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EXPERT REVIEW

Evolving Strategies for Target Selection for Antibody-Drug Conjugates

Marc Damelin • Wenyan Zhong • Jeremy Myers • Puja Sapra

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ABSTRACT Antibody-drug conjugates (ADCs) represent a promising modality for the treatment of cancer. The therapeutic strategy is to deliver a potent drug preferentially to the tumor and not normal tissues by attaching the drug to an antibody that recognizes a tumor antigen. The selection of antigen targets is critical to enabling a therapeutic window for the ADC and has proven to be surprisingly complex. We surveyed the tumor and normal tissue expression profiles of the targets of ADCs currently in clinical development. Our analysis demonstrates a surprisingly broad range of expression profiles and the inability to formalize any optimal parameters for an ADC target. In this context, we discuss additional considerations for ADC target selection, including interdependencies among biophysical properties of the drug, biological functions of the target and strategies for clinical development. The TPBG (5T4) oncofetal antigen and the anti-TPBG ADC A1-mcMMAF are highlighted to demonstrate the relevance of the target's biological function. Emerging platform technologies and novel biological insights are expanding ADC target space and transforming strategies for target selection.

KEY WORDS 5T4 · A1-mcMMAF · ADC · antibody-drug conjugate · site-specific conjugation

ABBREVIATIONS

ADC	Antibody-drug conjugate
ALCL	Anaplastic large-cell lymphoma
APEX	Absolute Protein Expression Measurements
BRCA	Breast invasive carcinoma
CCLE	Cancer Cell Line Encyclopedia
CD	Cluster of Differentiation
CEACAM	Carcinoembryonal antigen
COAD	Colon adenocarcinoma
CSC	Cancer stem cell
DLBC	Diffuse large B-cell lymphoma
DLT	Dose-limiting toxicity
EBV	Epstein-Barr Virus
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERBB2	erb-b2 receptor tyrosine kinase 2 (also known as HER2)
G2/M	Gap2 / mitosis
GPI	Glycosylphosphatidylinositol
GTEx	Genotype-Tissue Expression database
HL	Hodgkin's lymphoma
HSC	Hematopoietic stem cell
iBAQ	Intensity-Based Absolute Quantification
KIRC	Kidney renal clear cell carcinoma
LAML	Acute myeloid leukemia
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
mAb	Monoclonal antibody
MESO	Mesothelioma
MMAF	Monomethylauristatin F
MTI	Microtubule inhibitor
NSCLC	Non-small cell lung cancer
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PDX	Patient-derived xenograft
PRAD	Prostate adenocarcinoma
PSMA	Prostate-specific membrane antigen (also known as FOLH1)
RPKM	Reads per kilobase per million
SKCM	Skin Cutaneous Melanoma
TCGA	The Cancer Genome Atlas
T-DM1	Trastuzumab emtansine
TIC	Tumor-initiating cell
TMDD	Target-mediated drug disposition
TPBG	Trophoblast glycoprotein (also known as 5T4)
TPM	Transcripts per million

M. Damelin • W. Zhong • J. Myers • P. Sapra (✉)
Pfizer Inc., Oncology Research Unit, 401 N. Middletown Rd., Bldg. 200-4502, Pearl River, NY 10965, USA
e-mail: puja.sapra@pfizer.com

THE ANTIBODY-DRUG CONJUGATE MODALITY

While cytotoxic chemotherapy remains the standard of care for many tumor types, patient treatment is limited by non-tolerated and undesired toxicities. This problem was illustrated by tissue distribution studies in tumor-bearing mice, which demonstrated that paclitaxel and doxorubicin had higher exposure in normal tissues than in the tumor [1]. Antibody-drug conjugates (ADCs) were designed to overcome the limitations of chemotherapy by directing the chemotherapy to the tumor. An ADC consists of a potent cytotoxic drug, an antibody such as one that recognizes a tumor antigen, and a chemical linker. The therapeutic rationale is that the antibody confers specificity upon the cytotoxic drug and thus minimizes the drug's exposure in normal tissues. Clinical data have substantiated this rationale. For example, when the ADC trastuzumab emtansine (T-DM1) was compared to lapatinib plus capecitabine, patients treated with T-DM1 had relatively fewer adverse events (Grade 3 or above) and longer overall survival [2]; a similar trend was also observed when T-DM1 was compared to unconjugated trastuzumab plus docetaxel [3]. The recent approvals of trastuzumab emtansine (Kadcyla™, T-DM1) and brentuximab vedotin (Adcetris™, SGN-35) have validated the therapeutic strategy of ADCs for solid tumors and hematological malignancies. To date the clinical development of ADCs has been limited to oncology, but certain other disease indications might be successfully treated with ADCs in the future.

Most of the ADCs currently in clinical development are based on microtubule inhibitors (MTIs), while some are based on DNA damaging agents [4, 5]. Both of these mechanisms include multiple drugs and chemical linkers; in addition, there is a rapidly growing array of bioconjugation strategies and technologies. An optimized ADC therapeutic is a unified partnership of therapeutic target, monoclonal antibody (mAb), cytotoxic drug, chemical linker and bioconjugation method, as well as clinical development strategy. This article focuses on the selection of ADC therapeutic targets for oncology, with an emphasis on strategies that integrate all of the ADC's critical components.

The once-straightforward approach to ADC target selection has evolved substantially with the accumulation of clinical data, the expansion of ADC platform technologies and the deepened understanding of tumor biology. Historically, target selection was driven by three simple criteria: higher expression in tumors *versus* normal tissues, localization to the plasma membrane of tumor cells, and internalization into cells to enable drug release. While these characteristics are still important in most cases, exceptions, qualifications and additional parameters can now be appreciated and used to inform a more sophisticated, integrative target selection strategy.

GENE EXPRESSION PROFILES OF TARGETS OF CLINICAL ADCS

The expression profiles of candidate ADC targets have long been a fundamental aspect of target selection. To identify trends that might inform selection of future targets, we surveyed the expression profiles of targets of ADCs that are currently approved or in clinical development, as listed recently [4] and subsequently published for two additional targets [6, 7]. The survey was based on an analysis of publicly available mRNA expression databases, The Cancer Genome Atlas (TCGA; [8]) and Gene-Tissue Expression (GTEx; [9]). The TCGA database is a rich resource for genomic characterizations across major tumor types including gene expression measurement by RNA-Seq, which measures gene expression more accurately than the microarray platform [10].

In general, mRNA expression data must be interpreted with some degree of caution since there may be cases in which mRNA is not translated to protein, protein is produced but not presented on the cell surface, protein is presented on the cell surface but does not internalize, or there are differential internalization kinetics in tumor cells vs normal cells. While a survey of cell surface protein expression would be ideal, such data is not available for as many targets in as many tissue types. In general mRNA levels correlate with protein levels [11]. We observed reasonable correlation between mRNA expression in CCLE [12] and cell surface protein expression [13] for the ADC targets CD22 and CD79b, with Pearson correlation coefficients of $r=0.8$ and 0.6 respectively.

Normal Tissue Expression

Our survey of ADC target expression in normal tissues revealed a surprisingly broad range of profiles. Expression data in a panel of 45 normal tissues was compiled from GTEx, and the median value of a given gene in each tissue was determined (Fig. 1). The GTEx database includes multiple regions of the brain; the expression values of the targets surveyed were very similar across all brain regions (data not shown), so only Brain–Cortex was included in Fig. 1 to reduce the complexity of the graph. Data from Cells–EBV-transformed lymphocytes and Cells–Transformed fibroblasts were excluded since they do not represent normal cells. Figure 1 demonstrates the broad range of ADC target expression profiles in normal tissues: some targets have very low mRNA values across the panel; other targets have moderate expression in certain tissues, and some targets have moderate expression across nearly all the normal tissues.

The expression data was subjected to further analysis in an effort to reduce the normal tissue expression to a single parameter that could be used to search for correlations with various clinical observations. A parameter termed NormScore was calculated as the number of normal tissues in which the median target expression level was in the top quartile of all genes in that

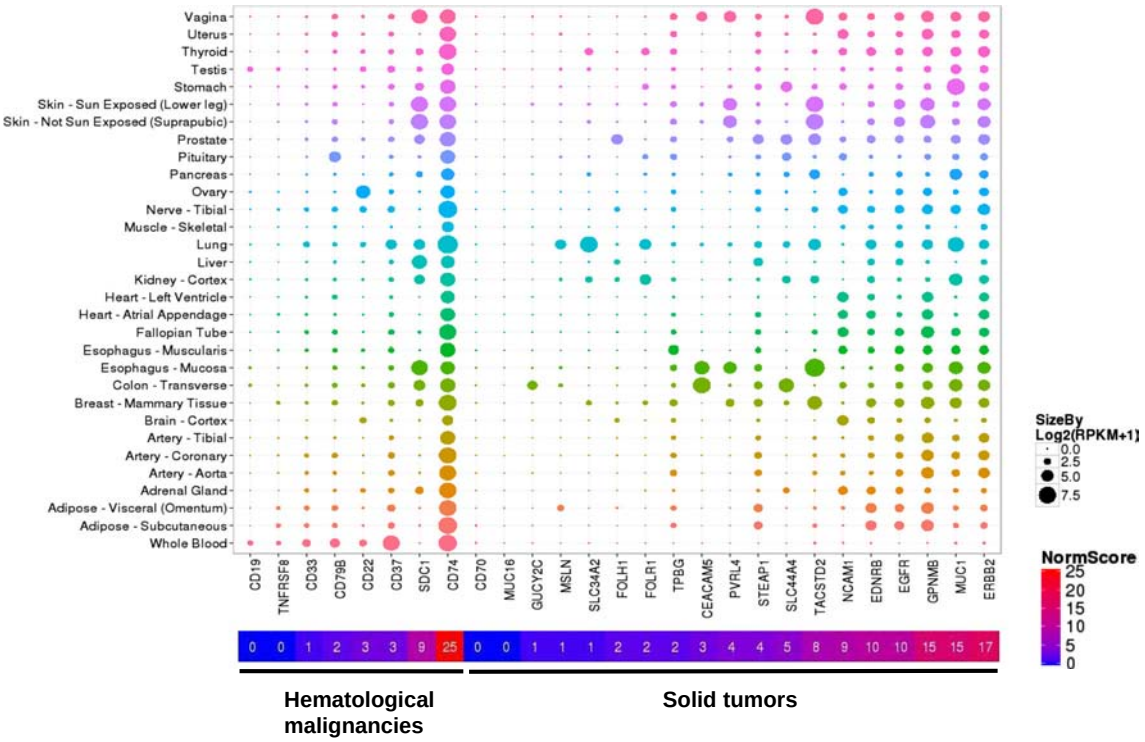

Fig. 1 mRNA expression of clinical ADC targets in normal tissues from GTEx database. Values indicate the median mRNA expression of the gene in each normal tissue. Bottom panel: NormScore of clinical ADC targets calculated based on their normal tissue expression from GTEx database. The NormScore was calculated as the number of normal tissues in which the median target expression level was in the top quartile of all genes in that tissue. See text for more details. *RPKM* reads per kilobase per million.

tissue. Each tissue was assigned a value of 0 or 1; for tissues that are represented in GTEx by multiple regions, expression in the top quartile of any region was translated to a score of 1, since expression even in one region could potentially elicit toxicity. The NormScores for current ADC targets spanned a broad range from 0 to 25 and are provided in Fig. 1. The values generally correlate with the trends observed in the full data set (Fig. 1), and thus NormScore offers a reasonable approach to reducing vast quantities of expression data; threshold adjustments and weighted averages could also be explored. Notably, the top quartile of gene expression has a large dynamic range, and thus the NormScore approach compromises some granularity of absolute scale.

Tumor Expression

The expression levels of current ADC targets in tumors were compiled from the TCGA. Figure 2 shows the distribution of mRNA expression values in patient samples from current clinical indications of each ADC (according to [4, 7, 14]), with corresponding values from matched normal tissue where available. Similar to the above survey of expression across normal tissues, this survey of expression in tumors and matched normal tissues revealed a broad range of profiles for ADC targets. There was a ~125-fold range of median expression values of targets in their clinical indications, and some distributions were narrow (*e.g.*, CD19 in DLBC) while others were broad (*e.g.*, ERBB2 in BRCA). There was a ~1000-fold range in median expression values in matched normal tissues. The relative expression in tumor *versus* matched normal tissue also varied greatly among the targets, with a ~64-fold difference between median CD70 in tumor *versus* normal and less than a 2-fold difference between median ERBB2 (HER2) levels in tumor *versus* normal.

The expression data for ERBB2 relative to other ADC targets – in particular, the seemingly less favorable expression profile – may be surprising at first in light of the recent approval of the anti-ERBB2 ADC trastuzumab emtansine (T-DM1). ERBB2 expression in breast tumors overall is barely higher than in corresponding normal breast tissue (Fig. 2), and its expression across normal tissues is generally higher than that of most other ADC targets (Fig. 1). However, as described below, ERBB2 has several distinguishing features that explain the success of T-DM1 despite apparently challenging pattern of antigen expression in tumors and normal tissues.

PROTEOMICS APPROACHES IN ADC TARGET SELECTION

Mass spectrometry-based proteomics has the potential to transform ADC target selection by providing direct

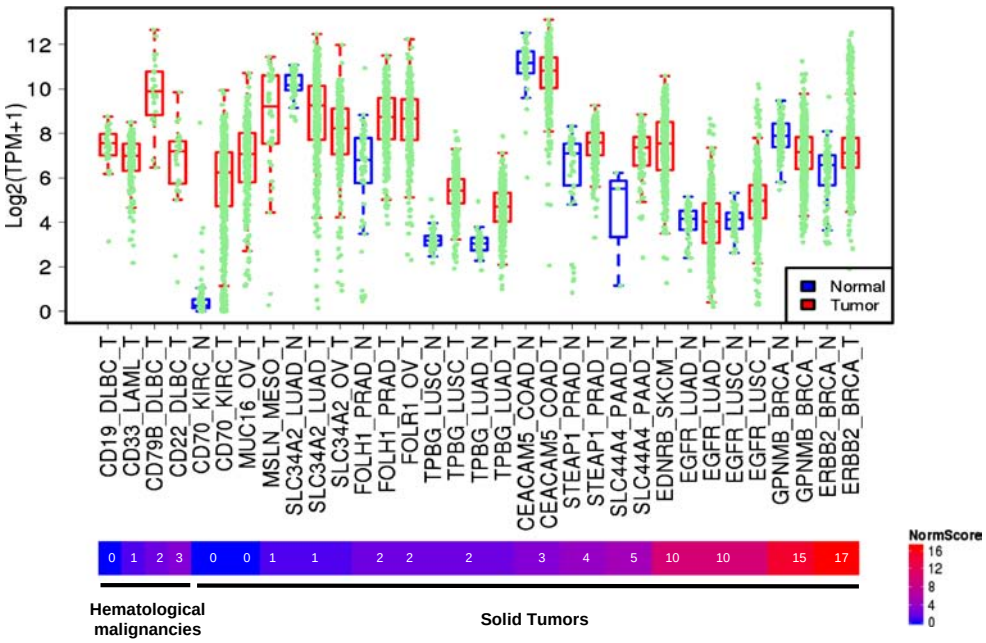

Fig. 2 mRNA expression of clinical ADC targets in tumor and matched normal tissue from TCGA database. The box plot represents the mRNA expression values in their current clinical indications (based on 4); red = tumor tissue (T), and blue = matched normal tissue (N). All data points are shown in green. Each box spans the 25th-75th percentile, and the line inside the box indicates the median value; the whiskers demarcate the data points within 1.5 times the interquartile range within the box. The NormScore parameter of normal tissue expression from GTEx database is also shown (also see Fig. 1). *TPM* transcripts per million, *DLBC* diffuse large B cell lymphoma, *LAML* acute myeloid leukemia, *KIRC* Kidney renal clear cell carcinoma, *OV* ovarian serous cystadenocarcinoma, *MESO* mesothelioma, *PRAD* prostate adenocarcinoma, *COAD* colon adenocarcinoma, *PAAD* pancreatic adenocarcinoma, *SKCM* skin cutaneous melanoma, *LUAD* lung adenocarcinoma, *LUSC* lung squamous cell carcinoma, *BRCA* breast invasive carcinoma.

measurements of protein levels. Improvements in sample preparation, instrumentation, and data analysis now allow the identification of thousands of antigens from a single sample with quantitative data to differentiate proteins expression between samples. While mRNA expression has successfully driven historical target selection efforts and still constitutes the richest datasets available across tumor types, proteomics approaches are complementary and overcome certain limitations of mRNA-based analysis, such as alternative splicing, post-transcriptional regulation, post-translational modification and regulation of trafficking to the cell surface.

We used a recent study of the human proteome in normal tissues [15] to evaluate the protein expression levels of current ADC targets and compare them to the mRNA-based analysis above. A web application (http://www.humanproteomemap.org) that accesses the draft human proteome data was used to generate a heatmap of protein expression data for ADC targets (Fig. 3). The heat map represents mean spectral counts that were normalized across tissues and then rescaled from 0 to 1; a value of 0 indicates no detected peptides and is colored white, while a value of 1 indicates the highest levels of detection and is colored red. Notably, this method of normalization does not enable a direct comparison across targets, in contrast to the RNAseq data. Nevertheless, similar trends are apparent in the proteomics and mRNA-based data, for instance among the targets in hematological malignancies, SDC1 and CD74 show the broadest expression profile in normal tissues (Figs. 1 and 3). Consistent with the above mRNA-based analysis, the proteomics data indicates that targets in hematological malignancies exhibit lower expression in organ tissues than in cell populations from whole blood, while the majority of targets for solid tumors exhibit relatively broad expression in normal organ tissues (Fig. 3). Thus proteomic data can also prove useful for ADC target selection and accessing safety liabilities. In summary, our survey of the mRNA and protein expression levels of ADC targets revealed a broad range of profiles and no obvious set of criteria that could be directly applied to selection of novel targets.

Mass spectrometry-based proteomic approaches based on enriched plasma membrane fractions provide an opportunity to generate highly relevant data for ADC target selection. These approaches include specific sample preparations that can be categorized as labeled-based enrichment, which uses chemical modification to enrich plasma membrane proteins [16, 17], and cell fractionation, which uses biochemical approaches to enrich plasma membrane proteins [18, 19]; label-based enrichment is typically more specific, while cell fractionation is typically more sensitive, as shown in Fig. 4. On-cell proteolysis enables the empirical determination of membrane conformation, which can define epitopes for antibody generation (Fig. 4).

Along with technical advances in modern mass spectrometry-based proteomics platforms and sample

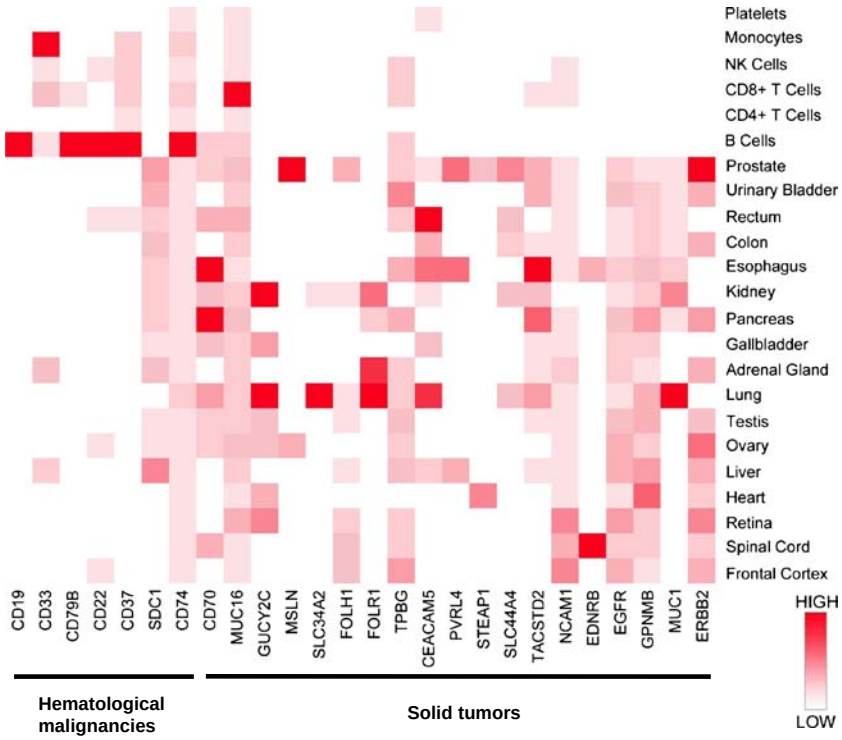

Fig. 3 Protein expression of clinical ADC targets from the Human Proteome Map. The heat map represents mean spectral counts that were normalized across tissues for each target and then rescaled from 0 to 1; a value of 0 indicates no detected peptides and is colored white, while a value of 1 indicates the highest levels of detection and is colored red. Image generated from the Human Proteome Map portal (http://www.humanproteomemap.org/). The sequence of targets is the same as in Fig. 1; no proteomics data was available for TNFRSF8.

preparation, improvements in data analysis allow investigators to move to absolute measurement of proteins abundance. The possibility of generating actual copy number estimates for cell surface proteins using mass spectrometry-based proteomics data is an exciting prospect. A recent study in a human cancer cell line has shown that the addition of isotopic standards enables copy number estimates for thousands of proteins [20]. Label-free approaches also offer opportunities to deliver actual protein abundance measurements. One example is the use of Intensity-Based Absolute Quantification (iBAQ) to estimate absolute protein abundance measurements of over two thousand proteins from the mouse embryonic fibroblast line NIH3T3 [11]. iBAQ uses a normalized sum of peptide ion intensities for each protein corrected to a protein standard. It works well for most proteins with intermediate or high abundance, because multiple peptides with varying ionization efficiencies are identified for each protein, and therefore iBAQ intensity metrics are not likely distorted by peptide specific ionization efficiency bias. iBAQ-based quantitation of lower abundant proteins may be distorted by peptide-specific ionization differences; however, in this case the accuracy may be improved by corrections such as for peptide peak intensities, similar to those described in the absolute protein expression measurements (APEX) method [21]. Together these advances in proteomics and absolute protein quantitation methods may enable robust copy number estimation for plasma membrane proteins in virtually any tumor tissue sample, and thus the emergence of mass spectrometry-based proteomics as a powerful tool for ADC target selection.

EVOLVING CONSIDERATIONS FOR ADC TARGET SELECTION

Our survey of mRNA and protein expression levels of ADC targets suggests that many additional factors besides a target's expression profile can influence the viability of a cognate ADC. In this section, we discuss additional interpretations of target expression profiles as well as other considerations for target selection, with a focus on perspectives that are evolving with emerging platform technologies and clinical experiences.

A Broader Context for Target Expression Profiles

The commonly used term "target overexpression" can have two meanings with distinct implications: overexpression in tumor relative to the corresponding normal tissue (and independent of other tissues); and overexpression in tumor relative to all normal tissues. While the former may imply biological rationale, the latter may be critical for assessing therapeutic rationale. Target expression in normal tissues can reduce ADC exposure in the tumor *via* target-mediated drug disposition (TMDD), and thus impact efficacy as well as safety. Moreover, many gene expression databases are limited to solid tissues, but expression of a candidate target in blood cells must be considered as it could significantly impact drug exposure and increase the risk of immunogenicity.

Trends in recent clinical data from microtubule inhibitor (MTI)-based ADCs in solid tumor indications suggest that responses are more commonly observed in patients whose

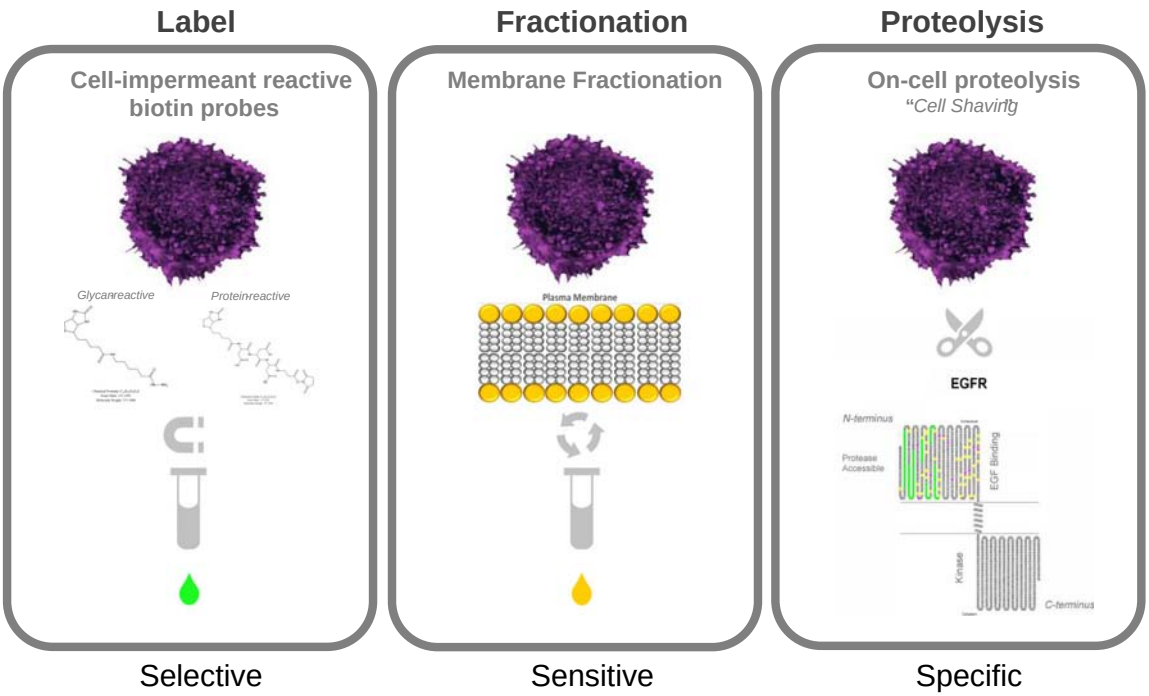

Fig. 4 Cell surface enrichment approaches for proteomics. Labeled-based approaches (left) typically use protein-glycan or protein reactive cell membrane-impermeant biotin probes to label cell surface proteins for subsequent enrichment. Generally more selective for cell surface proteins relative to other approaches, label-based approaches provide a high level of cell surface specificity but require relatively more material than other methods due to limitations in labeling. Label-based methods are suitable for direct tissue analysis and cell culture. Biochemical fractionation approaches (middle) use differential extraction to isolate cell membranes and are generally more sensitive than other methods for the detection of proteins but sacrifice cell surface selectivity due to contamination of organelle membranes and require in silico filtering of proteins. Fractionation approaches are relatively simple to execute, robust and suitable for tissue and cell culture. On-cell proteolysis (right) employs proteases to "shave" proteins from the cell surface. Although highly cell surface selective, on-cell proteolysis is most suitable to tissue culture-adapted cells or established cell lines since proteolysis and collection of liberated protein is less efficient from tissues samples than from cultured cells. One benefit of on-cell digest is that in addition to identifying differentially expressed cell surface proteins, the approach can provide confirmation of extracellular domains for single-pass and multi-pass transmembrane proteins for which the extracellular domains and protein orientation are not known. This information can be valuable in defining epitopes that may be specific to tumor cells and can guide antibody development strategies.

tumors exhibit high antigen levels [6, 22]. Trastuzumab emtansine has shown significant response rates in cases of ERBB2 overexpression but much lower response rates in cases of low-moderate ERBB2 expression [23, 24]. Thus, a substantial ratio of target overexpression in tumor *versus* normal may not be sufficient if the absolute levels in tumors are only modest. The implication for target selection, at least when MTIs are employed, could be that absolute expression levels should be considered as well as relative increases over normal tissue. Analysis of outliers might be more beneficial than analysis of averages; ERBB2 is a prime example in that its expression level is dramatically higher in tumor *versus* normal in the subpopulation of breast tumors defined by ERBB2 amplification [25], but only modestly higher across all breast tumors on average (Fig. 2). Importantly, the linker and drug used in the ADC can significantly alter the thresholds of target expression level that are needed to achieve efficacy and to cause toxicity. For example, antigens with low expression levels in tumors and normal tissues might be amenable to ADCs based on a highly potent DNA-damaging agent such as calicheamicin.

In hematological malignancies – in contrast to solid tumors – target overexpression may not be required to achieve a therapeutic window with an ADC, presumably due to the rapid and continual turnover of the normal hematological compartment. Thus even if normal blood cells are depleted by the ADC, the populations are consistently replenished from the hematopoietic stem cells (HSCs). In this respect it is important to consider whether a candidate ADC target is expressed on HSCs, especially if the drug impacts non-proliferative cells. For example, the expression of CD22 is restricted to the B-cell lineage with no expression on HSCs or antibody-producing plasma cells; thus minimal effects on long-term immune functions would be expected with CD22-targeted therapeutics. An anti-CD22 calicheamicin-based ADC is in clinical development for acute lymphoblastic leukemia [26] and an anti-CD22 MTI-based ADC is in clinical development for lymphomas [4].

An advantage shared by many ADC targets expressed on malignant hematological cells is their relatively homogeneous expression among patients with a given indication. This characteristic seems to naturally follow from the lack of requirement for overexpression and thus the adoption of many lineage markers as ADC targets. In many cases, patient selection biomarkers may not be necessary for ADCs in hematological

malignancies, although the CD19 antigen represents a possible exception as described below. Other advantages of ADC targets in hematological malignancies typically include low levels of expression in normal solid tissue organs (Figs. 1 and 3), higher drug exposure in the blood (tumor) compartment relative to normal solid tissue organs, and the lack of requirement for the ADC to penetrate into a solid tumor tissue in order to achieve pharmacological effects.

Brentuximab vedotin targets CD30, which displays an ideal expression profile for an ADC target in hematological malignancies. CD30 is closely associated with disease etiology as it was originally identified as a marker of Reed-Sternberg cells in Hodgkin's lymphoma (HL) and continues to serve as a primary diagnostic marker for HL. The antigen is also expressed broadly in anaplastic large-cell lymphoma (ALCL) and a subset of other lymphomas. In contrast, there is little or no expression of CD30 on normal tissues outside the immune system or on resting lymphocytes and monocytes. Rapid internalization of brentuximab vedotin was observed on clinical samples [27]. Indeed, the therapeutic has achieved extraordinary response rates in HL and ALCL [28, 29], and as a further advantage, no companion diagnostic has been implemented since antigen expression is assessed during routine clinical diagnosis.

Predicting ADC toxicity based on normal tissue expression is surprisingly challenging and still largely empirical. The anti-CD44v6-ADC (bivatuzumab mertansine) caused toxicity in the skin, which was consistent with the characterized expression of the target in skin keratinocytes [30]. In contrast, SLC34A2 (NaPi-2b) has notable expression in normal lung tissue (Figs. 1, 2, 3), yet the anti-SLC34A2-ADC (DNIB0600A) has shown encouraging safety and pharmacokinetics in the clinic [6, 14]. ERBB2 is expressed at relatively high levels in normal tissues compared to other ADC targets (Figs. 1 and 3), and trastuzumab emtansine was the first ADC approved for solid tumors, with dose-limiting toxicities that are generally not attributed to target expression on normal tissues. Several parameters may determine the translation of target expression to a dose-limiting toxicity (DLT), such as the biodistribution of ADC and exposure in various tissues, the linker and drug used in the ADC, and the particular cell type that expresses the target. For example, non-proliferative cells may not be susceptible to an MTI-based ADC since the drug's mechanism of action is dependent on cell cycle progression, whereas proliferative cells could be susceptible. The interplay of target-dependent and target-independent toxicities, which can vary based on these parameters, further complicates the predictions.

While it is still difficult to predict which toxicities will be dose-limiting, the growing knowledge base from preclinical and clinical studies with many ADCs should improve the ability to do so. Integrated preclinical studies can help to elucidate potential therapeutic implications associated with the expression of ADC targets on normal tissues (*e.g.*, [31]. A key distinction between ADCs and unconjugated antibodies that informs toxicology predictions is that target expression in normal tissues may impact tissue distribution of ADCs and unconjugated antibodies to different degrees: target-mediated drug disposition is typically dose-dependent, and the clinical dose levels of ADCs are substantially lower than those of most unconjugated antibodies. Of course, the lower dose levels of ADCs cannot be assumed to translate to lower toxicities than unconjugated antibodies due to the distinct mechanisms of action of the two modalities.

Complexity of Internalization Parameters

The specific activity of ADCs against target-expressing cells can be conferred by internalization into those cells prior to release of the drug, thus avoiding exposure of the drug to target-negative cells. However, in certain cases, it may be possible to achieve anti-tumor activity with a non-internalizing ADC that recognizes an antigen on tumor or stromal cells and releases drug that permeates the membranes of nearby cells [32]. The linker chemistry by which drug is attached to and released from the antibody must complement the internalization and trafficking of the antigen.

An illustrative example in which ADC potency directly correlated with internalization is CD19. The internalization of anti-CD19 antibody was observed only in some B cell lines, and an anti-CD19 ADC elicited cytotoxicity only in those cell lines that internalized the antibody [33]. Strikingly, the internalization of CD19 was inversely correlated with the expression of another cell surface protein, CD21, which suggested that the ADC would be effective only against CD21-negative tumors [33]. In addition to its potential ramifications for anti-CD19 ADCs, this study elucidated potential complexities that might apply to other targets, and indeed other dependencies have been observed *e.g.*, the regulation of β1 integrin internalization by caveolin-1 [34]. The complexities could hinder target validation and clinical development efforts but could also be exploited to establish biomarker strategies and to identify novel targets, for instance one without dramatic overexpression but with differential internalization in tumor *versus* normal tissue.

The variety of intracellular trafficking routes can complicate studies of antibody internalization, which is most readily analyzed at steady state. The recycling of antigen-antibody complexes to the cell surface can underestimate internalization kinetics, and brief exposure of an ADC to intracellular compartments may be sufficient for drug release, especially when a protease-cleavable linker is used. Pharmacological manipulation of intracellular trafficking (*e.g.*, [35]) and real-time imaging with co-staining of antibody and intracellular compartments can be used to more rigorously study antibody

recycling patterns. The evaluation of target internalization in solid tumors can face the challenge of potential differences in protein trafficking between standard two-dimensional culture conditions and intact solid tissue; three-dimensional culture and specialized matrices may effectively bridge this gap (*e.g.*, [36]).

Most historical and current ADC targets are integral membrane proteins, but a growing list of alternatives includes glycosylphosphatidylinositol (GPI)-anchored proteins on tumor cells and proteins in the tumor microenvironment [4, 37]. The internalization of GPI-anchored proteins as a class has been debated, but anti-CEACAM5, anti-Cripto and anti-mesothelin ADCs exhibited encouraging preclinical activity and entered clinical development [38–40]; these observations advise caution regarding assumptions – about integral membrane proteins and GPI-anchored proteins alike – that certain characteristics apply across the entire category. Similarly, a recent study demonstrated preclinical efficacy of ADCs that bind to certain fibronectin isoforms in the tumor microenvironment (not on tumor cells) and do not internalize [32]. While there are no published examples of ADCs that target secreted ligands of tumor cell-associated receptors, a related concept is the modality in which a drug is directly linked to the ligand, for instance folate conjugates that are internalized upon binding to the folate receptor [41].

Biological Function May Prove to be Important

Since the primary mechanism of action of an ADC is mediated by the cytotoxic drug and independent of target function, the target need not have a direct role in tumorigenesis. This characteristic does not apply to many other therapeutic modalities and is generally considered an advantage of ADCs, yet it is becoming increasingly apparent that tumorigenic functions of ADC targets may be beneficial and that a deeper understanding of target function can impact ADC discovery and development.

For the purposes of this review, the biological function of the target in tumorigenesis will be considered broadly from four aspects: molecular, genetic, cellular and stromal/vascular. Molecular basis includes oncogenic drivers as well as less direct contributors. ERBB2 exemplifies this category as it is an oncogene required for the growth of certain breast tumors [42]. Similarly, multiple ADCs in early clinical development target EGFR [4], an oncogenic driver that is closely related to ERBB2. A potential advantage of an oncogenic driver target is constraining the paths by which the tumor could become refractory to treatment; for instance, downregulation of target expression to reduce ADC engagement would also be deleterious to the tumor. However it should be noted that therapeutic resistance to trastuzumab emtansine is expected in the clinic and has been modeled in preclinical studies [43–45]. Many other targets of ADCs in the clinic have documented roles in tumorigenesis but not to the extent of being oncogenic drivers; hematological malignancies are underrepresented in this class but that is not surprising in light of the above discussion of expression profiles. It will take some time to accumulate sufficient clinical data with ADCs to fully understand the advantage conferred by targets with varying degrees of molecular basis.

Several ADC targets in hematological malignancies do not have molecular basis in tumorigenesis *per se* but serve biological functions that are well matched to the ADC modality. For example, CD79b is a component of the B-cell receptor signaling complex that efficiently internalizes and traffics to the lysosome and upon cross-linking of the signaling complex. The lysosome is a highly preferable destination of internalized ADCs since its acidic environment degrades the ADC and releases the drug [46]. An anti-CD79b ADC showed potent preclinical activity and is currently in the clinic [13]. Similarly, CD74 is an invariant chain of the major histocompatibility complex and efficiently internalizes and traffics to the lysosome; an anti-CD74 ADC showed excellent preclinical activity and is currently in the clinic [47].

Genetic basis refers to amplification or oncogenic mutation of the target gene and generally describes a subset of targets with molecular basis. ERBB2 has genetic basis in that the gene is amplified in those tumors [25], and to date, trastuzumab emtansine has primarily been used for tumors with amplification of the target. Thus ERBB2 is distinguished from many current ADC targets by multiple features that are considered to be advantageous. EGFR also has a genetic basis due to oncogenic mutations that are frequently found in lung adenocarcinoma and colorectal cancer [48]. There are many cases of EGFR overexpression without mutation, and it will be interesting to compare the clinical response of anti-EGFR ADCs in tumors with and without oncogenic mutations (but with similar EGFR expression levels).

While a genetic basis is generally confined to a subpopulation of patients with a given tumor type, appropriate patient selection strategies can be employed in the clinic to take advantage of this desirable target feature. During target selection, the evaluation of gene copy number variation in public databases such as TCGA [8] can complement the evaluation of target expression, especially in the context of the outlier analysis discussed above.

Cellular basis indicates a biological function in specific cell populations within the tumor and reflects growing appreciation of intratumoral heterogeneity and its therapeutic application [49]. In many tumors, cancer stem cells (CSCs) comprise the subpopulation of tumor cells that are responsible for driving tumor growth and metastasis and potentially for conferring therapeutic resistance; the eradication of CSCs may be critical to achieve long-term remissions [50, 51]. Thus proteins that are expressed on CSCs but not on other tumor cell populations could still represent clinically relevant targets.

Most of the methods used to identify tumor antigens are based on bulk tumor tissues, not cell subpopulations, and may not identify candidate targets that are only expressed in a small fraction of cells. For this reason cellular basis is complementary to molecular and genetic bases, with notable overlap. The example of the CSC-targeting anti-TPBG (5T4) ADC will be discussed below. In general, the consideration of CSC-specific targets should be integrated closely with the selection of the ADC linker and drug. The bystander effect, in which target-negative cells in close proximity to target-positive cells are exposed to drug, may be especially desirable. In some cases CSCs are less proliferative and may be less sensitive to cell cycle-dependent drugs such as MTIs.

Stromal and vascular targets are not expressed on tumor cells but rather on non-epithelial cell types that comprise the tumor microenvironment. Tumor cells are dependent on their microenvironment, and the clinical success of many anti-angiogenic agents has suggested the opportunity to target tumor vasculature with ADCs [52]. An ADC that targets the stroma or vasculature could act by dismantling the tumor microenvironment, by exposing the tumor cells to drug indirectly *via* the bystander effect, or both. While this class of targets presents challenges for achieving ADC-induced tumor regressions especially as single agent therapy, recent preclinical studies have highlighted its potential [32, 53]. Targeting endothelial cells may prove to be limited to internalizing antigens such that the release of drug into the bloodstream is minimized, whereas targeting non-endothelial stromal cells may be amenable to non-internalizing antigens. Splice isoforms of fibronectin represent an appealing class of non-internalizing stromal targets as evidenced by preclinical activity of several prototype ADCs [32, 54]. The growing catalog of linkers, payloads and bioconjugation methods may help realize the potential of ADCs that target the tumor stroma and tumor vasculature and thus challenge the historical constraint of tumor cell-based target expression. Some current ADC targets such as PSMA are expressed on both tumor cells and tumor vasculature [55], which could enable multiple mechanisms of action of the ADC. It should also be noted that radioimmunoconjugates and antibody-cytokine fusions, which are related to but distinct from ADCs, have shown encouraging data in this area (*e.g.*, [56, 57]).

In addition to the four general categories of biological function described above, additional characteristics are appealing to consider in candidate ADC targets. Tumor-specific variants of proteins, for instance generated by alternative splicing or post-translational modifications, could confer a high degree of specificity to the ADC for tumor *versus* normal tissue. EGFRvIII, a splice isoform most notable in glioblastoma, is the target of at least two clinical-stage ADCs [4]. The stromal targets fibronectin-EDA and -EDB also result from alternative splicing [32, 54]. However, in some cases it seems that the absolute expression levels of tumor-specific isoforms can be substantially lower than those of the standard isoforms, which might limit ADC activity despite the higher degree of tumor specificity. This observation echoes the concept of absolute *versus* relative expression levels, and candidate targets in this class should be considered together with drugs and bioconjugation methods that might confer additional potency.

Other aspects of a target's physical structure can potentially be exploited to maximize efficacy of an ADC. MUC16 contains repeat sequences that can serve as multiple binding sites for an antibody and thus lead to increased ADC binding to cells. In preclinical studies an anti-MUC16 ADC that bound to a repeated epitope was substantially more potent than an anti-MUC16 ADC with a single binding site per target molecule [58]. While MUC16 is highly expressed in tumors, it is conceivable that in general targeting a repeat sequence could overcome the limitation imposed by lower absolute expression levels. However, increased cell binding would also be expected to occur in normal tissue as well, which may potential limit the overall improvement in the therapeutic index.

Another feature of MUC16 that is shared by ADC targets GPNMB and Cripto is the presence of soluble circulating antigen forms as a consequence of shedding. Antigen shedding can adversely affect drug exposure and safety, though studies of anti-MUC16 in a rat model did not reveal any such effects [58]. In fact, a recent computer simulation suggested that antigen shedding may increase drug activity by increasing the local concentration of drug in the tumor [59]; this effect might be especially relevant in cases of higher expression levels of the target in the tumor relative to normal tissue.

Target selection can be further guided by inherent properties of the proteins including size and potential immunogenicity of extracellular domains, motifs that modulate internalization either through direct protein interactions or post-translationally regulated protein interactions [60]. Using the human reference protein of the UniprotKB database (Release 2014_05), we found that about 70% of ADC targets in clinical development are single-pass transmembrane, 19% are multi-pass and 11% are GPI-anchored. Among the entire set of proteins with extracellular domains in the UniprotKB human reference proteome, 45% are single-pass, 51% are multi-pass and 4% are GPI-anchored. Our analysis indicates an overrepresentation of single-pass transmembrane and GPI-anchored proteins among current ADC targets; it is not clear what drives this bias, but it is possible that multi-pass transmembrane proteins do not internalize as readily or are less amenable to antibody development, for example due to relatively small extracellular loops and challenge of generating recombinant antigens. Indeed, it is useful to consider technical aspects of antibody isolation and characterization, based on antigen structure, sequence homology in relevant toxicology species and the advantages and limitations of the available antibody technologies.

Finally, there are examples of the modulation of antigen expression levels by various tumor-specific factors, with varying implications for ADC discovery and development. In prostate cancer, expression of PSMA is increased after common androgen-deprivation therapy, which could have implications for the clinical development of anti-PSMA ADC [61, 62]. Chemotherapy can induce the expression of various tumor antigens, many of which are stress factors. However such increases in target expression may vary from patient to patient and may also be transient; these aspects can be observed in preclinical models and pose a daunting challenge of translation to the clinic. The sometimes transient nature of target induction may also complicate patient selection strategies since the test material is often archival biopsies that were obtained prior to initial treatments. Importantly, the effects of chemotherapy on gene expression can be similar in tumor and normal tissues, which could diminish potential benefits. Fortunately in the case of PSMA, increased expression upon antiandrogenic treatment would likely be restricted to prostate tumors and not normal tissue [63].

TPBG (5T4) AND A1-MCMMAF ADC: EXAMPLE AND PERSPECTIVES

A case study of Trophoblast Glycoprotein (TPBG or 5T4) will serve as an example of several concepts discussed above. TPBG is commonly referred to as 5T4, but use of the official gene name minimizes confusion. TPBG is the therapeutic target of several anti-cancer agents currently in clinical development. A1-mcMMAF is an MTI-based anti-5 T4 ADC [7]. MVA-5 T4 (TroVax™) is a vaccine based on a modified vaccinia virus engineered to express the target [64]. ABR-217620 (Anyara™) consists of superantigen fused to a moiety of anti-5 T4 antibody [65].

The general appeal of TPBG as a therapeutic target in oncology is driven by its high expression in many tumor types and low expression levels in normal adult tissues [66, 67]. Similar to the carcinoembryonal antigens (CEACAMs), TPBG is referred to as an oncofetal antigen on account of its expression in tumors and during embryonic development [68] but not in normal adult tissues. The expression of TPBG is associated with worse clinical outcome and/or more aggressive disease in lung, colorectal, ovarian and gastric cancers [69–72]. It is important to note that many of these historical studies classify the tumor samples as "positive" or "negative" for TPBG expression, but as discussed above, the level of target expression (*e.g.*, low positive *versus* high positive) can impact the activity of an ADC, and therefore more detailed indication profiling in addition to early clinical data will be required to inform the clinical development of A1-mcMMAF.

In addition to its favorable expression profile and internalization kinetics, TPBG has a molecular basis in tumorigenesis, though it has not been described as an oncogenic driver. TPBG has been associated with cell migration and the epithelial-mesenchymal transition (EMT), a process that occurs during development and tumor metastasis [73–75]. TPBG has also been implicated in Wnt signaling [76], which can be oncogenic. In a mouse model of gastrointestinal stromal tumors, TPBG expression was induced by the c-kit K641E oncogene [77]. Other studies have elucidated additional functions of TPBG that are less directly related to tumorigenesis [78, 79].

Recent preclinical studies have identified a cellular basis of the TPBG contribution to tumorigenesis, and the finding has enhanced interest in this therapeutic target. In non-small cell lung cancer (NSCLC), the expression of TPBG was enriched on CSCs and was associated with EMT and poor clinical outcome [69]. Importantly, and in contrast to many other molecular markers of CSCs, the connection between TPBG and CSCs was established by several independent approaches, including primary serum-free culture, patient-derived xenografts (PDX), conventional cancer cell lines and primary tumors [69]. Molecular aspects of TPBG that were previously described, including a role in cell migration [75] and expression in proliferating progenitor cells during embryonic development [68], are consistent with its association with CSCs, yet the precise function of TPBG in CSCs still must be elucidated.

A1-mcMMAF is comprised of the anti-TPBG humanized IgG1 mAb (A1), a maleimidocaproyl linker and the MTI monomethylauristatin F [7]. Approximately one-half of ADCs in clinical development are based on auristatins [4, 5], which are synthetic pentapeptide inhibitors of tubulin polymerization that induce G2/M cell cycle arrest and cell death. A1-mcMMAF exhibited specificity for the target and efficacy against a panel of lung and breast tumor xenografts; pharmacodynamic biomarker studies demonstrated the increased incidence of the mitotic marker phospho-histone H3, consistent with the expected mechanism of action [7]. The clinical compound was identified by integrated optimization of antibody, linker, drug and bioconjugation method [7], as opposed to a more restricted process that can sometimes arise from limitations on available antibodies, linkers and drugs as well as general resources.

An innovative experiment with A1-mcMMAF demonstrated the potential therapeutic advantage of a target with expression on CSCs [7]. In a tumor-initiating cell (TIC) frequency assay, PDX-bearing mice were treated with A1-mcMMAF, a control non-binding ADC or vehicle, and then more than a week after the last dose administration, tumors were harvested, and live human tumor cells were isolated by flow cytometry and reimplanted into naïve animals. The reimplanted cells from A1-mcMMAF-treated animals were less tumorigenic than those from vehicle- or control ADC-treated animals; thus, the therapeutic targeting of CSC-

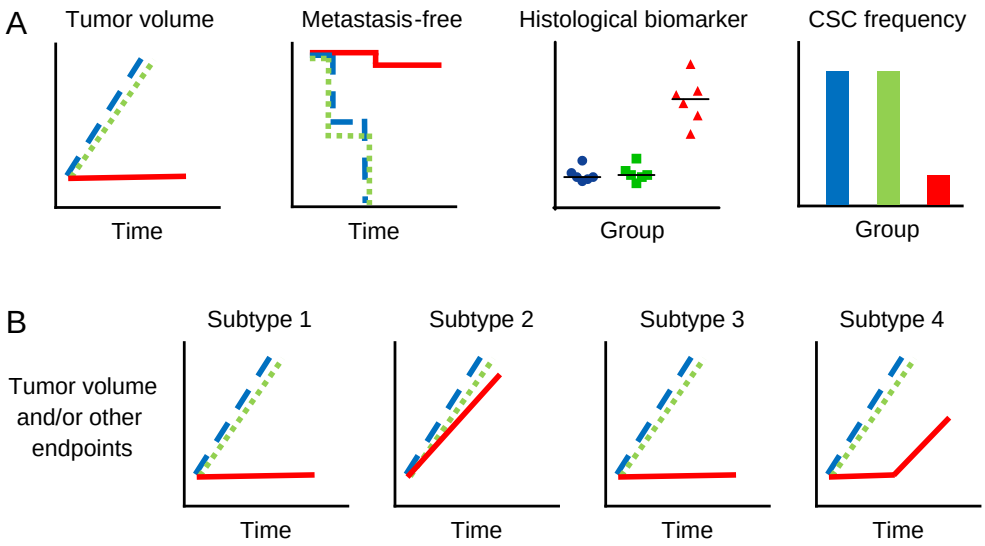

Fig. 5 Efficacy study endpoints for ADC discovery. (**a**), ADC efficacy studies are frequently limited to measurements of tumor volume and sometimes include pharmacodynamic biomarkers. Additional study endpoints that complement the target's biological function should be considered in order to inform clinical development strategy. All endpoints can also be evaluated in the context of a combination treatment of ADC plus another agent. Red data points indicate targeted ADC; green data points indicate a control non-binding ADC; blue data points indicate vehicle control. (**b**), Efficacy studies can be conducted across tumor subtypes in a given indication in order to inform clinical development strategy. The color scheme is the same as in A.

associated TPBG significantly reduced tumor relapse. Importantly, these results were observed with an MTI-based ADC, which could reflect the proliferative state of many TPBG-expressing cells including progenitors [7, 69]. In some cases, perhaps especially in hematological malignancies, a cell cycle-independent drug might be required to significantly impact CSCs.

Preclinical studies with ADCs should complement that target's biological function and thereby explore the potential for clinical development strategies that embrace efficacy determinants beyond target expression [49]. TIC frequency, metastasis and histological biomarkers represent endpoints that complement the measurement of tumor volume and may provide additional information on the compound's mechanism of action (Fig. 5a). Conducting preclinical studies in a panel of models that represent tumor subtypes could also inform clinical strategy (Fig. 5b). Any of these endpoints could also be evaluated in the context of a combination treatment of ADC plus another agent. The next generation of ADC targets is likely to be more nuanced – as a result of accumulated knowledge and improved technology – and preclinical study design will have to match the new level of sophistication.

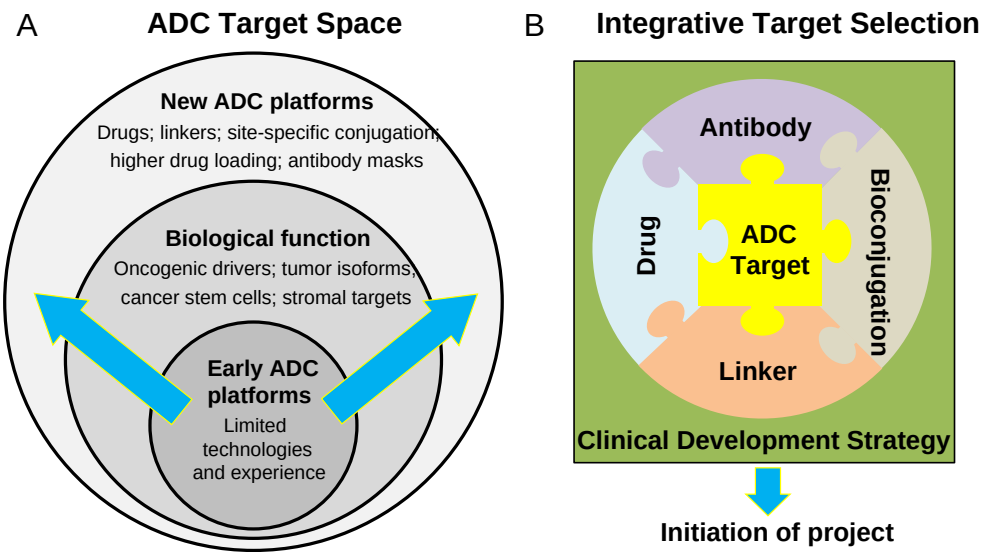

Fig. 6 Evolving strategies for ADC target selection. (**a**), ADC target space continues to expand due to novel platform technologies as well as the discovery of targets based on novel insights into biological function. (**b**), An integrative approach to ADC target selection considers biophysical aspects of the ADC and clinical development strategy prior to initiation of the project. The approach has been enabled by knowledge gained from preclinical and clinical ADC programs.

CONCLUSIONS

The number of targets that are amenable to the ADC modality ("ADC target space") continues to expand due to new technologies and strategies for target identification as well as emerging ADC platform technologies (Fig. 6a). Genomics and proteomics are poised to reveal candidate targets that were obscured by mRNA profiling. A battery of drugs with various mechanisms of action and improved linkers and bioconjugation methods could lower the threshold expression level required for activity. ADCs with masking peptides that block antigen binding in normal tissue but are cleaved in tumor tissue could overcome problems of biodistribution and toxicity caused by normal tissue expression (*e.g.*, [80]). There will certainly be unknowns in the next era as well; for instance, improved bioconjugation methods that enable site-specific linkage [81–83] and/or increased drug loading per antibody [84] may alter the balance of target-dependent *versus* target-independent DLTs, which could change the interpretation of normal tissue expression patterns.

This review has highlighted the evolution of strategies, considerations and methods for ADC target selection. The field is now poised to select targets by a highly integrative approach that considers biophysical properties and clinical aspects together with emerging ADC platform technologies *in a prospective manner* (Fig. 6b). Candidate targets that might have been rejected based on historical criteria or might have failed with early platform technologies could prove therapeutically viable in the context of specific drugs and linkers or innovative clinical development strategies. The confluence of novel technologies, evolving strategies and accruing clinical experience indicates a bright future for ADCs.

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