Chapter 3
Selecting an Optimal Antibody for Antibody-Drug Conjugate Therapy

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3.1 Target Selection for an ADC

3.1.1 Characteristics of an Optimal ADC Target

Selection of appropriate targets is likely the most important consideration for the success of an antibody–drug conjugate (ADC) development program. Target selection for ADCs can be classified into two principal approaches: target-first and antibody-first approach or agnostic approach (Fig. 3.1). In the target-first approach, a target is chosen based on a set of factors, including expression pattern, abundance and internalization properties, and an antibody-generation campaign is focused on isolation of antibodies against this target. In the agnostic approach, antibodies capable of binding to and internalizing in tumor cells are isolated, followed by retrospective identification of their targets. While the target-first approach may have the advantage of a higher degree confidence in the suitability and novelty of the target based on prior knowledge of target properties, it requires significant work before internalizing antibodies become available. By contrast, the agnostic approach leads quickly to reagents suitable for testing hypotheses directly, although this strategy tends to favor highly expressed surface antigens, which are likely to have been described previously.

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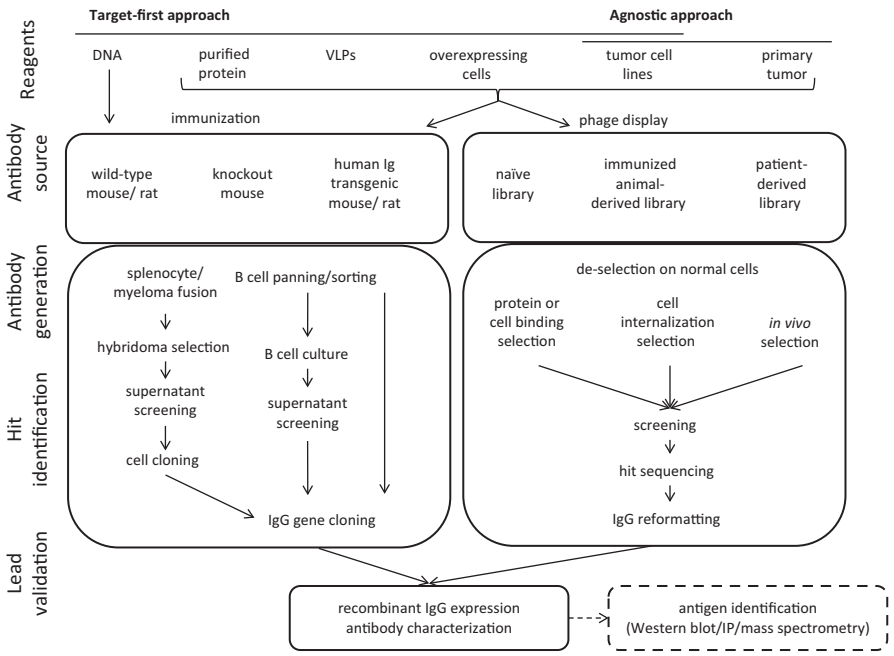

Fig. 3.1 Methods for antibody isolation and development via the agnostic and target-first approaches. Antibody isolation and validation can be performed with specific targets in mind ("target-first approach") or on tumor cells of interest without bias toward specific targets ("agnostic approach"). When the target is known, reagents for antibody generation can be as diverse as cDNA constructs for DNA immunization; purified target protein (full-length or domain); engineered cells or cell surrogates: virus-like particles (*VLPs*) or other target-enriched membranes; or tumor cells known to express the target. A target-agnostic approach will usually utilize primary tumor cells or tumor lines for antibody generation. The source for antibodies can be immunized animals, cancer patients, or phage display libraries with antibodies from naïve or tumor-exposed sources using a range of techniques, from conventional hybridoma generation or phage panning to B cell sorting and V gene isolation from immune sources or selection of phage binding to or internalizing in cells expressing the target. Once lead antibodies are cloned and selected, the antibodies can be recombinantly expressed and validated. If the target of an internalizing antibody is not known, target identification can often be done through mass spectrometry once a recombinant antibody is expressed

3.1.2 Selection and Validation of Tumor-Associated Targets Using Omics Approaches

Most strategies used for the identification of targets amenable to the ADC approach resemble strategies used to identify tumor-associated cell-surface antigens for other oncology biotherapeutic approaches, though with a few notable differences. The ADC concept of arming antibodies or antibody fragments with toxins or cytotoxic payloads relies on the selectivity of the antibody towards the target antigen to increase efficacy, while reducing toxicity compared to nontargeted delivery. Thus,

the ideal ADC target will be expressed highly in tumor cells and minimally across normal tissues. Multiple methods have been employed to identify differentially expressed proteins. Usually, this involves comparison of tumor samples (tissue biopsies, tumor cell lines, patient-derived xenografts, etc.) with closely-related nontumor cells. Comparison can be done at the DNA, messenger RNA (mRNA) or protein levels using genomic (e.g., continuous stacking hybridization (CSH) and fluorescence in situ hybridization (FISH)), transcriptomic (microarray) and/or proteomic techniques (two-dimensional gel electrophoresis/mass spectrometry (2DE/MS) and isotope-coded affinity tag (ICAT)) reviewed elsewhere (Guo 2003; Shiio and Aebersold 2006; Yaziji and Gown 2004). The output of such omics studies is typically a list of genes for which expression is increased in tumor samples relative to normal tissues.

The actual number of tumor-associated targets that are identified using such techniques is dependent on the scale and the methodology used in the selection process. It is quite common for a single study to identify tens to hundreds of potential targets that meet the criteria for tumor association. However, since selectivity is only one of multiple critical factors involved in target selection for ADCs, such a list is often just a starting point for validation of multiple targets in parallel.

Narrowing the list of potential targets can be achieved by additional diligence around each target to assess druggability, internalization, and other factors. For ADCs, the target molecule must reside on tumor cells in a location accessible to a systemically-administered therapeutic antibody. Confirmation that a target is localized to the plasma membrane and contains extracellular regions that could be targeted with an antibody is a critical early validation step. For annotated proteins, assessment of topology may be performed using a protein knowledge base, such as UniProt (UniProt 2013), to ensure that the protein contains at least one predicted transmembrane region, or is anchored to the cell surface through a glycosylphosphatidylinositol (GPI) link. If the target is novel, the expert protein analysis system (EXPASY) bioinformatic portal (www.expasy.org; Gasteiger et al. 2003) contains numerous structure prediction tools that can be used to define features such as transmembrane helices, secondary structural elements such as intra-/extracellular domains, as well as sites for predicted protein modification.

For confirmation of cell-surface localization, flow cytometry is commonly used if flow-compatible detection antibodies are available. Quantitative flow analysis can be useful to provide data on antigen density as the level of tumor (vs. normal) expression is an important component of target selection. For targets for which flow-compatible tools are unavailable, recombinant expression using genetic fusion to epitope tags (such as FLAG or myc) can be used. Alternatively, the target protein can be fused to green fluorescent protein and subsequently probed by microscopy.

Determining whether (and to what extent) a given antigen can be shed from the cell membrane is another important consideration. Many cell-surface receptors are shed, resulting in a portion (usually the extracellular domain(s)) being released into the circulation. Shed target protein at high levels may function as a sink, diverting the therapeutic antibody and its associated payload away from the tumor, and thereby lowering the potency of the ADC and potentially causing increased

liver clearance and associated systemic toxicity (Lin and Tibbitts 2012). It is known that a number of tumor-specific surface antigens, such as carcinoma antigen 125 (CA125), prostate-specific antigen (PSA), human epidermal growth factor receptor 2 (HER2), and others, are actively shed from cancer cells (Kulasingam and Diamandis 2008). The presence of circulating antigen can be expected to have a significant influence on the delivery of ADC payloads that use these antigens as the delivery target. While it is generally thought that targets that are shed from the cell surface should be avoided for the development of ADCs, the clinical efficacy of Kadcyla (anti-HER2–ADC; Burris et al. 2011) indicates that the presence of shed extracellular domain need not rule out an otherwise-promising ADC target. Indeed, a recent study suggests that the presence of soluble antigen may be beneficial for targeting solid tumors (Pak et al. 2012).

3.2 Antibody Characteristics

3.2.1 Optimal Antibody Characteristics

In addition to the careful selection of the tumor-specific antigen, characteristics of the antibody, such as affinity and molecular size, will have a significant impact on the efficacy of an ADC. Accordingly, there are a vast number of strategies available to characterize antibody characteristics. While high-affinity antibodies often stand out in screening campaigns, it is worth noting that high affinity does not always correlate with high efficacy, as a high-affinity antibody binding to a rapidly internalizing target may be rapidly eliminated from circulation. High affinity may also reduce the ability of the antibody to penetrate far within the tumor due to tight binding to and/or rapid internalization into the first cells it encounters, a phenomenon termed the binding-site barrier (Adams et al. 2001; Rudnick et al. 2011). A recent study has examined how efficiently a panel of anti-HER2 antibodies with differing affinities can penetrate through a solid tumor. All of these antibodies bind to the same epitope of HER2, but do so with log-fold differences in affinity (Rudnick et al. 2011). The antibody with the lowest affinity (~200 nM K_D) penetrated through the tumor with the highest efficiency; however, this antibody possessed the lowest tumor retention due to its low affinity and resultant inability to remain bound to the receptor (Rudnick et al. 2011). Conversely, antibodies with the higher affinity ($K_D < 1$ nM) for HER2 were largely confined to perivascular space of the tumor (Rudnick et al. 2011). These high-affinity antibodies exhibited lower penetration because they are rapidly internalized and catabolized by the first cells encountered after extravasation. Conversely, the lower-affinity antibodies were not bound long enough to internalize, and thus were able to continue moving through the tumor space (Rudnick et al. 2011). Accordingly, there exists a threshold whereby antibodies that possess a k_{off} faster than the K_e of receptor internalization can avoid internalization and catabolization by the cell, whereas

antibodies with k_{off} slower than the K_e of receptor internalization are internalized and catabolized more readily (Rudnick et al. 2011). A balance of the two rates, allowing effective delivery of toxin throughout the tumor, appears to be achieved by antibodies of moderate affinity (between 7 and 23 nM, in the case of the HER2 antibodies examined in Rudnick et al. (2011)).

Modeling and experimental analyses have shown that there is a fine balance between molecular size and affinity of a therapeutic protein and the degree of tumor retention and penetration. Accordingly, a complex correlation exists in which modalities of intermediate size (~25 kDa) have the lowest tumor uptake, while therapeutic modalities of extremely small (<20-kDa) and large (>100-kDa) size exhibit higher tumor uptake and retention (Schmidt and Wittrup 2009). This is due to the fact that small biotherapeutics can penetrate through a solid tumor rapidly without constraint, although a high affinity for the target antigen is required for the therapeutic to be retained (Schmidt and Wittrup 2009). However, while these smaller modalities can penetrate further through the tumor, they often suffer from faster serum clearance and shorter half-lives (Schmidt and Wittrup 2009). Alternatively, larger proteins usually exhibit reduced tumor penetration; however, they can achieve high accumulation due to their increased serum half-life (Schmidt and Wittrup 2009). Accordingly, intermediate-sized therapeutics (~25 kDa) appear to be the least optimal because they suffer from both a degree of restriction in tumor penetration and possess a reduced serum half-life.

Species cross-reactivity is also a practical consideration for many antibody discovery programs and is particularly important for ADC development due to the complex nature of toxicology studies. Alignment of the target amino acid sequence with that of its orthologs in other species can be useful to determine the identity or homology between human and another species such as nonhuman primate or rodent target. Sequence identity serves as a rough predictor of the likelihood for obtaining cross-reactive antibodies (i.e., there is a higher probability for an antibody to be cross-reactive to an ortholog when the antigens share high sequence identity). Similarly, it is difficult to raise cross-reactive antibodies when target identities are low (60 % identical or lower). Thus, when faced with multiple potential targets coming from -omics studies, priority may be given to follow up on targets with high-sequence homology. Linear sequence identity, however, does not always translate to the three-dimensional structure of a protein. For example, a ligand-binding site may be structurally well conserved across species, despite lower overall homology in other areas of the protein. For this reason, it is a common practice during an antibody generation campaign to screen for antibody candidates that bind to the human target and its nonhuman primate and/or rodent orthologs.

Finally, as cellular uptake of an antibody is a critical component of its efficacy, assessment of a target's ability to internalize and subsequently shuttle its antibody and payload to the appropriate intracellular compartment (usually the lysosome) is of the utmost importance. Strategies to measure internalization and trafficking of ADCs are presented in subsequent sections.

3.2.2 Engineering pH Dependence of Antibody Binding

Recently, the successful engineering of antibodies to have dramatically lower affinity at pH 6.0 than at neutral pH has attracted interest as a method for manipulation of the kinetics of antibody delivery to the lysosome (Chaparro-Riggers et al. 2012). The pH dependence is achieved through the introduction of histidine residues at or near the antibody/antigen binding interface. These histidine residues are protonated $<$pH 6.0 and deprotonated $>$pH 6.0 and, if placed correctly, can have a profound effect on binding affinity in different pH environments. Antibodies against PCSK9 and IL-6R have been engineered to dissociate from their targets in the early endosome (pH$\leq$6.0), while retaining binding to the FcRn receptor, which returns them to the cell surface and prevents degradation in the lysosome (Chaparro-Riggers et al. 2012). A similar approach has been used to *increase* antibody trafficking to the lysosome in cells lacking endosomal FcRn expression, which would be desirable for the delivery of ADCs requiring degradation for the release of the payload. However, the tumor microenvironment is often weakly acidic, which would reduce the cell-surface binding of pH-sensitive ADCs. Further investigation is required to understand the optimal degree of pH dependence for ADCs targeted to solid tumors, such that binding at the cell surface is not compromised.

3.2.3 Engineering Fc-Mediated Interactions

Fc-mediated effector function accounts for most of the in vivo efficacy imparted by unconjugated mAbs in cancer therapy. However, the role of Fc effector function in inhibition of tumor growth by an ADC in the clinic remains unclear. Several strategies have been employed to enhance immune-mediated effector functions of unconjugated mAbs, including afucosylation as well as mutations in the antibody-Fc region, both of which can enhance FcγR binding and amplify antibody-dependent cell-mediated cytotoxicity (ADCC). While this could potentially increase the potency of an ADC, effector mechanisms may lead to undesirable side effects such as cross-linking-related agonistic effects, cytokine storm, or platelet aggregation (Chatenoud et al. 1990; Wing et al. 1996; Langer et al. 2005). Thus, effector functions may not be desirable when the targeted delivery of the payload is sufficient for antitumor activity.

Antibody isotypes IgG2 and IgG4 have inherently reduced binding to FcgRII. Accordingly, these isotypes have been utilized in ADCs where effector function is not required for tumor killing (e.g., calicheamicin-based ADCs). However, given the high potency of these payloads, the concentration at which these ADCs are dosed in patients is low enough (1.8–9 mg/m^2) where eliciting effector function is likely to be minimal. However, ADCs possessing less potent payloads, such as microtubule inhibitors, may be dosed at higher levels (1–5 mg/kg) in the clinic, in which case effector function may come into play.

While advances in antibody engineering have allowed for the enhancement and elimination of effector function-mediated cytotoxicity of a tumor, little has been done to dissect what role(s), if any, effector function has in ADC-mediated tumor killing. As a result, three isotypes (IgG1, IgG2, and IgG4) are being utilized for ADC development programs. One preclinical study of an anti-CD70 antibody conjugated to maleimidocaproyl monomethyl auristatin F (mcMMAF) compared the activity of this ADC with isotype variants (IgG1, IgG2, and IgG4) and mutations that eliminate FcγR binding (IgG1v1 and IgG4v3). Interestingly, the IgG1v1-ADC lacking FcγIIIa binding was the most potent ADC in preclinical models of renal cancer and glioblastoma. Compared to the parental IgG1 ADC, this mutant displayed enhanced tumor exposure of the drug and an improved therapeutic index in mice (McDonagh et al. 2008). Accordingly, the importance of eliciting effector function in ADC-mediated tumor killing remains an important, unanswered question.

3.3 Agnostic Approach to Antibody Production

Whole-cell immunization has been used for decades to identify antigens located on the surface of cells (Williams et al. 1977). Immunophenotyping of hematopoietic cells based on surface markers identified by antibodies raised against cells at different stages of growth became the basis for the "clusters of differentiation" (or CD antigen) classification system (Bernard and Boumsell 1984). Targets with selective expression on tumor cells can also be identified using hybridomas generated via immunization with tumor cells. Many well-known tumor antigens such as prostate-specific membrane antigen (PSMA; Israeli et al. 1993) or Lewis^Y (Hellstrom et al. 1990) were identified in this manner. Typically, this includes a differential binding screen on tumor cells and a related nontumorigenic cell line. Screening can be performed using cell-based enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS) based methods using intact cells.

The efficiency of identifying tumor antigens via whole-cell immunization approaches can be improved using techniques such as subtractive immunization (Bickel et al. 2000; Sleister and Rao 2002). Subtractive immunization is a technique that can be used to produce monoclonal antibodies specific for antigens (such as tumor antigens) that are present in low abundance in a protein mixture or are poorly immunogenic due to the presence of immunodominant epitopes present within the sample. Here, neonatal mice are tolerized by injection of nontumorigenic tissue before thymic selection occurs, followed by more traditional immunization with tumor tissues after maturation of the mouse immune system.

Phage display has been used to identify panels of antibodies to surface antigens on a large variety of tumor cell lines, primary tumors, tumor tissue sections, cells captured by laser capture microdissection (Ruan et al. 2006), and even primary tumors in cancer patients, the latter injected with phage several hours prior to tumor surgery (Shukla et al. 2013). In order to identify tumor-specific antibodies, libraries are typically exposed to nontarget-bearing cells (e.g., noncancerous cell lines,

normal cells from the same tissue, different cancer types from the same tissues, etc.; Poul et al. 2000; Liu et al. 2004) in order to remove antibodies to common antigens, although this step is often applied following an initial round of selection on tumor cells to avoid depletion of rare antibodies (Zhou et al. 2012). Methods for isolating tumor cell-binding phage include simple washing and elution with standard low- and high-pH buffers (Zhou and Marks 2012; Poul et al. 2000; Becerril et al. 1999), centrifugation through an organic solvent to separate cells from unbound phage (Akahori et al. 2009), and internalization (see below). Some extensive campaigns have examined over 50 tumor lines and over 10,000 resulting scFv (Bouchard et al. 2009; Kurosawa et al. 2009).

Selection for phage that internalize into tumor cells can reduce the recovery of antibodies that bind nontumor-specific or noninternalizing targets and has been used successfully on many tumor types (Liu et al. 2004); reviewed in (Zhou et al. 2012); also (Zhou and Marks 2012; Poul et al. 2009; Fransson and Borrebaeck 2009). Selection for internalizing phage involves allowing phage to bind to the cell surface at 4 °C to prevent internalization, transfer to 37 °C for a brief period (typically 15–60 min) to allow internalization, followed by washing and stripping surface-bound phage with a low-pH buffer, and recovery of internalized phage by cell lysis and infection of *Escherichia coli* with cell lysate. Factors that can favor recovery of internalized phage include high abundance of the target on the cell surface, rapid constitutive target internalization, high affinity, accessible epitope, and multivalent phage (Burris et al. 2011; Zhou and Marks 2012; Zhou et al. 2012). While multivalent display (usually five copies per phage) allows greater recovery of lower affinity clones than does monovalent display (Burris et al. 2011), and, therefore, is likely to lead to pools of higher diversity, numerous examples exist of monovalent phagemid systems giving rise to internalizing clones from naïve human scfv library selections (McDonagh et al. 2008; Osborn et al. 2013; Sapra et al. 2013).

Identification of antibody targets from whole-cell phage selection or whole-cell immunization can be challenging. Antigens are frequently identified by immunoprecipitation with the isolated cell-surface-binding antibodies, followed by proteolytic digestion and mass spectrometry to identify bound peptides (Kurosawa et al. 2008, 2009); see also (Poul et al. 2000; Goenaga et al. 2007). Overexpression of the candidate gene on a test cell or reduction of the candidate gene expression via small interfering RNA (siRNA) can be used to confirm the identity of the candidate antigen. In one example, selection for phage internalizing in MDA-MB-231 breast cancer cells was followed by selection on yeast cells expressing either of two candidate proteins, leading to the isolation of antibodies recognizing the two candidates (Zhou et al. 2010). The authors suggest that this method could be applied on a larger scale to isolate cell-binding antibodies to a large panel of candidate genes.

Large-scale tumor antibody screening has required a series of steps to narrow down candidates. Kurosawa and coworkers (Kurosawa et al. 2008, 2009, 2012) first used patterns of binding to multiple tumor cell lines or tissue sections to narrow down a panel of several hundred antibodies into clusters, followed by a combination of ELISA and western blotting against known antigens and immunoprecipitation/MS to identify new antigens. Liu et al. (2004) selected and characterized a large

panel of internalizing antibodies on prostate cancer cells, similarly using binding profiles to group cells into different epitope clusters.

Studies that have attempted to address the spectrum of tumor surface proteins from several approaches in parallel have suggested that certain common antigens are detected by different technologies (e.g., phage display, hybridoma, and proteomic approaches) but that the majority of antigens identified are different, pointing to the biases and technical limitations of relying on any single method (Rust et al. 2013).

3.4 Target-First Approach to Antibody Generation

3.4.1 Considerations and Preparation of Target for Antibody Screening

A panel of high-quality reagents is key to a successful campaign. Many of the tools used for target validation may also be useful during antibody generation and screening. Such tools typically include target DNA, expression plasmids, native and recombinant cell lines, purified proteins, and control or reference antibodies. When generating screening reagents, it is important to consider generating target orthologs in parallel which can be used to test for species cross-reactivity of antibody hits.

The nature and inherent characteristics of target molecules selected for ADC intervention vary considerably. Nearly all are expressed on the cell surface, whether directly on tumor cells or tumor vasculature. As integral membrane proteins, they are permanently attached to the cell membrane, and typically contain one or more membrane-spanning alpha helices. Such proteins can only be separated from the biological membranes using detergents, nonpolar solvents, or denaturing agents. For this reason, the vast majority of protein antigens used for antibody generation and screening are broken into smaller soluble subdomains, or extracellular domains of cell-surface receptors. These fragments can be designed to be recombinantly expressed for use as immunogens or selection reagents. The precise determination of the domain boundaries is often critical, and small changes in an expression construct may result in drastically different expression yields.

Recombinant production of target antigens can be done using either prokaryotic or eukaryotic hosts. The choice of host likely depends on the nature of target molecule. Most importantly, the yield should be a homogenous, properly folded antigen such that the critical epitopes are accessible (Ebersbach and Geisse 2012). For rapid generation of material, transient expression in HEK293 or Chinese hamster ovary (CHO) cells is preferred. For many proteins, addition of tags, such as influenza hemagglutinin (HA), FLAG, or His_6, will allow easy purification from complex cell culture media. Fc-based fusion proteins, composed of an immunoglobulin Fc domain genetically fused to the target antigen, may also be used. The Fc domain folds independently and can improve the solubility and stability of the partner molecule both in vitro and in vivo, while also providing a convenient purification handle for protein A/G affinity chromatography.

For oncology targets, transformed cell lines expressing the target are often readily available. These cell lines are potent immunogens for the generation of antibodies, resulting in a strong immune response in animals (Kung et al. 1979). Whole cells are complex antigens, made up of many cell-surface proteins, each a potential antigen. Thus, extensive screening is often required to generate antibodies specific for a singular antigen of interest. To reduce complexity during cell-based screening, recombinant overexpressing cell lines can be used. The full-length target antigen can be expressed in commonly used cell systems such as HEK293 or CHO cells. Nontransfected, parental control cells expressing low or no target molecule can be used for counter screening.

To maximize the specific antibody response in the mouse upon immunization with whole cells, human target antigens can also be overexpressed on the surface of mouse cell lines derived from a strain syngenic to the immunization host (Ashley et al. 1997; Uchida et al. 2004). Unlike human cells, murine cells expressing recombinant antigen will typically exhibit a reduced background response as their entire cell-surface components (apart from the transfected and expressed target molecule) are of mouse origin and, therefore, tolerized in the animal. An ideal antibody generation campaign may employ native-expressing tumor cell lines and recombinant proteins as well as engineered cell lines on different backgrounds.

3.4.2 Approaches to Antibody Isolation

Many of the currently marketed therapeutic antibodies have been generated using traditional hybridoma technology developed by Kohler and Milstein (1975). Hybridoma cell lines are generated from the stable fusion of immortalized myeloma cells with immunoglobulin-producing B cells, which is inherently an inefficient process. Despite its limitations, hybridoma technology has been highly successful and has historically been the most widely used method for antibody generation. More recently, methods to identify antibodies in a more efficient manner have been employed. These include culture and expansion of antibody-producing B cells from immunized rodents using CD40/CD40L (Wen et al. 1987; Weber et al. 2003) as well as flow cytometry-based sorting of antibody-producing cells (APCs) using fluorescently labeled antigen followed by reverse transcription polymerase chain reaction (RT-PCR) from cells (Berry et al. 2009; Kurosawa et al. 2012).

Each of the above methods relies on the immunization of rodents or other animals as a source of antibody-producing immune cells. Mice are often used for immunization due to their well-defined genetics, short generation times, and low maintenance costs. The use of knockout mouse strains that have a targeted deletion of a particular gene of interest makes it easier to raise an immune response to some protein targets that are highly conserved among species. When knockout mice are not available, rats or other species can be used to facilitate the generation of human/mouse cross-reactive antibodies.

Human immunoglobulin transgenic rodents provide a method of obtaining fully human monoclonal antibodies using conventional hybridoma technology (Fishwild et al. 1996; Mendez et al. 1997; Osborn et al. 2013; Ma et al. 2013). Transgenic rodents offer a powerful way to generate antibodies to validated targets relatively rapidly without the need for humanization. The benefits of transgenic rodents must be balanced, however, against generally weaker immune responses, requiring more effort to generate equivalent panels of antibodies compared to wild-type rodents (Lonberg 2005). The relatively high technology access cost and limited availability of transgenic animals further contribute to the continued preference for nontransgenic animals among most researchers.

Immunization methods vary depending on the nature of the target and the reagents available during an antibody campaign. When the target is novel, there may be few available tools at the outset of a project. Requiring only plasmid DNA, genetic immunization can be initiated before other tools such as recombinant protein or engineered cell lines can be generated. DNA can be delivered by several means, including injection, a gene gun using DNA-coated gold beads, or intramuscular electroporation (Robinson and Pertmer 2001). The encoded antigen is taken up, translated and presented by immune cells such as dendritic cells, and elicits both a strong humoral and cellular immune response. Coadministration of DNA-encoding immune mediators such as granulocyte-macrophage colony stimulating factor (GMCSF) and Flt3L has been shown to increase the number of infiltrating dendritic cells at the immunization site, resulting in overall increased antigen-specific responses.

Recent improvements to the technique have shown that DNA immunization is capable of generating immune responses against antigens such as G protein-coupled receptors (GPCRs) and ion channels, which are typically considered intractable to antibody generation (Hazen et al. 2014). Generally, immune responses from DNA immunization take longer to develop than those elicited by other methods, but the delayed response time is often offset by the time required to generate immunogens for more traditional approaches.

Rapidly generating a panel of antibodies against multiple targets can be very useful for ADC target validation efforts. Typical rodent immunization methods require two or more months of injections before immune responses are of sufficient titer to be used for antibody generation. Rapid immunization methods such as repetitive immunization, multiple sites (RIMMS) can be used to reduce the period of time between initiation and screening for a panel of hits, without sacrificing either the quantity or the quality of hits in a given campaign (Kilpatrick et al. 1997). RIMMS capitalizes on rapid hypermutation and affinity maturation events that occur in secondary lymphatic tissue early in response to antigenic challenges. Small concentrations of antigen are injected at several subcutaneous sites three times per week over the course of 2–3 weeks. Following antigen challenge, draining lymph nodes from each of the injection sites are collected and used for fusion or B cell-based antibody screening.

Phage display provides the opportunity to guide antibody populations toward those with particular characteristics: for example, toward antibodies binding to a

tumor cell type and not to a set of normal cells, toward antibodies binding to a known epitope on a known target, toward antibodies that are internalized rapidly, or a combination of these traits. Phage-derived antibodies generally have lower affinities than those derived from immunized animals (K_D ranging from 1 to 100 nM vs 0.1 to 10 nM), but due to considerations of the binding-site barrier described above and the likelihood that moderate-affinity antibodies will avoid internalization into low-expressing normal cells, while still internalizing in high-expressing tumor cells, antibodies in the low double-digit nanomolar range are likely to be suitable for ADCs (Rudnick et al. 2011).

The most straightforward phage display approaches have used purified or recombinant antigens for phage selection in either solution or solid phase, followed by screening for binding to the antigen expressed on the surface of engineered cells or tumor cells (Zhou et al. 2012). The advantage of this approach is that the target specificity of the antibodies is known, and binding to the more complex cell surfaces is only confirmatory. The chief disadvantages are that the approach requires the generation of high-quality protein reagents, and even following successful selections, antibodies selected for binding to purified antigen frequently fail to recognize the target in the context of the tumor cell. Selection of phage for binding to cells, including tumor cell lines, tumor tissue samples, or mammalian expression lines engineered to overexpress a specific target, reduces the risk that clones will fail to bind cell-surface target, but as cell-surface complexity increases, the task of separating clones that bind to targets of interest from clones that do not becomes increasingly challenging.

Selection on cells overexpressing the surface protein of interest is also a path to isolating antibodies of known specificity. The advantage of this method is that it favors the isolation of antibodies that bind specifically to the cell surface form of a known target (Hoogenboom et al. 1999). Generation of stable mammalian cell lines can be time consuming, but often a transfected cell line and its untransfected parent can be used for both selection and screening of antibodies and for providing clear evidence for target specificity. Recombinant lines may overestimate the ability of low-affinity antibodies to bind cells expressing lower levels of target, and may have altered internalization mechanics, glycosylation, or association with other proteins, so validation of antibody properties on tumor cells is essential.

Often a combination of methods is appropriate. If the target is known, phage selections using alternating rounds on purified proteins and cells can improve the likelihood that the resulting clones will bind specifically to the desired target as presented on tumor cells. Similarly, combining selection on purified protein and internalizing selections on tumor cells can focus the selected pool on clones of the desired phenotype. It is difficult to predict what characteristics observed for scFv of Fab displayed on phage will translate to characteristics in immunoglobulin G (IgG) format, and for this reason, it is often advantageous to convert as diverse a set of primary isolates to IgG as possible to confirm their binding and internalization profiles.

3.5 Screening for Internalization and Intracellular Trafficking

3.5.1 Screening by Microscopy and Flow Cytometry

Current linker chemistry has been designed such that cytotoxic payloads are cleaved from the antibody, via an assortment of mechanisms, within the intracellular space of the cell, while remaining highly stable in the extracellular space. Thus, it is necessary to demonstrate efficient internalization of the selected antibodies. Depending on reagent availability, antibody binding and specificity can be determined in relatively high throughput by ELISA or other protein-binding assays, and in somewhat lower throughput by cell-based ELISA or flow cytometry on target-expressing engineered cell lines or tumor lines. Internalization is frequently tested in a subsequent step by either fluorescence-based internalization assays (Fig. 3.2a) or by testing the ability of the antibody to deliver toxins intracellularly (Fig. 3.2b).

When using fluorescence-based internalization assays, the screened antibodies can either be complexed with a fluorophore-conjugated modality (such as a Fab fragment or liposome) or directly conjugated with a fluorophore. The use of fluorophore-labeled secondary antibodies is the quickest way to tag an antibody or pool of antibodies. However, since the size and valency of a ligand can effect internalization, the presence of a secondary antibody may alter the internalization properties of the primary antibody (Nielsen et al. 2006). Alternatively, antibodies can be biotinylated with NHS-SS-biotin and subsequently complexed with streptavidin-linked fluorophore moieties (Nielsen et al. 2006). While this process has a higher throughput than direct labeling, it also carries a risk of altering binding affinity of the antibody to its antigen (Vira et al. 2010). The direct conjugation of a fluorophore to an antibody, while suitable only for a small number of antibodies in parallel, allows the user to monitor internalization and intracellular trafficking in real time and, importantly, also reduces the background noise that often arises from the use of secondary antibodies. Antibodies can be directly labeled with fluorophores via a number of commercially available conjugation kits that utilize random lysine or cysteine coupling of the fluorophore. Direct conjugation carries with it the risk of placing fluorophores at the antigen-binding interface, which may interfere with binding (Vira et al. 2010). Accordingly, after direct conjugation, it is important to verify that the binding affinity of the antibody has not been compromised (Vira et al. 2010).

To measure internalization, the fluorescent antibodies are added to cells seeded in a 96-well plate for a period of time at 4 ℃ to allow for binding without internalization (Nielsen et al. 2006; Harper et al. 2013). The cells are then moved to 37 ℃ to allow for target-mediated internalization (Nielsen et al. 2006; Harper et al. 2013). After a selected period of time, noninternalized fluorescent antibodies are gently removed from the cell surface (e.g., by washing with a low pH buffer or, if NHS-SS-biotin was utilized to conjugate the fluorophore, by addition of a reducing agent), or the extracellular fluorescence is quenched by an antifluorophore antibody (Nielsen et al. 2006; Harper et al. 2013). Internalized fluorescence can then be quantified via

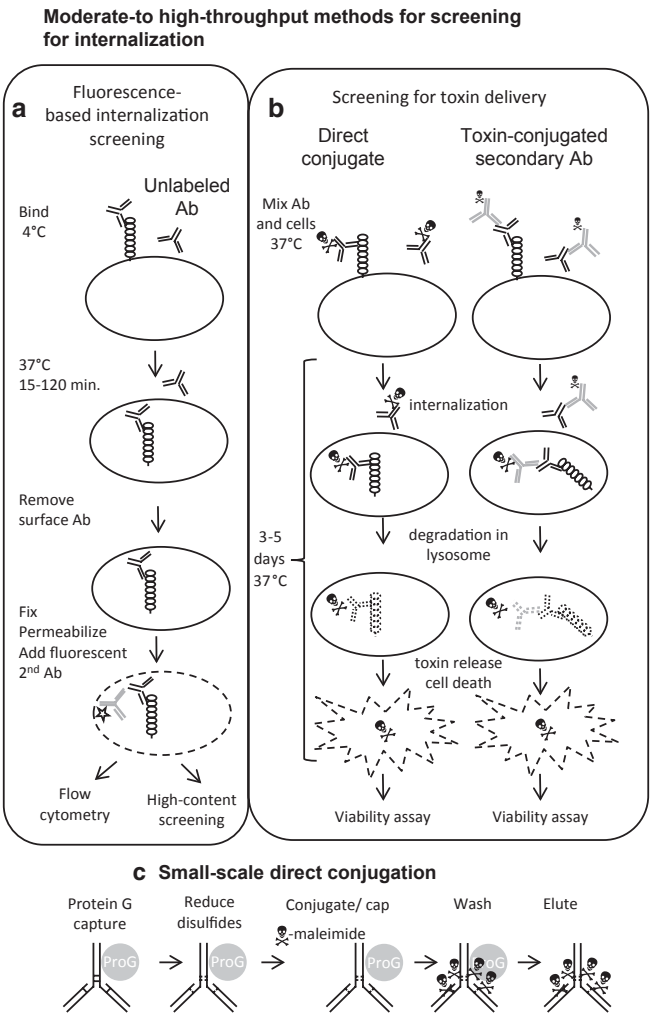

Fig. 3.2 Screening for internalizing antibodies. **a** Screening for internalization using fluorescence-based approaches, In fluorescence-based assays, target cells can be bound to antibodies at 4 ℃, warmed to 37 ℃ to allow for internalization, washed to remove extracellular antibodies, and fixed and stained with fluorescent secondary reagents to visualize internalized antibodies. Alternatively (not shown), antibodies directly conjugated to fluorophores can be used for internalization, and secondary antibodies omitted. Internalization can be measured qualitatively or quantitatively by microscopy or flow cytometry. **b** Screening for internalization by testing for intracellular toxin delivery. For target-mediated toxin delivery assays (*right panel*), antibodies can be directly conjugated to a toxin or can be complexed with a secondary antibody that is directly conjugated to a toxin. The conjugated antibody is then added to target cells for a period of 3–5 days and cell viability is assessed. **c** Small-scale direct conjugation of antibodies. In a method described by Lyon et al. (2012), IgG is captured onto protein G beads from small volumes of tissue culture medium and coupled to toxins via maleimide chemistry, while remaining immobilized on the beads. A mixture of toxin/linker and a sulfhydryl-capping reagent keeps the drug–antibody ratio (DAR) within a controlled range.

a high-content fluorescence reader or flow cytometer, and the degree of internalization can be assessed by comparison with cells kept at 4 ℃ (Nielsen et al. 2006). High-resolution images can also be captured via confocal microscopy to verify the results (Fransson et al. 2004).

The engineering of second-generation ADCs that possess noncleavable, stable payload linkages to the antibody requires delivery to the lysosomal compartment of the cell for liberation of the payload from the antibody and subsequent delivery to the cell in an active form. Accordingly, screening for this intracellular delivery has become of paramount importance and can also be achieved with moderately high throughput. This methodology requires the fluorescent labeling of the intracellular compartment of interest as well as the labeling of the internalizing antibodies with a different fluorophore by the methods described above. Organelle labeling can be achieved through various cell-permeable organelle fluorescent probes, through indirect immunostaining of the organelles, or through ectopic expression of a fluorophore-tagged organelle resident protein (Morelli et al. 2006; Starkuviene and Pepperkok 2007). Similarly to the approach described above, cells are exposed to fluorescently labeled antibodies at 4 ℃ and are then moved to 37 ℃ for a period of time before fixation. However, this method does not require quenching of noninternalized antibodies (Morelli et al. 2006; Starkuviene and Pepperkok 2007). The plates are read in a high-content fluorescence reader, and the magnitude of colocalization of the labeled antibody and the labeled organelle of interest are quantified (Starkuviene and Pepperkok 2007).

Conjugation to liposomes has also been used to measure antibody internalization (Nielsen et al. 2006; An et al. 2008). The antibodies can be coupled to fluorophore-labeled liposomes via either His_6/Ni-NTA or thiol coupling (An et al. 2008; Harper et al. 2013). In this method, conjugated liposomes are incubated with cells for a period of time, before noninternalized mAb/liposomes are gently removed and the cells are lysed (Harper et al. 2013). The remaining internalized fluorescence is measured via a microfluorimeter (Harper et al. 2013). Alternatively, liposomes can be loaded with a cytotoxin or immunotoxin in order to measure the ability of the antibody to confer target-mediated killing (An et al. 2008; Harper et al. 2013). However, the high valency of conjugated liposomes may result in internalization and trafficking properties that differ significantly from those of conventional IgG.

3.5.2 Screening for Intracellular Toxin Delivery

The ability of antibodies to internalize and deliver toxins to the cell type of interest can also be measured directly, and increasing numbers of methods are becoming available to allow rapid screening of hundreds to thousands of antibodies. Some investigators starting with antibodies derived from phage display have screened for internalization by generating genetic fusions of scFv to protein toxins such as *Pseudomonas* exotoxin and testing for cytotoxicity of the resulting immunotoxins (Cizeau et al. 2011; Yoshikawa et al. 2013). Expression in *E. coli* makes this method suitable

for screening of large numbers of scFv, although (Yoshikawa et al. 2013) showing that the cytotoxic potency of the selected fusion proteins was ~100-fold lower than that of the same molecules as neocarzinostatin-conjugated IgG, suggesting that only the most potent internalizing antibodies may be identified by this method.

The development of toxin-conjugated secondary reagents has facilitated screening of antibodies derived from both hybridoma and phage display. Conjugates of protein toxins to anti-IgG secondary reagents have been reported, either chemical conjugates to ricin A (Till et al. 1988) or pokeweed antiviral protein (Weltman et al. 1987) or genetic fusions of *Pseudomonas* exotoxin A to an antikappa single-domain antibody (Kellner et al. 2011) or to the Z domain of *Staphylococcus* protein A (Mazor et al. 2007). Commercially available secondary antibodies conjugated to the ribosome-inactivating protein saporin have been widely used to confirm antibody internalization (Kohls and Lappi 2000; Nguyen et al. 2006; Fransson and Borrebaeck 2009; Sawada et al. 2011). Antibodies can be incubated with saporin-conjugated antimouse, antirat, or antihuman reagents and then incubated with tumor cells for 3–4 days. Uptake of the complexes and release of saporin from the endosome into the cytoplasm leads to cell death (Fig. 3.2b), which can be measured in high-throughput format by viability reagents such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or CellTiterGlo (Promega). The assay can be increased in sensitivity through the use of bivalent saporin-conjugated secondary IgG, which allows higher order cross-linking of surface receptors and may enhance internalization, while Fab–saporin secondary conjugates maintain the level of cross-linking arising from the targeting antibody itself. For His-tagged scfv or Fabs, a saporin-conjugated anti-His_6 antibody provides a tool suitable for screening internalization of His_6-tagged antibody fragments such as scFv. In addition to their use in antibody screening, saporin-conjugated secondary antibodies provide an opportunity to validate the internalization of newly identified cell-surface proteins. For example, Fransson et al. (Fransson and Borrebaeck 2006) used a saporin conjugate to confirm the surface exposure and internalization of the nuclear protein Ku70/80.

The sensitivity of assays with secondary toxin conjugates can be further increased by using potent small-molecule toxins. Klussman et al. (2004) showed that the cytotoxicity of an anti-$Lewis^Y$ antibody complexed to vcMMAE-conjugated secondary antibodies was 1–2 orders of magnitude greater than cytotoxicity measured with the equivalent saporin-conjugated secondary antibodies. In addition, a vcMMAE-conjugated secondary antibody conferred nearly identical cytotoxicity to that of the direct vcMMAE conjugate, indicating that a high-throughput screening with the secondary antibody may be predictive of the activity of the final conjugate. This high-sensitivity approach has recently been facilitated by the commercial availability of secondary reagents conjugated with representative payloads such as Monomethyl auristatin E (MMAE), Monomethyl auristatin F (MMAF), duocarmycin, and mertansine (DM1) (Moradec, LLC, San Diego, CA).

Direct conjugation to a linker payload is frequently a late step in screening for antibodies, carried out on only a small number of candidate antibodies. However, small-scale methods for direct conjugation of toxin to antibodies are coming into increasingly widespread use, allowing early-stage screening of large panels of

candidate antibodies. Lyon et al. (2012) describe a method for small-scale thiol-based conjugation of mcMMAF (Fig. 3.2c), capturing antibodies on Protein G resin and reducing interchain disulfides with tris(2-carboxyethyl)phosphine (TCEP) to expose free thiols. The process uses less than 5 ml of conditioned medium in a 96-well block format, allowing for early-phase assessment of antibodies as ADC carriers prior to hybridoma subcloning, and by inclusion of both a maleimide linker payload and a capping agent is designed to minimize variations in drug–antibody ratios (DARs) despite the variable concentrations of antibody expected in crude supernatants. This critical step allows direct comparison of antibodies, since they carry similar levels of toxin. Lyon et al. describe preparation of 120 antibodies in parallel with a narrow range of (DARs) slightly under 4. Other methods have been published for thiol-based conjugation at the 5-mg scale (Stefano et al. 2013). Site-specific conjugation via several alternative strategies (see below; reviewed in Behrens and Liu (2013)) also hold the potential for application at early screening phases.

3.6 Payload Conjugation Strategies

The classes of cytotoxins currently being utilized to design ADCs fall into two categories: Those that target microtubules and those that target DNA. The method by which a payload is conjugated to an antibody is of critical importance to the overall efficacy of an ADC. The chemistry involved and placement of the payload linkage on the antibody can have a profound effect on binding, tolerability, pharmacokinetic (PK) properties and potency of an ADC.

Conventional conjugation strategies involve random conjugation to either lysine amines (as for Mylotarg; gemtuzumab ozogamicin), CMC-544, and Kadcyla; T-DM1) or sulfhydryl groups on cysteines (for SGN-35 and CDX-011) of the antibody. Due to the high availability of multiple exposed lysines on the antibody surface (~70–90 per IgG1), lysine-based conjugation methods can produce a high degree of conjugation heterogeneity. For example, analysis of the recently approved ADC Kadcyla showed the presence of various drug-to-Ab ratio species ranging from 0 to 7 payloads, with an average of 3.5 payloads per antibody (Junutula et al. 2010), whereas Mylotarg possessed a large mixture of unconjugated species and conjugates with an average loading of 2.5 (Hamann et al. 2002). Alternatively, conventional cysteine-based random conjugation methods produce a more uniform degree of antibody loading. The presence of four reducible disulfide bonds within the IgG structure that covalently link the heavy and light chains together is an attractive feature that allows for selective attachment of the drug to up to eight distinct sites using a thiol-reactive handle. The cysteine-conjugated Adcetris (SGN-35; brentuximab vedotin) contains a lower level heterogeneous mixture of drug-loaded variants with an average of four payloads per antibody (Wakankar et al. 2011; Okeley et al. 2010). In theory, each of the loaded species in an ADC can represent a unique conjugate and thereby exhibits distinct PK and other biological properties.

In fact, for anti-CD30–vcMMAE decreasing drug loading from eight to four or two drug molecules per Ab leads to slower ADC clearance and improvements in therapeutic index (Hamblett et al. 2004). In addition, batch-to-batch consistency in ADC production can be challenging and may require diligent manufacturing capabilities. Site-specific conjugation, in which a known number of linker drugs are consistently conjugated to defined sites, is one way to overcome these challenges.

One of the first site-specific conjugation processes was established by Junutula et al. at Genentech who developed a cysteine-based site-specific conjugation approach called "THIOMAB drug conjugates" (TDCs; Junutula et al. 2008). The authors engineered anti-Mucin-16 antibodies with additional cysteines on light and heavy chains that provide reactive thiol groups and did not perturb immunoglobulin folding and assembly, or alter antigen binding. The engineered Abs (ThiomAbs) were then reacted with maleimide functionalized toxins, thereby yielding nearly homogeneous conjugates, TDCs (Junutula et al. 2008). TDCs possessed comparable antitumor activity as the conventionally conjugated ADCs, despite having a lower DAR (Junutula et al. 2008). Furthermore, the TDCs were tolerated at higher doses in Sprague Dawley rats and cynomolgus monkeys, and had an improved PK profile compared to conventional conjugates (Junutula et al. 2008). However, this method includes a reduction–reoxidation step that can potentially lead to unpaired, reactive sulfhydryl groups. Furthermore, depending on the location of the engineered cysteines, maleimide exchange processes could lead to drug loss and thus decrease the potency of the ADC.

Recently, the use of nonnatural amino acids has also been employed for site-specific conjugation. In one study, a redundant stop codon was introduced at specific locations within the coding sequence of an anti-HER2 antibody, such that an amber suppressor, tRNA/aminoacyl-tRNA synthetase gene pair can incorporate p-acetylphenylalanine into these locations. This allowed for the site-specific conjugation of an auristatin derivative through a stable oxime linkage. This ADC possessed excellent in vitro and in vivo potency with similar clearance rates as the parental IgG in rats (Axup et al. 2012). In a separate study, this technology was also used to produce an anti-5T4 and an anti-HER2 site-specific ADC, containing two monomethyl auristatin D (MMAD) payloads per antibody (Sapra et al. 2013; Tian et al. 2014). These particular site-specific ADCs (NDCs) also possessed improved in vitro cytotoxicity, superior in vivo efficacy, increased PK stability relative to the conventional conjugates, and were well tolerated in preclinical toxicology studies (Sapra et al. 2013; Tian et al. 2014). Additionally, when NDCs were produced with protease-cleavable linkers, the site of conjugation on the antibody had a considerable effect on the stability of these rationally designed prodrug linkers, highlighting the importance of selecting suitable conjugation sites within the antibody (Tian et al. 2014).

In a different methodology to produce site-specific conjugates, the enzyme transglutaminase (mTG) from *Streptoverticillium mobaraense* was used to enzymatically conjugate payloads to antibodies in a site-specific manner and subsequently determine whether the site of conjugation affects the characteristics of the ADC (Strop et al. 2013). mTG does not recognize any of the naturally occurring glutamine residues in the Fc region of glycosylated antibodies, but does recognize

a "glutamine tag" that can be engineered into an antibody (Strop et al. 2013). The glutamine tag, LLQG, was engineered into different sites in the constant domain of an antibody targeting the epidermal growth factor receptor. mTG was then used to conjugate these sites with fluorophores or monomethyl dolastatin 10 (MMAD), and several sites were found to have good biophysical properties and a high degree of conjugation (Strop et al. 2013). Additionally, mTG was able to conjugate to glutamine tags present on anti-Her2 and anti-M1S1 antibodies (Strop et al. 2013). An anti-M1S1-vc-MMAD site-specific conjugate displayed strong in vitro and in vivo activity, suggesting that conjugation using this method does not alter antibody binding or affinity and demonstrates the utility of this approach in the site-specific conjugation of ADCs. The authors also showed that the site of conjugation has a significant impact on ADC stability and PKs in a species-dependent manner (Strop et al. 2013). These differences were attributed to the position of the linkage rather than the chemical instability, as was observed with a maleimide linkage. The authors conclude that this method provides a conjugation strategy that produces homogeneous ADCs and allows for the adjustment of ADC properties in order to maximize the therapeutic window.

3.7 Conclusions and Perspectives

Current linker chemistry has enabled the development of ADCs that are highly stable in the circulation but are amenable to intracellular drug release after entry into the endosome or lysosomal compartment of the cell. While this approach may yield reduced toxicity, as the toxic drug is liberated from the antibody within the tumor cell, the advancement of linker chemistry may make it feasible to devise next-generation linkers that release the drug on the cell surface of cancer cells, but not in plasma. The successful implementation of such linker chemistry would open new avenues in target identification and discovery of noninternalizing antigens that are uniquely present or highly overexpressed in tumors. The notion of noninternalizing antigens has been explored since the early period of radioimmunotherapeutics development. Correspondingly, localized extracellular drug release by the ADCs is a direction being actively explored by several groups. However, it remains to be seen whether this approach will provide sufficient potency in preclinical settings and later in clinics.

Unique, tumor-activating antibody applications are also emerging as a popular strategy to enhance the potency and reduce the toxicity of monoclonal antibodies. In one such example, the authors developed a probody technology, in which antibody binding remains masked against antigen binding until becoming activated locally by proteases commonly active in the tumor microenvironment (Desnoyers et al. 2013). Hypothetically, this technology could help in developing ADCs against targets which have high normal tissue expression. Furthermore, the technology could enable the development of safer ADCs and/or ADCs with an increased serum half-life. However, the utility of such technologies in the clinic remains to be evaluated.

In general, antibody-based therapies (i.e., ADCs, immunotoxins, or immunoliposomes) have performed better in hematological tumor settings compared to solid tumors, despite the fact that the overall expression level of target antigens are frequently expressed at lower levels in liquid tumors. However, in liquid tumors, antigens are expressed more homogeneously and the entire tumor is more accessible for antibody-based therapy. Additionally, liquid tumors are usually more sensitive to cytotoxic compounds and have a more restricted antigen expression pattern on normal tissues. Indeed, a solid tumor environment poses unique challenges with regard to the number of antibody-based molecules that extravasate the tumor blood vessels and translocate through the tumor interstitium toward the surface of tumor cells (Rybak et al. 2007). Further, solid tumors have a heterogeneous blood supply and high interstitial pressures within tumor tissue, especially in necrotic zones, which may limit the diffusion of drugs or ADCs to poorly perfused areas (Stohrer et al. 2000). Additionally, the binding-site barrier hypothesis suggests that antibodies bind to the first target cells they encounter, impairing their penetration through the entire tumor, and limiting their therapeutic effects (Weinstein and van Osdol 1992; Rudnick et al. 2011). Therefore, the development of novel vehicles that allow increased penetration through the solid tumor mass may offer improved efficacy of therapeutic antibodies and ADCs.

The development of companion diagnostic tests will likely become an essential component of future ADC development programs. These diagnostic tests may help translate effective antibody-based therapeutics to successful, targeted treatments for populations that will best respond to the treatment, especially if the drug is being developed against an antigen that has heterogeneous distribution. Designing this companion diagnostics during the early stages of an ADC program may provide valuable information about the best indications to target, but will also allow for the use of novel antibody tools being generated during the antibody isolation stage of the ADC program. It will be interesting to follow whether the field will continue to use the "gold standard" immunohistochemistry-based approaches to identify patient populations or move to less invasive procedures, such as circulating tumor cells or imaging modalities.

It remains to be determined whether amino acid alterations introduced to specific sites within an antibody will present any challenges for immunogenicity or stability of corresponding ADCs in the clinic. Although the site-specific ADCs appear to improve upon conventional conjugation strategies currently used in the clinic, these conclusions are based on fairly limited preclinical data and require a more rigorous analysis in the clinic. Further improvements in site-specific conjugation strategies are still possible, such as homogeneous conjugates with greater than two drugs per antibody. While higher drug loading has been correlated with an increased rate of clearance, those conclusions have been drawn from ADCs constructed using nonspecific cysteine modification methods, and may not be valid for site-specific ADCs. It will also be interesting to follow whether site-specific conjugates possess different off-target toxicity profiles compared to conventional conjugates. Furthermore, site-specific conjugates also promise increased drug exposure, and while higher exposure may be advantageous to achieve higher efficacy, it remains to

be determined whether higher exposure results in more on-target toxicities. To our knowledge, none of the ADCs currently being evaluated in trials in humans used site-specific conjugation approaches; however, emerging preclinical data continue to build on the promise of site-specific conjugation technology to produce safer ADCs with controlled manufacturing and improved analytics.

ADCs have emerged as therapeutic modalities with great promise for the treatment of cancer. However, more may be needed to ensure the clinical success of this therapeutic class. Advances in antigen identification, antibody engineering, and linker and payload conjugation chemistry may help to design more efficacious and safer ADCs.

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