Transpo-mAb display: Transposition-mediated B cell display and functional screening of full-length IgG antibody libraries

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ABSTRACT
In vitro antibody display and screening technologies geared toward the discovery and engineering of clinically applicable antibodies have evolved from screening artificial antibody formats, powered by microbial display technologies, to screening of natural, full-IgG molecules expressed in mammalian cells to readily yield lead antibodies with favorable properties in production and clinical applications. Here, we report the development and characterization of a novel, next-generation mammalian cell-based antibody display and screening platform called Transpo-mAb Display, offering straightforward and efficient generation of cellular libraries by using non-viral transposition technology to obtain stable antibody expression. Because Transpo-mAb Display uses DNA-transposable vectors with substantial cargo capacity, genomic antibody heavy chain expression constructs can be utilized that undergo the natural switch from membrane bound to secreted antibody expression in B cells by way of alternative splicing of Ig-heavy chain transcripts from the same genomic expression cassette. We demonstrate that stably transposed cells co-express transmembrane and secreted antibodies at levels comparable to those provided by dedicated constructs for secreted and membrane-associated IgGs. This unique feature expedites the screening and antibody characterization process by obviating the need for intermediate sequencing and re-cloning of individual antibody clones into separate expression vectors for functional screening purposes. In a series of proof-of-concept experiments, we demonstrate the seamless integration of antibody discovery with functional screening for various antibody properties, including binding affinity and suitability for preparation of antibody-drug conjugates.

Introduction
Antibody-based therapeutics represent the fastest growing segment in the development of new drugs over the last decade, and currently generate over USD 80 billion global revenues, accounting for almost 10% of the total global market of pharmaceutical prescription drugs. Therefore, efficient and innovative technologies that allow the discovery of pharmaceutically active, well-tolerated monoclonal antibodies (mAbs) are in high demand. Whereas hybridoma technology has made mAbs highly accessible, the labor intensity of the method, together with a long-standing inability to adapt it to the generation of human mAbs, has inspired the development of a large number of additional, antibody library-based approaches. These include, for instance, phage display, retroviral display, bacterial display, yeast display and various mammalian cell display technologies, in combination with solid surface binding (panning) or other enrichment techniques.

While phage, prokaryotic and ribosome/mRNA display systems have been established and are widely adopted in the biotechnology industry and in academia for the identification of antibody fragments, they suffer from a variety of limitations, including the inability to express full-length antibodies, the absence of natural post-translational modifications, the lack of proper folding by vertebrate chaperones, and an artificially enforced heavy and light chain combination. Therefore, the “reformatting” of antibody fragments into full-length antibodies followed by manufacturing in mammalian cells frequently results in molecules with unfavorable biophysical properties (e.g., low stability, tendency to aggregate, diminished affinity). This not only leads to significant attrition rates in the development of lead antibody molecules generated by these methods, but also requires significant effort to correct the biophysical and molecular liabilities in these proteins for further downstream drug development.

As a consequence, substantial efforts have been made to develop antibody discovery technologies based on the expression of antibodies in eukaryotic cells. In particular, screening of antibody libraries displayed on the surface of yeast cells addresses some of the above mentioned biases prokaryotic systems impose, and has proven to be a very powerful method. In order to perform library screening under the most physiological conditions possible, numerous mammalian cell-based antibody expression platforms have been developed, involving simple transient transfection of antibody libraries or their viral transduction using vaccinia, sindbis, or retrovirus vectors. While these approaches are believed to deliver mAbs with favorable biophysical properties that
have a higher potential in drug development and therapeutic use, mammalian cell-based screening platforms are currently also associated with a number of limitations, which has led to a slow adoption of these technologies in academia and industry.

Most notably, efficient and precise identification of the genetic information underlying an antibody with desired binding or functional properties requires a tight coupling of antibody phenotype with its genotype provided by highly stable genetic modifications. Yet, mammalian cells are not as efficiently and stably genetically modified as prokaryotic or lower eukaryotic cells. This, together with the significantly higher culture volume requirements of mammalian versus prokaryotic expression systems, makes the generation and screening of complex antibody libraries a highly challenging process. Thus, there clearly is a need for a more efficient, more controllable and straightforward technology that allows the generation of high-quality and complex mammalian cell-based full-length antibody libraries.

Here, we describe Transpo-mAb Display (summarized in Fig. 1), a novel and highly efficient non-viral antibody discovery and engineering platform based on DNA transposition and full-length antibody display in cells of the B lineage. Due to a built-in switch between surface and secreted expression of IgGs characteristic for B lineage cells, Transpo-mAb Display allows for seamless integration of screening for a binding phenotype and functional screening without the need for re-cloning or reformating of selected antibodies.

Results

Transposition-mediated antibody surface display and secretion in B-lineage cells

To stably deliver antibody expression constructs into mammalian cells, we initially evaluated the class II transposon systems Tol2, SleepingBeauty and PiggyBac. While each of these systems has been reported to be capable of gene delivery into mammalian cells,17-19 we found the PiggyBac system involving a hyperactive version of the PiggyBac transposase20 to be most suitable for our purpose (data not shown). Hence, we designed plasmid vectors containing human antibody heavy chain (HC) or light chain (LC) expression cassettes that were flanked by PiggyBac recognition sites (inverted terminal repeats, ITRs), and, thus, after delivery into host cells along with PiggyBac transposase transient expression constructs, can be “cut” from vectors and “pasted” as transposable elements (TEs) into the host cell genome by transposition (Fig. 2A). We chose to generate independent transposable constructs for expression of antibody HC and LC, thus allowing more flexibility in shuffling HC and LC libraries and straightforward cloning. Antibody gene expression from TEs is driven by the strong EF1α promoter, which is constitutively active in a broad host-cell range and is not prone to silencing.21 To allow for selection of HC and LC gene expression, selectable markers are transcriptionally coupled to transgene expression via internal ribosomal entry sites (IRES). Constructs were designed in a modular fashion with individual elements flanked by unique restriction sites, allowing routine exchange of, for example, antibody variable regions to generate libraries. In addition to HC expression constructs designed to produce secreted (sec) and membrane-bound (mb) antibodies, we took advantage of the large cargo capacity of the PiggyBac system and generated a third HC expression construct bearing a genomic (gen) version of the human HC-gamma 1 constant region (~5kb, total TE ~10kb). This vector therefore should allow alternative mRNA splicing, known to occur in the natural switch from membrane-bound to secreted Ig expression during B cell differentiation,22,23 and result in expression of both membrane-bound and secreted antibody when co-transposed with LC constructs (Fig. 2B). As a host cell line for transposition, we chose a subclone (“L11”) of the Abelson murine leukemia virus (A-MuLV) transformed pre-B cell clone 63–12 that was originally derived from RAG-2 deficient mice.24 Due to the RAG-2 gene knockout, 63–12 cells

![Figure 1. Generation and screening of whole-IgG antibody libraries stably expressed in mammalian pre-B cells by transposition. L11 murine pre-B cells are electroporated with a tripartite vector system encompassing two PiggyBac-transposable plasmids (pPB) encoding for antibody heavy chains (HC) and light chains (LC), and a PiggyBac transposase expression vector. Transient expression of PiggyBac transposase results in "cut-and-paste" transposition of antibody-coding sequences including resistance markers from their cognate plasmids into the host-cell genome. Cells stably transposed with HC and LC expression cassettes are positively selected by antibiotic selection. Single cell clones displaying antibodies specific to a desired antigen can then be isolated by flow cytometry employing tagged antigen as bait, taking into account signal strength of antigen binding and antibody expression levels. Supernatants of sorted clones containing secreted antibody are directly used for screening of best candidates in binding and functional assays. Finally, antibody variable regions of favorite clones are retrieved by PCR and cloned into production vectors for large-scale expression and validation.](image-url)
and their subclone L11 used here are unable to initiate V(D)J recombination, and therefore cannot express endogenous antibody, thus making them ideal host cells for exogenous antibody expression.

To assess the speed and efficiency of transposition and subsequent antibody expression employing the above-mentioned components, we generated transposable HC and LC constructs bearing EGFP as a marker to detect the presence of TE within cells. As a model antibody, we used HC and LC variable regions of the anti-CD30 antibody cAc1025 inserted in-frame upstream of Ig gamma 1 and Ig kappa constant regions, respectively. L11 cells were electroporated with a mix of transposable HC and LC constructs, either including transposase expression vector (TP) or empty vector as a control for assessment of transient expression from transposable constructs. EGFP expression as well as antibody surface expression, detected using an APC-labeled kappa-LC-specific antibody, was then monitored over time by flow cytometry (Fig. 3A). We noted faint EGFP and surface antibody expression in a subset of cells one day after electroporation, which was independent of the presence of TP and completely disappeared over time, consistent with transient expression from TE that did not integrate into the host cell genome. Much stronger EGFP and surface antibody expression in a subset of cells was observed only in the presence of TP, suggesting that, upon transposase-mediated stable integration of the transposable HC and LC constructs into the genome, expression from TE is much more efficient compared to transient expression.

In addition to cells that displayed concomitant EGFP and antibody surface expression, and therefore had integrated both HC and LC TEs, we also observed EGFP-positive cells that did not express surface antibody. These cells most likely represent cells that had stably integrated only HC or LC TEs and were therefore unable to express antibody on their surface. Remarkably, transposition efficiencies obtained using either membrane-bound or genomic versions of HC constructs were virtually identical, despite the much larger size of the TE containing the genomic version of HC constant region. With both constructs, we routinely obtained around 6% of stably antibody-expressing cells when using a DNA weight ratio of 0.25:0.125:1 (HC:LC:TP).

Importantly, we found that transposition efficiencies can be modulated by varying HC:LC:TP DNA ratios used for electroporation (Fig. S1). Increasing the amount of HC and LC transposable constructs relative to TP construct leads to a higher percentage of transposed cells (Fig. S1A), with each cell showing integration of multiple HC and LC copies (Fig. S1B). In contrast, lowering the amount of HC and LC transposable constructs leads to a lower percentage of transposed cells (Fig. S1A), with a significant fraction of cells showing integration of a single HC and LC construct (Fig. S1B). Therefore, depending on the desired antibody screening strategy, less complex libraries with a majority of cells expressing mAbs can be prepared, or more complex cellular antibody libraries can be generated comprising cells that display multiple antibody clones. Together, these data show that one-step electroporation of transposable HC and LC construct along with a transposase expression construct leads to rapid genomic integration of transposable constructs, followed by robust antibody surface expression with comparable efficiencies using either membrane-bound or genomic versions of HC constructs.

As an alternative to one-step electroporation of both HC and LC constructs together with a TP construct, we found that HC and LC constructs can also be transposed sequentially, thereby adding yet another level of flexibility. With this
approach, only two plasmids, namely one transposable construct coding for HC or LC, along with the transposase expression plasmid, have to be delivered per cell at once. Transposition of a single HC or LC construct typically leads to stable integration in around 20% of cells (Fig. S2A). Since PiggyBac transposition is considered to be reversible, albeit at low frequencies,\textsuperscript{26,27} we evaluated whether this phenomenon could interfere with sequential transposition. We neither observed a significant loss of previously transposed chains upon transposition of a second chain (Fig. S2A), nor did re-expression of PiggyBac transposase in cells transposed with both HC and LC lead to significant loss of antibody expression (unselected) or with antibiotics starting 24 hours after electroporation for 5 d (selected). Both samples were then stained (6 d after electroporation) as follows and analyzed by flow cytometry: a PE-labeled polyclonal Fc-specific anti-human-IgG antibody was used to determine antibody surface expression, and binding of surface-expressed monoclonal antibody to its cognate antigen was assessed by adding soluble, strep-tagged antigen which was detected with fluorophore-labeled anti-strep-tag antibody (StrepMAB-classic Oyster 645). Selected cells were further cultured without antibiotics for 2 and 4 weeks and analyzed in the same manner as described above. (C) Antibody secretion of cells transposed with different HC construct variants. Cells transposed with the indicated HC versions (see Fig. 2) and selected as described in (B) were used to condition supernatants for 5 d (n = 2) in 6-well plates. IgG titer in supernatants (left axis) were determined by ELISA. For comparison, relative median fluorescence intensities (MFI) of surface-antibody expression of the same cell lines determined by flow cytometry are shown (right axis). (n.d., not determined).
IgG, and for antigen-binding using soluble, strep-tagged ROR1 that was detected using a fluorophore-labeled strep-tag-specific antibody. Flow cytometry analysis revealed that under non-selective conditions, a low percentage of surface-antibody expressing cells was present as expected, while virtually all cells double-selected with antibiotics for the presence of HC and LC TEs expressed surface-antibody (Fig. 3B). Moreover, labeling of cells for antigen binding was successful and signal strength of antigen-binding correlated with expression levels of the cognate antibody, as expected. Of note, under non-selective conditions we observed that some cells expressing surface antibody did not show antigen-binding, which most likely represented cells that expressed only 2A2 HC, being displayed on the cell surface due to pairing with endogenously expressed murine surrogate light chains that are characteristically expressed in early progenitor and precursor B cells. Consistently, after positive selection for HC and LC constructs, thereby eliminating any cells transposed with 2A2 HC or LC alone, all cells expressing surface antibody displayed antigen-binding. Collectively, these data show that single-step antibiotic selection for expression of antibiotic-resistance genes, and thus expression of HC and LC genes, effectively enriches for cells displaying functional antibody on their surface. Moreover, these results establish that surface-displayed antibodies can effectively be evaluated for antigen binding by flow cytometry applying a dual surface-IgG/antigen-binding staining approach that allows determination of antigen-binding independent of differences in surface expression levels between single cells.

To evaluate if transposed constructs stably confer expression or might be lost or silenced with time, we cultured selected cells in the absence of any antibiotic selection pressure for another 4 weeks and then re-analyzed them by flow cytometry after 2 and 4 weeks (Fig. 3B). Antibody expression and antigen binding in cells transposed with both HC constructs remained stable over 4 weeks, albeit a small population of cells expressing only low levels of antibody was detectable in cells transposed with the membrane-bound HC construct. Thus, once transposed, antibody expression cassettes remain stably integrated and functional over at least a month, and therefore phenotype-genotype coupling is preserved and amenable for repeated screening experiments, if desired.

Having established that antibody expression involving transposed genomic versions of HC TEs is readily detectable and functional on the surface of cells, we next analyzed the extent to which cells transposed with this TE produced secreted antibody. We therefore generated cells homogenously expressing 2A2 antibody by transposition and subsequent selection with antibiotics, using either membrane-bound, genomic or secreted versions of the HC (see Fig. 2A). After selection was complete, we cultured the cells for another 5 d without exchange of growth medium to generate conditioned cell culture supernatants, which we then analyzed for IgG titers by ELISA. While we could not detect secreted IgG in supernatants of cells transposed with a membrane-bound HC cDNA expression construct, we found that cells transposed with a secreted HC cDNA constructs produced ~1 μg/ml soluble IgG (Fig. 3C). Strikingly, cells transposed with the genomic HC construct secreted more than twice as much IgG, while expressing surface antibody at a level comparable to cells transposed with membrane-bound constructs, as determined by flow cytometry. We speculate that this enhanced expression is the result of the splicing of the HC construct in genomic configuration, which is known to positively affect mRNA stability or translation. Together, these data confirm that L11 cells that were transposed with the TE containing the genomic HC version simultaneously express membrane-bound and secreted antibody, up to an extent that closely matches the performance of cDNA expression constructs specifically designed for expression of either secreted or membrane-bound antibody.

**Generation of cellular libraries by transposition and direct screening of clone supernatants by ELISA**

After having characterized and validated the components used for transposition, surface display and secretion of IgGs, we next determined whether the technology thus far developed is suitable for screening of antibody libraries. As a proof-of-concept, we chose to humanize the murine mAb MN, specific for the cell surface glycoprotein mesothelin, by screening mini-libraries consisting of humanized HCs and LCs. Humanized variable regions were designed by grafting of the MN HC and LC complementarity-determining regions (CDRs) into a series of 47 different frameworks for each immunoglobulin chain (Fig. S3A). Three different classes of framework sequences most closely resembling the parental sequences were chosen: (1) human germline sequences identified by alignment of the parental mAb sequence with human germline sequences found in the IMGT public database, the most similar sequences that were found by next-generation sequencing (NGS) of a somatic human antibody repertoire, and (3) the parental mouse framework sequences, in which single residues were humanized according to a bioinformatic algorithm comparing the original framework sequences with human framework sequences derived from the above-mentioned human germline and NGS databases. After total gene synthesis, the variable regions were pooled and amplified by PCR. Transposable libraries were prepared by introducing the amplified sequences into antibiotic-selectable, transposable constructs in-frame with HC (genomic version) and LC constant regions, respectively. Library diversities were 7.4 × 10^2 (HCgen) and 2 × 10^6 (LC) (data not shown), and thus overrepresented the small theoretical diversities by more than 10,000-fold.

To generate a cellular library, one-step electroporation of HC and LC libraries along with transposase expression plasmid into L11 cells was performed, as described above, followed by selection in hygromycin B and puromycin one day after electroporation. We determined transposition efficiency by culturing a small part of the library without antibiotics for 3 d and analyzed antibody surface expression by flow cytometry (Fig. 4A). Consistent with previous results obtained after electroporating cells with the same DNA weight ratios (Fig. 3A and 3SA), ~7.5% of the cells expressed surface antibody when analyzed by flow cytometry. Thus, considering the small theoretical size of the mini-library (47 × 47 ≈ 2,209 variants) and the number of cells electroporated (3.2 × 10^5), we estimated that an ~1000-fold overrepresentation of the cDNA library was achieved. Antibiotic selection of the cellular library was complete after 4 d and found to be highly efficient, as judged by surface antibody staining analyzed by flow cytometry (Fig. 4B).
After subculturing in non-selective media for another day, thus allowing the cells to recover from antibiotic selection, we proceeded to staining of the library for antigen binding (Fig. 4C). Flow cytometry analysis of the library demonstrated that a large portion of the cellular library was able to bind soluble antigen as expected, although the majority of cells appeared to display weaker binding compared to cells expressing the parental antibody. Based on these observations, we directly proceeded to stringent FACS-sorting of single cells into 96-well plates. After 2 weeks, single cells had expanded and supernatants of 96 clones were harvested and analyzed by ELISA (Fig. 4D). The secreted IgG titers across clones ranged from 0.1 to 1 $\mu$g/ml, with an estimated average of around 0.3 $\mu$g/ml. The majority of cell-clone derived supernatants showed antigen binding similar to that of the parental mAb when binding was normalized to IgG secretion levels, while only a few showed little or no antigen binding and/or were devoid of IgG expression, demonstrating that single-cell sorting based on antigen-binding and surface-expression had occurred at high efficiency.

Sequence recovery, validation and affinities of top mesothelin binders

Figure 4. Generation and screening of MN humanization library by Transpo-mAb Display (A) Surface antibody expression of cellular huMN library after transposition. A transposable library encompassing 47 HC (genomic variant) $\times$ 47 LC was electroporated into $3.2 \times 10^7$ cells along with the transposase expression construct using DNA ratios as described in Fig. 3A. To determine transposition efficiency, 1/64 of the total cellular library was cultured without antibiotics for 3 d until transposition was complete, and surface expression was detected by staining with APC-coupled anti-human-kappa LC. Percentages of surface-expression positive cells are indicated. (B) Evaluation of selection efficiency. Surface antibody expression after 4 d of selection was determined as described above. Unselected cells were also stained as a control. (C) Antigen/surface-antibody double staining of selected cellular humanization library for FACS single-cell sort. The selected library (L11-huMN-library) was stained for antigen binding using strep-tagged antigen used at limiting concentration. Bound antigen was detected by fluorophore-conjugated anti-strep-tag antibody. Antibody surface expression was detected using a PE-labeled polyclonal Fc-specific anti-human-IgG antibody, allowing for the normalization of binding based on antibody expression. A control staining without antigen (-antigen) was included to discriminate binding from background. Untransposed cells (L11) and cells transposed with parental antibody MN (L11-MN (parental)) were stained as negative and positive controls, respectively. Single cells were sorted into 96-well plates according to the representative sorting gate shown in red. (D) Scatter-plot of single-cell clone supernatants analyzed in parallel for antigen-binding and IgG titer by ELISA. Serial dilutions of clonal supernatants grown in 96-well plates were directly used for assessing binding to ELISA plates coated with limiting concentrations of mesothelin to minimize avidity effects. IgG levels were determined by sandwich ELISA. EC50 values were calculated using standard curves obtained with known concentrations of parental mAb MN (green). Clone numbers chosen for sequence recovery are indicated (orange).
crossovers that presumably occurred between highly similar sequences during amplification of pooled library fragments, in line with our observation that crossed-over sequences were present in the cDNA library (data not shown). Clones containing these types of sequences were not pursued further.

To verify antigen-binding of individual HC/LC pairs recovered from each cell clone, we transiently transfected 293T cells with the respective combinations of HC/LC constructs that were generated during sequence recovery, along with the parental chimeric antibody as a control. Supernatants were then analyzed for antigen-binding and IgG titer by ELISA. This analysis demonstrated that all of the recovered sequences were indeed coding for functional mesothelin-binders (Fig. 5B). To determine individual affinities of the entire set of ELISA-validated humanized mAbs, we analyzed the same supernatants by surface plasmon resonance (SPR) (Fig. 5C and 5D, see Fig. S4 for response curves). The best humanized anti-mesothelin mAb clone showed an affinity (KD = 114 pM) that was only about 2-fold lower than the affinity of the parental murine mAb MN (KD = 51 pM). The remaining clones showed affinities between 176 and 1030 pM. Analysis of the degree of humanization among these clones was also performed. For this, we determined the similarity of each chain’s framework regions to those of the human germline sequence that was most closely related to the entire variable region sequence of the humanized mAbs (Fig. S5). Significantly, the clone with the lowest affinity in this set contained both HC and LC frameworks that were 99% identical to frameworks of the closest human germline sequence, while higher affinity clones deviated more strongly from the most closely related germline sequence. Overall, the average degree of “humanness” of the library and isolated mAbs compared well to humanized antibodies that have been clinically approved (Fig. S5), thus validating the humanization strategy. Collectively, the results demonstrate that cellular antibody libraries can be generated by transposition, and high-affinity antibodies can be isolated from stably transposed cells in a straightforward fashion by screening of cell clone supernatants directly after single-cell sorting by FACS for antigen binders.

**Transpo-mAb screening of humanized anti-ROR1 antibody libraries**

In a similar series of screens, we humanized a mouse (2A2) and a rabbit (R11) mAb specific for the cancer cell-associated surface marker ROR1. Here, we used sets of humanized variable regions designed by grafting of CDRs into framework regions derived from a database of somatically hypermutated human sequences, and generated transposable libraries as described before. The humanized libraries consisted of 64 VH
After having shown that antibody-containing supernatants from sorted L11 cell clones can directly be used for affinity measurements, we next investigated whether other functional properties, such as suitability of mAbs as antibody-drug conjugates (ADCs), can directly be evaluated as well. The seamless integration of antibody discovery and evaluation of ADC-dependent in vitro cell killing activity would greatly facilitate ADC discovery, which typically requires the screening of large numbers of clones until a suitable mAb is identified. Thus, as a proof-of-principle, we chose to test the antibody-containing supernatants generated during our MN humanization screen (Fig. 3) directly in secondary ADC cell killing assays on EMT6-Meso cells, a subclone of mouse EMT6 breast cancer cells overexpressing human mesothelin (Fig. 7A). For this, EMT6-Meso cells were plated in 96-well format and exposed to serial dilutions of supernatants. After a brief incubation, a commercially available secondary ADC reagent was added consisting of a polyclonal anti-human Fc antibody conjugated to monomethyl auristatin E (MMAE) via a cleavable linker. While incubation with secondary ADC alone did not lead to cell death even when used at the highest concentration, combined incubation with antibody-containing supernatants resulted in significantly reduced cell viability, indicating antigen-specific cell killing via mAb-binding and internalization of mAb-ADC complexes (Fig. 7B). Importantly, we are currently developing our own monoclonal, monovalent secondary ADC reagents for use in future screenings to eliminate a possible bias in internalization due to crosslinking of surface-bound antibodies by bivalent, polyclonal secondary ADC reagents. Taken together, these results suggest that Transpo-mAb Display is not only a powerful antibody discovery and engineering platform, but also allows for efficient integration of functional screening without the need for antibody re-formatting or re-cloning.

**Figure 6.** Humanization of mouse 2A2 and rabbit R11 anti-ROR1 antibodies by Transpo-mAb Display. Transposable humanized libraries consisted of 64 VH × 49 VL (mouse) and 101 VH × 82 VL (rabbit) sequences and were transposed using a DNA weight ratio of 4:2:1 (HC:LC:transposase). Selection of cellular library and FACS-sorting of single-cell clones was essentially done as described for humanization of MN antibody (Fig. 3). After sorting, clone supernatants were screened by SPR. Antibody sequences of 5 clones showing best KDs were recovered by RT-PCR and deconvoluted/validated by ELISA as described in Fig. 5D. Sequences not matching library design were not pursued further. The top 3 clones from this analysis were expressed in 293T cells, purified and affinities were measured by SPR. (A) Isoaffinity plot showing $k_a$ and $k_d$ determined by SPR with single-cell sorted clone supernatants (unicates) or purified parental mAbs (triplicates). (B) Isoaffinity plot of purified top 3 mAbs of each screen. (C) Table showing affinities showing affinities ($K_D = k_d / k_a$).
Discussion

An increasing number of in vitro antibody screening technologies are being developed, reflecting the need for more rapid and efficient discovery of therapeutically useful antibodies. A common denominator of these technologies is the expression and screening of antibody libraries in a high-throughput manner, with the ultimate goal to identify mAbs with desired specificities, and ideally exhibiting favorable properties for manufacturing and use as therapeutics in patients. Each of these systems has their inherent advantages and limitations with respect to: (1) the number of experimental steps involved, (2) the library diversities that can be generated and screened within a given time due to constraints of space and resources, and (3) the capability to express antibodies in formats and qualities that are relevant for the final product.

Here, we describe Transpo-mAb Display, a novel antibody expression and screening technology based on transposition of antibody libraries into mammalian cells, enabling stable surface display and straightforward identification of antibodies with desired binding and functional properties.

The platform described here allows expression and screening of antibodies in clinically relevant full-length IgG format. This is enabled by the choice of a mammalian host cell type that is naturally equipped with chaperones and other cellular factors required for efficient and proper full-length IgG folding and post-translational modification. Hence, expression and screening of full-length IgG libraries in this cell type serves as a superb quality control filter, ensuring negative selection of poorly pairing HC–LC combinations and difficult-to-fold antibodies. In addition, the pre-B cells used here express the signaling components CD79a and CD79b of the B cell receptor complex comprising the transmembrane-anchoring domains for IgGs, thus supporting rigid and high-level IgG surface expression. Indeed, while surface IgG expression can occur in mammalian cells in the absence of CD79a and CD79b, we have observed higher expression levels when CD79a and CD79b are expressed in host cells (data not shown). Generally, expression in mammalian cells allows screening of antibodies carrying post-translational modifications highly similar to those of commercially manufactured products administered to patients.

These advantages unique to mammalian cell display are one way in which Transpo-mAb counterbalances the relatively low diversities that can be screened relative to microbial display techniques. Moreover, the pre-B host cell line used here shows rapid proliferation (doubling time of 10–12 hours), and can be grown as a high-density culture (up to \(2.5 \times 10^6\) cells/ml under static conditions). On one hand, this considerably speeds up experimental workflows and minimizes cell culture volumes in comparison to other previously used mammalian host cell lines such as HEK293T or BHK. On the other hand, it is conceivable that with optimized sequential transposition of HC and LC vectors, which result in around 20% stably transposed cells (Fig. S2A), expansion to 10 L cultures at \(2.5 \times 10^6\) cells/ml would allow the generation and screening of antibody libraries with an approximate complexity of \(1 \times 10^9\).

Transpo-mAb Display is the first antibody discovery platform that utilizes DNA transposition for the generation of cellular antibody libraries, thereby drastically simplifying the library generation process in comparison to virus-based approaches. While generation of cellular libraries by transposition involves a simple transfection, generation of cellular libraries by viral infection usually requires a multitude of steps including transfection of packaging cells, generation of viral particle intermediates, and finally infection of library host cells with viral particles.

Transposition has been used as a research tool for gene delivery and mutagenesis in a variety of organisms, including mammalian cells. The PiggyBac transposon has been reported to be capable of moving DNA elements as large as 100kb in mammalian genomes, and large elements up to 14kb are transposed without apparent loss of efficiency, whereas SleepingBeauty transposition efficiency drastically decreases with size. These observations are consistent with
our observation that similar transposition efficiencies could be achieved with PiggyBac TEs containing membrane-bound or much larger genomic HC constructs with total TE sizes of ~5kb and ~10kb, respectively (Fig. 3A), whereas transposition efficiency with SleepingBeauty TEs was clearly size-dependent and highly ineffective with genomic HC constructs (data not shown). The large PiggyBac cargo tolerance hence is superior over other commonly employed systems capable of permanent gene transfer, such as retro- and lentiviral systems, which preclude the use of large sequences such as the genomic HC version used here, due to their limited cargo size tolerance of around 8 kb. Moreover, intron-containing cargo such as the genomic HC expression construct used herein cannot be delivered using the above-mentioned viral systems due to the likely loss of introns during splicing in virus-producing cell lines.

One challenging aspect in the generation and screening of mammalian cell-based antibody libraries is to precisely control the number of transgenes delivered per cell, to ensure screening of cell clones that express defined antibodies. In line with previously published data, we show here that the number of copies transposed per cell can be controlled by adapting plasmid DNA ratios used during library electroporation. We observed decreasing HC and LC copy numbers when decreasing amounts of transposable HC and LC vectors were electroporated, and near-monoclonal expression of antibodies could be achieved (Fig. S1B). Notably, we detected uniformly high antibody surface expression even when DNA ratios resulting in more than 50% monoclonal cells were used (Fig. S1A), illustrating that a single transposed copy of each HC and LC results in robust expression, and suggesting that most integrated TEs are capable of achieving similar expression levels. This observation is consistent with the preferential integration of PiggyBac TEs into transcriptionally active regions. Under the same conditions, around 5% of cells stably expressed antibody (Fig. S1A), which subsequently can be enriched to almost 100% surface-positive cells by a five-day antibiotic treatment. Thus, an estimated cellular library diversity of ~10^6 can be easily achieved and screened by a single person (electroporation of 3.2 x 10^8 cells and subsequent culture and selection in 10 T175 culture flasks), using the protocols described here. Notably, higher-diversity cellular libraries may be generated via increasing integration copy numbers by adapting DNA ratios or scaling up protocols.

PiggyBac-mediated transposition leads to robust and long-lived surface-display of antibodies from stably integrated TEs for at least one month (Fig. 3A), allowing, in principle, multiple rounds of screening. Hence, screening of highly diverse naïve or synthetic libraries using Transpo-mAb Display is achievable. Clearly, however, the strength of the technology described here lies in the rapid and efficient screening of smaller, less complex libraries, or by applying Transpo-mAb Display as a final screening/quality control tool in combination with other antibody platforms that can more readily harness larger complexity antibody libraries (e.g., phage, ribosome display). Importantly, FACS technology enables fine discrimination of differences in antigen-binding strengths, thus allowing the direct and selective isolation of only the best-binding clones, as exemplified here with the screening of small, pre-defined humanization libraries. Further, Transpo-mAb Display is likely to be useful for the screening of libraries prepared from immunized animals, particularly if B cells enriched for antigen binding are used for library generation. Currently ongoing work in our laboratory suggests that Transpo-mAb Display can indeed be successfully employed for isolation of high-affinity clones from immunized animals using splenocyte-derived antibody libraries (data not shown).

To circumvent tedious and time-consuming cloning and re-expression of candidate antibodies in a soluble form after isolation of binders by FACS, we implemented simultaneous antibody surface display and secretion by expression of antibodies using a genomic version of the HC constant region. This resulted not only in sufficient surface expression useful for labeling and isolation of binders by FACS, but also in secreted amounts of full-length IgG that are of sufficient quality and quantity to be useful for antigen-binding assays such as ELISA and SPR, as well as for functional assays such as 2^° ADC screening.

Other groups have used different approaches to obtain both surface expression as well as secretion of antibodies. One approach is based on semi-stable episomal HEK293T expression of whole-IgG or Fab antibodies using HC constructs containing artificial splice sites, resulting in expression of both membrane-bound and secreted antibody, which is used in an antibody discovery platform termed SHM-XEL (AnaptysBio Inc.). In a second approach, antibodies are anchored to the membrane of CHO cells with the help of a platelet-derived growth factor receptor transmembrane domain (PDGFR-TM) that is fused to the C-terminus of the HC. Secretion in this system is achieved by a Furin cleavage site located between the PDGFR-TM and the HC. Yet another approach is used in a mammalian display platform called Retrocyte Display developed by 4-Antibody, and is based on irreversible, cre-recombinase-mediated deletion of TM exons.

It would be interesting to determine whether our technology can be transferred to other widely established cell lines such as Chinese hamster ovary (CHO) or HEK293T, and to investigate the extent to which different host cell lines would be beneficial in terms of the library sizes that could be generated and screened, the ratio of membrane-bound vs. secreted IgG that could be achieved with these non-B cell lines, and finally how the properties of the isolated antibodies compare with antibodies isolated from the B cell host cell line employed here. Importantly, PiggyBac seems to be active in a wide range of mammalian cell lines, and thus could in principle be applied to other cell systems of choice.

Since the Transpo-mAb Display vector system described here is built to express HC and LC from separate transposable elements that are individually selectable (Fig. 2), and based on our observation that sequential transposition of individual chains can be achieved without loss of previously transposed TEs (Fig. S2), the Transpo-mAb Display system could also be exploited for guided selection approaches, useful, for example, for antibody humanization or affinity maturation.

The use for antibody screening is outlined in this study, but our transposition technology also represents a valuable research tool useful for straightforward delivery of any sort of transgene. For example, we have successfully generated several types of
cancer cell lines stably overexpressing proteins of interest by transposition and antibiotic selection, including the EMT6-Meso cell line used in this study (Fig. 7). One attractive future therapeutic application may be the use of PiggyBac transposition technology in the generation of patient-derived genetically engineered T cells that currently hold great promise as a strategy for combating cancer, e.g., CAR-T cells.\textsuperscript{56} Suitability of the PiggyBac system for generation of CAR-T cells has been demonstrated recently.\textsuperscript{57–59}

In summary, we developed a novel antibody discovery platform that allows the establishment of full-length IgG antibody libraries in mammalian B cells by transposition using simple protocols. Rapid isolation of top-binding candidates from these cellular antibody libraries is facilitated by display of antibodies on the cell surface and FACS-sorting, followed by direct analysis of soluble antibody molecules secreted into the supernatant of sorted single cell clones using analytical and functional assays of choice.

Materials and methods

Antibody production

Parental anti-ROR1 mouse mAb 2A2\textsuperscript{28} and rabbit mAb R11,\textsuperscript{37} as well as anti-mesothelin mouse mAb MN,\textsuperscript{60} were produced as chimeric, full-length IgG1 antibodies with human constant regions as follows: Variable region coding regions were produced by total gene synthesis (GenScript) using MNFLRLIFLVTLGKVQC as leader sequence, and were assembled with human IgH-\gamma-1 and IgL-\kappa constant regions in the expression vector pCB14b. This vector, a derivative of the episomal mammalian expression vector pCEP4 (Invitrogen), carries the Epstein-Barr virus (EBV) replication origin, encodes the EBV nuclear antigen (EBNA-1) to permit extrachromosomal replication, and contains a puromycin selection marker in place of the original hygromycin B resistance gene. Expression of antibodies was achieved by transfecting pCB14b-based expression constructs into HEK-293T cells and harvesting of cell supernatants, according to the following protocols:

For transient antibody expression, cells were transfected in 6-well plates using Lipofectamine LTX plus (LifeTechnologies). Per well, 2.5 \( \mu \)g of total DNA was transfected, and fresh growth medium was added the next day. Supernatants were harvested after 4 d of conditioning, sterile-filtered and stored at -20°C until analysis.

For semi-stable antibody expression, cells were transfected in 10 cm dishes using Lipofectamine LTX plus (LifeTechnologies). Expanded to 14 cm dishes coated with poly-L lysine and maintained in DMEM/F12 serum-free medium (Gibco) containing 161 \( \mu \)g/ml N-acetyl-L-cysteine, 10 mg/ml L-glutathione and 1 \( \mu \)g/ml puromycin. Supernatants were harvested twice a week, sterile-filtered and stored at 4°C until purification. Purification by FPLC was done on an Äkta purifier instrument (GE Life-sciences). After passing supernatants over Amersphere protein A columns (JWTT203CE, ICR Micro) and washing columns with phosphate-buffered saline (PBS), antibodies were eluted with 0.1 M Tris pH 2.5 and immediately neutralized with 1 M Tris pH 8.0. Buffer exchange with PBS was performed using Amicon Ultra-4 Centrifugal Filters (Merck Millipore).

Expression and purification of antigens

StrepII-tagged ROR1 extracellular domain encompassed the nucleotide sequence encoding the extracellular domain of human ROR1 (NP_005003, amino acids 1–406) and was C-terminally fused with a sequence encoding a strepII-tag (GWSPQFEK). StrepII-tagged mature mesothelin encompassed the nucleotide sequence encoding the mature human mesothelin isoform 2 (NP_037536, amino acids 296–605) fused to an N-terminal signal sequence (MNFGRLIFLVTLGKVQC) and a C-terminal strepII-tag. The entire nucleotide sequences of tagged, soluble antigens flanked with 5’NotI and 3’HindIII sites were produced by total gene synthesis (GenScript), assembled in the proprietary mammalian expression vector pEvi5 by Evitria (Schlieren, Switzerland) and verified by DNA sequencing. Expression of the proteins was performed in suspension-adapted CHO K1 cells by Evitria. Supernatants from pools of transfected CHO K1 cells were harvested by centrifugation and sterile filtered (0.2 \( \mu \)m) before FPLC-based affinity purification using StrepTactin columns (IBA GmbH).

Construction of Transpo-mAb display vectors

All DNA syntheses required to generate plasmids were performed by GenScript. The amino-acid sequence of hyperactive PiggyBac transposase (hyPB) according to Yusa et al.\textsuperscript{20} was codon-optimized for murine expression, synthesized and cloned into the pcDNA3.1 transient expression vector. Vectors bearing transposable elements for antibody expression contain PiggyBac inverted terminal repeats (ITRs) up- and downstream of antibody expression cassettes, that are composed of an EF1-\alpha promoter upstream of IgH and IgL open reading frames (ORFs) followed by an IRES sequence allowed co-expression of downstream selectable markers or reporter genes. Further, transposable constructs contain functional sequences such as synthetic introns, polyA signals and Woodchuck hepatitis post-transcriptional regulatory elements for improved expression yields. All functional elements are flanked by unique restriction sites to allow for modular exchange. A detailed description of Transpo-mAb Display vectors can be found in Patent WO2014013026A1. Transposable constructs were assembled by restriction digest and ligation of two fragments that were synthesized individually: the first fragment comprised ITR-flanked expression cassettes, the second represented backbone sequence containing an origin of replication and an ampicillin resistance gene.

Antibody ORFs were introduced into transposable vectors as follows: variable regions with an N-terminal leader sequence (MNFGRLIFLVTLGKVQC) were synthesized with flanking 5’NotI/3’Nhel (IgHV) or 5’NotI/3’BsiWI (IgkappaV) and were assembled in-frame with human constant regions that were synthesized with flanking 5’Nhel/3’BstBI (IgHC-gamma 1) or 5’BsiWI/3’BstBI (IgKC) restriction sites.

Library construction

Variable regions were synthesized by Gen9, Inc., pooled to equimolar amounts and amplified by PCR using Q5 DNA polymerase (NEB) with forward primer univ-Not1-SP-F
(GAGAGGCGGCGCCCATGAACTTTGGG) and reverse primers huCG1-B
(AAGACCGATGGCCCTTTGGT) for IgHV and huCK-B
(GAAACAGATGGTGCAGCCAC) for IgKV. Cycling conditions were: 98°C/60sec -> 25x (98°C/45sec, 56°C/45sec, 72°C/60sec) -> 72°C, 5min -> hold@ 4°C. Amplified fragments were column-purified, digested using NotI/Nhel (IgHV) or NotI/BsiWI (Ig kappa V) and cloned into transposable vectors by 2-way (HC constructs) or 3-way cloning (LC constructs): Vector fragments were prepared by digestion with NotI/Nhel (pPB-Hygro-HCg1-gen) or by digestion with NotI/BstBI, as well as with BsiWI/BstBI (pPB-Puro-LC). Library ligations were transformed into Neb5-α electrocompetent cells (Neb), pre-cultured for 1 hour and amplified overnight in selective LB-media containing 0.1mg/ml ampicillin. Plasmid DNA was subsequently isolated using a NucleoBond Xtra Maxi Plus kit (Macherey-Nagel). Library sizes were determined by plating out serial dilutions of the pre-culture onto selective agar plates (titration plates) and obtained clone numbers were backcalculated to obtain library sizes. At least 12 clones from titration plates were analyzed by restriction digest and sequencing of variable regions using primer pPBseq13 (GGCCAGCT TGGCACTTTGATG).

Cells
L11 cells represent an in-house generated subclone of the Abel-son murine leukemia virus (A-MuLV) transformed pre-B cell line 63–12 isolated from RAG-2 deficient mice, and were cultured in SF-IMDM media supplemented with 2% fetal calf serum, 2mM L-glutamine, 100 IU penicillin, 0.1mg/ml streptomycin (all from Amimed) and 50μM b-mercaptoethanol (Amresco) in screwcap bottles (Sarstedt) at 37°C under 7.5% CO2.

EMT6 cells (ATCC, CRL-2755), a kind gift of Prof. A. Zippelius (University of Basel, Switzerland) and 293T cells (ATCC, CRL-3216) were both grown in DMEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100IU penicillin, 0.1mg/ml streptomycin and 0.25μg/ml fungizone (all from Amimed) at 37°C under 5% CO2.

Transposition and selection of L11 cells
L11 cells were seeded one day before electroporation at a density of 0.2×106 cells/ml to obtain log-phase growing cells the next day. The entire procedure of electroporation was performed at room temperature. Cells were harvested by centrifugation at 1200 rpm for 6 min and resuspended in plain RPMI medium to obtain a cell suspension of 8×106 cells/ml. Per electroporation, 25μg of total DNA was diluted in 400μl RPMI (using HC/LC/transposase DNA weight ratios as shown in Fig. S1B), mixed with 400μl cell suspension and transferred to a 0.4cm gap gene pulser cuvette (BioRad). Electroporation was done with a BioRad GenePulser II equipped with capacitance extender set to 300 V and 950μF. After incubation for 5–10 min in cuvettes in order to allow pores to close, cells were washed once in complete SF-IMDM growth medium, resuspended in 64ml of complete SF-IMDM growth medium and transferred to a T175 tissue culture flask. For selection, 1μg/ml puromycin and 800μg/ml hygromycin (0240.4 and CP12.2, respectively; Carl Roth) were added simultaneously 24 hours after electroporation, and selection was allowed to proceed for 4–5 d without exchange of medium or subculturing.

Staining and sorting of cellular libraries
Cells were stained on ice in FACS-buffer (PBS supplemented with 2% FCS) at a concentration of 1×106 cells/ml. Washes between incubations were performed by pelleting cells by centrifugation at 1300 rpm for 3 min, resuspending in FACS-buffer using a 5-fold volume of staining reactions, pelleting again and resuspending in FACS buffer.

For analysis of surface-antibody expression, cells were stained using 1:200 diluted Ig-kappaLC-APC labeled antibody (MH10515, Life Technologies) for 30 minutes, followed by one wash and analysis by flow cytometry on a FACScalibur instrument (Becton-Dickinson).

For antigen/surface-expression double labeled, strepII-tagged antigens were added to cells at previously determined limiting concentrations (0.12 μg/ml mesothelin-strep or 0.25 μg/ml ROR1-strep) together with a PE-labeled, Fc gamma-specific anti-human-IgG antibody (ebioscience 12-4998-82) used at 1:250 dilution. After incubation for 30 min on ice, cells were washed once followed by incubation with 1:500 diluted Oyster-645-labeled anti-strep antibody (Strep-MAB classic Oyster-645, 2-1555-050, iba) for 30 minutes. After a final wash, cells were either analyzed by flow cytometry on a FACScalibur instrument (Becton-Dickinson), or filtered using cell strainer cap FACS tubes (BD Falcon) and sorted employing a FACSARiaII instrument (Becton-Dickinson).

ELISA
For determination of antigen-binding, Nunc-Immuno MaxiSorp 96-well plates (Thermo Scientific) were coated with antigens diluted in coating buffer (100 mM bicarbonate/carbonate buffer), using previously determined limiting concentrations of antigen to minimize avidity effects (0.25 μg/ml Meso-strep, 0.125 μg/ml ROR1-strep). For determination of IgG titers, plates were coated with 2 μg/ml AffiniPure F(ab')2 fragment donkey anti-human IgG (Jackson ImmunoResearch) diluted in coating buffer. Coating was done overnight at 4°C. Plates were then washed twice with PBS/0.05%Tween-20 (PBS-T), blocked for 1 hour at 37°C using PBS-T supplemented with 3% bovine serum albumin (BSA) (Carl Roth) and washed again 5 times with PBS/T. L11 clone supernatants were pre-diluted 3-fold in ELISA buffer (PBS-T containing 1% BSA), while supernatants from transiently transfected 293T cells were pre-diluted 50-fold. Parental mAbs used to generate standard curves were diluted to a starting concentration of 0.5 μg/ml. Samples were added to plates as 3.5-fold serial dilutions in ELISA buffer and were incubated for 1 hour at 37°C. After 5 washes with PBS/T, HRPO-conjugated F(ab')2 anti-human FC-gamma (Jackson ImmunoResearch) diluted 10,000-fold in ELISA buffer was added, and plates were incubated for 1 hour at 37°C. After 5 final washes with PBS/T, 5μl of Sigmafast OPD Peroxidase substrate (Sigma-Aldrich) was added, and reactions were stopped by adding 50μl of 2 M H2SO4. Absorption was measured at 490 nm. OD50 values of standards with known concentrations and samples...
determined by 4-point curve fitting models were used to calculate half-maximal concentrations (EC50).

**SPR**

Affinities were determined using a Biacore T200 instrument (GE Healthcare) and data was evaluated using Biacore Evaluation T200 V2.0 software. To capture mAbs, goat α-human Fc-gamma-specific IgG (Jackson ImmunoResearch, # 109-005-098) was covalently immobilized on a CM5 chip (GE Healthcare, # BR-1005-30).

For determination of huMN affinities by multicyle SPR, 293T supernatants containing mAbs were diluted to 10 μg/ml IgG with running buffer HBS-EP+ pH 7.4 (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05 % Tween 20) and captured for 60s with a flow of 10 μl/min. Mesothelin-strep was diluted in running buffer using 2-fold serial dilutions ranging from 10 nM to 1.25 nM. Association was measured at a flow rate of 30 μl/min for 120s, and dissociation was followed for 1000s. Capture levels ranged from 90.0 RU to 493.6 RU.

For single-concentration SPR screening of hu2A2 and huR11 L11-clone supernatants, IgG titers were determined by ELISA, and supernatants were diluted to IgG concentrations of 0.3 μg/ml with complete SF-IMDM cell culture medium. Capturing was done for 120s with a flow of 10 μl/min. ROR1-strep was diluted to 40 nM in running buffer and association was measured at a flow rate of 30 μl/min for 120s, then dissociation was followed for 200s. Curves were fitted using 30s dissociation due to upper plateau formation at later timepoints. Capture levels ranged from 29.1 RU to 57.7 RU.

For determination of hu2A2 and huR11 affinities by multicyle SPR, purified mAbs were diluted to 0.3 μg/ml with running buffer and captured for 120s with a flow of 10 μl/min. ROR1-strep was diluted in running buffer using 2-fold serial dilutions ranging from 20 nM to 2.5 nM. Association was measured at a flow of 30 μl/min for 120s, and dissociation was followed for 200s. Curves were fitted using 30s dissociation due to upper plateau formation at later timepoints. Capture levels ranged from 29.1 RU to 57.7 RU.

All measurements were performed at 25°C. All curves were fitted using a 1:1 binding model with RI = 0. Regeneration was done for 90s using 100 mM H3PO4 at a flow of 30 μl/min.

**Sequence recovery**

RNA was extracted from ~2x10⁶ cells grown in 24-well plates using Tri-Reagent (Sigma-Aldrich) and was reverse transcribed with ProtoScriptII Reverse transcriptase (Neb) using random nonamers, according to the manufacturer’s instructions. Variable regions were amplified by PCR using Q5 DNA polymerase (Neb) by means of forward primer EF1aNotI_F (CCATTT-CAGGTTGCAGTAC) and reverse primers CG-revseq-1 (GGTTCGG GGAAGTAGTCCTTG) for VH and Intron-rev-1 (GGTGGATCTGAGAAGAGGCTG) for VL. Cycling conditions were 98°C/30sec -> 30x (98°C/20sec, 58°C/20sec, 72°C/15sec) -> 72°C/5min -> hold@ 4°C. PCR products were purified by column purification (Macherey-Nagel), digested and again purified by agarose-gel purification. Recovered variable region fragments were assembled with human IgH-γ 1 and IgL-κ constant regions in the expression vector pCB14b. To determine VH and VL sequences, several bacterial clones per library clone were sequenced (Microsynth AG) using the pCB14b sequencing primer CMVseq2 (GCAATTCATCTCGAGCAGTAC). Variable region sequences were compared to designed library sequences using Geneious R8 Software (Biomatters).

**Secondary ADC assay**

Mesothelin-overexpressing EMT6-Meso cells were plated in 96-well plates in 75 μl growth medium at a density of 1000 cells per well and incubated under growth conditions. One day after plating, growth medium was replaced with 50 μl L11 supernatants that were serially diluted 3.5-fold in complete DMEM growth medium. After a 30 min incubation under growth conditions, 3.5-fold serial dilutions of secondary ADC reagent (aHFc-CL-MMAE, MORADEC AH-102PN), ranging from 10 μg/ml to 0.1 ng/ml, were added to supernatants. After incubation for 3 d under growth conditions, plates were removed from the incubator, equilibrated to room temperature for 30 minutes, and 100 μl CellTiter-Glo Luminescent Solution (Promega, Cat.No G7570) was added to each well. After shaking the plates at 650 rpm for 5 min, followed by a 10 min incubation without shaking, luminescence was measured on a Tecan Infinity F200 with an integration time of 1 second per well.

**Statistical analysis**

Statistical analysis was generated using GraphPad Prism Software v. 6.0 (GraphPad Software Inc., San Diego, CA).

**Disclosure of potential conflicts of interest**

LW, IH, CKG, FIW, UG and RRB are employees of NBE-Therapeutics AG. RBB and UG hold stocks of NBE-Therapeutics AG. This work has been included in a patent application by NBE-Therapeutics AG. The authors have no additional financial interests.

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