjugated ch2448 suppresses tumour cell proliferation in a tumour xenograft mouse model, *in vivo*.

References

1. Chames P, Van Regenmortel M, Weiss E, Baty D (2009) Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol* 157:220–233. doi: 10.1111/j.1476-5381.2009.00190.x
2. Fridman WH (2006) Principles of the therapeutic use of monoclonal antibodies in oncology. *C R Biol* 329:255–259. doi: 10.1016/j.crvi.2005.12.009
3. Nelson AL, Dhimolea E, Reichert JM (2010) Development trends for human monoclonal antibody therapeutics. *Nat Rev Drug Discov* 9:767–774. doi: 10.1038/nrd3229
4. Pillay V, Gan HK, Scott AM (2011) Antibodies in oncology. *New Biotechnol* 28:518–529. doi: 10.1016/j.nbt.2011.03.021
5. Schrama D, Reisfeld RA, Becker JC (2006) Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* 5:147–159. doi: 10.1038/nrd1957
6. Scott AM, Wolchok JD, Old LJ (2012) Antibody therapy of cancer. *Nat Rev Cancer* 12:278–287. doi: 10.1038/nrc3236
7. Shuptrine CW, Surana R, Weiner LM (2012) Monoclonal antibodies for the treatment of cancer. *Semin Cancer Biol* 22:3–13. doi: 10.1016/j.semcancer.2011.12.009
8. Weiner LM, Surana R, Wang S (2010) Antibodies and cancer therapy: versatile platforms for cancer immunotherapy. *Nat Rev Immunol* 10:317–327. doi: 10.1038/nri2744
9. Carter P, Smith L, Ryan M (2004) Identification and validation of cell surface antigens for antibody targeting in oncology. *Endocr Relat Cancer* 11:659–687. doi: 10.1677/erc.1.00766
10. Loo DT, Mather JP (2008) Antibody-based identification of cell surface antigens: targets for cancer therapy. *Curr Opin Pharmacol* 8:627–631. doi: 10.1016/j.coph.2008.08.011
11. Apweiler R, Hermjakob H, Sharon N (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* 1473:4–8.
12. Khoury GA, Baliban RC, Floudas CA (2011) Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Sci Rep* 1:90. doi: 10.1038/srep00090
13. Christiansen MN, Chik J, Lee L, et al (2014) Cell surface protein glycosylation in cancer. *Proteomics* 14:525–546. doi: 10.1002/pmic.201300387

14. Dall'Olio F, Malagolini N, Trinchera M, Chiricolo M (2012) Mechanisms of cancer-associated glycosylation changes. *Front Biosci Landmark Ed* 17:670–699.
15. Paszek MJ, DuFort CC, Rossier O, et al (2014) The cancer glycocalyx mechanically primes integrin-mediated growth and survival. *Nature* 511:319–325. doi: 10.1038/nature13535
16. Schriebl K, Satianegara G, Hwang A, et al (2012) Selective removal of undifferentiated human embryonic stem cells using magnetic activated cell sorting followed by a cytotoxic antibody. *Tissue Eng Part A* 18:899–909. doi: 10.1089/ten.TEA.2011.0311
17. Tan HL, Fong WJ, Lee EH, et al (2009) mAb 84, a Cytotoxic Antibody that Kills Undifferentiated Human Embryonic Stem Cells via Oncosis. *STEM CELLS* 27:1792–1801. doi: 10.1002/stem.109
18. Choo AB, Tan HL, Ang SN, et al (2008) Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells Dayt Ohio* 26:1454–1463. doi: 10.1634/stemcells.2007-0576
19. Ng VY, Ang SN, Chan JX, Choo ABH (2010) Characterization of epithelial cell adhesion molecule as a surface marker on undifferentiated human embryonic stem cells. *Stem Cells Dayt Ohio* 28:29–35. doi: 10.1002/stem.221
20. Hanahan D, Weinberg RA (2011) Hallmarks of Cancer: The Next Generation. *Cell* 144:646–674. doi: 10.1016/j.cell.2011.02.013
21. Almendro V, Marusyk A, Polyak K (2013) Cellular Heterogeneity and Molecular Evolution in Cancer. *Annu Rev Pathol Mech Dis* 8:277–302. doi: 10.1146/annurev-pathol-020712-163923
22. Cancer Genome Atlas Research Network, Weinstein JN, Collisson EA, et al (2013) The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet* 45:1113–1120. doi: 10.1038/ng.2764
23. Harrison C (2012) Cancer: Intratumour heterogeneity. *Nat Rev Drug Discov* 11:354–354. doi: 10.1038/nrd3741
24. Kleppe M, Levine RL (2014) Tumor heterogeneity confounds and illuminates: assessing the implications. *Nat Med* 20:342–344. doi: 10.1038/nm.3522
25. Vogelstein B, Papadopoulos N, Velculescu VE, et al (2013) Cancer genome landscapes. *Science* 339:1546–1558. doi: 10.1126/science.1235122
26. Are C, Rajaram S, Are M, et al (2013) A review of global cancer burden: Trends, challenges, strategies, and a role for surgeons. *J Surg Oncol* 107:221–226. doi: 10.1002/jso.23248

27. Ferlay J, Soerjomataram I, Dikshit R, et al (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136:E359-386. doi: 10.1002/ijc.29210
28. Brody H (2014) Cancer. *Nature* 509:S49–S49. doi: 10.1038/509S49a
29. Guppy AE, Nathan PD, Rustin GJS (2005) Epithelial ovarian cancer: a review of current management. *Clin Oncol R Coll Radiol G B* 17:399–411.
30. Siegel RL, Miller KD, Jemal A (2016) Cancer statistics, 2016. *CA Cancer J Clin* 66:7–30. doi: 10.3322/caac.21332
31. Lowe KA, Chia VM, Taylor A, et al (2013) An international assessment of ovarian cancer incidence and mortality. *Gynecol Oncol* 130:107–114. doi: 10.1016/j.ygyno.2013.03.026
32. Bowtell DDL (2010) The genesis and evolution of high-grade serous ovarian cancer. *Nat Rev Cancer* 10:803–808. doi: 10.1038/nrc2946
33. Prat J (2015) FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication. *J Gynecol Oncol* 26:87–89. doi: 10.3802/jgo.2015.26.2.87
34. Cannistra SA (2004) Cancer of the Ovary. *N Engl J Med* 351:2519–2529. doi: 10.1056/NEJMra041842
35. Kurman RJ, Shih I-M (2010) The Origin and Pathogenesis of Epithelial Ovarian Cancer: A Proposed Unifying Theory: *Am J Surg Pathol* 34:433–443. doi: 10.1097/PAS.0b013e3181cf3d79
36. Bottoni P, Scatena R (2015) The Role of CA 125 as Tumor Marker: Biochemical and Clinical Aspects. In: Scatena R (ed) *Adv. Cancer Biomark*. Springer Netherlands, pp 229–244
37. Goff BA (2013) Advanced ovarian cancer: what should be the standard of care? *J Gynecol Oncol* 24:83–91. doi: 10.3802/jgo.2013.24.1.83
38. Vaughan S, Coward JI, Bast RC, et al (2011) Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 11:719–725. doi: 10.1038/nrc3144
39. Bast RC, Hennessey B, Mills GB (2009) The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* 9:415–428. doi: 10.1038/nrc2644
40. Yap TA, Carden CP, Kaye SB (2009) Beyond chemotherapy: targeted therapies in ovarian cancer. *Nat Rev Cancer* 9:167–181. doi: 10.1038/nrc2583
41. Polyak K (2011) Heterogeneity in breast cancer. *J Clin Invest* 121:3786–3788. doi: 10.1172/JCI60534

42. Perou CM, Sørli T, Eisen MB, et al (2000) Molecular portraits of human breast tumours. *Nature* 406:747–752. doi: 10.1038/35021093
43. Sørli T, Perou CM, Tibshirani R, et al (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98:10869–10874. doi: 10.1073/pnas.191367098
44. van 't Veer LJ, Dai H, van de Vijver MJ, et al (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415:530–536. doi: 10.1038/415530a
45. Arteaga CL, Sliwkowski MX, Osborne CK, et al (2012) Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat Rev Clin Oncol* 9:16–32. doi: 10.1038/nrclinonc.2011.177
46. Tauxe W (2015) A tumour through time. *Nature* 527:S102–S103. doi: 10.1038/527S102a
47. Torre LA, Siegel RL, Ward EM, Jemal A (2016) Global Cancer Incidence and Mortality Rates and Trends—An Update. *Cancer Epidemiol Prev Biomark* 25:16–27. doi: 10.1158/1055-9965.EPI-15-0578
48. Bianchini G, Balko JM, Mayer IA, et al (2016) Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nat Rev Clin Oncol*. doi: 10.1038/nrclinonc.2016.66
49. Foulkes WD, Smith IE, Reis-Filho JS (2010) Triple-Negative Breast Cancer. *N Engl J Med* 363:1938–1948. doi: 10.1056/NEJMra1001389
50. Lengyel E (2010) Ovarian Cancer Development and Metastasis. *Am J Pathol* 177:1053–1064. doi: 10.2353/ajpath.2010.100105
51. Nguyen DX, Bos PD, Massagué J (2009) Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* 9:274–284. doi: 10.1038/nrc2622
52. Steeg PS (2016) Targeting metastasis. *Nat Rev Cancer* 16:201–218. doi: 10.1038/nrc.2016.25
53. Mani SA, Guo W, Liao M-J, et al (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133:704–715. doi: 10.1016/j.cell.2008.03.027
54. Wellner U, Schubert J, Burk UC, et al (2009) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol* 11:1487–1495. doi: 10.1038/ncb1998
55. Kajiyama H, Shibata K, Terauchi M, et al (2007) Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* 31:277–283.

56. Miow QH, Tan TZ, Ye J, et al (2015) Epithelial-mesenchymal status renders differential responses to cisplatin in ovarian cancer. *Oncogene* 34:1899–1907. doi: 10.1038/onc.2014.136
57. Lamouille S, Xu J, Derynck R (2014) Molecular mechanisms of epithelial–mesenchymal transition. *Nat Rev Mol Cell Biol* 15:178–196. doi: 10.1038/nrm3758
58. Thiery JP, Acloque H, Huang RYJ, Nieto MA (2009) Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 139:871–890. doi: 10.1016/j.cell.2009.11.007
59. Craene BD, Berx G (2013) Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 13:97–110. doi: 10.1038/nrc3447
60. Tam WL, Weinberg RA (2013) The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med* 19:1438–1449. doi: 10.1038/nm.3336
61. Iwatsuki M, Mimori K, Yokobori T, et al (2010) Epithelial-mesenchymal transition in cancer development and its clinical significance. *Cancer Sci* 101:293–299. doi: 10.1111/j.1349-7006.2009.01419.x
62. Nieto MA (2011) The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu Rev Cell Dev Biol* 27:347–376. doi: 10.1146/annurev-cellbio-092910-154036
63. Nieto MA, Huang RY-J, Jackson RA, Thiery JP (2016) EMT: 2016. *Cell* 166:21–45. doi: 10.1016/j.cell.2016.06.028
64. Davidson B, Tropé CG, Reich R (2012) Epithelial–Mesenchymal Transition in Ovarian Carcinoma. *Front Oncol.* doi: 10.3389/fonc.2012.00033
65. Huang RY-J, Chung VY, Thiery JP (2012) Targeting pathways contributing to epithelial-mesenchymal transition (EMT) in epithelial ovarian cancer. *Curr Drug Targets* 13:1649–1653.
66. Vergara D, Merlot B, Lucot J-P, et al (2010) Epithelial–mesenchymal transition in ovarian cancer. *Cancer Lett* 291:59–66. doi: 10.1016/j.canlet.2009.09.017
67. Akalay I, Janji B, Hasmim M, et al (2013) Epithelial-to-mesenchymal transition and autophagy induction in breast carcinoma promote escape from T-cell-mediated lysis. *Cancer Res* 73:2418–2427. doi: 10.1158/0008-5472.CAN-12-2432
68. Foroni C, Broggin M, Generali D, Damia G (2012) Epithelial-mesenchymal transition and breast cancer: role, molecular mechanisms and clinical impact. *Cancer Treat Rev* 38:689–697. doi: 10.1016/j.ctrv.2011.11.001

69. Tomaskovic-Crook E, Thompson EW, Thiery JP (2009) Epithelial to mesenchymal transition and breast cancer. *Breast Cancer Res BCR* 11:213. doi: 10.1186/bcr2416
70. Chua K-N, Poon KL, Lim J, et al (2011) Target cell movement in tumor and cardiovascular diseases based on the epithelial–mesenchymal transition concept. *Adv Drug Deliv Rev* 63:558–567. doi: 10.1016/j.addr.2011.02.003
71. Micalizzi DS, Farabaugh SM, Ford HL (2010) Epithelial-Mesenchymal Transition in Cancer: Parallels Between Normal Development and Tumor Progression. *J Mammary Gland Biol Neoplasia* 15:117–134. doi: 10.1007/s10911-010-9178-9
72. Tan TZ, Miow QH, Miki Y, et al (2014) Epithelial-mesenchymal transition spectrum quantification and its efficacy in deciphering survival and drug responses of cancer patients. *EMBO Mol Med* 6:1279–1293. doi: 10.15252/emmm.201404208
73. Zhang J, Ma L (2012) MicroRNA control of epithelial-mesenchymal transition and metastasis. *Cancer Metastasis Rev* 31:653–662. doi: 10.1007/s10555-012-9368-6
74. Davis FM, Stewart TA, Thompson EW, Monteith GR (2014) Targeting EMT in cancer: opportunities for pharmacological intervention. *Trends Pharmacol Sci* 35:479–488. doi: 10.1016/j.tips.2014.06.006
75. DeVita VT, Chu E (2008) A History of Cancer Chemotherapy. *Cancer Res* 68:8643–8653. doi: 10.1158/0008-5472.CAN-07-6611
76. Werner HMJ, Mills GB, Ram PT (2014) Cancer Systems Biology: a peek into the future of patient care? *Nat Rev Clin Oncol* 11:167–176. doi: 10.1038/nrclinonc.2014.6
77. Imai K, Takaoka A (2006) Comparing antibody and small-molecule therapies for cancer. *Nat Rev Cancer* 6:714–727. doi: 10.1038/nrc1913
78. Adams JL, Smothers J, Srinivasan R, Hoos A (2015) Big opportunities for small molecules in immuno-oncology. *Nat Rev Drug Discov* 14:603–622. doi: 10.1038/nrd4596
79. Zhang J, Yang PL, Gray NS (2009) Targeting cancer with small molecule kinase inhibitors. *Nat Rev Cancer* 9:28–39. doi: 10.1038/nrc2559
80. Deininger M, Buchdunger E, Druker BJ (2005) The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 105:2640–2653. doi: 10.1182/blood-2004-08-3097
81. Druker BJ, Tamura S, Buchdunger E, et al (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 2:561–566.

82. Druker BJ, Talpaz M, Resta DJ, et al (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 344:1031–1037. doi: 10.1056/NEJM200104053441401
83. Druker BJ, Guilhot F, O'Brien SG, et al (2006) Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med* 355:2408–2417. doi: 10.1056/NEJMoa062867
84. Vidarsson G, Dekkers G, Rispens T (2014) IgG Subclasses and Allotypes: From Structure to Effector Functions. *Front Immunol*. doi: 10.3389/fimmu.2014.00520
85. Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495–497.
86. Mould DR, Sweeney KRD (2007) The pharmacokinetics and pharmacodynamics of monoclonal antibodies--mechanistic modeling applied to drug development. *Curr Opin Drug Discov Devel* 10:84–96.
87. Hoelder S, Clarke PA, Workman P (2012) Discovery of small molecule cancer drugs: successes, challenges and opportunities. *Mol Oncol* 6:155–176. doi: 10.1016/j.molonc.2012.02.004
88. Reichert JM (2010) Metrics for antibody therapeutics development. *mAbs* 2:695–700. doi: 10.4161/mabs.2.6.13603
89. Kola I, Landis J (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* 3:711–715. doi: 10.1038/nrd1470
90. Ecker DM, Jones SD, Levine HL (2014) The therapeutic monoclonal antibody market. *mAbs* 7:9–14. doi: 10.4161/19420862.2015.989042
91. (Rob) Aggarwal S (2014) What's fueling the biotech engine—2012 to 2013. *Nat Biotechnol* 32:32–39. doi: 10.1038/nbt.2794
92. Chartrain M, Chu L (2008) Development and production of commercial therapeutic monoclonal antibodies in Mammalian cell expression systems: an overview of the current upstream technologies. *Curr Pharm Biotechnol* 9:447–467.
93. Alkan SS (2004) Monoclonal antibodies: the story of a discovery that revolutionized science and medicine. *Nat Rev Immunol* 4:153–156. doi: 10.1038/nri1265
94. Wold ED, Smider VV, Felding BH (2016) Antibody Therapeutics in Oncology. *Immunother. Los Angel. Calif* 2:
95. Gronski P, Seiler FR, Schwick HG (1991) Discovery of antitoxins and development of antibody preparations for clinical uses from 1890 to 1990. *Mol Immunol* 28:1321–1332. doi: 10.1016/0161-5890(91)90034-H

96. Strebhardt K, Ullrich A (2008) Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer* 8:473–480. doi: 10.1038/nrc2394
97. Smith S (1996) Ten years of Orthoclone OKT3 (muromonab-CD3): a review. *J Transpl Coord* 6:109–121. doi: 10.7182/prtr.1.6.3.8145l3u185493182
98. Glennie MJ, Johnson PWM (2000) Clinical trials of antibody therapy. *Immunol Today* 21:403–410. doi: 10.1016/S0167-5699(00)01669-8
99. Jones PT, Dear PH, Foote J, et al (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321:522–525. doi: 10.1038/321522a0
100. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci U S A* 81:6851–6855.
101. Brekke OH, Sandlie I (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov* 2:52–62. doi: 10.1038/nrd984
102. Carter PJ (2006) Potent antibody therapeutics by design. *Nat Rev Immunol* 6:343–357. doi: 10.1038/nri1837
103. McCafferty J, Griffiths AD, Winter G, Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348:552–554. doi: 10.1038/348552a0
104. Brüggemann M, Caskey HM, Teale C, et al (1989) A repertoire of monoclonal antibodies with human heavy chains from transgenic mice. *Proc Natl Acad Sci U S A* 86:6709–6713.
105. Green LL, Hardy MC, Maynard-Currie CE, et al (1994) Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat Genet* 7:13–21. doi: 10.1038/ng0594-13
106. Lonberg N, Taylor LD, Harding FA, et al (1994) Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 368:856–859. doi: 10.1038/368856a0
107. Evans JB, Syed BA (2014) From the analyst's couch: Next-generation antibodies. *Nat Rev Drug Discov* 13:413–414. doi: 10.1038/nrd4255
108. Presta LG (2008) Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* 20:460–470. doi: 10.1016/j.coi.2008.06.012
109. Walsh G (2014) Biopharmaceutical benchmarks 2014. *Nat Biotechnol* 32:992–1000. doi: 10.1038/nbt.3040

110. Giezen TJ, Mantel-Teeuwisse AK, Straus SMJM, et al (2008) Safety-related regulatory actions for biologicals approved in the United States and the European Union. *JAMA* 300:1887–1896. doi: 10.1001/jama.300.16.1887
111. Hansel TT, Kropshofer H, Singer T, et al (2010) The safety and side effects of monoclonal antibodies. *Nat Rev Drug Discov* 9:325–338. doi: 10.1038/nrd3003
112. Force T, Krause DS, Van Etten RA (2007) Molecular mechanisms of cardiotoxicity of tyrosine kinase inhibition. *Nat Rev Cancer* 7:332–344. doi: 10.1038/nrc2106
113. Bianchini D, Jayanth A, Chua YJ, Cunningham D (2008) Epidermal growth factor receptor inhibitor-related skin toxicity: mechanisms, treatment, and its potential role as a predictive marker. *Clin Colorectal Cancer* 7:33–43. doi: 10.3816/CCC.2008.n.005
114. Adams GP, Weiner LM (2005) Monoclonal antibody therapy of cancer. *Nat Biotechnol* 23:1147–1157. doi: 10.1038/nbt1137
115. Weiner GJ (2015) Building better monoclonal antibody-based therapeutics. *Nat Rev Cancer* 15:361–370. doi: 10.1038/nrc3930
116. Reichert JM (2016) Antibodies to watch in 2016. *mAbs* 8:197–204. doi: 10.1080/19420862.2015.1125583
117. Hassan MS, Abedi-Valugerdi M, Lefranc G, et al (1991) Biological half-life of normal and truncated human IgG3 in scid mice. *Eur J Immunol* 21:1319–1322. doi: 10.1002/eji.1830210534
118. Stapleton NM, Andersen JT, Stemerding AM, et al (2011) Competition for FcRn-mediated transport gives rise to short half-life of human IgG3 and offers therapeutic potential. *Nat Commun* 2:599. doi: 10.1038/ncomms1608
119. Salfeld JG (2007) Isotype selection in antibody engineering. *Nat Biotechnol* 25:1369–1372. doi: 10.1038/nbt1207-1369
120. Brüggemann M, Williams GT, Bindon CI, et al (1987) Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 166:1351–1361.
121. Lohse S, Derer S, Beyer T, et al (2011) Recombinant dimeric IgA antibodies against the epidermal growth factor receptor mediate effective tumor cell killing. *J Immunol Baltim Md* 1950 186:3770–3778. doi: 10.4049/jimmunol.1003082
122. Rasche L, Duell J, Morgner C, et al (2013) The Natural Human IgM Antibody PAT-SM6 Induces Apoptosis in Primary Human Multiple Myeloma Cells by Targeting Heat Shock Protein GRP78. *PLoS ONE* 8:e63414. doi: 10.1371/journal.pone.0063414

123. Deckert PM (2009) Current constructs and targets in clinical development for antibody-based cancer therapy. *Curr Drug Targets* 10:158–175.
124. Holliger P, Hudson PJ (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23:1126–1136. doi: 10.1038/nbt1142
125. Nelson AL (2010) Antibody fragments. *mAbs* 2:77–83.
126. Nelson AL, Reichert JM (2009) Development trends for therapeutic antibody fragments. *Nat Biotechnol* 27:331–337. doi: 10.1038/nbt0409-331
127. Kontermann RE, Brinkmann U (2015) Bispecific antibodies. *Drug Discov Today* 20:838–847. doi: 10.1016/j.drudis.2015.02.008
128. Sheridan C (2015) Amgen's bispecific antibody puffs across finish line. *Nat Biotechnol* 33:219–221. doi: 10.1038/nbt0315-219
129. Mullard A (2015) FDA approves first bispecific. *Nat Rev Drug Discov* 14:7–7. doi: 10.1038/nrd4531
130. Sun LL, Ellerman D, Mathieu M, et al (2015) Anti-CD20/CD3 T cell-dependent bispecific antibody for the treatment of B cell malignancies. *Sci Transl Med* 7:287ra70. doi: 10.1126/scitranslmed.aaa4802
131. Wolf E, Hofmeister R, Kufer P, et al (2005) BiTEs: bispecific antibody constructs with unique anti-tumor activity. *Drug Discov Today* 10:1237–1244. doi: 10.1016/S1359-6446(05)03554-3
132. Linke R, Klein A, Seimetz D (2010) Catumaxomab. *mAbs* 2:129–136.
133. Nagorsen D, Kufer P, Baeuerle PA, Bargou R (2012) Blinatumomab: a historical perspective. *Pharmacol Ther* 136:334–342. doi: 10.1016/j.pharmthera.2012.07.013
134. Seimetz D (2011) Novel Monoclonal Antibodies for Cancer Treatment: The Trifunctional Antibody Catumaxomab (Removab®). *J Cancer* 2:309–316.
135. Byrne H, Conroy PJ, Whisstock JC, O'Kennedy RJ (2013) A tale of two specificities: bispecific antibodies for therapeutic and diagnostic applications. *Trends Biotechnol* 31:621–632. doi: 10.1016/j.tibtech.2013.08.007
136. Garber K (2014) Bispecific antibodies rise again. *Nat Rev Drug Discov* 13:799–801. doi: 10.1038/nrd4478
137. Spiess C, Zhai Q, Carter PJ (2015) Alternative molecular formats and therapeutic applications for bispecific antibodies. *Mol Immunol* 67:95–106. doi: 10.1016/j.molimm.2015.01.003

138. Adinolfi M, Lessof MH (1985) Cancer, oncogenes and oncofetal antigens. *Q J Med* 54:193–204.
139. Brewer BG, Mitchell RA, Harandi A, Eaton JW (2009) Embryonic vaccines against cancer: an early history. *Exp Mol Pathol* 86:192–197. doi: 10.1016/j.yexmp.2008.12.002
140. Magdelénat H (1992) Tumour markers in oncology: past, present and future. *J Immunol Methods* 150:133–143.
141. Wright AJ, Andrews PW (2009) Surface marker antigens in the characterization of human embryonic stem cells. *Stem Cell Res* 3:3–11. doi: 10.1016/j.scr.2009.04.001
142. Terentiev AA, Moldogazieva NT (2013) Alpha-fetoprotein: a renaissance. *Tumor Biol* 34:2075–2091. doi: 10.1007/s13277-013-0904-y
143. Gold P, Freedman SO (1965) DEMONSTRATION OF TUMOR-SPECIFIC ANTIGENS IN HUMAN COLONIC CARCINOMATA BY IMMUNOLOGICAL TOLERANCE AND ABSORPTION TECHNIQUES. *J Exp Med* 121:439–462.
144. Bast RC, Feeney M, Lazarus H, et al (1981) Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 68:1331–1337.
145. Diamandis EP, Bast RC, Gold P, et al (2013) REFLECTION ON THE DISCOVERY OF CARCINOEMBRYONIC ANTIGEN, PROSTATE-SPECIFIC ANTIGEN AND CARBOHYDRATE ANTIGENS CA125 and CA19.9. *Clin Chem* 59:22–31. doi: 10.1373/clinchem.2012.187047
146. Beauchemin N, Arabzadeh A (2013) Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. *Cancer Metastasis Rev* 32:643–671. doi: 10.1007/s10555-013-9444-6
147. Hammarström S (1999) The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol* 9:67–81. doi: 10.1006/scbi.1998.0119
148. Ho MF Mitchell (2014) Glypican-3 antibodies: a new therapeutic target for liver cancer. *FEBS Lett* 588:377–382. doi: 10.1016/j.febslet.2013.10.002
149. Ho M, Kim H (2011) Glypican-3: a new target for cancer immunotherapy. *Eur J Cancer Oxf Engl* 1990 47:333–338. doi: 10.1016/j.ejca.2010.10.024
150. Mizejewski GJ (2002) Biological role of alpha-fetoprotein in cancer: prospects for anticancer therapy. *Expert Rev Anticancer Ther* 2:709–735. doi: 10.1586/14737140.2.6.709

151. Taketa K, Endo Y, Sekiya C, et al (1993) A collaborative study for the evaluation of lectin-reactive alpha-fetoproteins in early detection of hepatocellular carcinoma. *Cancer Res* 53:5419–5423.
152. Toyoda H, Kumada T, Tada T, et al (2011) Clinical utility of highly sensitive Lens culinaris agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/mL. *Cancer Sci* 102:1025–1031. doi: 10.1111/j.1349-7006.2011.01875.x
153. Campbell BJ, Finnie IA, Hounsell EF, Rhodes JM (1995) Direct demonstration of increased expression of Thomsen-Friedenreich (TF) antigen in colonic adenocarcinoma and ulcerative colitis mucin and its concealment in normal mucin. *J Clin Invest* 95:571–576. doi: 10.1172/JCI117700
154. Govindan SV, Cardillo TM, Moon S-J, et al (2009) CEACAM5-targeted therapy of human colonic and pancreatic cancer xenografts with potent labetuzumab-SN-38 immunoconjugates. *Clin Cancer Res Off J Am Assoc Cancer Res* 15:6052–6061. doi: 10.1158/1078-0432.CCR-09-0586
155. Stein R, Goldenberg DM (2004) A humanized monoclonal antibody to carcinoembryonic antigen, labetuzumab, inhibits tumor growth and sensitizes human medullary thyroid cancer xenografts to dacarbazine chemotherapy. *Mol Cancer Ther* 3:1559–1564.
156. Chen Y, Clark S, Wong T, et al (2007) Armed antibodies targeting the mucin repeats of the ovarian cancer antigen, MUC16, are highly efficacious in animal tumor models. *Cancer Res* 67:4924–4932. doi: 10.1158/0008-5472.CAN-06-4512
157. Eskander RN, Tewari KS (2013) Epithelial cell-adhesion molecule-directed trifunctional antibody immunotherapy for symptom management of advanced ovarian cancer. *Clin Pharmacol Adv Appl* 5:55–61. doi: 10.2147/CPAA.S45885
158. McClintock SD, Warner RL, Ali S, et al (2015) Monoclonal antibodies specific for oncofetal antigen--immature laminin receptor protein: Effects on tumor growth and spread in two murine models. *Cancer Biol Ther* 16:724–732. doi: 10.1080/15384047.2015.1026484
159. Borcherdig N, Kusner D, Liu G-H, Zhang W (2014) ROR1, an embryonic protein with an emerging role in cancer biology. *Protein Cell* 5:496–502. doi: 10.1007/s13238-014-0059-7
160. Shabani M, Naseri J, Shokri F (2015) Receptor tyrosine kinase-like orphan receptor 1: a novel target for cancer immunotherapy. *Expert Opin Ther Targets* 19:941–955. doi: 10.1517/14728222.2015.1025753
161. Freire-de-Lima L, Gelfenbeyn K, Ding Y, et al (2011) Involvement of O-glycosylation defining oncofetal fibronectin in epithelial-mesenchymal

- transition process. *Proc Natl Acad Sci U S A* 108:17690–17695. doi: 10.1073/pnas.1115191108
162. Nielsen JS, McNagny KM (2008) Novel functions of the CD34 family. *J Cell Sci* 121:3683–3692. doi: 10.1242/jcs.037507
 163. Sassetti C, Van Zante A, Rosen SD (2000) Identification of endoglycan, a member of the CD34/podocalyxin family of sialomucins. *J Biol Chem* 275:9001–9010.
 164. Kaucká M, Krejčí P, Plevová K, et al (2011) Post-translational modifications regulate signalling by Ror1: Post-translational modification of Ror1. *Acta Physiol* 203:351–362. doi: 10.1111/j.1748-1716.2011.02306.x
 165. Shaw DM, Woods AM, Myers KA, et al (2002) Glycosylation and epitope mapping of the 5T4 glycoprotein oncofoetal antigen. *Biochem J* 363:137–145.
 166. Weerasinghe P, Buja LM (2012) Oncosis: an important non-apoptotic mode of cell death. *Exp Mol Pathol* 93:302–308. doi: 10.1016/j.yexmp.2012.09.018
 167. Majno G, Joris I (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 146:3–15.
 168. Chua JX, Vankemmelbeke M, McIntosh RS, et al (2015) Monoclonal Antibodies Targeting LecLex-Related Glycans with Potent Antitumor Activity. *Clin Cancer Res* 21:2963–2974. doi: 10.1158/1078-0432.CCR-14-3030
 169. Hernandez AM, Rodriguez N, Gonzalez JE, et al (2011) Anti-NeuGcGM3 Antibodies, Actively Elicited by Idiotypic Vaccination in Nonsmall Cell Lung Cancer Patients, Induce Tumor Cell Death by an Oncosis-Like Mechanism. *J Immunol* 186:3735–3744. doi: 10.4049/jimmunol.1000609
 170. Loo D, Pryer N, Young P, et al (2007) The glycotope-specific RAV12 monoclonal antibody induces oncosis in vitro and has antitumor activity against gastrointestinal adenocarcinoma tumor xenografts in vivo. *Mol Cancer Ther* 6:856–865. doi: 10.1158/1535-7163.MCT-06-0581
 171. Patz M, Isaeva P, Forcob N, et al (2011) Comparison of the in vitro effects of the anti-CD20 antibodies rituximab and GA101 on chronic lymphocytic leukaemia cells. *Br J Haematol* 152:295–306. doi: 10.1111/j.1365-2141.2010.08428.x
 172. Ferrara N, Adamis AP (2016) Ten years of anti-vascular endothelial growth factor therapy. *Nat Rev Drug Discov* 15:385–403. doi: 10.1038/nrd.2015.17
 173. Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. *Nature* 438:967–974. doi: 10.1038/nature04483

174. Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. *Cell* 146:873–887. doi: 10.1016/j.cell.2011.08.039
175. Samant RS, Shevde LA (2011) Recent Advances in Anti-Angiogenic Therapy of Cancer. *Oncotarget* 2:122–134.
176. Folkman J (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med* 285:1182–1186. doi: 10.1056/NEJM197111182852108
177. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 6:273–286. doi: 10.1038/nrd2115
178. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6:392–401. doi: 10.1038/nrc1877
179. Pietras K, Sjöblom T, Rubin K, et al (2003) PDGF receptors as cancer drug targets. *Cancer Cell* 3:439–443.
180. Folkman J (1990) What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82:4–6.
181. Kerbel R, Folkman J (2002) Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2:727–739. doi: 10.1038/nrc905
182. Ellis LM, Hicklin DJ (2008) VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* 8:579–591. doi: 10.1038/nrc2403
183. Ferrara N (2016) Commentary on “Humanization of an Anti-VEGF Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders.” *Cancer Res* 76:4913–4915. doi: 10.1158/0008-5472.CAN-16-1973
184. Kim KJ, Li B, Winer J, et al (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362:841–844. doi: 10.1038/362841a0
185. Brack SS, Silacci M, Birchler M, Neri D (2006) Tumor-Targeting Properties of Novel Antibodies Specific to the Large Isoform of Tenascin-C. *Clin Cancer Res* 12:3200–3208. doi: 10.1158/1078-0432.CCR-05-2804
186. Czajkowsky DM, Shao Z (2009) The human IgM pentamer is a mushroom-shaped molecule with a flexural bias. *Proc Natl Acad Sci U S A* 106:14960–14965. doi: 10.1073/pnas.0903805106
187. Gaboriaud C, Thielens NM, Gregory LA, et al (2004) Structure and activation of the C1 complex of complement: unraveling the puzzle. *Trends Immunol* 25:368–373. doi: 10.1016/j.it.2004.04.008

188. Ricklin D, Hajishengallis G, Yang K, Lambris JD (2010) Complement - a key system for immune surveillance and homeostasis. *Nat Immunol* 11:785–797. doi: 10.1038/ni.1923
189. Diebolder CA, Beurskens FJ, de Jong RN, et al (2014) Complement Is Activated by IgG Hexamers Assembled at the Cell Surface. *Science* 343:1260–1263. doi: 10.1126/science.1248943
190. Idusogie EE, Presta LG, Gazzano-Santoro H, et al (2000) Mapping of the C1q Binding Site on Rituxan, a Chimeric Antibody with a Human IgG1 Fc. *J Immunol* 164:4178–4184. doi: 10.4049/jimmunol.164.8.4178
191. Idusogie EE, Wong PY, Presta LG, et al (2001) Engineered antibodies with increased activity to recruit complement. *J Immunol Baltim Md* 150:2571–2575.
192. Michaelsen TE, Sandlie I, Bratlie DB, et al (2009) Structural difference in the complement activation site of human IgG1 and IgG3. *Scand J Immunol* 70:553–564. doi: 10.1111/j.1365-3083.2009.02338.x
193. Cragg MS, Morgan SM, Chan HTC, et al (2003) Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood* 101:1045–1052. doi: 10.1182/blood-2002-06-1761
194. Weiner GJ (2010) Rituximab: mechanism of action. *Semin Hematol* 47:115–123. doi: 10.1053/j.seminhematol.2010.01.011
195. Nimmerjahn F, Ravetch JV (2008) Fcγ receptors as regulators of immune responses. *Nat Rev Immunol* 8:34–47. doi: 10.1038/nri2206
196. Hayes JM, Cosgrave EFJ, Struwe WB, et al (2014) Glycosylation and Fc receptors. *Curr Top Microbiol Immunol* 382:165–199. doi: 10.1007/978-3-319-07911-0_8
197. Carson WE, Parihar R, Lindemann MJ, et al (2001) Interleukin-2 enhances the natural killer cell response to Herceptin-coated Her2/neu-positive breast cancer cells. *Eur J Immunol* 31:3016–3025. doi: 10.1002/1521-4141(2001010)31:10<3016::AID-IMMU3016>3.0.CO;2-J
198. Hubert P, Heitzmann A, Viel S, et al (2011) Antibody-Dependent Cell Cytotoxicity Synapses Form in Mice during Tumor-Specific Antibody Immunotherapy. *Cancer Res* 71:5134–5143. doi: 10.1158/0008-5472.CAN-10-4222
199. Clynes RA, Towers TL, Presta LG, Ravetch JV (2000) Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med* 6:443–446. doi: 10.1038/74704
200. Weng W-K, Levy R (2003) Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients

with follicular lymphoma. *J Clin Oncol Off J Am Soc Clin Oncol* 21:3940–3947. doi: 10.1200/JCO.2003.05.013

201. Cartron G, Dacheux L, Salles G, et al (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood* 99:754–758. doi: 10.1182/blood.V99.3.754
202. Koene HR, Kleijer M, Algra J, et al (1997) Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. *Blood* 90:1109–1114.
203. Musolino A, Naldi N, Bortesi B, et al (2008) Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol* 26:1789–1796. doi: 10.1200/JCO.2007.14.8957
204. Bibeau F, Lopez-Crapez E, Di Fiore F, et al (2009) Impact of Fc{gamma}RIIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. *J Clin Oncol Off J Am Soc Clin Oncol* 27:1122–1129. doi: 10.1200/JCO.2008.18.0463
205. Mellman I, Coukos G, Dranoff G (2011) Cancer immunotherapy comes of age. *Nature* 480:480–489. doi: 10.1038/nature10673
206. Lee L, Gupta M, Sahasranaman S (2016) Immune Checkpoint inhibitors: An introduction to the next-generation cancer immunotherapy. *J Clin Pharmacol* 56:157–169. doi: 10.1002/jcph.591
207. Page DB, Postow MA, Callahan MK, et al (2014) Immune Modulation in Cancer with Antibodies. *Annu Rev Med* 65:185–202. doi: 10.1146/annurev-med-092012-112807
208. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12:252–264. doi: 10.1038/nrc3239
209. Topalian SL, Drake CG, Pardoll DM (2015) Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 27:450–461. doi: 10.1016/j.ccell.2015.03.001
210. Weiner LM, Murray JC, Shuptrine CW (2012) Antibody-based immunotherapy of cancer. *Cell* 148:1081–1084. doi: 10.1016/j.cell.2012.02.034
211. Senter PD (2009) Potent antibody drug conjugates for cancer therapy. *Curr Opin Chem Biol* 13:235–244. doi: 10.1016/j.cbpa.2009.03.023

212. Sievers EL, Senter PD (2013) Antibody-drug conjugates in cancer therapy. *Annu Rev Med* 64:15–29. doi: 10.1146/annurev-med-050311-201823
213. Teicher BA, Chari RVJ (2011) Antibody conjugate therapeutics: challenges and potential. *Clin Cancer Res Off J Am Assoc Cancer Res* 17:6389–6397. doi: 10.1158/1078-0432.CCR-11-1417
214. Thomas A, Teicher BA, Hassan R (2016) Antibody-drug conjugates for cancer therapy. *Lancet Oncol* 17:e254-262. doi: 10.1016/S1470-2045(16)30030-4
215. Wu AM, Senter PD (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol* 23:1137–1146. doi: 10.1038/nbt1141
216. Dumontet C, Jordan MA (2010) Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat Rev Drug Discov* 9:790–803. doi: 10.1038/nrd3253
217. Oroudjev E, Lopus M, Wilson L, et al (2010) Maytansinoid-antibody conjugates induce mitotic arrest by suppressing microtubule dynamic instability. *Mol Cancer Ther* 9:2700–2713. doi: 10.1158/1535-7163.MCT-10-0645
218. Chari RV, Martell BA, Gross JL, et al (1992) Immunoconjugates containing novel maytansinoids: promising anticancer drugs. *Cancer Res* 52:127–131.
219. Hamblett KJ, Senter PD, Chace DF, et al (2004) Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res Off J Am Assoc Cancer Res* 10:7063–7070. doi: 10.1158/1078-0432.CCR-04-0789
220. Bidard F-C, Trédan O (2013) Trends in cancer-targeted antibody-drug conjugates. *Target Oncol*. doi: 10.1007/s11523-013-0302-9
221. Alley SC, Okeley NM, Senter PD (2010) Antibody-drug conjugates: targeted drug delivery for cancer. *Curr Opin Chem Biol* 14:529–537. doi: 10.1016/j.cbpa.2010.06.170
222. Diamantis N, Banerji U (2016) Antibody-drug conjugates--an emerging class of cancer treatment. *Br J Cancer* 114:362–367. doi: 10.1038/bjc.2015.435
223. Junutula JR, Raab H, Clark S, et al (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol* 26:925–932. doi: 10.1038/nbt.1480
224. Panowski S, Bhakta S, Raab H, et al (2014) Site-specific antibody drug conjugates for cancer therapy. *mAbs* 6:34–45. doi: 10.4161/mabs.27022

225. Deng C, Pan B, O'Connor OA (2013) Brentuximab vedotin. *Clin Cancer Res Off J Am Assoc Cancer Res* 19:22–27. doi: 10.1158/1078-0432.CCR-12-0290
226. Katz J, Janik JE, Younes A (2011) Brentuximab Vedotin (SGN-35). *Am Assoc Cancer Res* 17:6428–6436. doi: 10.1158/1078-0432.CCR-11-0488
227. Senter PD, Sievers EL (2012) The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nat Biotechnol* 30:631–637. doi: 10.1038/nbt.2289
228. Amiri-Kordestani L, Blumenthal GM, Xu QC, et al (2014) FDA approval: ado-trastuzumab emtansine for the treatment of patients with HER2-positive metastatic breast cancer. *Clin Cancer Res Off J Am Assoc Cancer Res* 20:4436–4441. doi: 10.1158/1078-0432.CCR-14-0012
229. Lambert JM, Chari RVJ (2014) Ado-trastuzumab Emtansine (T-DM1): An Antibody–Drug Conjugate (ADC) for HER2-Positive Breast Cancer. *J Med Chem* 57:6949–6964. doi: 10.1021/jm500766w
230. Desjarlais JR, Lazar GA, Zhukovsky EA, Chu SY (2007) Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective. *Drug Discov Today* 12:898–910. doi: 10.1016/j.drudis.2007.08.009
231. Kubota T, Niwa R, Satoh M, et al (2009) Engineered therapeutic antibodies with improved effector functions. *Cancer Sci* 100:1566–1572. doi: 10.1111/j.1349-7006.2009.01222.x
232. Jong RN de, Beurskens FJ, Verploegen S, et al (2016) A Novel Platform for the Potentiation of Therapeutic Antibodies Based on Antigen-Dependent Formation of IgG Hexamers at the Cell Surface. *PLOS Biol* 14:e1002344. doi: 10.1371/journal.pbio.1002344
233. Moore GL, Chen H, Karki S, A G (2010) Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. *mAbs* 2:181–189.
234. Igawa T, Ishii S, Tachibana T, et al (2010) Antibody recycling by engineered pH-dependent antigen binding improves the duration of antigen neutralization. *Nat Biotechnol* 28:1203–1207. doi: 10.1038/nbt.1691
235. Kuo TT, Aveson VG (2011) Neonatal Fc receptor and IgG-based therapeutics. *mAbs* 3:422–430. doi: 10.4161/mabs.3.5.16983
236. Jiang X-R, Song A, Bergelson S, et al (2011) Advances in the assessment and control of the effector functions of therapeutic antibodies. *Nat Rev Drug Discov* 10:101–111. doi: 10.1038/nrd3365

237. Natsume A, Niwa R, Satoh M (2009) Improving effector functions of antibodies for cancer treatment: Enhancing ADCC and CDC. *Drug Des Devel Ther* 3:7–16.
238. Beck A, Reichert JM (2012) Marketing approval of mogamulizumab: a triumph for glyco-engineering. *mAbs* 4:419–425. doi: 10.4161/mabs.20996
239. Jefferis R (2009) Glycosylation as a strategy to improve antibody-based therapeutics. *Nat Rev Drug Discov* 8:226–234. doi: 10.1038/nrd2804
240. Lee H-Z, Miller BW, Kwitkowski VE, et al (2014) U.S. Food and drug administration approval: obinutuzumab in combination with chlorambucil for the treatment of previously untreated chronic lymphocytic leukemia. *Clin Cancer Res Off J Am Assoc Cancer Res* 20:3902–3907. doi: 10.1158/1078-0432.CCR-14-0516
241. Gerdes CA, Nicolini VG, Herter S, et al (2013) GA201 (RG7160): A Novel, Humanized, Glycoengineered Anti-EGFR Antibody with Enhanced ADCC and Superior In Vivo Efficacy Compared with Cetuximab. *Clin Cancer Res* 19:1126–1138. doi: 10.1158/1078-0432.CCR-12-0989
242. Junttila TT, Parsons K, Olsson C, et al (2010) Superior In vivo Efficacy of Afucosylated Trastuzumab in the Treatment of HER2-Amplified Breast Cancer. *Cancer Res* 70:4481–4489. doi: 10.1158/0008-5472.CAN-09-3704
243. Shields RL, Lai J, Keck R, et al (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcγ₃ and antibody-dependent cellular toxicity. *J Biol Chem* 277:26733–26740. doi: 10.1074/jbc.M202069200
244. Cerny T, Borisch B, Introna M, et al (2002) Mechanism of action of rituximab. *Anticancer Drugs* 13 Suppl 2:S3-10.
245. Di Gaetano N, Cittera E, Nota R, et al (2003) Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol Baltim Md* 1950 171:1581–1587.
246. Johnson P, Glennie M (2003) The mechanisms of action of rituximab in the elimination of tumor cells. *Semin Oncol* 30:3–8. doi: 10.1053/sonc.2003.50025
247. Kennedy AD, Beum PV, Solga MD, et al (2004) Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol Baltim Md* 1950 172:3280–3288.
248. Valabrega G, Montemurro F, Aglietta M (2007) Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. *Ann Oncol Off J Eur Soc Med Oncol* 18:977–984. doi: 10.1093/annonc/mdl475

249. Breccia M, Lo-Coco F (2011) Gemtuzumab ozogamicin for the treatment of acute promyelocytic leukemia: mechanisms of action and resistance, safety and efficacy. *Expert Opin Biol Ther* 11:225–234. doi: 10.1517/14712598.2011.543895
250. Alinari L, Lapalombella R, Andritsos L, et al (2007) Alemtuzumab (Campath-1H) in the treatment of chronic lymphocytic leukemia. *Oncogene* 26:3644–3653. doi: 10.1038/sj.onc.1210380
251. Chinn PC, Leonard JE, Rosenberg J, et al (1999) Preclinical evaluation of 90Y-labeled anti-CD20 monoclonal antibody for treatment of non-Hodgkin's lymphoma. *Int J Oncol* 15:1017–1025.
252. Davies AJ (2005) A review of tositumomab and I131 tositumomab radioimmunotherapy for the treatment of follicular lymphoma. *Expert Opin Biol Ther* 5:577–588. doi: 10.1517/14712598.5.4.577
253. Beers SA, Chan CHT, James S, et al (2008) Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. *Blood* 112:4170–4177. doi: 10.1182/blood-2008-04-149161
254. Grothey A, Galanis E (2009) Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. *Nat Rev Clin Oncol* 6:507–518. doi: 10.1038/nrclinonc.2009.110
255. Mukohara T, Engelman JA, Hanna NH, et al (2005) Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. *J Natl Cancer Inst* 97:1185–1194. doi: 10.1093/jnci/dji238
256. Schneider-Merck T, Bueren JJL van, Berger S, et al (2010) Human IgG2 Antibodies against Epidermal Growth Factor Receptor Effectively Trigger Antibody-Dependent Cellular Cytotoxicity but, in Contrast to IgG1, Only by Cells of Myeloid Lineage. *J Immunol* 184:512–520. doi: 10.4049/jimmunol.0900847
257. Yang XD, Jia XC, Corvalan JR, et al (1999) Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. *Cancer Res* 59:1236–1243.
258. Zhang B (2009) Ofatumumab. *mAbs* 1:326–331.
259. Doronina SO, Toki BE, Torgov MY, et al (2003) Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat Biotechnol* 21:778–784. doi: 10.1038/nbt832
260. Francisco JA, Cervený CG, Meyer DL, et al (2003) cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood* 102:1458–1465. doi: 10.1182/blood-2003-01-0039

261. Oflazoglu E, Kissler KM, Sievers EL, et al (2008) Combination of the anti-CD30-auristatin-E antibody-drug conjugate (SGN-35) with chemotherapy improves antitumour activity in Hodgkin lymphoma. *Br J Haematol* 142:69–73. doi: 10.1111/j.1365-2141.2008.07146.x
262. Okeley NM, Miyamoto JB, Zhang X, et al (2010) Intracellular activation of SGN-35, a potent anti-CD30 antibody-drug conjugate. *Clin Cancer Res Off J Am Assoc Cancer Res* 16:888–897. doi: 10.1158/1078-0432.CCR-09-2069
263. Peggs KS, Quezada SA, Korman AJ, Allison JP (2006) Principles and use of anti-CTLA4 antibody in human cancer immunotherapy. *Curr Opin Immunol* 18:206–213. doi: 10.1016/j.coi.2006.01.011
264. Adams CW, Allison DE, Flagella K, et al (2006) Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer Immunol Immunother* 55:717. doi: 10.1007/s00262-005-0058-x
265. Franklin MC, Carey KD, Vajdos FF, et al (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* 5:317–328.
266. Fischer K, Busch R, Engelke A, et al (2013) Head-To-Head Comparison Of Obinutuzumab (GA101) Plus Chlorambucil (Clb) Versus Rituximab Plus Clb In Patients With Chronic Lymphocytic Leukemia (CLL) and Co-Existing Medical Conditions (Comorbidities): Final Stage 2 Results Of The CLL11 Trial. *Blood* 122:6–6.
267. Golay J, Roit FD, Bologna L, et al (2013) Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. *Blood* 122:3482–3491. doi: 10.1182/blood-2013-05-504043
268. Mössner E, Brünker P, Moser S, et al (2010) Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood* 115:4393–4402. doi: 10.1182/blood-2009-06-225979
269. Dineen SP, Lynn KD, Holloway SE, et al (2008) Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice. *Cancer Res* 68:4340–4346. doi: 10.1158/0008-5472.CAN-07-6705
270. Lu D, Jimenez X, Zhang H, et al (2002) Selection of high affinity human neutralizing antibodies to VEGFR2 from a large antibody phage display library for antiangiogenesis therapy. *Int J Cancer* 97:393–399.
271. Lu D, Shen J, Vil MD, et al (2003) Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial

- growth factor receptor 2 for enhanced neutralizing activity. *J Biol Chem* 278:43496–43507. doi: 10.1074/jbc.M307742200
272. Hamid O, Robert C, Daud A, et al (2013) Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* 369:134–144. doi: 10.1056/NEJMoa1305133
 273. Wang C, Thudium KB, Han M, et al (2014) In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer Immunol Res* 2:846–856. doi: 10.1158/2326-6066.CIR-14-0040
 274. Löffler A, Kufer P, Lutterbüse R, et al (2000) A recombinant bispecific single-chain antibody, CD19 x CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. *Blood* 95:2098–2103.
 275. Krejcik J, Casneuf T, Nijhof IS, et al (2016) Daratumumab depletes CD38+ immune-regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma. *Blood* blood-2015-12-687749. doi: 10.1182/blood-2015-12-687749
 276. Overdijk MB, Verploegen S, Marijn B, et al (2012) Phagocytosis Is A Mechanism of Action for Daratumumab. *Blood* 120:4054–4054.
 277. de Weers M, Tai Y-T, van der Veer MS, et al (2011) Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol Baltim Md* 1950 186:1840–1848. doi: 10.4049/jimmunol.1003032
 278. Collins SM, Bakan CE, Swartzel GD, et al (2013) Elotuzumab directly enhances NK cell cytotoxicity against myeloma via CS1 ligation: evidence for augmented NK cell function complementing ADCC. *Cancer Immunol Immunother CII* 62:1841–1849. doi: 10.1007/s00262-013-1493-8
 279. Hsi ED, Steinle R, Balasa B, et al (2008) CS1, a Potential New Therapeutic Antibody Target for the Treatment of Multiple Myeloma. *Clin Cancer Res* 14:2775–2784. doi: 10.1158/1078-0432.CCR-07-4246
 280. Tai Y-T, Dillon M, Song W, et al (2008) Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood* 112:1329–1337. doi: 10.1182/blood-2007-08-107292
 281. Deng R, Bumbaca D, Pastuskovas CV, et al (2016) Preclinical pharmacokinetics, pharmacodynamics, tissue distribution, and tumor penetration of anti-PD-L1 monoclonal antibody, an immune checkpoint inhibitor. *mAbs* 8:593–603. doi: 10.1080/19420862.2015.1136043

282. Herbst RS, Soria J-C, Kowanetz M, et al (2014) Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 515:563–567. doi: 10.1038/nature14011
283. Powles T, Eder JP, Fine GD, et al (2014) MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 515:558–562. doi: 10.1038/nature13904
284. Slamon DJ, Clark GM, Wong SG, et al (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235:177–182.
285. Slamon DJ, Godolphin W, Jones LA, et al (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244:707–712.
286. Slamon DJ, Leyland-Jones B, Shak S, et al (2001) Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. *N Engl J Med* 344:783–792. doi: 10.1056/NEJM200103153441101
287. Press MF, Sauter G, Buyse M, et al (2016) HER2 Gene Amplification Testing by Fluorescent In Situ Hybridization (FISH): Comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials. *J Clin Oncol Off J Am Soc Clin Oncol*. doi: 10.1200/JCO.2016.66.6693
288. Révillion F, Bonnetterre J, Peyrat JP (1998) ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Cancer Oxf Engl* 34:791–808.
289. Wolff AC, Hammond MEH, Hicks DG, et al (2013) Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *J Clin Oncol Off J Am Soc Clin Oncol* 31:3997–4013. doi: 10.1200/JCO.2013.50.9984
290. Stopeck AT, Brown-Glaberman U, Wong HY, et al (2012) The role of targeted therapy and biomarkers in breast cancer treatment. *Clin Exp Metastasis* 29:807–819. doi: 10.1007/s10585-012-9496-y
291. Ghatalia P, Morgan CJ, Je Y, et al (2015) Congestive heart failure with vascular endothelial growth factor receptor tyrosine kinase inhibitors. *Crit Rev Oncol Hematol* 94:228–237. doi: 10.1016/j.critrevonc.2014.12.008
292. Pàez-Ribes M, Allen E, Hudock J, et al (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15:220–231. doi: 10.1016/j.ccr.2009.01.027

293. Pujade-Lauraine E, Hilpert F, Weber B, et al (2014) Bevacizumab combined with chemotherapy for platinum-resistant recurrent ovarian cancer: The AURELIA open-label randomized phase III trial. *J Clin Oncol Off J Am Soc Clin Oncol* 32:1302–1308. doi: 10.1200/JCO.2013.51.4489
294. Aghajanian C, Blank SV, Goff BA, et al (2012) OCEANS: a randomized, double-blind, placebo-controlled phase III trial of chemotherapy with or without bevacizumab in patients with platinum-sensitive recurrent epithelial ovarian, primary peritoneal, or fallopian tube cancer. *J Clin Oncol Off J Am Soc Clin Oncol* 30:2039–2045. doi: 10.1200/JCO.2012.42.0505
295. Burger RA, Brady MF, Bookman MA, et al (2011) Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N Engl J Med* 365:2473–2483. doi: 10.1056/NEJMoa1104390
296. Becker G, Galandi D, Blum HE (2006) Malignant ascites: systematic review and guideline for treatment. *Eur J Cancer Oxf Engl* 1990 42:589–597. doi: 10.1016/j.ejca.2005.11.018
297. Smolle E, Taucher V, Haybaeck J (2014) Malignant ascites in ovarian cancer and the role of targeted therapeutics. *Anticancer Res* 34:1553–1561.
298. Sapra P, Shor B (2013) Monoclonal antibody-based therapies in cancer: Advances and challenges. *Pharmacol Ther.* doi: 10.1016/j.pharmthera.2013.03.004
299. Tse BWC, Collins A, Oehler MK, et al (2013) Antibody-based immunotherapy for ovarian cancer: where are we at? *Ann Oncol mdt*405. doi: 10.1093/annonc/mdt405
300. Vacchelli E, Pol J, Bloy N, et al (2015) Trial watch: Tumor-targeting monoclonal antibodies for oncological indications. *Oncoimmunology* 4:e985940. doi: 10.4161/2162402X.2014.985940
301. Leone Roberti Maggiore U, Bellati F, Ruscito I, et al (2013) Monoclonal antibodies therapies for ovarian cancer. *Expert Opin Biol Ther* 13:739–764. doi: 10.1517/14712598.2013.767328
302. Choo AB, Tan HL, Ang SN, et al (2008) Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells Dayt Ohio* 26:1454–1463. doi: 10.1634/stemcells.2007-0576
303. Lee J, Gan HT, Latiff SMA, et al (2012) Principles and applications of steric exclusion chromatography. *J Chromatogr A* 1270:162–170. doi: 10.1016/j.chroma.2012.10.062
304. Ho SCL, Bardor M, Feng H, et al (2012) IRES-mediated Tricistronic vectors for enhancing generation of high monoclonal antibody

- expressing CHO cell lines. *J Biotechnol* 157:130–139. doi: 10.1016/j.jbiotec.2011.09.023
305. Jimenez L, Rana N, Travers K, et al (2010) Evaluation of the Endosafe(R) Portable Testing System™ for the Rapid Analysis of Biopharmaceutical Samples. *PDA J Pharm Sci Technol* 64:211–221.
 306. Malyala P, Singh M (2008) Endotoxin limits in formulations for preclinical research. *J Pharm Sci* 97:2041–2044. doi: 10.1002/jps.21152
 307. Ng VY, Ang SN, Chan JX, Choo ABH (2010) Characterization of Epithelial Cell Adhesion Molecule as a Surface Marker on Undifferentiated Human Embryonic Stem Cells. *STEM CELLS* 28:29–35. doi: 10.1002/stem.221
 308. Sjögren J, Olsson F, Beck A (2016) Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis. *The Analyst* 141:3114–3125. doi: 10.1039/c6an00071a
 309. Kohls MD, Lappi DA (2000) Mab-ZAP: a tool for evaluating antibody efficacy for use in an immunotoxin. *BioTechniques* 28:162–165.
 310. Woodward MP, Young WW, Bloodgood RA (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. *J Immunol Methods* 78:143–153. doi: 10.1016/0022-1759(85)90337-0
 311. Maley F, Trimble RB, Tarentino AL, Plummer TH (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Anal Biochem* 180:195–204.
 312. Tretter V, Altmann F, März L (1991) Peptide-N4-(N-acetyl-beta-glucosaminyl)asparagine amidase F cannot release glycans with fucose attached alpha 1----3 to the asparagine-linked N-acetylglucosamine residue. *Eur J Biochem* 199:647–652.
 313. Brandish PE, Kimura KI, Inukai M, et al (1996) Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. *Antimicrob Agents Chemother* 40:1640–1644.
 314. Elbein AD (1987) Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu Rev Biochem* 56:497–534. doi: 10.1146/annurev.bi.56.070187.002433
 315. Dricu A, Carlberg M, Wang M, Larsson O (1997) Inhibition of N-linked glycosylation using tunicamycin causes cell death in malignant cells: role of down-regulation of the insulin-like growth factor 1 receptor in induction of apoptosis. *Cancer Res* 57:543–548.

316. Bull VH, Thiede B (2012) Proteome analysis of tunicamycin-induced ER stress. *Electrophoresis* 33:1814–1823. doi: 10.1002/elps.201100565
317. Pimlott NJ, Miller RG (1986) The use of tunicamycin to study the role of cell surface oligosaccharides in lymphocyte recognition. *J Immunol Baltim Md 1950* 137:2455–2459.
318. Powell LD (2001) Inhibition of N-Linked Glycosylation. *Curr. Protoc. Immunol.*
319. Kepp O, Galluzzi L, Lipinski M, et al (2011) Cell death assays for drug discovery. *Nat Rev Drug Discov* 10:221–237. doi: 10.1038/nrd3373
320. Cheng ZJ, Garvin D, Paguio A, et al (2014) Development of a robust reporter-based ADCC assay with frozen, thaw-and-use cells to measure Fc effector function of therapeutic antibodies. *J Immunol Methods* 414:69–81. doi: 10.1016/j.jim.2014.07.010
321. Parekh BS, Berger E, Sibley S, et al (2012) Development and validation of an antibody-dependent cell-mediated cytotoxicity-reporter gene assay. *mAbs* 4:310–318. doi: 10.4161/mabs.19873
322. Adie EJ, Kalinka S, Smith L, et al (2002) A pH-sensitive fluor, CypHer 5, used to monitor agonist-induced G protein-coupled receptor internalization in live cells. *BioTechniques* 33:1152–1154, 1156–1157.
323. Riedl T, van Boxtel E, Bosch M, et al (2016) High-Throughput Screening for Internalizing Antibodies by Homogeneous Fluorescence Imaging of a pH-Activated Probe. *J Biomol Screen* 21:12–23. doi: 10.1177/1087057115613270
324. Ke N, Wang X, Xu X, Abassi YA (2011) The xCELLigence system for real-time and label-free monitoring of cell viability. *Methods Mol Biol Clifton NJ* 740:33–43. doi: 10.1007/978-1-61779-108-6_6
325. Drukker M, Muscat C, Weissman IL (2007) Generation of a monoclonal antibody library against human embryonic stem cells. *Methods Mol Biol Clifton NJ* 407:63–81. doi: 10.1007/978-1-59745-536-7_6
326. Li Y, Zeng H, Xu R-H, et al (2009) Vaccination with human pluripotent stem cells generates a broad spectrum of immunological and clinical responses against colon cancer. *Stem Cells Dayt Ohio* 27:3103–3111. doi: 10.1002/stem.234
327. Zwaka TP (2010) Stem cell vaccination against cancer: fighting fire with fire? *Mol Ther J Am Soc Gene Ther* 18:8–9. doi: 10.1038/mt.2009.287
328. Heimburg-Molinaro J, Lum M, Vijay G, et al (2011) Cancer Vaccines and Carbohydrate Epitopes. *Vaccine* 29:8802–8826. doi: 10.1016/j.vaccine.2011.09.009

329. National Research Council (US) Committee on Assessing the Importance and Impact of Glycomics and Glycosciences (2012) Transforming Glycoscience: A Roadmap for the Future. National Academies Press (US), Washington (DC)
330. Pinho SS, Reis CA (2015) Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer* 15:540–555. doi: 10.1038/nrc3982
331. Kannagi R, Yin J, Miyazaki K, Izawa M (2008) Current relevance of incomplete synthesis and neo-synthesis for cancer-associated alteration of carbohydrate determinants—Hakomori's concepts revisited. *Biochim Biophys Acta BBA - Gen Subj* 1780:525–531. doi: 10.1016/j.bbagen.2007.10.007
332. Daniotti JL, Vilcaes AA, Torres Demichelis V, et al (2013) Glycosylation of Glycolipids in Cancer: Basis for Development of Novel Therapeutic Approaches. *Front Oncol*. doi: 10.3389/fonc.2013.00306
333. Dalziel M, Crispin M, Scanlan CN, et al (2014) Emerging principles for the therapeutic exploitation of glycosylation. *Science* 343:1235681. doi: 10.1126/science.1235681
334. Drake RR (2015) Glycosylation and cancer: moving glycomics to the forefront. *Adv Cancer Res* 126:1–10. doi: 10.1016/bs.acr.2014.12.002
335. Fuster MM, Esko JD (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat Rev Cancer* 5:526–542. doi: 10.1038/nrc1649
336. Hakomori S-I, Cummings RD (2012) Glycosylation effects on cancer development. *Glycoconj J* 29:565–566. doi: 10.1007/s10719-012-9448-4
337. Rabu C, McIntosh R, Jurasova Z, Durrant L (2012) Glycans as targets for therapeutic antitumor antibodies. *Future Oncol Lond Engl* 8:943–960. doi: 10.2217/fon.12.88
338. Eastham AM, Spencer H, Soncin F, et al (2007) Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res* 67:11254–11262. doi: 10.1158/0008-5472.CAN-07-2253
339. Kim Y-S, Yi B-R, Kim N-H, Choi K-C (2014) Role of the epithelial–mesenchymal transition and its effects on embryonic stem cells. *Exp Mol Med* 46:e108. doi: 10.1038/emm.2014.44
340. Stern PL, Brazzatti J, Sawan S, McGinn OJ (2014) Understanding and exploiting 5T4 oncofoetal glycoprotein expression. *Semin Cancer Biol* 29:13–20. doi: 10.1016/j.semcancer.2014.07.004
341. Ward CM, Eastham AM, Stern PL (2006) Cell surface 5T4 antigen is transiently upregulated during early human embryonic stem cell differentiation: effect of 5T4 phenotype on neural lineage formation. *Exp Cell Res* 312:1713–1726. doi: 10.1016/j.yexcr.2006.02.006

342. Baeuerle PA, Gires O (2007) EpCAM (CD326) finding its role in cancer. *Br J Cancer* 96:417–423. doi: 10.1038/sj.bjc.6603494
343. Gao J, Yan Q, Wang J, et al (2015) Epithelial-to-Mesenchymal Transition Induced by TGF- β 1 Is Mediated by AP1-Dependent EpCAM Expression in MCF-7 Cells. *J Cell Physiol* 230:775–782. doi: 10.1002/jcp.24802
344. Martowicz A, Spizzo G, Gastl G, Untergasser G (2012) Phenotype-dependent effects of EpCAM expression on growth and invasion of human breast cancer cell lines. *BMC Cancer* 12:501. doi: 10.1186/1471-2407-12-501
345. Munz M, Fellingner K, Hofmann T, et al (2008) Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM. *Front Biosci J Virtual Libr* 13:5195–5201.
346. Cavallaro U, Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* 4:118–132. doi: 10.1038/nrc1276
347. Seidel B, Braeg S, Adler G, et al (2004) E- and N-cadherin differ with respect to their associated p120ctn isoforms and their ability to suppress invasive growth in pancreatic cancer cells. *Oncogene* 23:5532–5542. doi: 10.1038/sj.onc.1207718
348. Spencer HL, Eastham AM, Merry CLR, et al (2007) E-Cadherin Inhibits Cell Surface Localization of the Pro-Migratory 5T4 Oncofetal Antigen in Mouse Embryonic Stem Cells. *Mol Biol Cell* 18:2838–2851. doi: 10.1091/mbc.E06-09-0875
349. Bates RC, Mercurio AM (2003) Tumor Necrosis Factor- α Stimulates the Epithelial-to-Mesenchymal Transition of Human Colonic Organoids. *Mol Biol Cell* 14:1790–1800. doi: 10.1091/mbc.E02-09-0583
350. Dong R, Wang Q, He XL, et al (2007) Role of nuclear factor kappa B and reactive oxygen species in the tumor necrosis factor- α -induced epithelial-mesenchymal transition of MCF-7 cells. *Braz J Med Biol Res* 40:1071–1078. doi: 10.1590/S0100-879X2007000800007
351. Barr TA, Heath AW (2001) Functional activity of CD40 antibodies correlates to the position of binding relative to CD154. *Immunology* 102:39–43.
352. Benedict CA, MacKrell AJ, Anderson WF (1997) Determination of the binding affinity of an anti-CD34 single-chain antibody using a novel, flow cytometry based assay. *J Immunol Methods* 201:223–231. doi: 10.1016/S0022-1759(96)00227-X
353. Hulspas R, Dombkowski D, Preffer F, et al (2009) Flow cytometry and the stability of phycoerythrin-tandem dye conjugates. *Cytom Part J Int Soc Anal Cytol* 75:966–972. doi: 10.1002/cyto.a.20799

354. Rathanaswami P, Babcook J, Gallo M (2008) High-affinity binding measurements of antibodies to cell-surface-expressed antigens. *Anal Biochem* 373:52–60. doi: 10.1016/j.ab.2007.08.014
355. Pollard TD (2010) A Guide to Simple and Informative Binding Assays. *Mol Biol Cell* 21:4061–4067. doi: 10.1091/mbc.E10-08-0683
356. Trinkle-Mulcahy L, Boulon S, Lam YW, et al (2008) Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *J Cell Biol* 183:223–239. doi: 10.1083/jcb.200805092
357. Bharadwaj A, Bydoun M, Holloway R, Waisman D (2013) Annexin A2 heterotetramer: structure and function. *Int J Mol Sci* 14:6259–6305. doi: 10.3390/ijms14036259
358. Croset A, Delafosse L, Gaudry J-P, et al (2012) Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells. *J Biotechnol* 161:336–348. doi: 10.1016/j.jbiotec.2012.06.038
359. Goulet F, Moore KG, Sartorelli AC (1992) Glycosylation of annexin I and annexin II. *Biochem Biophys Res Commun* 188:554–558. doi: 10.1016/0006-291X(92)91091-4
360. Liu A, Liu D, Zhao S, et al (2011) Up regulation of annexin A2 on murine H22 hepatocarcinoma cells induced by cartilage polysaccharide. *Cancer Epidemiol* 35:490–496. doi: 10.1016/j.canep.2010.10.004
361. Patchell BJ, Wojcik KR, Yang T-L, et al (2007) Glycosylation and annexin II cell surface translocation mediate airway epithelial wound repair. *Am J Physiol Lung Cell Mol Physiol* 293:L354-363. doi: 10.1152/ajplung.00412.2006
362. Phueaouan T, Chaiyawat P, Netsirisawan P, et al (2013) Aberrant O-GlcNAc-modified proteins expressed in primary colorectal cancer. *Oncol Rep* 30:2929–2936. doi: 10.3892/or.2013.2794
363. Wang H, Kachman MT, Schwartz DR, et al (2004) Comprehensive proteome analysis of ovarian cancers using liquid phase separation, mass mapping and tandem mass spectrometry: a strategy for identification of candidate cancer biomarkers. *Proteomics* 4:2476–2495. doi: 10.1002/pmic.200300763
364. Zhuang H, Tan M, Liu J, et al (2015) The expression of annexin II and Lewis y antigen in ovarian epithelial tumors and the correlation between them. *Tumour Biol J Int Soc Oncodevelopmental Biol Med* 36:2343–2349. doi: 10.1007/s13277-014-2841-9
365. Moremen KW, Tiemeyer M, Nairn AV (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol* 13:448–462. doi: 10.1038/nrm3383

366. Lokman NA, Elder ASF, Ween MP, et al (2013) Annexin A2 is regulated by ovarian cancer-peritoneal cell interactions and promotes metastasis. *Oncotarget* 4:1199–1211. doi: 10.18632/oncotarget.1122
367. Zheng L, Foley K, Huang L, et al (2011) Tyrosine 23 phosphorylation-dependent cell-surface localization of annexin A2 is required for invasion and metastases of pancreatic cancer. *PloS One* 6:e19390. doi: 10.1371/journal.pone.0019390
368. Gerke V, Moss SE (2002) Annexins: from structure to function. *Physiol Rev* 82:331–371. doi: 10.1152/physrev.00030.2001
369. Hedhli N, Falcone DJ, Huang B, et al (2012) The annexin A2/S100A10 system in health and disease: emerging paradigms. *J Biomed Biotechnol* 2012:406273. doi: 10.1155/2012/406273
370. Liu Y, Myrvang HK, Dekker LV (2015) Annexin A2 complexes with S100 proteins: structure, function and pharmacological manipulation. *Br J Pharmacol* 172:1664–1676. doi: 10.1111/bph.12978
371. Luo M, Hajjar KA (2013) Annexin A2 system in human biology: cell surface and beyond. *Semin Thromb Hemost* 39:338–346. doi: 10.1055/s-0033-1334143
372. Cesarman GM, Guevara CA, Hajjar KA (1994) An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). II. Annexin II-mediated enhancement of t-PA-dependent plasminogen activation. *J Biol Chem* 269:21198–21203.
373. Chung CY, Murphy-Ullrich JE, Erickson HP (1996) Mitogenesis, cell migration, and loss of focal adhesions induced by tenascin-C interacting with its cell surface receptor, annexin II. *Mol Biol Cell* 7:883–892.
374. Hajjar KA, Guevara CA, Lev E, et al (1996) Interaction of the fibrinolytic receptor, annexin II, with the endothelial cell surface. Essential role of endonexin repeat 2. *J Biol Chem* 271:21652–21659.
375. Liu W, Hajjar KA (2016) The annexin A2 system and angiogenesis. *Biol Chem* 397:1005–1016. doi: 10.1515/hsz-2016-0166
376. Karimi-Busheri F, Marcoux Y, Tredget EE, et al (2002) Expression of a releasable form of annexin II by human keratinocytes. *J Cell Biochem* 86:737–747. doi: 10.1002/jcb.10256
377. Uhlén M, Björling E, Agaton C, et al (2005) A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics MCP* 4:1920–1932. doi: 10.1074/mcp.M500279-MCP200
378. Munoz J, Low TY, Kok YJ, et al (2011) The quantitative proteomes of human-induced pluripotent stem cells and embryonic stem cells. *Mol Syst Biol* 7:550. doi: 10.1038/msb.2011.84

379. Xu X-H, Pan W, Kang L-H, et al (2015) Association of annexin A2 with cancer development (Review). *Oncol Rep* 33:2121–2128. doi: 10.3892/or.2015.3837
380. Wang C-Y, Lin C-F, Wang C-Y, Lin C-F (2014) Annexin A2: Its Molecular Regulation and Cellular Expression in Cancer Development, Annexin A2: Its Molecular Regulation and Cellular Expression in Cancer Development. *Dis Markers* 2014, 2014:e308976. doi: 10.1155/2014/308976, 10.1155/2014/308976
381. Lokman NA, Ween MP, Oehler MK, Ricciardelli C (2011) The role of annexin A2 in tumorigenesis and cancer progression. *Cancer Microenviron Off J Int Cancer Microenviron Soc* 4:199–208. doi: 10.1007/s12307-011-0064-9
382. Liu X, Ma D, Jing X, et al (2015) Overexpression of ANXA2 predicts adverse outcomes of patients with malignant tumors: a systematic review and meta-analysis. *Med Oncol Northwood Lond Engl* 32:392. doi: 10.1007/s12032-014-0392-y
383. El-Abd N, Fawzy A, Elbaz T, Hamdy S (2016) Evaluation of annexin A2 and as potential biomarkers for hepatocellular carcinoma. *Tumour Biol J Int Soc Oncodevelopmental Biol Med* 37:211–216. doi: 10.1007/s13277-015-3524-x
384. Fidel J, Kennedy KC, Dernell WS, et al (2015) Preclinical Validation of the Utility of BLZ-100 in Providing Fluorescence Contrast for Imaging Spontaneous Solid Tumors. *Cancer Res* 75:4283–4291. doi: 10.1158/0008-5472.CAN-15-0471
385. Kesavan K, Ratliff J, Johnson EW, et al (2010) Annexin A2 is a molecular target for TM601, a peptide with tumor-targeting and anti-angiogenic effects. *J Biol Chem* 285:4366–4374. doi: 10.1074/jbc.M109.066092
386. Ojeda PG, Wang CK, Craik DJ (2016) Chlorotoxin: Structure, activity, and potential uses in cancer therapy. *Pept Sci* 106:25–36. doi: 10.1002/bip.22748
387. Deng Y, Chen C, Hua M, et al (2015) Annexin A2 plays a critical role in epithelial ovarian cancer. *Arch Gynecol Obstet* 292:175–182. doi: 10.1007/s00404-014-3598-5
388. Lokman NA, Pyragius CE, Ruzskiewicz A, et al (2016) Annexin A2 and S100A10 are independent predictors of serous ovarian cancer outcome. *Transl Res J Lab Clin Med* 171:83–95.e2. doi: 10.1016/j.trsl.2016.02.002
389. Spijkers-Hagelstein J a. P, Mimoso Pinhanços S, Schneider P, et al (2013) Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. *Leukemia* 27:1063–1071. doi: 10.1038/leu.2012.372

390. Olwill SA, McGlynn H, Gilmore WS, Denis Alexander H (2005) Annexin II cell surface and mRNA expression in human acute myeloid leukaemia cell lines. *Thromb Res* 115:109–114. doi: 10.1016/j.thromres.2004.07.014
391. Syed SP, Martin A-M, Haupt HM, et al (2007) Angiostatin receptor annexin II in vascular tumors including angiosarcoma. *Hum Pathol* 38:508–513. doi: 10.1016/j.humpath.2006.09.011
392. Jeon YR, Kim SY, Lee EJ, et al (2013) Identification of annexin II as a novel secretory biomarker for breast cancer. *Proteomics* 13:3145–3156. doi: 10.1002/pmic.201300127
393. Sharma MR, Koltowski L, Ownbey RT, et al (2006) Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. *Exp Mol Pathol* 81:146–156. doi: 10.1016/j.yexmp.2006.03.003
394. Choi CH, Chung J-Y, Chung EJ, et al (2016) Prognostic significance of annexin A2 and annexin A4 expression in patients with cervical cancer. *BMC Cancer* 16:448. doi: 10.1186/s12885-016-2459-y
395. Hellman K, Alaiya AA, Becker S, et al (2009) Differential tissue-specific protein markers of vaginal carcinoma. *Br J Cancer* 100:1303–1314. doi: 10.1038/sj.bjc.6604975
396. Jin L, Shen Q, Ding S, et al (2012) Immunohistochemical expression of Annexin A2 and S100A proteins in patients with bulky stage IB-IIA cervical cancer treated with neoadjuvant chemotherapy. *Gynecol Oncol* 126:140–146. doi: 10.1016/j.ygyno.2012.04.005
397. Duncan R, Carpenter B, Main LC, et al (2008) Characterisation and protein expression profiling of annexins in colorectal cancer. *Br J Cancer* 98:426–433. doi: 10.1038/sj.bjc.6604128
398. Yang T, Peng H, Wang J, et al (2013) Prognostic and diagnostic significance of annexin A2 in colorectal cancer. *Colorectal Dis Off J Assoc Coloproctology G B Irel* 15:e373-381. doi: 10.1111/codi.12207
399. Pena-Alonso E, Rodrigo JP, Parra IC, et al (2008) Annexin A2 localizes to the basal epithelial layer and is down-regulated in dysplasia and head and neck squamous cell carcinoma. *Cancer Lett* 263:89–98. doi: 10.1016/j.canlet.2007.12.029
400. Rodrigo JP, García-Pedrero JM, Llorente JL, et al (2011) Down-regulation of annexin A1 and A2 protein expression in intestinal-type sinonasal adenocarcinomas. *Hum Pathol* 42:88–94. doi: 10.1016/j.humpath.2010.05.017
401. Li J, Li D, Zhang L, et al (2014) [Effects of CD147 gene silencing on protein expression of ANXA2, MMP-2 and TIMP-2 by thyroid medullary

carcinoma TT cells and biologic characteristics]. *Zhonghua Bing Li Xue Za Zhi* 43:103–108.

402. Qi Y-J, He Q-Y, Ma Y-F, et al (2008) Proteomic identification of malignant transformation-related proteins in esophageal squamous cell carcinoma. *J Cell Biochem* 104:1625–1635. doi: 10.1002/jcb.21727
403. Alonso-Alconada L, Santacana M, Garcia-Sanz P, et al (2014) Annexin-A2 as predictor biomarker of recurrent disease in endometrial cancer. *Int J Cancer J Int Cancer*. doi: 10.1002/ijc.29213
404. Emoto K, Sawada H, Yamada Y, et al (2001) Annexin II overexpression is correlated with poor prognosis in human gastric carcinoma. *Anticancer Res* 21:1339–1345.
405. Han Y, Ye J, Dong Y, et al (2015) Expression and significance of annexin A2 in patients with gastric adenocarcinoma and the association with E-cadherin. *Exp Ther Med* 10:549–554. doi: 10.3892/etm.2015.2565
406. Zhang Q, Ye Z, Yang Q, et al (2012) Upregulated expression of annexin II is a prognostic marker for patients with gastric cancer. *World J Surg Oncol* 10:103. doi: 10.1186/1477-7819-10-103
407. Zhai H, Acharya S, Gravanis I, et al (2011) Annexin A2 Promotes Glioma Cell Invasion and Tumor Progression. *J Neurosci Off J Soc Neurosci* 31:14346–14360. doi: 10.1523/JNEUROSCI.3299-11.2011
408. Cui J-W, Wang Y-L (2013) Expression and function of Annexin II in lung cancer tissue. *Asian Pac J Trop Med* 6:150–152. doi: 10.1016/S1995-7645(13)60012-7
409. Seckinger A, Meißner T, Moreaux J, et al (2012) Clinical and prognostic role of annexin A2 in multiple myeloma. *Blood* 120:1087–1094. doi: 10.1182/blood-2012-03-415588
410. Rodrigo JP, Lequerica-Fernández P, Rosado P, et al (2011) Clinical significance of annexin A2 downregulation in oral squamous cell carcinoma. *Head Neck* 33:1708–1714. doi: 10.1002/hed.21661
411. Zhong L, Wei K, Yang X, et al (2009) Increased expression of Annexin A2 in oral squamous cell carcinoma. *Arch Oral Biol* 54:17–25. doi: 10.1016/j.archoralbio.2008.08.006
412. Gillette JM, Chan DC, Nielsen-Preiss SM (2004) Annexin 2 expression is reduced in human osteosarcoma metastases. *J Cell Biochem* 92:820–832. doi: 10.1002/jcb.20117
413. Zhuang H, Tan M, Liu J, et al (2014) Human epididymis protein 4 in association with Annexin II promotes invasion and metastasis of ovarian cancer cells. *Mol Cancer*. doi: 10.1186/1476-4598-13-243

414. Díaz VM, Hurtado M, Thomson TM, et al (2004) Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro. *Gut* 53:993–1000.
415. Takano S, Togawa A, Yoshitomi H, et al (2008) Annexin II overexpression predicts rapid recurrence after surgery in pancreatic cancer patients undergoing gemcitabine-adjuvant chemotherapy. *Ann Surg Oncol* 15:3157–3168. doi: 10.1245/s10434-008-0061-5
416. Vishwanatha JK, Chiang Y, Kumble KD, et al (1993) Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers. *Carcinogenesis* 14:2575–2579.
417. Banerjee AG, Liu J, Yuan Y, et al (2003) Expression of biomarkers modulating prostate cancer angiogenesis: differential expression of annexin II in prostate carcinomas from India and USA. *Mol Cancer* 2:34. doi: 10.1186/1476-4598-2-34
418. Chetcuti A, Margan SH, Russell P, et al (2001) Loss of annexin II heavy and light chains in prostate cancer and its precursors. *Cancer Res* 61:6331–6334.
419. Inokuchi J, Narula N, Yee DS, et al (2009) Annexin A2 positively contributes to the malignant phenotype and secretion of IL-6 in DU145 prostate cancer cells. *Int J Cancer* 124:68–74. doi: 10.1002/ijc.23928
420. Liu J-W, Shen J-J, Tanzillo-Swartz A, et al (2003) Annexin II expression is reduced or lost in prostate cancer cells and its re-expression inhibits prostate cancer cell migration. *Oncogene* 22:1475–1485. doi: 10.1038/sj.onc.1206196
421. Xin W, Rhodes DR, Ingold C, et al (2003) Dysregulation of the annexin family protein family is associated with prostate cancer progression. *Am J Pathol* 162:255–261. doi: 10.1016/S0002-9440(10)63816-3
422. Yee DS, Narula N, Ramzy I, et al (2007) Reduced annexin II protein expression in high-grade prostatic intraepithelial neoplasia and prostate cancer. *Arch Pathol Lab Med* 131:902–908. doi: 10.1043/1543-2165(2007)131[902:RAIPEI]2.0.CO;2
423. Ohno Y, Izumi M, Kawamura T, et al (2009) Annexin II represents metastatic potential in clear-cell renal cell carcinoma. *Br J Cancer* 101:287–294. doi: 10.1038/sj.bjc.6605128
424. Yang S-F, Hsu H-L, Chao T-K, et al (2014) Annexin A2 in renal cell carcinoma: Expression, function, and prognostic significance. *Urol Oncol*. doi: 10.1016/j.urolonc.2014.08.015
425. Myrvang HK, Guo X, Li C, Dekker LV (2013) Protein interactions between surface annexin A2 and S100A10 mediate adhesion of breast

- cancer cells to microvascular endothelial cells. *FEBS Lett* 587:3210–3215. doi: 10.1016/j.febslet.2013.08.012
426. Shiozawa Y, Havens AM, Jung Y, et al (2008) Annexin II/annexin II receptor axis regulates adhesion, migration, homing, and growth of prostate cancer. *J Cell Biochem* 105:370–380. doi: 10.1002/jcb.21835
 427. Tressler RJ, Updyke TV, Yeatman T, Nicolson GL (1993) Extracellular annexin II is associated with divalent cation-dependent tumor cell-endothelial cell adhesion of metastatic RAW117 large-cell lymphoma cells. *J Cell Biochem* 53:265–276. doi: 10.1002/jcb.240530311
 428. de Graauw M, Cao L, Winkel L, et al (2014) Annexin A2 depletion delays EGFR endocytic trafficking via cofilin activation and enhances EGFR signaling and metastasis formation. *Oncogene* 33:2610–2619. doi: 10.1038/onc.2013.219
 429. Graauw M de, Tijdens I, Smeets MB, et al (2008) Annexin A2 Phosphorylation Mediates Cell Scattering and Branching Morphogenesis via Cofilin Activation. *Mol Cell Biol* 28:1029–1040. doi: 10.1128/MCB.01247-07
 430. Mai J, Finley RL, Waisman DM, Sloane BF (2000) Human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells. *J Biol Chem* 275:12806–12812.
 431. Madureira PA, Hill R, Miller VA, et al (2011) Annexin A2 is a novel Cellular Redox Regulatory Protein involved in Tumorigenesis. *Oncotarget* 2:1075–1093.
 432. Sharma MC, Sharma M (2007) The role of annexin II in angiogenesis and tumor progression: a potential therapeutic target. *Curr Pharm Des* 13:3568–3575.
 433. Babiychuk EB, Draeger A (2000) Annexins in cell membrane dynamics. Ca(2+)-regulated association of lipid microdomains. *J Cell Biol* 150:1113–1124.
 434. Schnitzer JE, Liu J, Oh P (1995) Endothelial caveolae have the molecular transport machinery for vesicle budding, docking, and fusion including VAMP, NSF, SNAP, annexins, and GTPases. *J Biol Chem* 270:14399–14404.
 435. Zhang W, Zhao P, Xu X-L, et al (2013) Annexin A2 promotes the migration and invasion of human hepatocellular carcinoma cells in vitro by regulating the shedding of CD147-harboring microvesicles from tumor cells. *PloS One* 8:e67268. doi: 10.1371/journal.pone.0067268
 436. Zhao P, Zhang W, Tang J, et al (2010) Annexin II promotes invasion and migration of human hepatocellular carcinoma cells in vitro via its interaction with HAb18G/CD147. *Cancer Sci* 101:387–395. doi: 10.1111/j.1349-7006.2009.01420.x

437. Brichory FM, Misek DE, Yim AM, et al (2001) An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc Natl Acad Sci U S A* 98:9824–9829. doi: 10.1073/pnas.171320598
438. Deora AB, Kreitzer G, Jacovina AT, Hajjar KA (2004) An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface. *J Biol Chem* 279:43411–43418. doi: 10.1074/jbc.M408078200
439. Semov A, Moreno MJ, Onichtchenko A, et al (2005) Metastasis-associated protein S100A4 induces angiogenesis through interaction with Annexin II and accelerated plasmin formation. *J Biol Chem* 280:20833–20841. doi: 10.1074/jbc.M412653200
440. Shetty P, Bargale A, Patil BR, et al (2016) Cell surface interaction of annexin A2 and galectin-3 modulates epidermal growth factor receptor signaling in Her-2 negative breast cancer cells. *Mol Cell Biochem* 411:221–233. doi: 10.1007/s11010-015-2584-y
441. Zimmermann U, Woenckhaus C, Pietschmann S, et al (2004) Expression of annexin II in conventional renal cell carcinoma is correlated with Fuhrman grade and clinical outcome. *Virchows Arch Int J Pathol* 445:368–374. doi: 10.1007/s00428-004-1103-4
442. Lu G, Maeda H, Reddy SV, et al (2006) Cloning and characterization of the annexin II receptor on human marrow stromal cells. *J Biol Chem* 281:30542–30550. doi: 10.1074/jbc.M607072200
443. McCready J, Sims JD, Chan D, Jay DG (2010) Secretion of extracellular hsp90alpha via exosomes increases cancer cell motility: a role for plasminogen activation. *BMC Cancer* 10:294. doi: 10.1186/1471-2407-10-294
444. Lei H, Romeo G, Kazlauskas A (2004) Heat shock protein 90alpha-dependent translocation of annexin II to the surface of endothelial cells modulates plasmin activity in the diabetic rat aorta. *Circ Res* 94:902–909. doi: 10.1161/01.RES.0000124979.46214.E3
445. Kpetemey M, Dasgupta S, Rajendiran S, et al (2015) MIEN1, a novel interactor of Annexin A2, promotes tumor cell migration by enhancing AnxA2 cell surface expression. *Mol Cancer*. doi: 10.1186/s12943-015-0428-8
446. Chao P-Z, Hsieh M-S, Cheng C-W, et al (2015) Dendritic cells respond to nasopharyngeal carcinoma cells through annexin A2-recognizing DC-SIGN. *Oncotarget* 6:159–170. doi: 10.18632/oncotarget.2700
447. Wang T, Yuan J, Zhang J, et al (2015) Anxa2 binds to STAT3 and promotes epithelial to mesenchymal transition in breast cancer cells. *Oncotarget* 6:30975–30992. doi: 10.18632/oncotarget.5199

448. Wang Y-Q, Zhang F, Tian R, et al (2012) Tyrosine 23 Phosphorylation of Annexin A2 Promotes Proliferation, Invasion, and Stat3 Phosphorylation in the Nucleus of Human Breast Cancer SK-BR-3 Cells. *Cancer Biol Med* 9:248–253. doi: 10.7497/j.issn.2095-3941.2012.04.005
449. Zhou S, Yi T, Liu R, et al (2012) Proteomics identification of annexin A2 as a key mediator in the metastasis and proangiogenesis of endometrial cells in human adenomyosis. *Mol Cell Proteomics MCP* 11:M112.017988. doi: 10.1074/mcp.M112.017988
450. Wu B, Zhang F, Yu M, et al (2012) Up-regulation of Anxa2 gene promotes proliferation and invasion of breast cancer MCF-7 cells. *Cell Prolif* 45:189–198. doi: 10.1111/j.1365-2184.2012.00820.x
451. Yamada A, Irie K, Hirota T, et al (2005) Involvement of the Annexin II-S100A10 Complex in the Formation of E-cadherin-based Adherens Junctions in Madin-Darby Canine Kidney Cells. *J Biol Chem* 280:6016–6027. doi: 10.1074/jbc.M408215200
452. Regnoui F, Sagot I, Delouche B, et al (1995) “In vitro” phosphorylation of annexin 2 heterotetramer by protein kinase C. Comparative properties of the unphosphorylated and phosphorylated annexin 2 on the aggregation and fusion of chromaffin granule membranes. *J Biol Chem* 270:27143–27150.
453. Lange T, Samatov TR, Tonevitsky AG, Schumacher U (2014) Importance of altered glycoprotein-bound N- and O-glycans for epithelial-to-mesenchymal transition and adhesion of cancer cells. *Carbohydr Res* 389:39–45. doi: 10.1016/j.carres.2014.01.010
454. Li X, Wang X, Tan Z, et al (2016) Role of Glycans in Cancer Cells Undergoing Epithelial–Mesenchymal Transition. *Front Oncol*. doi: 10.3389/fonc.2016.00033
455. Matsuura H, Hakomori S (1985) The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. *Proc Natl Acad Sci U S A* 82:6517–6521.
456. Menzin AW, Loret de Mola JR, Bilker WB, et al (1998) Identification of oncofetal fibronectin in patients with advanced epithelial ovarian cancer: detection in ascitic fluid and localization to primary sites and metastatic implants. *Cancer* 82:152–158.
457. Ding Y, Gelfenbeyn K, Freire-de-Lima L, et al (2012) Induction of epithelial-mesenchymal transition with O-glycosylated oncofetal fibronectin. *FEBS Lett* 586:1813–1820. doi: 10.1016/j.febslet.2012.05.020
458. Mandel U, Hamilton Therkildsen M, Reibel J, et al (1992) Cancer-associated changes in glycosylation of fibronectin. *Immunohistological*

localization of oncofetal fibronectin defined by monoclonal antibodies. *APMIS Acta Pathol Microbiol Immunol Scand* 100:817–826.

459. Sharma M, Blackman MR, Sharma MC (2012) Antibody-directed neutralization of annexin II (ANX II) inhibits neoangiogenesis and human breast tumor growth in a xenograft model. *Exp Mol Pathol* 92:175–184. doi: 10.1016/j.yexmp.2011.10.003
460. Chaudhary P, Thamake SI, Shetty P, Vishwanatha JK (2014) Inhibition of triple-negative and Herceptin-resistant breast cancer cell proliferation and migration by Annexin A2 antibodies. *Br J Cancer* 111:2328–2341. doi: 10.1038/bjc.2014.542
461. Trauth BC, Klas C, Peters AM, et al (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 245:301–305.
462. Alduaij W, Ivanov A, Honeychurch J, et al (2011) Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. *Blood* 117:4519–4529. doi: 10.1182/blood-2010-07-296913
463. Herter S, Herting F, Mundigl O, et al (2013) Preclinical Activity of the Type II CD20 Antibody GA101 (Obinutuzumab) Compared with Rituximab and Ofatumumab In Vitro and in Xenograft Models. *Mol Cancer Ther* 12:2031–2042. doi: 10.1158/1535-7163.MCT-12-1182
464. Chan HTC, Hughes D, French RR, et al (2003) CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. *Cancer Res* 63:5480–5489.
465. Honeychurch J, Alduaij W, Azizyan M, et al (2012) Antibody-induced nonapoptotic cell death in human lymphoma and leukemia cells is mediated through a novel reactive oxygen species-dependent pathway. *Blood* 119:3523–3533. doi: 10.1182/blood-2011-12-395541
466. Elmore S (2007) Apoptosis: A Review of Programmed Cell Death. *Toxicol Pathol* 35:495–516. doi: 10.1080/01926230701320337
467. Hockenbery D (1995) Defining apoptosis. *Am J Pathol* 146:16–19.
468. Lim DYX, Ng Y-H, Lee J, et al (2011) Cytotoxic antibody fragments for eliminating undifferentiated human embryonic stem cells. *J Biotechnol* 153:77–85. doi: 10.1016/j.jbiotec.2011.03.017
469. Gadjeva MG, Rouseva MM, Zlatarova AS, et al (2008) Interaction of human C1q with IgG and IgM: revisited. *Biochemistry (Mosc)* 47:13093–13102. doi: 10.1021/bi801131h
470. Painter RH (1984) The C1q receptor site on human immunoglobulin G. *Can J Biochem Cell Biol Rev Can Biochim Biol Cell* 62:418–425.

471. Duncan AR, Winter G (1988) The binding site for C1q on IgG. *Nature* 332:738–740. doi: 10.1038/332738a0
472. Lubeck MD, Steplewski Z, Baglia F, et al (1985) The interaction of murine IgG subclass proteins with human monocyte Fc receptors. *J Immunol Baltim Md* 1950 135:1299–1304.
473. Sawada R, Sun S-M, Wu X, et al (2011) Human Monoclonal Antibodies to Sialyl-Lewis a (CA19.9) with Potent CDC, ADCC and Anti-Tumor Activity. *Clin Cancer Res Off J Am Assoc Cancer Res* 17:1024–1032. doi: 10.1158/1078-0432.CCR-10-2640
474. Niwa R, Hatanaka S, Shoji-Hosaka E, et al (2004) Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 is independent of FcγRIIIa functional polymorphism. *Clin Cancer Res Off J Am Assoc Cancer Res* 10:6248–6255. doi: 10.1158/1078-0432.CCR-04-0850
475. Higel F, Seidl A, Sörgel F, Friess W (2016) N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. *Eur J Pharm Biopharm* 100:94–100. doi: 10.1016/j.ejpb.2016.01.005
476. Zhang P, Woen S, Wang T, et al Challenges of glycosylation analysis and control: an integrated approach to producing optimal and consistent therapeutic drugs. *Drug Discov Today*. doi: 10.1016/j.drudis.2016.01.006
477. Kanda Y, Yamada T, Mori K, et al (2007) Comparison of biological activity among nonfucosylated therapeutic IgG1 antibodies with three different N-linked Fc oligosaccharides: the high-mannose, hybrid, and complex types. *Glycobiology* 17:104–118. doi: 10.1093/glycob/cwl057
478. Shinkawa T, Nakamura K, Yamane N, et al (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* 278:3466–3473. doi: 10.1074/jbc.M210665200
479. Yamane-Ohnuki N, Satoh M (2009) Production of therapeutic antibodies with controlled fucosylation. *mAbs* 1:230–236. doi: 10.4161/mabs.1.3.8328
480. Junttila TT, Li G, Parsons K, et al (2011) Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat* 128:347–356. doi: 10.1007/s10549-010-1090-x
481. Zhang N, Liu L, Dumitru CD, et al (2011) Glycoengineered *Pichia* produced anti-HER2 is comparable to trastuzumab in preclinical study. *mAbs* 3:289–298. doi: 10.4161/mabs.3.3.15532

482. Shibata-Koyama M, Iida S, Okazaki A, et al (2009) The N-linked oligosaccharide at FcγRIIIa Asn-45: an inhibitory element for high FcγRIIIa binding affinity to IgG glycoforms lacking core fucosylation. *Glycobiology* 19:126–134. doi: 10.1093/glycob/cwn110
483. Ferrara C, Stuart F, Sonderrmann P, et al (2006) The carbohydrate at FcγRIIIa Asn-162. An element required for high affinity binding to non-fucosylated IgG glycoforms. *J Biol Chem* 281:5032–5036. doi: 10.1074/jbc.M510171200
484. Mimura Y, Church S, Ghirlando R, et al (2000) The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol Immunol* 37:697–706.
485. Tao MH, Morrison SL (1989) Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol Baltim Md* 1950 143:2595–2601.
486. Haryadi R, Zhang P, Chan KF, Song Z (2013) CHO-gmt5, a novel CHO glycosylation mutant for producing afucosylated and asialylated recombinant antibodies. *Bioengineered* 4:90–94. doi: 10.4161/bioe.22262
487. Malphettes L, Freyvert Y, Chang J, et al (2010) Highly efficient deletion of FUT8 in CHO cell lines using zinc-finger nucleases yields cells that produce completely nonfucosylated antibodies. *Biotechnol Bioeng* 106:774–783. doi: 10.1002/bit.22751
488. Okeley NM, Alley SC, Anderson ME, et al (2013) Development of orally active inhibitors of protein and cellular fucosylation. *Proc Natl Acad Sci* 110:5404–5409. doi: 10.1073/pnas.1222263110
489. Becker DJ, Lowe JB (2003) Fucose: biosynthesis and biological function in mammals. *Glycobiology* 13:41R–53R. doi: 10.1093/glycob/cwg054
490. Sarkar S, Kantara C, Singh P (2012) Clathrin mediates endocytosis of progastrin and activates MAPKs: role of cell surface annexin A2. *Am J Physiol Gastrointest Liver Physiol* 302:G712–722. doi: 10.1152/ajpgi.00406.2011
491. Creutz CE, Snyder SL (2005) Interactions of annexins with the mu subunits of the clathrin assembly proteins. *Biochemistry (Mosc)* 44:13795–13806. doi: 10.1021/bi051160w
492. Futter CE, White IJ (2007) Annexins and endocytosis. *Traffic Cph Den* 8:951–958. doi: 10.1111/j.1600-0854.2007.00590.x
493. Polito L, Bortolotti M, Mercatelli D, et al (2013) Saporin-S6: a useful tool in cancer therapy. *Toxins* 5:1698–1722. doi: 10.3390/toxins5101698

494. Raju TS (2008) Terminal sugars of Fc glycans influence antibody effector functions of IgGs. *Curr Opin Immunol* 20:471–478. doi: 10.1016/j.coi.2008.06.007
495. Coloma MJ, Trinh KR, Wims LA, Morrison SL (1997) The hinge as a spacer contributes to covalent assembly and is required for function of IgG. *J Immunol Baltim Md* 158:733–740.
496. Patel D, Guo X, Ng S, et al (2010) IgG isotype, glycosylation, and EGFR expression determine the induction of antibody-dependent cellular cytotoxicity in vitro by cetuximab. *Hum Antibodies* 19:89–99. doi: 10.3233/HAB-2010-0232
497. Velders MP, van Rhijn CM, Oskam E, et al (1998) The impact of antigen density and antibody affinity on antibody-dependent cellular cytotoxicity: relevance for immunotherapy of carcinomas. *Br J Cancer* 78:478–483.
498. Baulida J, Kraus MH, Alimandi M, et al (1996) All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *J Biol Chem* 271:5251–5257.
499. de Goeij BECG, Peipp M, de Haij S, et al (2014) HER2 monoclonal antibodies that do not interfere with receptor heterodimerization-mediated signaling induce effective internalization and represent valuable components for rational antibody-drug conjugate design. *mAbs* 6:392–402. doi: 10.4161/mabs.27705
500. Li JY, Perry SR, Muniz-Medina V, et al (2016) A Biparatopic HER2-Targeting Antibody-Drug Conjugate Induces Tumor Regression in Primary Models Refractory to or Ineligible for HER2-Targeted Therapy. *Cancer Cell* 29:117–129. doi: 10.1016/j.ccell.2015.12.008
501. Acchione M, Kwon H, Jochheim CM, Atkins WM (2012) Impact of linker and conjugation chemistry on antigen binding, Fc receptor binding and thermal stability of model antibody-drug conjugates. *mAbs* 4:362–372. doi: 10.4161/mabs.19449
502. Erickson HK, Phillips GDL, Leipold DD, et al (2012) The Effect of Different Linkers on Target Cell Catabolism and Pharmacokinetics/Pharmacodynamics of Trastuzumab Maytansinoid Conjugates. *Mol Cancer Ther* 11:1133–1142. doi: 10.1158/1535-7163.MCT-11-0727
503. Klussman K, Mixan BJ, Cervený CG, et al (2004) Secondary mAb--vcMMAE conjugates are highly sensitive reporters of antibody internalization via the lysosome pathway. *Bioconjug Chem* 15:765–773. doi: 10.1021/bc049969t
504. Kato J, O'Donnell RT, Abuhay M, Tuscano JM (2012) Efficacy and toxicity of a CD22-targeted antibody-saporin conjugate in a xenograft

- model of non-Hodgkin's lymphoma. *Oncoimmunology* 1:1469–1475. doi: 10.4161/onci.21815
505. Kuroda K, Liu H, Kim S, et al (2010) Saporin toxin-conjugated monoclonal antibody targeting prostate-specific membrane antigen has potent anticancer activity. *The Prostate* 70:1286–1294. doi: 10.1002/pros.21164
506. Polito L, Bolognesi A, Tazzari PL, et al (2004) The conjugate Rituximab/saporin-S6 completely inhibits clonogenic growth of CD20-expressing cells and produces a synergistic toxic effect with Fludarabine. *Leukemia* 18:1215–1222. doi: 10.1038/sj.leu.2403378
507. Quadros EV, Nakayama Y, Sequeira JM (2010) Targeted delivery of saporin toxin by monoclonal antibody to the transcobalamin receptor, TCbIR/CD320. *Mol Cancer Ther* 9:3033–3040. doi: 10.1158/1535-7163.MCT-10-0513
508. Sharma MR, Rothman V, Tuszynski GP, Sharma MC (2006) Antibody-directed targeting of angiostatin's receptor annexin II inhibits Lewis Lung Carcinoma tumor growth via blocking of plasminogen activation: possible biochemical mechanism of angiostatin's action. *Exp Mol Pathol* 81:136–145. doi: 10.1016/j.yexmp.2006.03.002
509. Falcone DJ, Borth W, Khan KM, Hajjar KA (2001) Plasminogen-mediated matrix invasion and degradation by macrophages is dependent on surface expression of annexin II. *Blood* 97:777–784.
510. Ling Q, Jacovina AT, Deora A, et al (2004) Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J Clin Invest* 113:38–48. doi: 10.1172/JCI19684
511. Feng L, Wang W, Yao H-P, et al (2015) Human tumor xenografts in mouse as a model for evaluating therapeutic efficacy of monoclonal antibodies or antibody-drug conjugate targeting receptor tyrosine kinases. *Methods Mol Biol Clifton NJ* 1233:151–159. doi: 10.1007/978-1-4939-1789-1_14
512. Olive KP, Tuveson DA (2006) The Use of Targeted Mouse Models for Preclinical Testing of Novel Cancer Therapeutics. *Clin Cancer Res* 12:5277–5287. doi: 10.1158/1078-0432.CCR-06-0436
513. House CD, Hernandez L, Annunziata CM (2014) Recent technological advances in using mouse models to study ovarian cancer. *Front Oncol* 4:26. doi: 10.3389/fonc.2014.00026
514. Ricci F, Broggini M, Damia G (2013) Revisiting ovarian cancer preclinical models: implications for a better management of the disease. *Cancer Treat Rev* 39:561–568. doi: 10.1016/j.ctrv.2013.01.005

515. Richmond A, Su Y (2008) Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech* 1:78–82. doi: 10.1242/dmm.000976
516. Shaw TJ, Senterman MK, Dawson K, et al (2004) Characterization of intraperitoneal, orthotopic, and metastatic xenograft models of human ovarian cancer. *Mol Ther J Am Soc Gene Ther* 10:1032–1042. doi: 10.1016/j.ymthe.2004.08.013
517. Sharpless NE, DePinho RA (2006) The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* 5:741–754. doi: 10.1038/nrd2110
518. Connolly DC, Hensley HH (2009) Xenograft and Transgenic Mouse Models of Epithelial Ovarian Cancer and Non Invasive Imaging Modalities to Monitor Ovarian Tumor Growth In situ -Applications in Evaluating Novel Therapeutic Agents. *Curr Protoc Pharmacol* Editor Board SJ Enna Ed--Chief AI 45:14.12.1-14.12.26.
519. Loo D, Pryer N, Young P, et al (2007) The glycotope-specific RAV12 monoclonal antibody induces oncosis in vitro and has antitumor activity against gastrointestinal adenocarcinoma tumor xenografts in vivo. *Mol Cancer Ther* 6:856–865. doi: 10.1158/1535-7163.MCT-06-0581
520. Jefferis R, Lund J (2002) Interaction sites on human IgG-Fc for FcγR: current models. *Immunol Lett* 82:57–65.
521. Steplewski Z, Sun LK, Shearman CW, et al (1988) Biological activity of human-mouse IgG1, IgG2, IgG3, and IgG4 chimeric monoclonal antibodies with antitumor specificity. *Proc Natl Acad Sci U S A* 85:4852–4856.
522. Roskos LK, Davis CG, Schwab GM (2004) The clinical pharmacology of therapeutic monoclonal antibodies. *Drug Dev Res* 61:108–120. doi: 10.1002/ddr.10346
523. Ober RJ, Radu CG, Ghetie V, Ward ES (2001) Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *Int Immunol* 13:1551–1559.
524. Roopenian DC, Akilesh S (2007) FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol* 7:715–725. doi: 10.1038/nri2155
525. Neuber T, Frese K, Jaehrling J, et al (2014) Characterization and screening of IgG binding to the neonatal Fc receptor. *mAbs* 6:928. doi: 10.4161/mabs.28744
526. Guo K, Tang JP, Li J, et al (2012) Engineering the First Chimeric Antibody in Targeting Intracellular PRL-3 Oncoprotein for Cancer Therapy in Mice. *Oncotarget* 3:158–171.

527. Zeiderman MR, Egger ME, Kimbrough CW, et al (2014) Targeting of BRAF resistant melanoma via extracellular matrix metalloproteinase inducer receptor. *J Surg Res* 190:111–118. doi: 10.1016/j.jss.2014.02.021
528. Fan Q, Cai H, Yang H, et al (2014) Biological evaluation of ¹³¹I- and CF750-labeled Dmab(scFv)-Fc antibodies for xenograft imaging of CD25-positive tumors. *BioMed Res Int* 2014:459676. doi: 10.1155/2014/459676
529. Li X, Iida M, Tada M, et al (2014) Development of an anti-claudin-3 and -4 bispecific monoclonal antibody for cancer diagnosis and therapy. *J Pharmacol Exp Ther* 351:206–213. doi: 10.1124/jpet.114.216911
530. Sharma MC, Tuszynski GP, Blackman MR, Sharma M (2016) Long-term efficacy and downstream mechanism of anti-annexinA2 monoclonal antibody (anti-ANX A2 mAb) in a pre-clinical model of aggressive human breast cancer. *Cancer Lett* 373:27–35. doi: 10.1016/j.canlet.2016.01.013
531. Bugge TH, Kombrinck KW, Xiao Q, et al (1997) Growth and dissemination of Lewis lung carcinoma in plasminogen-deficient mice. *Blood* 90:4522–4531.
532. Hogarth PM, Pietersz GA (2012) Fc receptor-targeted therapies for the treatment of inflammation, cancer and beyond. *Nat Rev Drug Discov* 11:311–331. doi: 10.1038/nrd2909
533. Lux A, Nimmerjahn F (2013) Of mice and men: the need for humanized mouse models to study human IgG activity in vivo. *J Clin Immunol* 33 Suppl 1:S4-8. doi: 10.1007/s10875-012-9782-0
534. Flanagan SP (1966) “Nude”, a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 8:295–309.
535. Nimmerjahn F (2016) Translating Inhibitory Fc Receptor Biology into Novel Therapeutic Approaches. *J Clin Immunol* 36 Suppl 1:83–87. doi: 10.1007/s10875-016-0249-6
536. Overdijk MB, Verploegen S, Ortiz Buijsse A, et al (2012) Crosstalk between human IgG isotypes and murine effector cells. *J Immunol Baltim Md 1950* 189:3430–3438. doi: 10.4049/jimmunol.1200356
537. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL (2012) Humanized mice for immune system investigation: progress, promise and challenges. *Nat Rev Immunol* 12:786–798. doi: 10.1038/nri3311
538. Smith P, DiLillo DJ, Bournazos S, et al (2012) Mouse model recapitulating human Fc receptor structural and functional diversity. *Proc Natl Acad Sci* 109:6181–6186. doi: 10.1073/pnas.1203954109
539. Dahan R, Barnhart BC, Li F, et al (2016) Therapeutic Activity of Agonistic, Human Anti-CD40 Monoclonal Antibodies Requires Selective

FcγR Engagement. *Cancer Cell* 29:820–831. doi: 10.1016/j.ccell.2016.05.001

540. Johnson JI, Decker S, Zaharevitz D, et al (2001) Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. *Br J Cancer* 84:1424–1431. doi: 10.1054/bjoc.2001.1796
541. Fong MY, Kakar SS (2009) Ovarian cancer mouse models: a summary of current models and their limitations. *J Ovarian Res* 2:12. doi: 10.1186/1757-2215-2-12
542. Ackerman ME, Pawlowski D, Wittrup KD (2008) Effect of antigen turnover rate and expression level on antibody penetration into tumor spheroids. *Mol Cancer Ther* 7:2233–2240. doi: 10.1158/1535-7163.MCT-08-0067
543. Adams GP, Schier R, McCall AM, et al (2001) High affinity restricts the localization and tumor penetration of single-chain fv antibody molecules. *Cancer Res* 61:4750–4755.
544. Wang Y-C, Peterson SE, Loring JF (2014) Protein post-translational modifications and regulation of pluripotency in human stem cells. *Cell Res* 24:143–160. doi: 10.1038/cr.2013.151
545. Ng VY, Ang SN, Chan JX, Choo ABH (2010) Characterization of Epithelial Cell Adhesion Molecule as a Surface Marker on Undifferentiated Human Embryonic Stem Cells. *STEM CELLS* 28:29–35. doi: 10.1002/stem.221
546. Tan HL, Fong WJ, Lee EH, et al (2009) mAb 84, a cytotoxic antibody that kills undifferentiated human embryonic stem cells via oncosis. *Stem Cells Dayt Ohio* 27:1792–1801. doi: 10.1002/stem.109
547. Tuszynski GP, Sharma MR, Rothman VL, Sharma MC (2002) Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc Res* 64:448–462.
548. Sharma M, Ownbey RT, Sharma MC (2010) Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation. *Exp Mol Pathol* 88:278–286. doi: 10.1016/j.yexmp.2010.01.001
549. Sharma MR, Koltowski L, Ownbey RT, et al (2006) Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. *Exp Mol Pathol* 81:146–156. doi: 10.1016/j.yexmp.2006.03.003
550. Tuszynski GP, Sharma MR, Rothman VL, Sharma MC (2002) Angiostatin binds to tyrosine kinase substrate annexin II through the lysine-binding domain in endothelial cells. *Microvasc Res* 64:448–462.

551. Gillet J-P, Varma S, Gottesman MM (2013) The clinical relevance of cancer cell lines. *J Natl Cancer Inst* 105:452–458. doi: 10.1093/jnci/djt007
552. Holliday DL, Speirs V (2011) Choosing the right cell line for breast cancer research. *Breast Cancer Res BCR* 13:215. doi: 10.1186/bcr2889
553. Lacroix M, Leclercq G (2004) Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat* 83:249–289. doi: 10.1023/B:BREA.0000014042.54925.cc
554. Wilding JL, Bodmer WF (2014) Cancer Cell Lines for Drug Discovery and Development. *Cancer Res* 74:2377–2384. doi: 10.1158/0008-5472.CAN-13-2971
555. Dybdal N, Leiberman G, Anderson S, et al (2005) Determination of HER2 gene amplification by fluorescence in situ hybridization and concordance with the clinical trials immunohistochemical assay in women with metastatic breast cancer evaluated for treatment with trastuzumab. *Breast Cancer Res Treat* 93:3–11. doi: 10.1007/s10549-004-6275-8
556. de Paz JL, Seeberger PH (2012) Recent advances and future challenges in glycan microarray technology. *Methods Mol Biol Clifton NJ* 808:1–12. doi: 10.1007/978-1-61779-373-8_1
557. Smith DF, Song X, Cummings RD (2010) Chapter Nineteen - Use of Glycan Microarrays to Explore Specificity of Glycan-Binding Proteins. In: Fukuda M (ed) *Methods Enzymol*. Academic Press, pp 417–444
558. Hidalgo M, Amant F, Biankin AV, et al (2014) Patient-derived xenograft models: an emerging platform for translational cancer research. *Cancer Discov* 4:998–1013. doi: 10.1158/2159-8290.CD-14-0001
559. Whittle JR, Lewis MT, Lindeman GJ, Visvader JE (2015) Patient-derived xenograft models of breast cancer and their predictive power. *Breast Cancer Res BCR* 17:17. doi: 10.1186/s13058-015-0523-1
560. Siolas D, Hannon GJ (2013) Patient Derived Tumor Xenografts: transforming clinical samples into mouse models. *Cancer Res* 73:5315–5319. doi: 10.1158/0008-5472.CAN-13-1069
561. Tentler JJ, Tan AC, Weekes CD, et al (2012) Patient-derived tumour xenografts as models for oncology drug development. *Nat Rev Clin Oncol* 9:338–350. doi: 10.1038/nrclinonc.2012.61
562. Moreno-Bueno G, Peinado H, Molina P, et al (2009) The morphological and molecular features of the epithelial-to-mesenchymal transition. *Nat Protoc* 4:1591–1613. doi: 10.1038/nprot.2009.152
563. Blumenthal GM, Scher NS, Cortazar P, et al (2013) First FDA approval of dual anti-HER2 regimen: pertuzumab in combination with trastuzumab

and docetaxel for HER2-positive metastatic breast cancer. *Clin Cancer Res Off J Am Assoc Cancer Res* 19:4911–4916. doi: 10.1158/1078-0432.CCR-13-1212

- 564. Swain SM, Baselga J, Kim S-B, et al (2015) Pertuzumab, Trastuzumab, and Docetaxel in HER2-Positive Metastatic Breast Cancer. *N Engl J Med* 372:724–734. doi: 10.1056/NEJMoa1413513
- 565. Marshall DJ, Harried SS, Murphy JL, et al (2016) Extracellular Antibody Drug Conjugates Exploiting the Proximity of Two Proteins. *Mol Ther* 24:1760–1770. doi: 10.1038/mt.2016.119
- 566. Bagshawe KD, Sharma SK, Begent RHJ (2004) Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. *Expert Opin Biol Ther* 4:1777–1789. doi: 10.1517/14712598.4.11.1777
- 567. Barrett DM, Grupp SA, June CH (2015) Chimeric Antigen Receptor- and TCR-Modified T Cells Enter Main Street and Wall Street. *J Immunol Baltim Md 1950* 195:755–761. doi: 10.4049/jimmunol.1500751

Appendix