Phenotypic screening: the future of antibody discovery

Andrea L. Gonzalez-Munoz, Ralph R. Minter and Steven J. Rust

Department of Antibody Discovery and Protein Engineering, MedImmune, Milstein Building, Granta Park, Cambridge CB21 6GH, UK

Most antibody therapeutics have been isolated from high throughput target-based screening. However, as the number of validated targets diminishes and the target space becomes increasingly competitive, alternative strategies, such as phenotypic screening, are gaining momentum. Here, we review successful phenotypic screens, including those used to isolate antibodies against cancer and infectious agents. We also consider exciting advances in the expression and phenotypic screening of antibody repertoires in single cell autocrine systems. As technologies continue to develop, we believe that antibody phenotypic screening will increase further in popularity and has the potential to provide the next generation of therapeutic antibodies.

Introduction

During an era when drug discovery has been dominated by target-based screening, nearly 50 antibodies have been approved or are in late-stage review for clinical use. This number is also set to dramatically increase, with at least 39 antibodies in Phase III studies and many more in early-stage trials [1]. The influx of antibodies into the clinic is a direct result of advances, over the past 30 years, in genomics, molecular biology techniques, and target-based screening. This has led to the successful prosecution of many well-validated antibody-tractable targets [2–4], with the delivery of novel antibodies to the clinic. It has also enabled the engineering of antibodies that have functionality beyond that of a traditional antagonist or agonist, such as antibody drug conjugates, bi- and multispecific antibodies, and antibody fragments [5]. However, during this era, there has also been intense competition around antibody-tractable targets and the next wave of clinical antibodies includes many biosimilar and biosuperior candidates [6]. This increased competition highlights the importance of identifying the next generation of first-in-class antibody drugs against novel targets that will address unmet medical needs. In 2011, Swinney and Anthony [7] observed that, during an era dominated by target-based screening, phenotypic screens were more successful at identifying first-in-class small molecule drugs. Hence, it is reasonable to assume that phenotypic screening, which is target agnostic and identifies molecules based on functionality in cell-based systems before target elucidation, provides a means of identifying first-in-class antibody therapeutics against novel targets (Fig. 1).

Antibodies discovered by phenotypic screens

Unlike small-molecule drug discovery, antibody drug discovery does not have a consistent history of utilising phenotypic screens for the discovery of therapeutic molecules. However, this does not preclude their use to identify therapeutic antibodies that modulate targets that have not previously been associated with a phenotypic response. When developing a phenotypic screen, it should model human disease as accurately as possible; however, the complexity of the screen also has to be balanced against the ability to robustly screen large numbers of molecules. This ability is especially acute for antibody-based screens, where antibodies have to be selected from a population that can exceed one billion. To address this challenge, antibody populations are often enriched before functional screening; for hybridoma-based approaches, cell immunisation is used [8]; for combinatorial library-based approaches, affinity selection against cells or tissue is used [9]; and for human B cell and plasma-cell screens, selected donors are used [10]. There have been several successful phenotypic screens, performed on these enriched populations, which have generated antibodies against cancer cells and infectious agents. As early as 1995, de

Corresponding author:. Rust, S.J. (rusts@medimmune.com)

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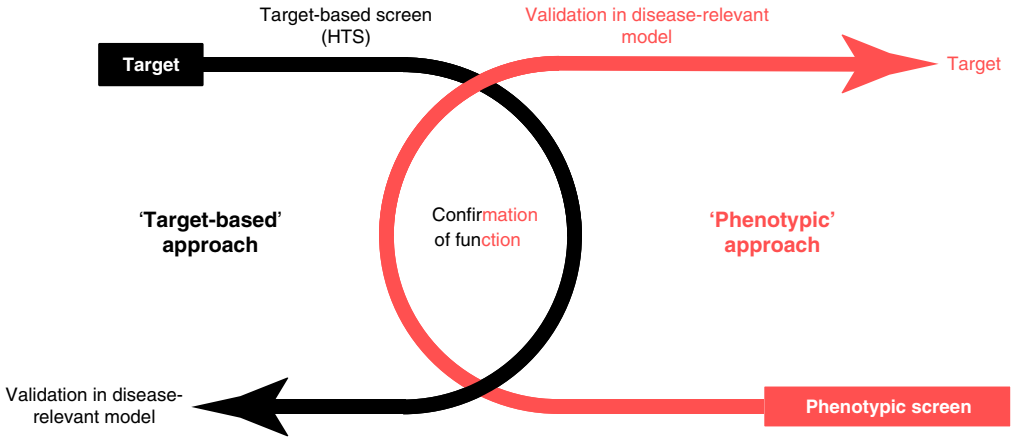

'Target-based' approach	'Phenotypic' approach
Advantages • Molecular target known • Mechanism of action known • Simple design of HTS • History of delivering clinical therapeutics	**Advantages** • Target linked to disease-relevant biological response • Antibodies bind to native target on cells • Multiple targets and/or mechanisms of action can be discovered • More novelty, less competition
Disadvantages • Target might not be important in disease • Literature data on target might not be reproducible • Biochemical and functional assays might not correlate • Single, known mechanism of action • More competition, less novelty	**Disadvantages** • Mechanism of action/target unknown at onset • Target identification can be challenging • Substantial target validation can be required

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FIGURE 1

A comparison of 'target-based' and phenotypic approaches to antibody drug discovery, highlighting the advantages and disadvantages of each. In particular, the phenotypic approach involves early functional testing of drug leads in a cell-based assay, while target-based screening predominantly uses target specificity for the early drug prioritisation step.

Kruif *et al.* [11] screened for target cell specificity and more recent examples have followed [12–14]. Functional screens have also been performed that have identified antibodies that induce apoptosis [15], inhibit cell proliferation [16], or internalise [17,18].

Phenotypic screening of hybridoma-derived antibodies

Historically, the first antibodies in the clinic were identified using hybridoma technology. The most notable antibody isolated using this technology coupled with a phenotypic screen was alemtuzumab (Box 1), a previously approved therapy for chronic lymphocytic leukaemia (CLL) and an approved therapy for relapsing multiple sclerosis. There have also been other antibodies identified using this strategy. The CD44 antagonist, RG7356 [19–22], was isolated using the function-FIRST™ platform [23]. Primary tumour cells were used to immunise mice and antibodies were subsequently screened in multiple functional assays for effects on cell signalling, proliferation, and apoptosis. RG7356 was evaluated in Phase I trials against acute myeloid leukaemia (AML) as well as solid tumours (late-stage, metastatic disease), although both trials were ultimately discontinued. In 2007, Loo *et al.* [24] described the isolation of RAV12. Human foetal progenitor stem cell lines were used to immunise mice and the subsequent functional characterisation of RAV12 revealed some interesting biological properties. RAV12 recognised an *N*-linked carbohydrate antigen (RAAG12) strongly expressed on solid tumours [25], and also induced morphological changes consistent with oncosis in COLO205 cells [24]. RAV12 was evaluated in Phase II trials against metastatic pancreatic cancer in combination with gemcitabine, although this trial was discontinued. These trial discontinuations are hard to rationalise without greater disclosure of the underlying reasons; however, they do reflect the challenges and

BOX 1

Alemtuzumab: the first phenotypic antibody

The use of phenotypic screening to find therapeutic antibodies is not a new phenomenon. Early antibody research during the 1980s that led to the discovery of the anticancer and multiple sclerosis antibody alemtuzumab (Lemtrada™/Campath®) was pioneering in many ways, not least because the clinical efficacy of the antibody was demonstrated many years before the antigenic target was identified [57]. Initially, with the motivation to find an antibody to deplete T cells as a treatment for graft versus host disease (GvHD), researchers from the Cambridge University Pathology Department (hence the name 'Campath') immunised rats with human T cells to raise antibodies specific for human T cells. The best of these was a rat immunoglobulin G (IgG)-2b antibody, which in 1989 was tested in two patients CLL and was effective in dramatically clearing tumour cells in each patient [58]. Groundbreaking work to humanise the antibody and to produce it recombinantly led to the final drug molecule, alemtuzumab, a previously approved therapy for CLL, withdrawn in USA and European Union in 2012, and an approved therapy for relapsing multiple sclerosis. However, despite the clinical efficacy in T cell depletion being demonstrated in GvHD in 1984 [59] and CLL in 1989 [58], the identification of the antigen as CD52 was not made until 1991 [60], making it a true example of a phenotypic antibody.

high 'attrition rate' associated with drug development [26]. If antibody phenotypic screens are to lead to greater success in the clinic, the initial screen needs to reflect the disease state as closely as possible [27]. Many more phenotypic-derived antibodies will have to be evaluated in the clinic before the impact on 'attrition rate' can be assessed. It is also worth highlighting that RAV12 targeted a carbohydrate. Carbohydrates are typically omitted from target-based screens, which are predominantly protein-target focused and, by this exclusion, these screens are not surveying the full diversity of antibody-tractable targets.

Phenotypic screening of combinatorial antibody libraries

Large combinatorial antibody libraries, for example those selected by phage display technology [28,29], have been a rich source of fully human monoclonal antibodies (mAbs) for clinical use and are highly amenable to target-based screening. However, they have also been successfully used in phenotypic screens: Sánchez-Martín *et al.* [9] recently reviewed target-agnostic selection strategies for isolating anticancer antibodies. In 2006, Fransson *et al.* [15] described the isolation of BI-505 using the FIRST^{TM} platform. Using this platform, three rounds of affinity selection were performed with a naïve antibody phage library against intact Ramos B lymphoma cells in the presence of membrane vesicles derived from Jurkat T leukaemia cells for deselection. Enriched antibodies were subsequently assayed for selective B lymphoma cell surface binding and induction of tumour cell apoptosis. Following the isolation of BI-505, its molecular target was identified as intercellular adhesion molecule 1 (ICAM-1). Interestingly, this was the first time that ICAM-1 had been reported to be involved in apoptosis in B lymphoma cells. Further characterisation of BI-505 revealed that ICAM-1 and the BI-505 epitope are strongly expressed in multiple myeloma (MM) and BI-505 was found to have a broad antimyeloma activity *in vivo* in clinically relevant models of MM [30]. Previous knowledge of ICAM-1 biology did not suggest that targeting ICAM-1 with an antibody would induce apoptosis or mediate antineoplastic activity in models of MM. Hence, it is not unreasonable to suggest that such an antibody would not have been identified using a target-led approach focussed on altering the known biological functions of this target [31]. This example illustrates the ability of phenotypic screens to expand the number of antibody-tractable targets, particularly when known targets become novel targets. A Phase I dose-escalation study of BI-505 in relapsed/refractory MM has recently been published [32]. This study concluded that BI-505 can be safely administered at doses that saturate myeloma cell ICAM-1 receptors in patients.

In 2012, DiGiandomenico *et al.* [33] reported the use of combinatorial antibody libraries and a phenotypic screen to isolate antibodies against *Pseudomonas aeruginosa*. In this example, multiple rounds of affinity selection were performed against *P. aeruginosa* using naïve and patient-derived antibody phage libraries. Enriched antibodies were assayed for binding to multiple clinically relevant serotypes. They were also assayed for their ability to promote opsonophagocytic killing, which involved incubating antibodies with complement to mediate bacterial killing by monocytes. Using *P. aeruginosa* knockout mutants, several functional antibodies were identified that bound epitopes associated with polysaccharide synthesis locus (Psl) exopolysaccharide. A high level of Psl expression was observed among *P. aeruginosa* clinical isolates, *in vitro* and *in vivo*, and these antibodies provided up to 100% protection in an acute *in vivo* model of bacteraemia. Subsequently, a multifunctional bispecific antibody (MEDI3902) was engineered to target Psl and the protein PcrV, with the Psl functionality coming from a phenotypic screen and the PcrV functionality coming from a target-based screen [34]. MEDI3902 is being investigated for the prevention of *P. aeruginosa* pneumonia and is currently in Phase I trials.

Phenotypic screening of human B cell repertoires

In addition to hybridoma and combinatorial library-derived antibodies, natural B cell repertoires are also being used for phenotypic screening. If patients can be identified who have themselves raised an effective antibody response to their disease, then this creates the opportunity to screen their B cell repertoire for potentially therapeutic antibodies (Fig. 2). For example, McLellan *et al.* [35] detailed the characterisation of an anti-Respiratory Syncytial Virus (RSV) antibody, D25, which was isolated from a healthcare worker with high exposure to RSV. D25 neutralises RSV by binding to a unique epitope on the prefusion structure of the F protein expressed on the virus coat, which was not identified when target-based screening was used. D25, now known as MEDI8897, has recently progressed into Phase I clinical trials. Several other examples exist in the infection area, given that antibodies are a key part of our natural defence against bacterial and viral infections, and these have been reviewed by Corti and Lanzavecchia [10]. Of particular note is the isolation of human antibodies that potently neutralise human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex, a previously unexplored therapeutic target [36]. There are also examples emerging in other diseases, such as AML. The antibody AT13-37 was derived by screening antibodies from three patients with exceptional clinical outcomes in AML for tumour binding and cell killing. After B cell immortalisation and screening, it was found that as many as 50% of antibodies could be cytotoxic towards tumour cells, highlighting the potential of this approach [37].

Increasing the throughput of phenotypic screening: single cell screening

To date, most antibodies identified by phenotypic screens have been found using a two-step process. First, antibody populations have been enriched against a target cell or tissue type before being screened for function [11–18]. A two-step process is dependent on the efficiency of both steps; however, the functional screen poses the greatest challenge and is often the bottleneck in the process. To be screened for function, antibodies have to be produced individually and there are significant constraints around their production in sufficient throughput and concentration to observe functional effects. This is particularly relevant when hit rate is low because of poor access to the cell surface target antigen. With the aim of increasing the number of antibodies that can be screened, Xu *et al.* [38] developed an antibody expression system that utilises adenoviral transduction of mammalian cells for the production of thousands of antibodies. However, this approach still falls short of being able to screen entire antibody repertoires for function. With a view to addressing this, Zhang *et al.* [39] developed an autocrine method that links antibody sequence and cellular phenotype using lentiviral infection of antibody repertoires in

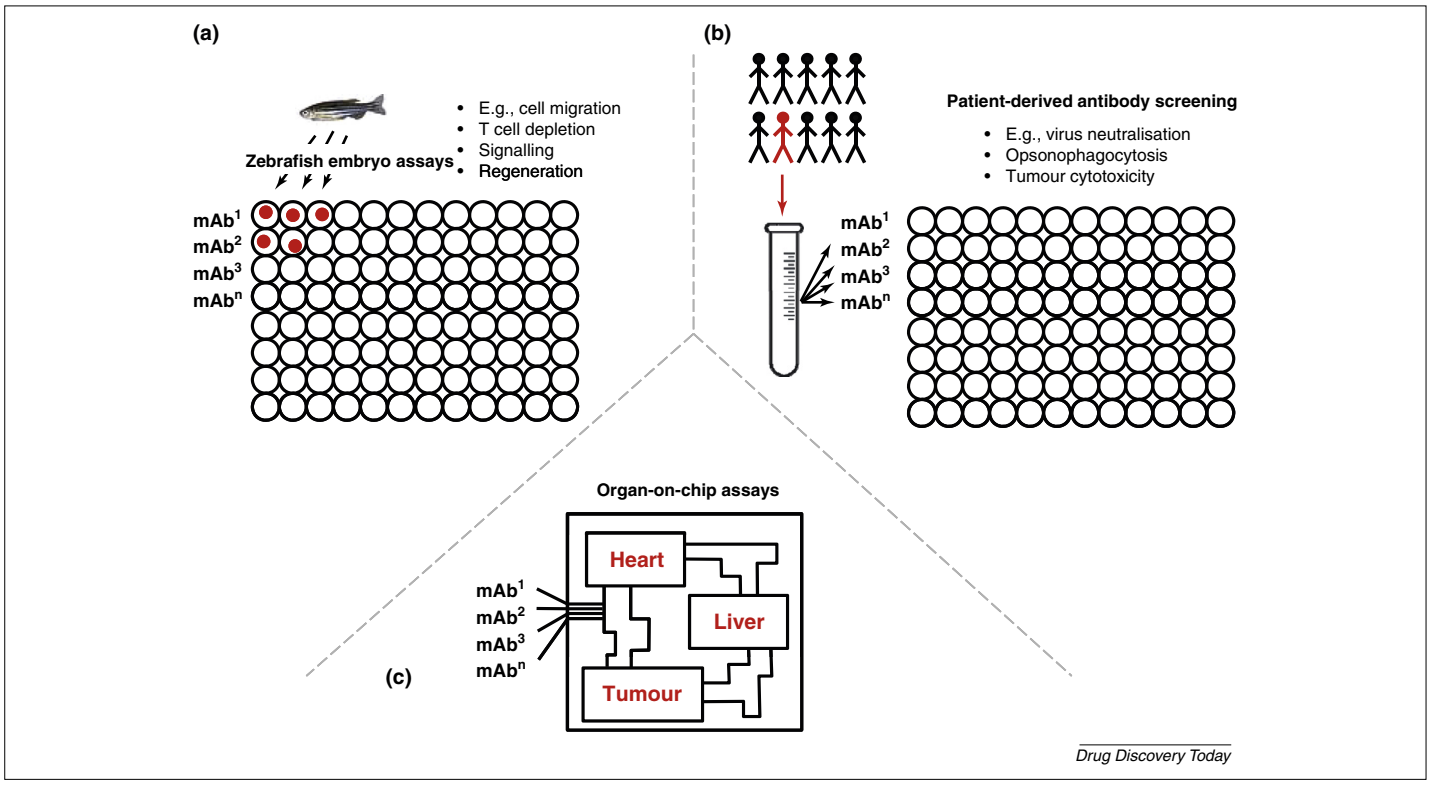

FIGURE 2

The future of phenotypic monoclonal antibody (mAb) discovery. Three examples of screening techniques that could impact phenotypic antibody discovery in the future. Zebrafish embryo screening **(a)** enables complex multi-organ processes, such as metastasis, to be assayed in high-throughput, while simultaneously accounting for aspects such as drug metabolism and toxicology. The use of patient-derived B-cell repertoires **(b)** can rapidly identify antibodies with therapeutic potential directly from patient donors who are responding favourably to their disease. Organ-on-chip technologies **(c)**, which reconstitute complex organ systems using primary *ex vivo* human cells grown in polymeric microchannels, have the potential to be highly predictive of patient responses while requiring only low doses of drug for effect.

eukaryotic cells. Such an approach allows for entire antibody repertoires to be screened for function and addresses the bottleneck of the two-step process. Antibodies against erythropoietin receptor (EpoR) were isolated from a combinatorial antibody library by affinity selection. The antibody population was subsequently transferred to lentiviruses and used to infect TF-1 cells that had been engineered to overexpress wild-type EpoR, which is essential to sustain their growth in the presence of erythropoietin (EPO). To determine whether any of the antibodies could substitute for EPO and promote growth, cells were plated in EPO-free soft agar to impair antibody diffusion and favour autocrine signalling, and observed for 14 days. This approach successfully identified a unique antibody whose agonist function was dependent on its bispecific nature. The discovery of this unique antibody is a result of the lentiviral system used, which allows for multiple viruses to infect a single cell and for combinatorial associations at the protein level. This approach also enables the screening of at least 1.0×10^7 antibodies, which increases further when multiple viruses infect a single cell. This is well in excess of anything that has been screened previously (Table 1). To retain the autocrine link between antibody and reporter cell, antibodies have been screened that have either been retained within the cell [39], retained at the cell surface [40], or secreted into semisolid growth media [41–43]. These examples have all focussed on functionally screening antibody populations pre-enriched for target binding to boost the hit rate, including those enriched on EpoR [39], thrombopoietin receptor [41], granulocyte colony-stimulating receptor (G-CSFR) [40], fibroblast growth factor 4, and fibroblast growth factor receptor 1β and 2β [42,43]. Xie *et al*. [40] used this approach to isolate an agonist antibody to G-CSFR that induced human $CD34^+$ stem cells to form neural progenitor cells via a transdifferentiation process. What is remarkable is that this antibody induced a phenotype that was not observed with the natural ligand. Hence, this screening approach, as discussed for the ICAM-1 antibody [31], has the potential to turn established targets into novel targets by identifying those unique antibodies that would otherwise be missed if the entire population was not screened for function.

More recently, there have also been successful attempts to directly screen antibody repertoires for function without enriching the population for binding first [44–46]. This direct, target-agnostic approach was used by Xie *et al*. [44], who described the isolation of intracellular antibodies capable of protecting cells from rhinovirus-induced cell death. A naïve antibody lentiviral library, containing 1.0×10^8 members, was used to infect HeLa cells before infection with rhinovirus. After infection, antibody sequences were recovered from surviving cells and used to construct a second-generation lentiviral library. The infection–survival cycle was subsequently repeated a further four times. This resulted in the isolation of two antibodies against human rhinovirus B 3C protease and both were capable of protecting against

TABLE 1

Advantages and disadvantages of single cell antibody phenotypic screening

Advantages	Disadvantages
Increased throughput: improvements in autocrine expression systems allow for more than 1×10^9 individual antibodies to be tested phenotypically	**Capacity:** 1×10^9 capacity does not cover size of the largest antibody repertoires
Agonist discovery: identification of agonists is favoured because screens are often based on gain of function assay readouts	**Technical challenges:** lentiviral systems require careful manipulation and production, and certain cell types (e.g., primary cells) might have reduced transduction rates
Truly phenotypic: no need for pre-enrichment based on binding	**Assay options:** requires a selectable phenotype at single cell level; multicellular assays could be complex and difficult to establish
Removes bottlenecks: no need to express and purify antibodies before screening	**High selection pressure:** given the strong selection pressure for a specific phenotype, it is possible that some antibodies dominate the hit population
Accesses combinations: screens can be adapted for bispecific or mixtures of antibodies	

rhinovirus-induced cell death. Although it can be argued that cell survival is one of the most selectable phenotypes, it can be envisaged that such a scheme could be used in other screens where gain-of-function phenotypes that are dependent on agonistic activity or strong morphological changes can be identified. For example, Yea *et al*. [45] described the use of an unbiased morphogenic selection to identify secreted antibodies capable of converting stem cells to dendritic cells. They used a naïve secreted scFv-Fc antibody lentiviral library in TF-1 erythroblast cells and observed morphogenic changes. In total, they observed four unique types of colony morphology and successfully recovered 20 unique antibody sequences. The targets for three of these antibodies were identified as voltage-gated hydrogen channel 1 (HVCN1), transient receptor potential cation channel, subfamily M member 7 (TRPM1), and integrin α3. Given the well-characterised nature of integrins, they further characterised the integrin α3 antibody and confirmed that it was capable of converting $CD34^+$ cells into $CD11c^+$ dendritic cells. Interestingly, the authors chose not to pursue the HVCN1 antibody or the TRPM1 antibody. Both of these receptors are complex multipass membrane proteins, which is a membrane receptor class that has proved challenging to isolate therapeutic antibodies against using target-based strategies [47,48]. Hence, the identification of these receptors and, in particular, the potential for these antibodies to be agonists, strengthen the rational for phenotypic screening approaches. To lend credence to this argument, Mazuc *et al*. [46] used a similar approach to identify a previously undescribed and novel protein, C12orf4, which is involved in rodent mast cell degranulation. All of these examples have successfully used an autocrine-based approach to functionally screen large antibody populations. Screening throughput has been dramatically increased and novel targets [46] and unexpected antibody functionality towards known targets has been observed [40]. We believe that this screening approach is an important development in the field and only time will tell if it translates into success in the clinic.

Increasing the relevance of drug screening: an aspiration for the future

Here, we have summarised current efforts to screen antibodies for particular functions or phenotypes and have highlighted some notable successes. Looking ahead, a major challenge still facing drug discovery, whether phenotype or target driven, is the poor level of physiological and patient relevance of *in vitro* and *in vivo* drug testing. If this challenge is not addressed, we will continue to see high attrition rates in the clinic because of the poor predictive power of 2D cell monolayer screens and preclinical disease models. As such, future phenotypic screens should aim to improve their physiological and patient relevance. Fortunately, there are some evolving technologies and strategies that show promise. Although many of these have not yet been implemented for antibody screening, we believe that they could be adapted for this purpose and offer significant opportunities in the future.

Whole-organism screening offers the opportunity to increase the physiological relevance of drug discovery. Given that the throughput of screening in rodent models has practical limitations, some researchers have begun performing phenotypic screens in the zebrafish (*Danio rerio*). The primary benefit of this approach is that zebrafish embryos can easily be assayed in 96-well plates (Fig. 2), enabling whole-organism profiling of many compounds [49]. This system allows simultaneous screening for efficacy, drug metabolism, and toxicology, while also being compatible with the latest fluorescence imaging methods, given that zebrafish larvae are translucent. Whereas small-molecule compounds are easily taken up by embryos from the surrounding media, antibodies would require transfection of expression plasmids. Although this has not yet been demonstrated for antibody libraries, it was recently shown that zebrafish embryos can be transfected with plasmids encoding CRISPR Cas9 nucleases [50]. Hence, there is now a precedent for screening transfected samples and, in the future, this will likely be applied to antibody testing. In an example of the opportunities offered by zebrafish screening, inhibitors of cell migration were identified using a high-throughput screen, which used automated fluorescence microscopy to visualise the migration of the posterior lateral primordium in larvae. This study identified a compound, the Src inhibitor SU6656, which inhibited cell migration and ultimately suppressed invasion in a mouse model of tumour metastasis [51]. Many other phenotypic screens have now been exemplified in zebrafish, including cardiac morphology, cell regeneration, angiogenesis, behavioural studies, and signalling via reporter genes [52]. An alternative approach, in the area of tumour targeting, is the use of *in vivo* phage display selection in rodent models of cancer to

identify tumour-homing antibodies, as reviewed elsewhere [9]. To address the question of patient relevance and the low predictive power of preclinical animal models, a further development in the field of drug screening is the use of more relevant *in vitro* screens. It is known that cells grown in 3D conditions, which attempt to recreate the *in vivo* tissue architecture, more closely reflect *in vivo* phenotypes than those grown in simple 2D monolayers [53]. 3D phenotypic screens are now being successfully applied to antibody discovery. Examples have highlighted the benefits of this approach and have successfully identified tumour-relevant targets, such as α2β1 integrin [16] and CDCP1 [54], whose relevance could be translated in subsequent disease models *in vivo*. 3D screening is a clear improvement to the phenotypic screening paradigm, provided that challenges around assay robustness and reproducibility can be overcome.

An extension of this approach for future phenotypic screens is 'organ-on-chip' technology (Fig. 2). These devices are made from transparent, polymeric microchannels in which *ex vivo* human cells are grown to mimic real tissue architecture. The transparent nature of the microchannels enables facile imaging in real time of cell phenotypes and their small size enables screening with reduced drug doses when compared with whole-animal screens. In one example of organ-on-chip screening, alveolar epithelial cells were grown in close proximity to human pulmonary microvascular endothelial cells, separated only by a thin, porous, and elastic membrane as a model of the alveolar–capillary interface of the lung. This system was used to successfully model the pulmonary oedema caused by the administration of interleukin-2, mimicking effects seen in the clinic and also in a mouse lung model [55]. In a separate study, a 3D assay of epithelial to mesenchymal transition, using lung cancer spheroids passing through an endothelial-lined microchannel, demonstrated a ranking of 12 model drugs that agreed with human studies but not with 2D assays, which were inaccurate in predicting *in vivo* potency by as much as three orders of magnitude [56]. However, some important challenges remain in this area. Polymers are relatively nonphysiological, can also be 'sticky', and there are barriers to entry, such as the high cost of materials and the requirement for specialist expertise. However, the promise of performing highly predictive, patient-relevant screens in real time with low drug doses and obviating the need for animal models is a strong motivation for developing this technology for phenotypic screening.

Concluding remarks

In summary, here we have described the benefits of using phenotypic screening to identify therapeutic antibodies against novel targets and highlighted several phenotypic drug leads that are either approved or progressing in clinical trials. Despite these successes, phenotypic drug discovery is still disfavoured by most biologics companies in favour of target-led antibody programs. However, we suggest that an increase in the prevalence of phenotypic screening would have the beneficial effect of increasing the diversity of targets tested clinically and reduce duplicated efforts between rival companies. In the future, it is predicted that new technology advances will combine to further increase the opportunities for phenotypic screening, including the use of patient B cell repertoires, single cell autocrine screening methods, and more physiological- and patient-relevant *in vitro* models.

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