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Ian N. Foltz
Kannan Gunasekaran
Chadwick T. King

Discovery and bio-optimization of human antibody therapeutics using the XenoMouse® transgenic mouse platform

Authors' addresses
Ian N. Foltz[1], *Kannan Gunasekaran*[2], *Chadwick T. King*[1]
[1]Amgen British Columbia, Burnaby, BC, Canada.
[2]Amgen Inc., Thousand Oaks, CA, USA.

Correspondence to:
Chadwick T. King
Amgen British Columbia
7990 Enterprise St
Burnaby, BC, Canada V5A 1V7
Tel.: +1 604 415-1800
e-mail: chaking@amgen.com

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Summary: Since the late 1990s, the use of transgenic animal platforms has transformed the discovery of fully human therapeutic monoclonal antibodies. The first approved therapy derived from a transgenic platform – the epidermal growth factor receptor antagonist panitumumab to treat advanced colorectal cancer – was developed using XenoMouse® technology. Since its approval in 2006, the science of discovering and developing therapeutic monoclonal antibodies derived from the XenoMouse® platform has advanced considerably. The emerging array of antibody therapeutics developed using transgenic technologies is expected to include antibodies and antibody fragments with novel mechanisms of action and extreme potencies. In addition to these impressive functional properties, these antibodies will be designed to have superior biophysical properties that enable highly efficient large-scale manufacturing methods. Achieving these new heights in antibody drug discovery will ultimately bring better medicines to patients. Here, we review best practices for the discovery and bio-optimization of monoclonal antibodies that fit functional design goals and meet high manufacturing standards.

Keywords: *antibody therapeutics, XenoMouse®, drug discovery, monoclonal antibodies, bio-optimization*

Introduction and overview of XenoMouse®

The field of therapeutic monoclonal antibodies was revolutionized in the 1990s with the development of transgenic animal systems capable of producing high-quality human antibody repertoires. The original transgenic mouse platforms capable of producing human antibodies were the XenoMouse®, developed by the biotechnology company Cell-Genesys (later Abgenix Inc., and now part of Amgen Inc., Thousand Oaks, CA, USA), and the HuMAb-Mouse®, developed by Genpharm Inc. (later Medarex Inc., now part of Bristol-Myers Squibb Inc., New York, NY, USA). This invention has had a significant effect on human health, with more than 10 human antibody therapeutics derived from transgenic animals currently approved for human use (1). The technology used to develop these transgenic platforms

has been reviewed by Larry Green, coinventor of XenoMouse® technology and the Ablexis (San Francisco, CA, USA) platform (2).

XenoMouse® and the other related transgenic technologies for generating fully human or human chimeric antibodies are incredibly powerful platforms for generating lead molecules with functional properties meeting rigorous therapeutic design goals. Design goals may include functional modulation of the activity of a given biological system. Essential to the design goal is the potency at which a molecule can effect its action on a given system. More specific design goals include the precise epitope with which an antibody interacts, its specific affinity of interaction, or its degree of cross-binding to paralog or ortholog antigens. Ideally these design goals are established at the outset of a therapeutic discovery campaign and form the basis for the screening strategy used to identify lead molecules.

Leveraging our considerable experience developing antibody therapeutics with XenoMouse® (*Table 1*), we recognize the difference between a lead antibody candidate and a viable antibody therapeutic that can be successfully manufactured, developed through clinical trials, and delivered to patients. Although lead candidates can be discovered that match theoretical functional design goals, there is a wide range of additional biophysical properties that affect the therapeutic potential of an antibody molecule, including mammalian production yield, purification efficiency, physical stability, aggregation propensity, and viscosity.

Patient experience is a key factor driving the development of large molecule therapeutics and innovative devices that can be used to deliver them. Consequently, guidelines for biophysical properties may also change depending on desired delivery of the therapeutic to patients. As new devices emerge there may be increasingly stringent requirements on the biophysical properties of molecules suitable to be delivered using them. Another major design consideration is the final structural format of the antibody or antibody-like molecule, such as a modified immunoglobulin

***Table 1.* Antibodies derived using XenoMouse®**

Mechanism of action	Target	Target class	Therapeutic area	Reference
Antagonist	PCSK9*	Soluble	Metabolic	(3)
Antagonist	IL-13	Soluble	Inflammation	(4)
Antagonist	PTH	Soluble	Metabolic	(5)
Antagonist	Hepcidin	Soluble	Hematology	(6)
Antagonist	EGFR*	Type I TM	Oncology	(7, 8)
Antagonist	PD-L1	Type I TM	Oncology	(9)
Antagonist	RANKL*	Type II TM	Metabolic	(10)
Antagonist	αVβ6	Integrin	Oncology	(11)
Antagonist	α4β7	Integrin	Inflammation	(12)
Antagonist	CD-20	Tetraspanner	Oncology	(13)
Antagonist	CGRPR	GPCR	Neurology	(14)
Antagonist	CXCR4	GPCR	Oncology	(15)
Antagonist	GluR	GPCR	Metabolic	(16)
Antagonist	Orai1	Ion Channel	Inflammation	(17)
Antagonist	SARS	Viral Proteins	Infectious Disease	(18)
Agonist	β-Klotho	Type I TM	Metabolic	(19)
Agonist	EPOR	Type I TM	Hematology	(20)
Agonist	GITR	Type I TM	Oncology	(21)
Agonist	TRAILR2	Type I TM	Oncology	(22)
Targeting	BCMA	Type III TM	Oncology	(23)
Targeting	dEGFR	Type I TM	Oncology	(24)
Targeting	CDH-19	Type I TM	Oncology	(25)
Enzyme activator	LCAT	Soluble	Metabolic	(26)
Enzyme inhibitor	Heparanase	Soluble	Oncology	(27)
Enzyme inhibitor	Matriptase	Type II TM	Oncology	(28)
Binder	Cocaine	Small molecule	Addiction	(29)
Human antibody reference	EPO	Soluble	Biomarker	(30)

α4β7 = integrin α4β7; αVβ6 = integrin αVβ6; BCMA = B-cell maturation antigen; CDH-19 = cadherin 19; CD-20 = cell determinant-20; CGRPR = calcitonin gene–related peptide receptor; CXCR4 = chemokine C-X-C motif receptor 4; (d)EGFR = (delta) epidermal growth factor receptor; EPO = erythropoietin; EPOR = erythropoietin receptor; GITR = glucocorticoid-induced tumor necrosis factor receptor; IL-13 = interleukin 13; GluR = glucagon receptor; GPCR = G-protein–coupled receptor; LCAT = lecithin-cholesterol acyltransferase; Orai1 = calcium release-activated calcium channel protein 1; PCSK9 = proprotein convertase subtilisin/kexin type 9; PD-L1 = programmed death-ligand 1; PTH = parathyroid hormone; RANKL = receptor activator of nuclear factor kappa-B; SARS = severe acute respiratory syndrome; TRAILR2 = tumor necrosis factor–related apoptosis-inducing ligand receptor 2; TM = transmembrane.
*Therapeutic molecules derived from XenoMouse® approved against these targets.

(IgG) format or diverse non-IgG formats, such as bispecific T-cell engager (BiTE®; Amgen Inc.) molecules, or multispecific antibodies. The nature of the lead candidates selected for engineering can affect the functional and biophysical properties of the final molecule, again potentially changing the probability of developing a successful therapeutic.

To mitigate the many potential points of failure, our antibody discovery strategy has evolved to maximize antibody diversity at the epitope and sequence levels. Following the identification of a diverse set of antibodies, a rigorous screening process is applied to identify a panel of lead candidates matching a predetermined set of therapeutic design goals. The lead candidates identified through this methodology are then advanced to a recombinant engineering process that defines the final structure of the molecule to provide the ultimate desired functionality. This is followed by the bio-optimization process through which a minimal number of changes are made at the sequence level to modify the overall biophysical properties of the molecule. The addition of the bio-optimization process greatly improves the probability of advancing functionally active molecules that can withstand the rigors of an industrial scale manufacturing process and be compatible with state-of-the-art drug delivery devices. The ultimate goal is to deliver an optimal medicine into the hands of physicians to treat patients with grievous medical need (*Fig. 1*).

Repertoire generation

Transgenic animal platforms leverage the natural *in vivo* system that generates immune responses to foreign antigens.

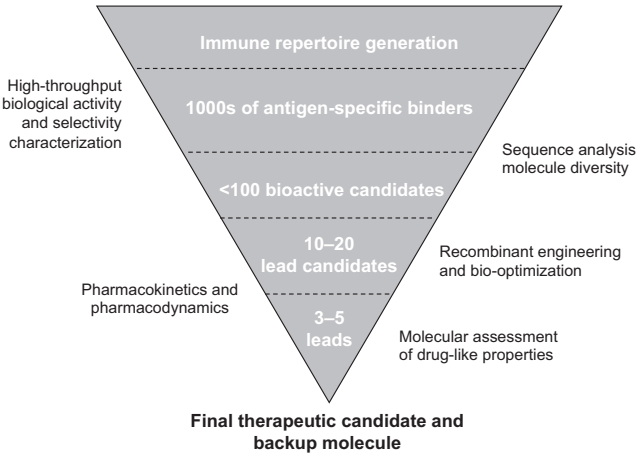

***Fig. 1.* Ideal selection process for antibody candidates.**

Immune repertoires derived in this way use natural processes to create diversity through recombination and mutational processes that combine with a selective pressure on expression and secretion of antibodies. With XenoMouse®, the recombination and mutational processes have been recapitulated to generate fully human antibodies by the transplantation of all or portions of the human genetic loci into a mouse (31). This process largely recapitulates normal human gene regulation and B-cell development. Thus, for XenoMouse® and *in vivo* platforms in general there is an innate selective pressure on the properties of antibodies for expression (31). The B-cell development process selects for antibodies that are secreted and can present on the surface as part of the B-cell receptor complex (32). This is unlike *in vitro* systems, such as phage display, that create diversity through molecular engineering methods and potentially create molecules that do not exist in natural human antibody repertoires (33). For example, XenoMouse® maintains pairing of variable heavy chains (V_H) and variable kappa light chains (33). The repertoire obtained using phage display, although often derived from human sequences, often loses this pairing (33) and have no selection pressure applied to enable expression or secretion from mammalian cells.

Transgenic animals capable of expressing fully human or chimeric immunoglobulins have been developed in mice (32,34,35), rabbits (36), rats (37), cows (38), and other animals, each with significant differences in their design. However, there is no published evidence that repertoire quality is superior to the newer platforms compared with the originators. A wide diversity of antibody therapeutics with extreme potency and selectivity has been derived from XenoMouse® (*Table 1*). In developing this large panel of therapeutic candidates, we have found that the successful discovery and development of a therapeutic antibody relies on starting with a high-quality repertoire of human antibodies. This is followed by an optimally designed screening campaign, performed on a large scale to yield multiple diverse candidate molecules that can then be bio-optimized to result in therapeutic molecules with desirable functional attributes that are compatible with industrial scale manufacturing processes.

Immunization: creating relevant diversity

When using XenoMouse® to create immune libraries of fully human antibodies, the goal is to generate a repertoire of antigen-specific antibodies with maximum possible epitope and sequence diversity. Diversity can be generated in

multiple ways. One way is to present multiple forms of antigen, each presenting the target of interest to the immune system (16). Slightly different presentations and methods of delivery can create a repertoire of functionally interesting candidate antibodies with non-overlapping epitopes and diverse sequences. The second way to develop repertoire diversity is to sample many repertoires from individual host animals. The size of the human antibody repertoire achievable in an individual mouse does not match that achievable in a human or the theoretical maximal antibody diversity, owing in part to the physical size of the total B-cell compartment in a mouse. Therefore, the antigen-specific repertoire raised in one animal can be very different from another. To probe the full theoretical diversity of a human antibody immune response, repertoires from many different animals need to be either individually or collectively sampled. In cases in which the epitope or function of interest is rare, the results can be stochastic, requiring the sampling of large numbers of repertoires to find a suitable antibody candidate.

The first step in generating a high-quality immune repertoire in a transgenic animal host is to design a high-quality immunogen. The immunogen is an antigen preparation that is capable of raising a humoral immune response. To design an optimal immunogen, the primary consideration is the degree of native structure that has been preserved in the immunogen preparation. In the case of soluble proteins produced in recombinant expression systems, evidence of native structure can be determined using bioactivity or direct biophysical interactions with known receptors and antibody controls. Similar biophysical interactions and functional activity can be assayed for membrane receptors, but there are also situations, in particular with membrane targets, in which confirmation of native structure or properties can pose challenges. One method to eliminate the ambiguity of generating immunogens via recombinant production methods is genetic immunization. Genetic immunization is accomplished by delivery of a DNA vector encoded with the gene of interest, resulting in *in vivo* expression of the receptor on host cells (39). This approach enables native presentation of targets without confirmation of the structure. In some cases, the desire to present native epitopes to the immune system can be a significant challenge. For example, complex membrane receptors, such as ion channels, are difficult to express at high levels on most cell types. This type of challenge requires a compromise that involves maintaining native structures as best as possible while using engineering techniques to improve expression levels.

Another consideration to take into account when devising an immunization strategy is immunologic tolerance: T-cell immune tolerance can be a significant challenge when attempting to raise an immune response to human targets in a mouse. Many human targets have a high degree of sequence and/or structural identity to the mouse homolog. Therefore, these human proteins can look like 'self' proteins to the host immune system and evade humoral immune responses. Strategies can be employed to circumvent this type of T-cell tolerance, including engineering of foreign T-cell epitopes into the immunogen (40) and transgenic deletion of the murine homolog gene from the host animals' genome (41).

Hybridoma technology

Once immune repertoires have been generated and are determined to contain antigen-specific antibodies, often numbering in the thousands (*Fig.* 1), many different technologies are available for recovering the antibodies of interest. Factors that should be considered when selecting the best technology for isolating antibodies from transgenic animals include the following:

- *Quantity*: Does the process produce enough antibodies to support the complex screening campaign required to isolate antibodies having the design goals of interest?
- *Scale*: Can the process be done in a massively parallel format to allow extensive repertoire sampling?
- *Efficiency*: Is the process highly efficient at generating a stable source of the antibodies?
- *Accuracy*: Does the process enable the evaluation of functional properties in natural bivalent IgG format?
- *Screening*: Is the process compatible with cell-based binding and functional screens that are essential for recovering antibodies to membrane receptor targets?
- *Assay*: Does the process produce a quantifiable amount of antigen-specific antibody that facilitates early comparison of functional potency on a large scale?

Hybridoma fusion is one of the oldest described antibody generation technologies (42). This process involves the fusion of the B-cell population with an immortalized myeloma cell to generate a hybrid cell capable of secreting antibody and continually dividing. Through many modern improvements, hybridoma generation has been developed as a process that fits all of the essential considerations mentioned above. Historically, however, hybridoma fusion efficiency has been low owing to the poor frequency of fusion events, resulting in poor sampling of the total available B-cell repertoire (43).

The generation of B-cell hybridomas via cellular fusion is typically done *en masse* in large cell culture pools, followed by cell culture in microtiter plates. The resulting hybridoma cultures are polyclonal because they produce and secrete many different antibodies, some of which specifically bind to the target of interest. A common approach is to grow these polyclonal cultures in microtiter plates and then test the supernatants in various binding and functional screens. However, because of the lack of clonality, the specific antibody concentration is not known and therefore the functional potency of any given antibody in the mix cannot be quantified. At this stage, a polyclonal hybridoma line of interest requires subcloning, a process whereby the hybridomas are replated into microtiter plates at a density of one cell per well and then grown up over a period of several weeks. The antibody produced from these subcloned cultures can then be quantitated and retested in potency determination assays. Subcloning can be performed accurately and reproducibly using limit dilution plating or by single-cell sorting using fluorescence-activated cell sorting (FACS). However, this is a burdensome and time-consuming process that can limit the scale of a therapeutic antibody discovery campaign and delay progression of projects significantly. Therefore, clonal hybridoma processes are highly preferred (43).

Several strategies exist to allow upfront clonal plating of the hybridoma cell lines enabling a robust screening workflow based on quantified amounts of specific antibody. One is the enrichment of B cells before hybridoma fusion. This can be achieved through removal of cells that will not result in hybridomas secreting IgG. Immune cells are often obtained from spleens and can contain T cells, macrophages, monocytes, and IgM memory B cells. Eliminating these cell types before fusion enriches IgG B cells and significantly increases the frequency of hybridomas expressing antigen-specific antibodies (44). This enrichment makes it feasible to consider clonal plating of hybridoma cultures. Without this type of enrichment at the primary immune cell stage, the size of the hybridoma pools would prevent full repertoire screening even in industrial screening facilities. Enrichment can also be achieved through antigen-specific selection of surface IgG-positive B cells or a combination of both enrichment processes.

The use of FACS has greatly improved the clonal plating of antigen-specific hybridomas. Hybridomas generated with this process naturally express sufficient antibody on their cell surface to allow their identification. Others have reported that the levels of surface IgG can be further enhanced through the overexpression of immunoglobulin α (45). The use of FACS has the distinct advantage of requiring significantly fewer plates to be handled during the initial plating process to identify antibody diversity. Additionally, the clonal plating of hybridomas provides significant improvements to the screening process, namely quantitation and DNA sequencing of the resulting monoclonal antibodies. Typically, hybridoma lines are plated as polyclonal cell mixtures that secrete multiple different antibody specificities. The polyclonal nature of the secreted antibody prevents specific quantitation of any one antibody in the mix that may have been determined to be antigen-specific and therefore of interest (43). However, with clonal hybridomas, antigen-specific antibodies can be quantitated early in the process and exhausted supernatants can be normalized to a single concentration before performing any secondary characterization assays. This allows direct quantitative comparison of molecules with assays that were previously only qualitative in nature. This offers enormous advantages in early characterization of initial panels of hybridoma-generated antibodies. After identifying the initial antibodies of interest, the samples can then be titrated at known quantities to generate activity curves, allowing accurate interpretation of antibody potency in a functional assay or binding profile in an affinity assay. Thus, the generation of clonal hybridomas has advanced antibody screening from an 'art' to a science and provided confidence in the activity of the initial panel of antibodies at the screening stage. This also eliminates the requirement of a subcloning step, followed by antibody scale-up later in the process to appropriately understand the antibody activity.

Lead candidate selection

Therapeutic design goals

After a successful immunization campaign, the antibody generation process changes from generating a diverse panel of antibodies to identifying those with the best properties. Unlike small molecule screening or *in vitro* antibody selection processes, antibodies derived from transgenic animal platforms generally are imbued with sufficient affinity and biological activity to advance as therapeutic leads without further optimization of these properties (2). Therefore, it is essential to establish the right therapeutic design goals early. As seen in *Fig. 1*, the selection of lead candidates is one of the most significant culls in the antibody screening process, and the diversity resulting from the immunization step is heavily reduced at this stage.

Considerations for developing design goal parameters

Comprehensive antibody design goals are important to the overall therapeutic profile of an antibody. These goals need to encompass the different criteria needed for an antibody to pass this stage of the screening process and include sufficient foresight to support downstream screens, which are primarily early preclinical models (43). Because each target is different, there is no 'one size fits all' approach to antibody design. However, there are some basic concepts that should be considered before beginning antibody screening. These include the nature of the target, known interacting proteins and their affinity for the target, functional activity requirements, and the preferred mechanism of action and the preclinical screening plan. The downstream assays, typically binding and functional screening assays, used to examine the antibody panel should reflect the fulfillment of these concepts.

Antigen-specific binding

The development of assays to identify antigen-specific antibodies is the critical first step in the development of therapeutic antibodies using transgenic animal platforms. This assay will be used during the immunization process to monitor the serologic response of the XenoMouse® animals to the different immunization strategies and identify the hyperimmune animals to initiate the hybridoma generation process. Subsequently, after the generation and culture of the hybridoma pool, this binding assay is required to identify the thousands of antigen-specific binders from the hybridoma panel (*Fig. 1*).

For a therapeutic antibody campaign, the initial binding assay is generally against the human ortholog of the target antigen. This screen should be performed on a native form of the antigen; therefore, the nature and structure of this antigen needs to be carefully considered. For example, some targets require coreceptors for cell surface expression and proper conformation (e.g. calcitonin gene-related peptide receptor) (14), whereas other targets require inclusion of metal ions in the binding assays to maintain proper structure (e.g. CDH19 or integrins) (11, 12, 25). For soluble proteins or single transmembrane proteins with large extracellular domains, techniques such as enzyme-linked immunosorbent assays or bead-based binding assays can be used. For such proteins, antibody candidates can be identified using native assays in which the antibody binds to its target expressed on cell surface using high-throughput FACS or CellInsight™ (Thermo Fisher Scientific, Waltham, MA, USA) screening methods. When possible, these binding assays should also be performed on antigen that has been derived or is expressed on primary cells or cell lines to confirm accurate identification of antibodies against the bona fide target (4, 43). The next aspect of design involves defining the antibody specificity for the target antigen, its cross-reactivity for other related human proteins (i.e. homologs), and its cross-reactivity to orthologs in other species. The specificity is already minimally defined in the original binding screen, but it can be refined to a small region of a protein when exquisite specificity is required. For example, neo-epitopes created by protein splice junctions can allow the creation of junction-specific antibodies that have no appreciable binding to the wildtype protein (24). The specificity of the antibody can be further refined through the requirement to have cross-reactivity to other highly related human proteins that may share a common biology (46) or through the elimination of antibodies with cross-reactivity to highly related proteins that are not desired owing to concerns of interfering with off-target biology or toxicity (5).

Finally, therapeutic design goals need to consider species cross-reactivity, which is necessary for the preclinical screening plan. Antibodies with high specificity and cross-reactivity to highly conserved orthologs (>90% identity) can prove difficult to identify during screening. If a minimal level cross-reactivity is not already present, it will be extremely difficult to achieve binding specificity through engineering afterward. Species cross-reactivity to the ortholog in cynomolgus monkeys and in a non-murine, non-primate species, such as rat or pig, is usually required for pharmacokinetic and pharmacodynamic preclinical assays. Antibodies with these features can be selected through binding assays, but these screens can greatly reduce the diversity of the antibody panel so it is important to restrict these binding requirements to those orthologs that are truly important for clinical development. In the absence of the required species cross-reactivity, the translation of the antibody to the clinic can be significantly delayed and difficult, often requiring the generation of surrogate antibodies with similar affinities and mechanisms of action to provide the necessary safety and pharmacokinetic data. As with the initial binding assays, it is important to confirm cross-reactivity on the endogenously expressed antigen, particularly with primary cells obtained from cynomolgus monkeys when possible. Significant differences in post-translational modifications between recombinant and endogenous forms of the cynomolgus protein, e.g. can lead to reduced or absent binding between the two forms of the antigen (47).

Affinity determination and affinity ranking

Antibody design goals also need to include the concept of the antibody affinity for its target (48). The affinity requirement of an antibody for its target needs to consider many concepts, such as the affinity of known interacting proteins and the expression level of the target on cells or in plasma (48). Poor biodistribution of the antibody to the target's location in the body (49) or a poor pharmacokinetic profile of an antibody may necessitate a higher affinity antibody (48). Simply put, the antibody has to have sufficient affinity to deliver its biological effect at the concentration it will ultimately achieve through dosing at the target location.

Assays to formally determine the affinity of an antibody for its target are well described in the literature and typically include such methods as KinExA® (Sapidyne Instruments, Inc., Boise, ID, USA), BiaCore™ (GE Healthcare Life Sciences, Little Chalfont, UK), and Octet (ForteBio, Menlo Park, CA, USA). Identifying antibodies with high affinity is particularly important for antibodies with mechanisms of action related to their targeting properties (such as a BiTE® or antibody drug conjugate molecule) or a requirement to antagonize another protein–protein interaction in the body. Although still relevant, affinity ranking is less important for functional antibodies because the biological potency of the antibody will primarily drive the lead selection.

In contrast to *in vitro* selection-based antibody generation techniques, antibodies derived from transgenic platforms such as XenoMouse® generally do not need engineering to modify their affinity because single-digit pM- to sub-nM–affinity antibodies can usually be identified directly from the original screening campaigns. However, there are situations in which it may be necessary to have high affinity for an antigen at physiologic pH and lower affinity for the antigen at acidic pH. These pH-sensitive antibodies may facilitate clearance of highly expressed soluble proteins or prevent target-mediated disposition of antibodies against transmembrane proteins coexpressed in cells expressing neonatal fragment crystallizable receptor (50). pH-sensitive antibodies may be found through screening in the natural immune repertoire or be created through antibody engineering (6, 50).

Affinity analysis is also important for toxicology predictions in cynomolgus monkeys. Therapeutic antibodies for the toxicity study species are generally required to have an affinity within approximately 10-fold that of the human protein. This affinity gap is primarily set because these cross-species affinity differences can be overcome through increased antibody dosing. Although larger affinity differences can be tolerated, particularly for high-affinity antibodies, the affinities of the antibody for the cynomolgus protein and the human target should be as similar as possible to be able to properly predict the results from the human studies.

Functional characterization

The goal of functional screening assays is to determine if the antibody of interest has the function specified during the design process. Several strategies are available for these assays. The most common is a basic functional screen using a receptor–ligand (R-L) style competition assay (e.g. proprotein convertase/subtilase kinase 9 antibody–low-density lipoprotein-C receptor) (3). The sensitivity of this style of assay and its ability to screen antibodies effectively is limited by the affinity between the R-L pair. For lower affinity interactions, these assays may not be as useful for screening owing to the concentration of proteins needed to visualize the interaction. These low-affinity interactions generally result in qualitative data, and there may be a need to increase the affinity or avidity of the interaction to miniaturize the assay effectively to address quantitative differences in the antibody panel. These assays can be performed by FACS on cell lines or on beads using technologies such as Alphascreen® (PerkinElmer, Waltham, MA, USA).

For targets with a clear mechanism of action, the blockade of a known protein–protein interaction and relatively high affinity can be sufficient to allow lead selection. However, there is still value in screening in a functional assay that uses a relevant biological readout, such as proliferation or cytokine release. This type of screening can be established with more stringent conditions, such as biologically relevant concentrations of stimulus and longer time courses (43). Furthermore, these assays can also identify other mechanisms of action that an antibody might require to inhibit a biological function, such as receptor dimerization (e.g. 2C4 antibody against receptor tyrosine-protein kinase erbB-2) (51), inhibition of ligand-independent signaling owing to overexpression of the target (e.g. trastuzumab) (52), or receptor internalization or receptor downregulation. Antibodies having these mechanisms of action might not have been identified with basic R-L assays and represent productive means to inhibit a biological function. Therefore, it is good practice to include both a functional assay and an R-L interaction style assay to understand potential functional diversity in an antibody panel and provide a means to identify novel mechanisms to inhibit the desired target's biology.

Functional assays can also be used to screen for indirect biological activities that an antibody can redirect onto a target cell through their binding specificities. Antibodies with human IgG1 isotypes can be directly used to assess natural killer cell-mediated killing on target expressing target cells through antibody-dependent cellular cytotoxicity (53). Tumor cell killing can also be mediated through antibodies through the facilitation of toxin internalization in a process involving antibody drug conjugates (54). Finally, with engineering of the antibody specificity into a bispecific antibody, one can redirect cytotoxic killing of cytotoxic T lymphocytes with BiTE® (55).

Antibodies can also agonize a receptor to deliver a biological signal or stimulus to cells. In rare cases, this activity may be due to monovalent binding of an antibody to a cell surface receptor (e.g. tropomyosin receptor kinase A) (56). Usually this induced signaling is due to the bivalent nature of an antibody leading to the homodimerization of a receptor and subsequent signal transduction (19). This kind of agonist antibody signaling often requires additional cross-linking for maximal activity that is generally provided *in vitro* through a secondary cross-linking agent and *in vivo* through the fragment crystallizable (Fc) receptors for IgG (AMG 655, anti-CD40) (22, 57). Recently, it was recognized that the IgG2 disulfide isoforms have different agonistic activity (58); thus, antibody structure as well as the epitope it recognizes can affect the agonist activity of these antibodies.

Epitope mapping

For the selection of targeting antibodies for antibody drug conjugate development or for the development of recombinant molecules that require engineering before functional activity testing, such as the bispecific T-cell engager (BiTE®; Amgen Inc.) molecules (59), the design goal changes from identifying the epitope with the best functional properties to the identification of multiple epitopes so that each one can be analyzed. There are several techniques recognized to allow the identification of these epitope bins in the antibody pool. Chimeric molecules can be used to epitope-map antibody interaction sites (*Fig. 2A*). This is usually done through mouse-human chimeric proteins because mouse-based immunizations generally lack cross-reactivity to the murine ortholog of the target, either because of tolerance or lack of sequence identity to the human target protein, and can provide information about the binding location of these antibodies (19). This method can also be done with structurally related molecules, such as human paralogs. In addition to chimeric molecules, deletion mutants of target antigens can be generated either as soluble or transmembrane proteins and, when positive binding is observed, can provide great confidence in the binding epitopes of an antibody (*Fig. 2B*). Although these approaches can identify the part of the target that the antibody binds, they do not

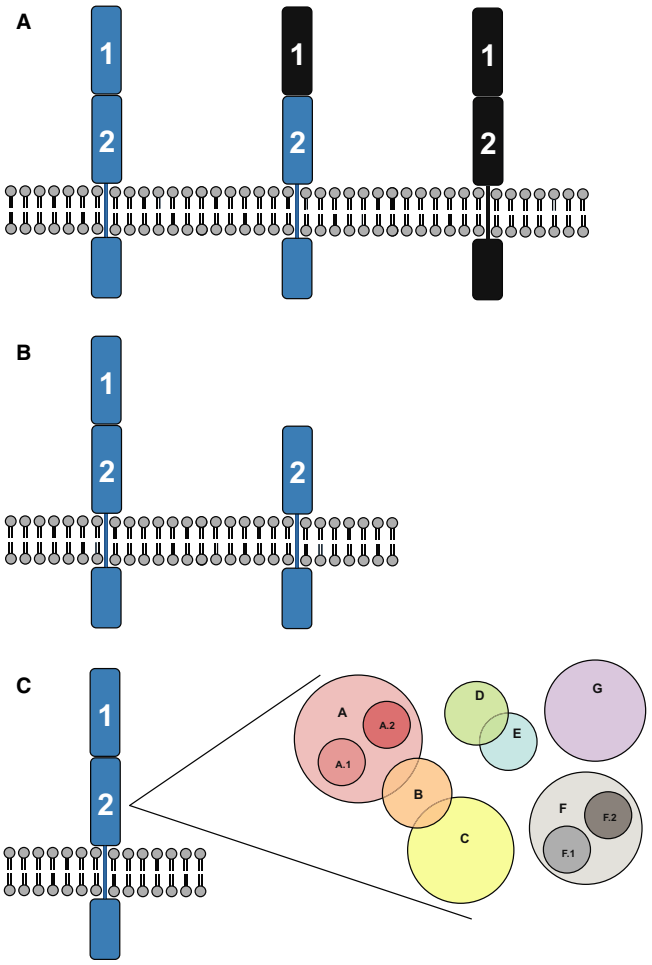

Fig. 2. Epitope mapping of antibodies against such a receptor could start with grossly mapping the antibody epitope to domain 1 or 2 through several possible methods including (a) domain swapping with ortholog or paralog proteins or (b) deletion mutations. (c) Antibodies that fall within a given domain (e.g. domain 2) can be further differentiated by comparing antibodies to each other. The binding data from each antibody can then be used to create a conceptual map of the epitope space covered within that particular domain. The magnified section illustrates the conceptual epitope space. Overlapping circles show antibodies with overlapping epitope space; non-overlapping circles indicate epitopes that are mutually exclusive. This method does not necessarily landmark the epitopes onto the domain unless at least one of the antibodies being compared has been defined.

directly address the true epitope space of a particular antibody versus other antibodies in the panel. For this level of definition, binning against other known monoclonal antibodies (either commercially sourced or identified through previous campaigns) can be performed (*Fig. 2C*). Binning-based methods against other antibodies can be accomplished through BiaCore™ or other label-free approaches (60). For many new therapeutic targets, there may be few existing antibodies available for landmarking distinct epitopes. In these situations, higher throughput label-free methods to compare large panels of unknown antibodies against themselves can be valuable to assess epitope space. Such approaches can be important for antibody targeting modalities such as bispecific T-cell engager (BiTE®; Amgen Inc.) molecules (59). Understanding the inter-relationship of each antibody's binding can guide lead candidate selection based on an epitope bin sampling strategy. Once the engineered recombinant molecules have been made, functional testing can be performed to identify the epitope bin that provides the greatest biological activity; additional antibodies from the functional bin can be sampled to identify the functional lead candidate. These kinds of screening paradigms allow the identification of the best bins of antibodies to advance as the lead panel, all of which cover a maximum amount of relevant epitope space (*Fig. 2*).

Sequence analysis

Sequencing of the cDNAs encoding the variable region gene regions of the human antibody produced by a hybridoma clone is a key step in enabling the molecular cloning of the antibody and facilitating its development into a therapeutic molecule. This sequence information also provides valuable insights into understanding the success of the immunization campaign and the screening campaign. For instance, this sequence information reveals the lineage diversity of the antibody clones discovered. Consider a situation in which a rigorous functional screening regimen has been applied to an antibody repertoire; this can result in a lead panel with highly restricted epitope diversity and potential clonal dominance. Therefore, sequencing is required to reveal the molecular diversity of the lead panels. It is important to obtain this information early because it can show if the process has been successful and yielded a diverse candidate panel ready to advance to recombinant engineering and further development. Alternatively, data such as these can reveal that the process has not succeeded to generate the necessary diversity to ensure a high probability of success in further development. In these cases, the generation of more hybridomas and additional screening campaigns will be required. Further, new immunization strategies can be put in place based on the sequence information, which can lead to broader repertoire diversity.

Variable gene (V-gene) sequencing is essential to understand the quality of a lead candidate panel and therefore should be integrated into the screening campaign as early as possible. However, the hybridoma sequencing process can be rate-limiting and, therefore, is best applied after the antibody panel has been culled, through binding or functional assays, to a panel of interesting putative lead molecules (hundreds of antibodies). This formal linkage of primary sequence to binding and functional activity is made possible by the clonal hybridoma plating process. Incorporating sequencing early in a discovery campaign allows this information to be considered as another upfront screen rather than the last step in the process, such as during traditional hybridoma approaches that use polyclonal hybridoma plating.

Isotype and effector functions

Among therapeutic antibodies currently on the market, all are of the IgG isotype (1). There are four isotypes of IgG (IgG1, IgG2, IgG3, and IgG4) that vary in their hinge sequence and structure, abundance, and ability to elicit effector function through specific interaction with Fc receptors (61, 62). The choice of isotype largely depends on the desired effector function, which can affect the biology of the targeted pathway as well as the safety profile of the drug. For example, targets against surface expressed receptor, particularly in the non-oncology therapeutic areas, may require an IgG2 or IgG4 isotype that has no or limited effector functions to prevent depletion of the cells on which the target is expressed. Alternatively, the IgG1 isotype is used when the effector function is desired because it can impart antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis and complement-dependent cytotoxicity.

Recombinant antibody engineering

An immunization and screening campaign resulting in the identification of a diverse panel of antibodies meeting design goals marks the successful completion of the first stages of antibody development. The output from this stage is the data package and sequence of the variable light and heavy chain regions from the most potent sequence-diverse antibodies from the immunization and screening campaigns.

The variable regions will be cloned onto a human Fc region with the desired properties. Generally this will be an IgG1 isotype for antibodies that require effector function or an IgG2 or IgG1 engineered to lack effector functions for antibodies without this mechanism of action (63). The selection of the Fc region for the antibody panel is the last consideration of this portion of the antibody design goals and sets the scaffold for the next stage of the therapeutic antibody generation process.

Bio-optimization of monoclonal antibodies

The screening campaign as described ideally results in 10 - 20 sequence-diverse antibodies that meet the binding and activity criteria. However, antibodies generated through immunization may still have variable and unpredictable biophysical and biochemical properties (64). Antibody aggregation or particulation can lead to immunogenicity. High viscosity can be challenging for a subcutaneous route of administration and can also pose challenges during the process development stages. Chemical modifications, such as isomerization, deamidation, and oxidation that can be frequently present in the complementarity-determining regions, can affect activity and or lead to lengthy process optimization to control the levels during storage and maintain consistency between lots during production (65). Although computational and experimental screening methods are being developed to identify antibodies with fewer incompatibilities to manufacturing, immunization methods do not always lead to a large number of highly potent sequence-diverse antibodies. Thus, antibody engineering and bio-optimization is a critical step in developing not only biologically active but also manufacturable therapeutic antibodies. An example of this process is shown in *Table* 2, which shows the manufacturing attributes for the parent antibody (Antibody A) and its variants (Antibody A Optimized Variant 1 and Antibody A Optimized Variant 2) optimized for development and two other parent antibodies that are diverse in sequence. All antibodies bind to the same target. The parent non-engineered Antibody A has low production yield, poor purification fit, and poor biophysical stability, whereas its two variants are optimized for development. Antibody B and Antibody C have a different V-gene subtype compared with Antibody A. Of the three parental antibodies, only Antibody C has reasonable development characteristics but is still not optimal because of low production level, which could increase cost.

Attempts have been made to improve pharmaceutical properties, such as solubility and stability of antibodies, through changes in molecule primary sequence. These engineering strategies include mutating residues to the most frequent ones based on the alignment of homologous antibody sequences, engineering β-turns with amino acids that have high propensity to form turn conformations, increasing hydrophilicity of the solvent-exposed residues, adding additional hydrogen bonds or disulfide bonds, library-based screening of a large number of variants, and directed evolution by *in vitro* or *in vivo* methods. Methods that combine many of these approaches have been reported (66, 67). In another engineering method, the complementarity-determining regions from a poorly expressed antibody or single-chain fragment variable domains were grafted onto a preferred framework that has favorable biophysical properties (68–70). Although each of these methods alone or in combination has been met with limited success in increasing stability, none are guaranteed to work in all cases of antibodies against different targets.

Table* 2. Comparison of manufacturing attributes for parent (antibody A) and its variants (antibody optimized variant 1 and antibody optimized variant 2) optimized for development and two other parent antibodies that are diverse in sequence

Development Attributes	Antibody A	Antibody A — Optimized Variant 1	Antibody A Optimized Variant 2	Antibody B	Antibody C
Production level	No	Yes	Yes	Yes	Moderate
Purification fit	No	Yes	Yes	No	Moderate
Photostable	No	No	Yes	No	Yes
Absence of particulation	No	Yes	Yes	No	Yes
Biochemically stable	Yes	Yes	Yes	No	Yes
CDR sequence	Similar	Similar	Similar	Different	Different
V_H/V_L subtype	$V_{H3}/V_{\kappa 1}$	$V_{H3}/V_{\kappa 3}$	$V_{H1}/V_{\kappa 1}$	$V_{H6}/V_{\kappa 1}$	$V_{H4}/V_{\kappa 4}$

CDR = complementarity-determining region; V_H = variable heavy chain; Vκ = variable kappa light chain; V_L = variable light chain.
*All antibodies bind to same target. The parental non-engineered antibody A has low production yield and purification fit and poor biophysical stabilities as opposed to its two variants, which are optimized for development. Antibody B and antibody C have different V-gene subtype compared with antibody A.

Recent advances in *in silico* sequence analysis enable us to implement knowledge gained from the previous assessments of antibodies (71). Large numbers of candidates can be rapidly screened for potential liabilities, commonly referred to as sequence hotspots. The type of computational analyses typically performed and the processes are shown in *Figs 3 and 4*, respectively. *Fig.* 5 depicts examples of hotspots on an IgG antibody structure. Computational analysis is performed on the antibody variable domain sequences to identify hotspots such as non-standard (or free) cysteines, N-linked glycosylation sites, chemical modification sites such as deamidation and isomerization, and covariance violations (72). Each type of hotspot violation can lead to manufacturability issues and/or negatively affect functional activity. For example, free cysteines can lead to protein aggregation. Deamidation and isomerization sites occurring in the complementarity-determining regions can potentially affect activity and create inconsistency (or heterogeneity) among different lots during production.

Within a panel of antibodies exhibiting desirable binding or activity attributes, sequences are evaluated using sequence and structure computational analysis methods. The sequences can first be aligned structurally using the AHo numbering scheme (69). This provides structural meaning to individual residue positions and allows comparison across multiple antibodies. From this alignment, a sequence-based clustering is performed, which illustrates diversity across the antibody panel and allows for the evaluation of closely related antibodies, termed siblings, at the sequence level to aid analysis and potential engineering (73). An antibody representing each cluster is then selected, and a structure model of the variable or antigen-binding fragments is built for each using the antibody modeler method within the Molecular Operating Environment (i.e. MOE; Chemical Computing Group Inc., Montreal, QC, Canada). This structural model can then be used for further analyses to optimize the molecules.

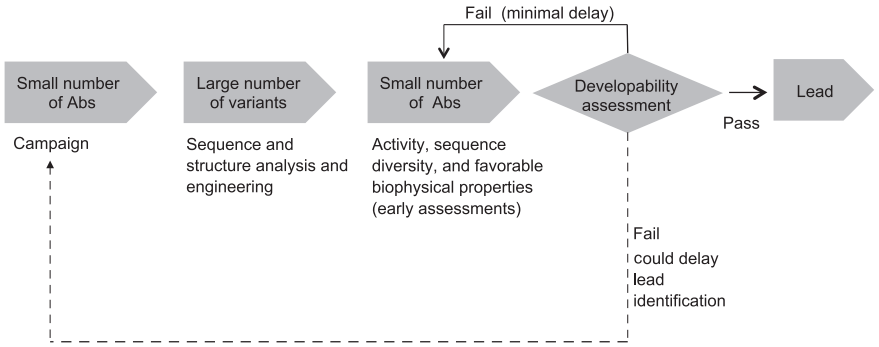

***Fig.* 3. Schematic of the typical engineering and developability assessment process.** Abs = antibodies.

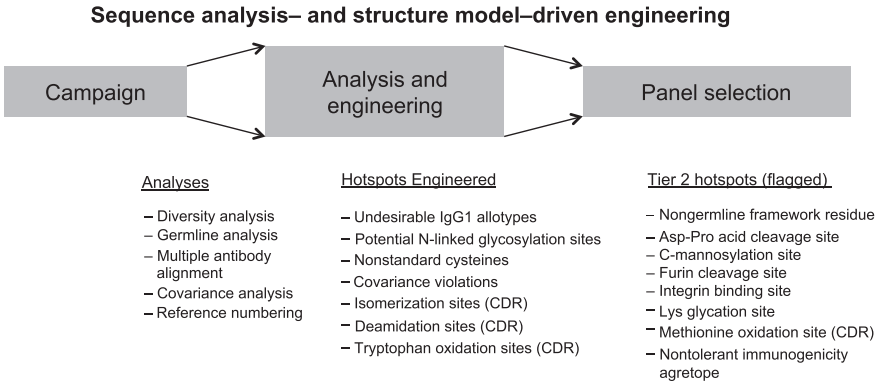

***Fig.* 4. Engineering and optimization work flow.** Engineering begins with a diverse set of computational analyses, such as cladding and germline analysis. This step is followed by prediction of potential problems such as physical and chemical hotspots, free cysteines, and non-consensus N-glycosylation sites. These sites are always engineered. If a hotspot cannot be fixed through engineering and if modification or effect is experimentally confirmed, then additional process development time or change in target product profile may be warranted. The tier 2 hotspots are not engineered but are flagged for further investigation in the downstream activities. CDR = complementarity-determining region. CDR3 = complementarity-determining region 3.

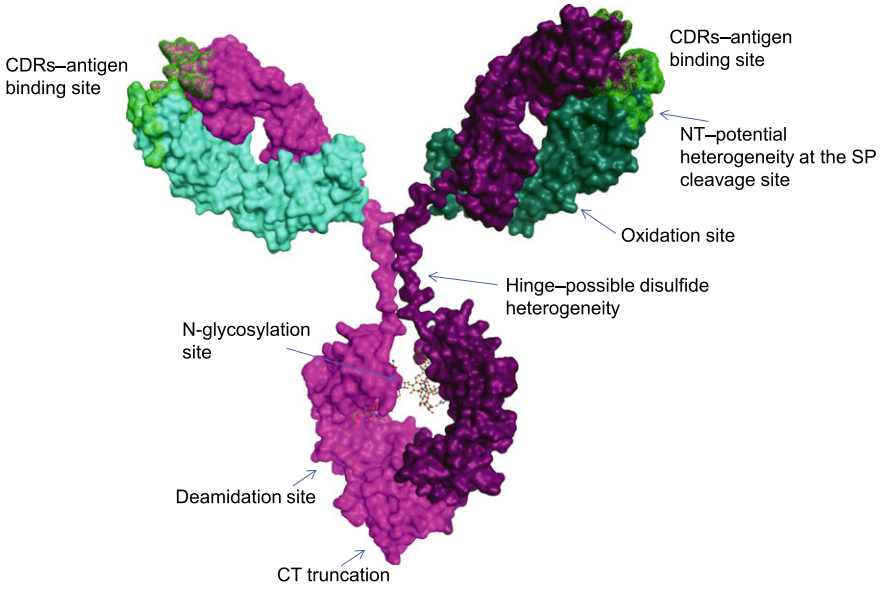

Fig. 5. Modeled structure of an antibody. Fab and Fc crystal structures were used to model the full antibody structure. The carbohydrate structure at the N-glycosylation site is shown in a ball-and-stick model; the molecule in surface representation. Some of the typical hotspot regions and potential heterogeneities are highlighted. CDR = complementarity-determining region; CT = C-terminus; Fab = fragment antigen binding; Fc = fragment crystallizable; NT = N-terminus; SP = signal peptide.

Another method of improving the ability to manufacture or develop antibodies through a computational approach is covariance analysis (72). Covariance analysis entails identification of pairwise conserved residue positions based on the physiochemical properties of the residues evaluating how the antibody sequence of interest deviates from pairwise conservation and substituting the deviating position(s) with amino acids found at the equivalent positions in germline or related germline sequences. Pairwise conserved residues can be identified by (i) assigning a germline subtype to the antibody variable domain of interest; (ii) aligning framework regions of multiple variable domains belonging to the same germline subtype identified in (i); (iii) classifying the amino acid at each position within an aligned variable domain as small hydrophobic, aromatic, neutral polar, positively charged, negatively charged, or glycine/deletion; (iv) calculating a conservation score for each pairwise position; and (v) determining covarying or correlated mutational pairs or pairwise conserved residue positions based on a threshold calculation. A preferred method of determining a conservation score includes calculating number of pairs belonging to the same physiochemical characteristics and subtracting that from the sum of pairs belonging to different physiochemical characteristics. Deviations within the antibody variable domain of interest can be determined by comparing amino acid pairs from the target sequence of interest with the correlated (or covarying pairs) identified from the multiple sequence alignment. In other words, deviations (or covariance violations) in the target sequence are those that differ from the observed pattern of pairwise conserved positions that are identified using the database of variable domain sequences.

The covariance analysis method often identifies positions that could cause conformational stability issues and suggests they be replaced with germline or related germline residues. This method can also identify issues with germline residues and suggest a related germline residue as a better replacement (e.g. a V-gene subtype that shares a slightly lower percentage of sequence identify with the antibody sequence of interest). This computational method has been applied to more than 50 antibodies against various antigens. The suggested single- and multiple-point mutations have led to consistent improvement in one or more physical and chemical properties and expression. Characteristics that may be improved through covariance analysis include expression within transiently or stably transfected host cells, thermostability, resistance to aggregation, *in vivo* half-life, storage shelf-life, folding efficiency, resistance to light-induced oxidation, reduced clippings during storage conditions, reduced sensitivity to pH changes, and reduced chemical and physical degradation.

More complex engineering involves switching or replacing a rare framework to a more prevalent subtype, such as variable gene subtype switching from Vκ6 to Vκ1. This is similar to humanization, in which the complementarity-determining region sequences are grafted onto a human

framework. Such level of engineering would require multiple rounds and/or a large number of variants to regain the activity of the parent antibody.

Variant generation and testing

Computational analysis is performed on the antibody variable domain sequences to identify hotspots such as non-standard (or free) cysteines, N-linked glycosylation sites, chemical modification sites such as deamidation and isomerization, and covariance violations. The parental sequences are then modified into multiple sequence variants using the desired IgG isotype in an attempt to remove the hotspot sites without affecting the binding or activity of the antibody. Both sequence and structure play a role in the engineering of these hotspots. Solvent exposure, atomic interactions, and sibling residue information are taken into account in the variant design. The engineered hotspot remediated variants that retain activity are further subjected to biophysical and stability analyses to select lead candidates for scale-up and extensive developability assessments (*Fig.* 3). This stage of the process ends with the selection of 3–5 sequence-unique antibody variants meeting the therapeutic design goals to advance for process development to support large scale expression and purification.

Conclusions

In conclusion, transgenic animal technologies, such as XenoMouse®, that enable human antibody generation can be enormously powerful for creating high-quality repertoires of human antibodies. When this technology is combined with well-designed immunization and screening strategies, these efforts can generate large and diverse lead panels meeting stringent therapeutic antibody design goals. To proactively mitigate the many challenges posed by the development process, the molecule discovery process can be complemented with a bio-optimization step. The addition of this engineering step can increase the probability of successful development and commercialization of a molecule as well as potentially delivering a superior patient experience in the end.

Future drug discovery efforts will be increasingly shaped by human genetic validation data. As new therapeutic targets emerge from these genomics efforts, the technical challenges to antibody drug discovery will increase. The challenges encountered may include complex target classes, design goals that include high selectivity and potency requirements, and new drug delivery devices. All of these will require a continuous evolution of the methods used to generate immune repertoires in transgenic animals, screening strategies used for lead selection, and enhancement to the biophysical properties of our molecules.

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