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Utilizing non-clonal CHO cell derived materials for preclinical studies of complex molecules

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Abstract

Background The use of non-clonal CHO cell derived materials for preclinical studies has been found to be a valuable approach to accelerate the development of monoclonal antibodies (mAbs) for first-in-human (FIH) studies. In a comprehensive study, we assessed the culture performance, productivity, and product quality of non-clonal cell lines compared with clonal cell lines expressing various biologic modalities to determine if this approach can be applied to complex molecules.

Results We evaluated a multi-specific antibody, a cytokine-Fc fusion protein, and a mAb produced using the stable pool, the pool of top clones, and the lead clone utilizing transposase-mediated integration. The results indicated that the attributes were comparable regardless of the source of cells. Building upon these findings, the study progressed to the preclinical manufacturing of two multi-specific antibodies using both the pool of top clones and the lead clone. Subsequently, clinical manufacturing of these multi-specific antibodies was performed using the lead clone. The batches produced with the pool of clones and the lead clone demonstrated a high level of comparability in culture performance, productivity, and product quality.

Conclusions In conclusion, non-clonal CHO cell derived materials can be effectively utilized for preclinical studies of complex molecules without compromising their quality, allowing for accelerated development for FIH studies.

Keywords Preclinical study, Complex molecules, First-in-human development, Speed to clinic, Non-clonal material for Tox

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Background

Biotherapeutics are commonly produced in Chinese hamster ovary (CHO) cells [1, 2]. Cell line development is the first step in the development workflow and remains a significant bottleneck in the overall timeline to first-in-human (FIH) studies. A typical CHO cell line development process starts from transfection and generation of stable pools, progresses to single cell cloning, and then multiple rounds of screening of hundreds of clones until the selection of a lead clone with desired growth, productivity, product quality, and cell line stability profile. The process from single cell cloning to selection of lead clone is the most time-consuming and typically requires approximately 6 months. Nevertheless, establishment of a clonal cell line for biologic manufacturing remains a key control strategy to ensure consistent product quality [3, 4].

Typically, materials for critical preclinical studies, such as the Good Laboratory Practice (GLP) toxicology (Tox) study to evaluate drug safety, are produced using the lead clone which is also used in Good Manufacturing Practice (GMP) clinical manufacturing. Using the same clone for preclinical and clinical material generation minimizes development risk and safety concerns. However, the relatively long cell line development timeline to lead clone identification can become a bottleneck for downstream preclinical activities such as process development, manufacturing, and Tox studies. Using stable pool or pool of clones can expedite the production of material for Tox studies by avoiding the time-consuming process of selecting and characterizing a single lead clone. It allows for Tox studies to occur sooner while cell line development activities continue in parallel to significantly reduce the FIH timeline (Fig. 1).

A key enabler for this "pool for Tox" strategy is cell culture performance and product quality comparability between the pool and the lead clone. Previous works on "pool for Tox" have shown that either stable pool or pool of clones can produce material with product quality that is representative of the clonal material for standard monoclonal antibodies (mAbs) [5–11]. However, complex molecules, including bispecific antibodies, fusion proteins, and non-Fc containing proteins, often present non-platform technical challenges due to their structural complexity. They may have additional critical product quality attributes (e.g. mispairing, fragmentation) and complex post-translational modifications (e.g. N-glycan structures) [12, 13]. Thus, it is more challenging to apply "pool for Tox" approach for complex molecules. So far, there has not been any report on implementing this strategy for complex molecules.

Traditionally cell line development relies on random integration of transgene in the CHO host cell genome. The inherent "random" nature of integration leads to stable pools consisting of clones with diverse integration sites, growth, productivity, and product quality. Semi-random (e.g. transposase-mediated) or targeted integration have experienced increased popularity in recent years due to advantages of shortened cell line development timeline, improved product quality, and increased cell line stability [14–16]. While targeted integration generates genetically homogenous stable pools, transposase-mediated integration preferentially targets transcriptionally active genomic sites. In this study, we

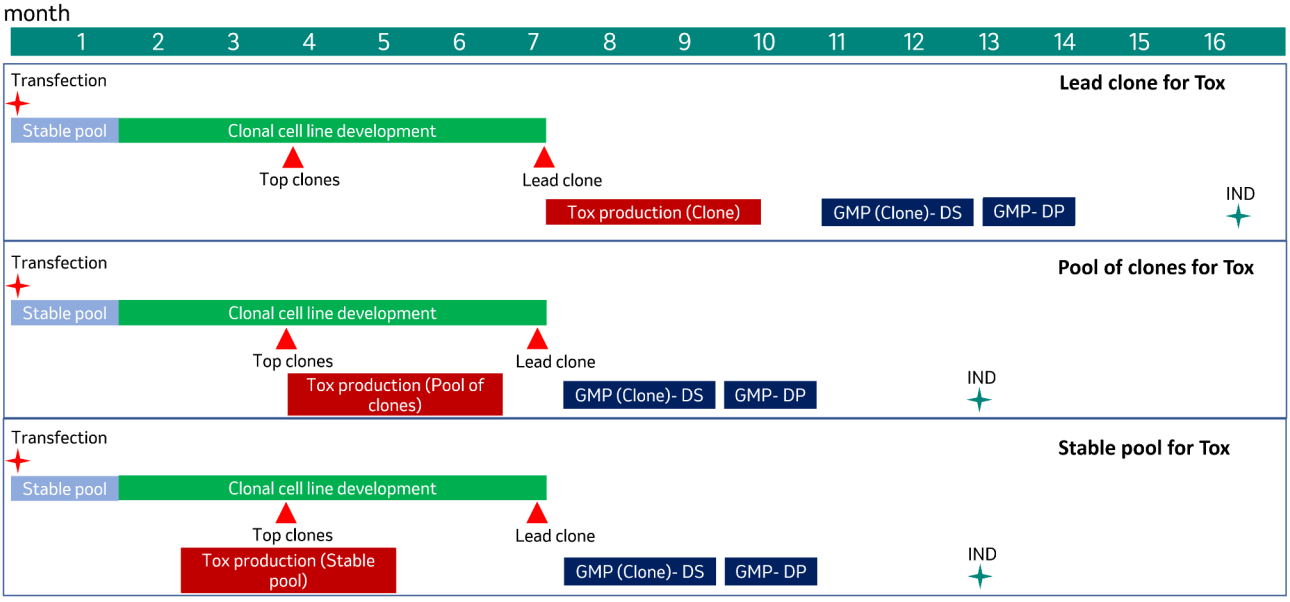

Fig. 1 Comparison of traditional Chemistry, Manufacturing, and Control (CMC) timeline utilizing lead clone for Tox and accelerated timelines utilizing pool of clones or stable pool for Tox

evaluated the suitability of leveraging our transposase-derived stable pool or pool of clones to produce pre-clinical materials for three different biologic modalities including mAb, Fc-fusion protein, and multi-specific antibody. Furthermore, we used a pool of clones to generate Tox materials at 500L scale and demonstrated comparability to the clinical material produced by lead clone at 2000L scale for two multi-specific antibody programs. The results indicate that the "pool for Tox" approach can be applied for standard mAbs as well as complex molecules, leading to timeline saving of approximately 3 months from transfection to FIH regulatory filing.

Methods

Pool and clone generation

The recombinant CHO cell lines in this study were generated using our company's proprietary CHO-K1 derived glutamine synthetase (GS) knockout host that was developed in-house. CHO cells were cultured in shaker incubators (Kuhner) at 37 °C, 80% humidity, and 5% CO_2. Cells were passaged at a seeding density of $0.2–0.5 \times 10^6$ viable cells/mL every 2–3 days.

Our company's in-house expression vectors were designed with PiggyBac inverted terminal repeats (ITRs) [17–18]. The transgene expression cassettes containing cytomegalovirus promoter and SV40 polyadenylation signal were placed between ITRs. The host cells were co-transfected with one or two expression vectors and PiggyBac transposase mRNA [19] using Neon electroporation system (Thermo Fisher). The transfected cells were passaged in selection media until cell viabilities recovered to generate stable pools. The stable pools were single cell cloned into 96-well plates by fluorescence activated cell sorting (BD Biosciences). Clones were selected for expansion through multi-well plates and suspension culture based on titer. The top 48 clones were evaluated in fed batch culture and the top 6 or top 3 clones were selected based on growth, titer, and product quality. A lead clone is selected following both clone stability study and bioreactor process evaluation.

Shake flask fed batch production and protein a purification

Research cell bank vials of stable pool and top 6 clones were thawed, and cells were passaged in shake flasks. The top 6 clones were passaged separately and pooled by equal cell number 5 passages prior to N-1 to generate the early pooling population. The early pooling population were subsequently passaged 5 times until N-1. In parallel, the 6 clones were separately passaged until N-1, and then pooled by equal cell number at the N-1 stage to generate the late pooling population. The stable pool, early pooling, late pooling, and lead clone cultures were inoculated in a platform fed batch process using chemically defined media. Product titer was quantified via HPLC using a POROS Protein A ID Sensor Cartridge (Thermo Scientific). Cell culture samples were clarified by centrifugation at 1000 rpm for 20 min and filtrated through 0.22-µm PES filters. Recombinant proteins were then captured using MabSelect SuRe protein A affinity chromatography and eluted in 20 mM sodium acetate, pH 3.5. Protein concentration was assessed by UV spectroscopy (Spectramax M5e; Molecular Devices) at 260/280 nm. Protein A purified material was used for various analytical assays.

Bioreactor fed batch production and purification process

Single-use bioreactors were used for fed batch production at the 500 L and 2000 L scales. The bioreactors were inoculated at a target viable cell density of 2×10^6 cells/mL. The production bioreactor temperature, pH, agitation, dissolved oxygen (DO), air, and pCO_2 were monitored and controlled throughout the process. Antifoam was added as needed to prevent foaming. The culture pH was controlled using sparged CO_2 gas and the addition of sodium hydroxide. The culture DO was controlled around a target set point using sparged air and oxygen. Feed media and glucose were added at predefined intervals. The purification process for MsAb-A and MsAb-B used a three-column process: a protein A affinity chromatography (Cytiva Life Sciences, MabSelect SuRe) followed by anion exchange chromatography (Thermo Fisher Scientific, POROS 50 HQ) and cation exchange chromatography (Thermo Fisher Scientific, POROS 50 HS) [20]. Purification was performed on AKTA™ process skids controlled by UNICORN™ software. Column chromatography was run applying the same residence time across scales. Column delta pressure was maintained within acceptable operational parameters.

Product quality analysis

Product quality analysis was performed using the methods described below.

Ultra-performance size exclusion chromatography (UP-SEC) UP-SEC was used to determine the size heterogeneity of the samples. UP-SEC was performed using a Waters Acquity H-class® instrument.

Reduced sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) The purity of the samples was determined using a gradient (4–20%) precast polyacrylamide gel stained with a highly sensitive fluorescent stain (SYPRO Ruby). The purity of monoclonal antibodies was determined by resolving the light chain (LC), the heavy chain (HC) and their degradation products according to their size in a capillary containing a replaceable SDS-gel matrix.

Non-reduced sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) The purity of IgG monoclonal antibodies and total low molecular weight (LMW) species were determined by resolving the intact IgG from impurities or degradation products according to their size in a capillary containing a replaceable SDS-gel matrix.is the samples were treated with an alkylating agent (NEM) and the anionic surfactant SDS prior to heating.

Imaged capillary isoelectric focusing (iCIEF) Samples were analyzed to determine the charge profile using a Maurice Simple® iCIEF instrument. The charge variants were grouped into acidic, main and basic species.

Reverse-Phase Ultra-Performance liquid chromatography (RP-UPLC) The purity and critical impurities, including IgG homodimer and Single Chain Variable Fragment (ScFv) related impurities, were analyzed by a RP-UPLC method using a Waters Acquity H-Class instrument with UV detection and Empower software.

N-glycan by ultra Performance-Hydrophillic interaction chromatography (UP-HILIC) A UP-HILIC method was developed to evaluate the N-linked glycan profile of antibodies. Prior to UP-HILIC analysis, samples are prepared using Waters GlycoWorks™ N-Glycan Kit and procedure. In this method N-linked glycans are enzymatically released from the antibody using a rapid PNGase F. The free glycans are subsequently labeled with RapiFluor-MS reagent and purified by solid-phase extraction (SPE) utilizing GlycoWorks HILIC µElution™ Plate. The labeled glycans are then analyzed using UPLC with fluorescence detection and Empower software.

Enzyme-Linked immunosorbant assay (ELISA) A dual-specific ELISA was developed to measure relative potency of the MsAbs. A reference standard was designated from the 500 L Single Clone batch and used to assess relative potency.

Cell based assay (CBA) A mechanism of action-reflective CBA was developed to measure relative potency of the MsAb-A. A reference standard was designated from the 500 L Single Clone batch and was used to assess relative potency.

Forced degradation study

Three batches of drug substance MsAb-A were exposed to extreme conditions for a short period of time to better understand their degradation pathways. Samples were staged and tested according to standard practice outlined in International Conference on Harmonization (ICH) Q1A, Q1B and Q2B. Exposure to light, heat, pH and oxidative stress conditions were conducted for the prescribed times. Chemical stressed samples were buffer-exchanged immediately at the end of each timepoint, followed by storage at -70 °C.

Results

Stable pool, pool of clones, and lead clone had similar growth profiles in fed batch production for mAb, Fc-fusion protein, and multi-specific antibody

We utilized three different programs to evaluate the suitability of using the stable pool or a pool of top 6 clones to produce Tox material. The three programs include a mAb, a cytokine-Fc fusion protein, and a multi-specific antibody. The pool of clones was generated by combining the top 6 clones either at the N-6 passage (early) or N-1 passage (late), where N denotes production. The top clones used for pooling were selected based on cell growth, productivity, and product quality attributes such as aggregates, N-glycans, and heterodimer purity for complex molecules. For each program, we compared the stable pool, the pool of clones, and the lead clone using a fed batch process in shake flasks where the cell growth, metabolite profiles, productivity and product quality were monitored.

Growth and viability of stable pool, pool of clones, and lead clone for all three molecules were within the expected range, with peak viable cell densities reaching $20–30 \times 10^6$ cells/mL (Fig. 2). For the mAb and Fc-fusion

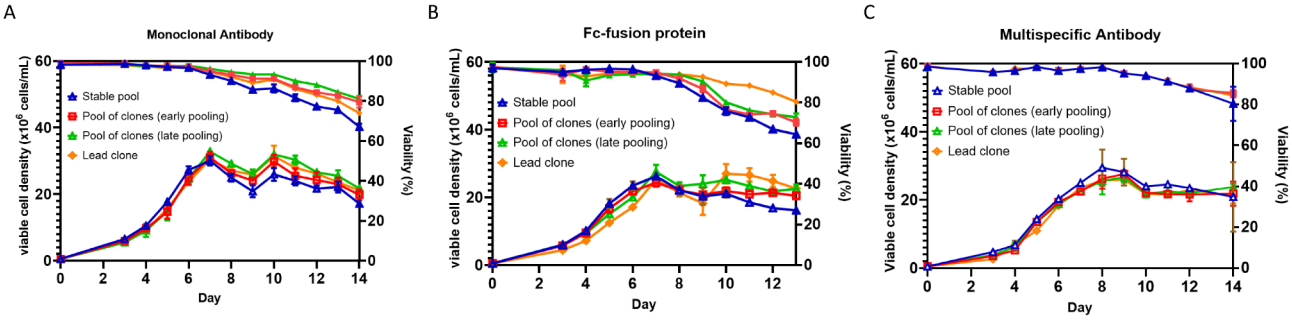

Fig. 2 Viable cell density and viability cell count results during fed batch production for **A** monoclonal antibody (mAb), **B** Fc-fusion protein, and **C** multi-specific antibody. Each symbol represents the average of triplicate fed batch experiment. Each error bar represents 1 standard deviation

protein, the lead clone and pool of clones had higher harvest viabilities at around 70–80% than stable pool at around 60–65%. In addition, the two clone-pooling approaches (early vs. late) showed no difference in culture performance.

Comparable productivity and product quality among stable pool, pool of clones, and lead clone

Cell culture harvests from fed batch production were measured for titer, purified by Protein A chromatography, and subsequently analyzed for product quality. For all three molecules, the titer of pool of 6 clones and lead clone were similar but higher than the stable pool (Fig. 3A). The purity levels assessed by size exclusion chromatography (SEC) were comparable among stable pool, pool of clones, and lead clone for each of the three molecules (Fig. 3B). While the SEC main peak was >98% for mAb, the main peak % was lower for the complex molecule programs at 81–85% for cytokine-Fc fusion protein and 72–80% for multi-specific antibody. Both the cytokine-Fc fusion protein and multi-specific antibody have a knob-into-hole Fc domain and can form product-related impurities such as homodimers and half molecules. These product-related impurities were detected as distinct peaks in reverse phase High Performance Liquid Chromatography (RP-HPLC). The product purity levels analyzed by RP-HPLC were comparable among stable pool, pool of clones, and lead clone and ranged from 74 to 80% for cytokine-Fc fusion protein and 87–95% for multi-specific antibody (Fig. 3C).

Glycosylation of biologics often plays an important role in its biological function or pharmacokinetics. For instance, afucosylation on antibody Fc domain has been shown to greatly enhance antibody-dependent cellular cytotoxicity (ADCC) activity, and afucosylation level may need to be tightly controlled for a molecule with ADCC as a mechanism of action [21]. High abundance of high mannose glycan species on mAbs and lower level of sialylation on fusion molecules have been linked to faster clearance of the molecule, potentially reducing the exposure [21]. We compared the N-glycan profile of the mAb, cytokine-Fc fusion protein, and multi-specific antibody produced from the stable pool, pool of clones, and lead clone and found them highly similar (Fig. 3D-F). The three molecules all have N-glycosylation in their Fc domain. Their N-glycan cores were mostly fucosylated, with G0F being the major glycoform, and the afucosylation levels were similar. The Man5 levels were around 5% or lower and were deemed low risk.

We demonstrated in all three programs acceptable product quality comparability among stable pool, pool of top 6 clones, and lead clone. We also observed that the early pooling and late pooling of top 6 clones had no

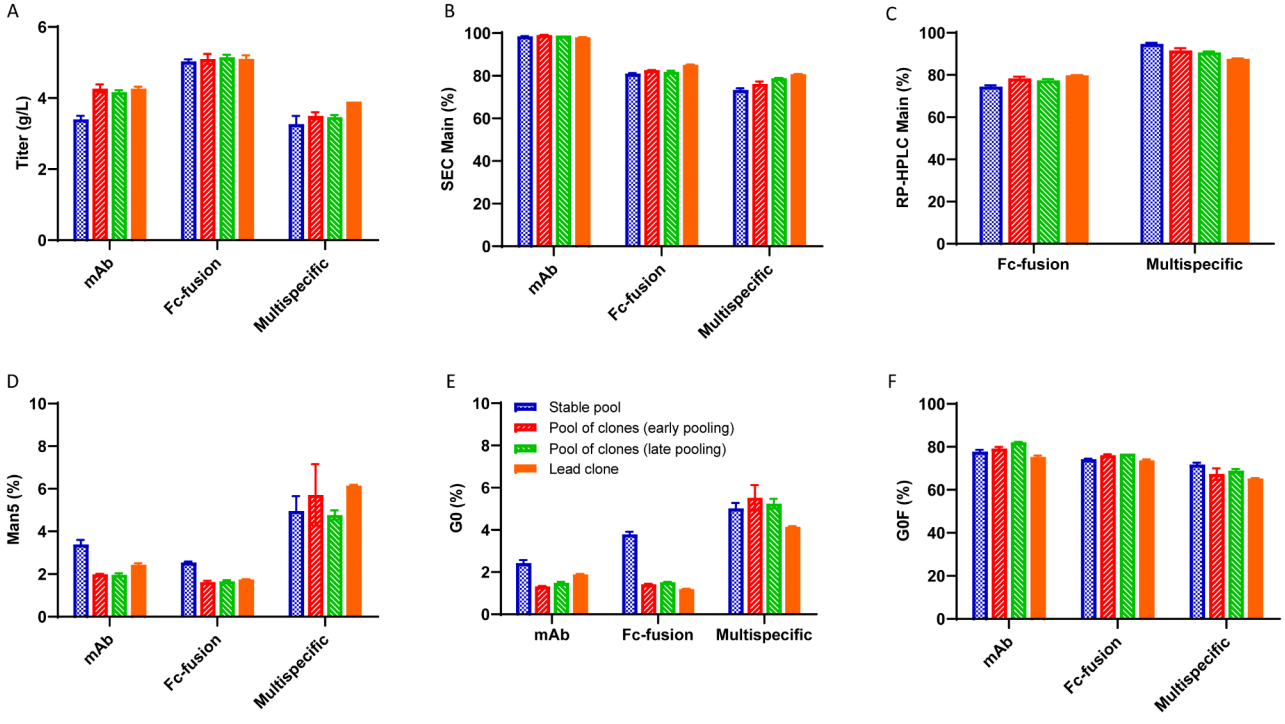

Fig. 3 Stable pool, pool of clones, lead clone titer and product quality attributes for monoclonal antibody (mAb), Fc-fusion protein and multi-specific antibody. **A** Titer, **B** Main peak % by UP-SEC, **C** Main peak % by reverse phase HPLC, **D**-**F** N-glycan Man5, G0, G0F. Each symbol represents the average of triplicate fed batch experiment. Each error bar represents 1 standard deviation

significant differences in productivity and product quality (Figs. 2 and 3).

Cell culture growth, metabolites, and titer were acceptable utilizing pool of clones at manufacturing scale production for two multi-specific antibody programs

Encouraged by the results in the small-scale study across the three programs, we implemented the use of non-clonal cell line for preclinical Tox production in two multi-specific antibody programs (MsAb-A and MsAb-B). We chose to use pool of clones instead of stable pools for Tox production. As shown in Fig. 1, using the stable pool for Tox can lead to much earlier availability of material for preclinical Tox study. However, selection of the lead clone and GMP manufacturing of clinical supply are on the critical path to investigational new drug (IND) filing and their timelines remain the same whether stable pool or pool of clones is used for Tox. Thus, using the stable pool for Tox does not lead to a faster IND filing.

Materials for Tox studies were generated by pooling the top 6 clones for MsAb-A and pooling the top 3 clones for MsAb-B at the 500 L scale. Another 500 L batch was run using the lead clone for each program. The GMP clinical batch was run using the lead clone at the 2000 L scale. The cell growth and viability profiles were similar among the 500 L preclinical batches and 2000 L clinical batch in both programs (Fig. 4A). The culture metabolites including glucose, lactate, and ammonium had similar levels among the pool of clones and lead clone batches (Fig. 4B-D). The harvest titers of the batches were very consistent within each program, except for the MsAb-B Tox batch (Fig. 4E). The Tox production batch of MsAb-B was harvested earlier on day 11 instead of day 14 to accommodate facility schedule, which explains the lower titer of this specific batch. The same purification conditions were used for all three batches of MsAb-A and MsAb-B.

Product quality profiles were similar between pool of clones and lead clone at manufacturing scale production for MsAb-A and MsAb-B

Various analytical methods were utilized to assess the product quality of purified MsAb-A and MsAb-B drug substances. The purity of both molecules was analyzed by UP-SEC, non-reduced CE-SDS, and potency was determined by binding ELISA. The results were highly comparable among the pool of clones and lead clone batches (Table 1). In addition, we performed a comprehensive comparability study for MsAb-A including a large panel of attributes. Effector function of Fc is expected to contribute to the mechanism of action of MsAb-A, thus afucosylation is considered a critical quality attribute of this molecule. No significant difference was observed in N-glycan species including afucosylation between the pool of clones and lead clone batches. Potency of the molecule determined by binding ELISA, Biacore, and a cell-based assay, which recapitulates the biological mechanism of action of MsAb-A, were also highly comparable

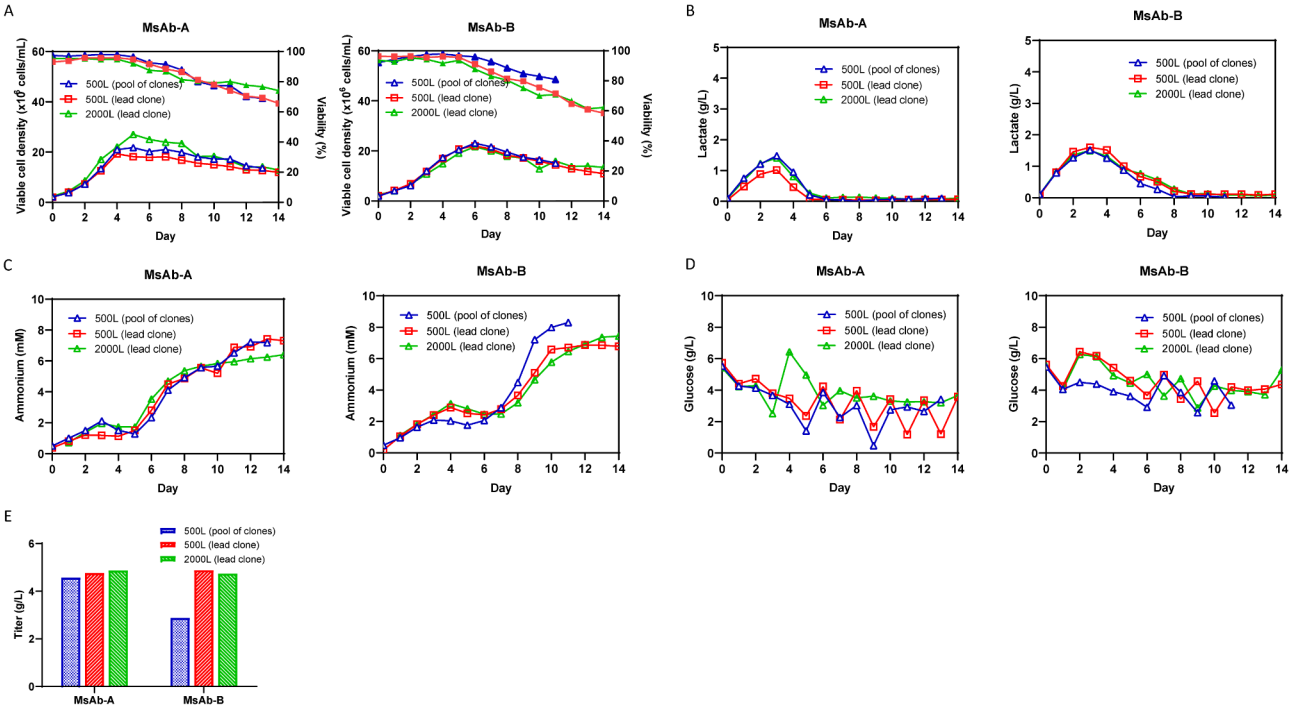

Fig. 4 Large scale production runs of MsAb-A and MsAb-B comparing pool of clones at 500 L, lead clone at 500 L, and lead clone at 2000 L. **A** Viable cell density and viability, **B** Lactate profile, **C** Ammonium profile, **D** Glucose profile, **E** Titer

Table 1 Analytical data across various batches for MsAb-A and MsAb-B

Assay	MsAb-A Batch		
	500 L (pool of clones)	500 L (lead clone)	2000 L (lead clone)
UP-SEC (%Monomer)	99.0	99.4	99.3
UP-SEC (%High Molecular Weight)	0.5	0.5	0.4
iCIEF (%acidic)	23.6	24.6	26.0
iCIEF (%main)	70.8	69.4	70.0
iCIEF (%basic)	5.6	6.0	4.1
CE-SDS non reduced (%Intact IgG)	97.9	98.0	98.6
CE-SDS non reduced (%Low Molecular Weight)	2.1	2.0	1.4
Reduced CE-SDS (%HC + LC + ScFv-Fc)	98.9	99.4	99.1
Reduced CE-SDS (%total impurities)	1.1	0.6	0.9
Residual DNA (pg/mg)	< 0.406	< 0.045	< QL
Residual HCP (ng/mg)	< QL	< QL	< 0.5
Residual ProA (ng/mg)	< QL	0.809	< 0.3
pH	5.6	5.5	5.7
Binding ELISA (% relative potency)	100	100	96
FcgRIII	89	90	86
Cell-based assay (% relative potency)	98	90	86
N-glycan (G0-GlcNAc) (%area)	3.8	4.5	2.4
N-glycan (G0F-GlcNAc) (%area)	8.5	11.1	10.5
N-glycan (G0) (%area)	7.9	6.0	4.5
N-glycan (G0F) (%area)	61.6	58.5	64.2
N-glycan (Man5/G1F-GlcNAc/G1) (%area)	6.2	8.2	7.7
N-glycan (G1F) (%area)	4.4	4.1	4.5
N-glycan (G1F') (%area)	1.9	1.8	2.2
Assay	**MsAb-B Batch**		
	500 L (pool of clones)	500 L (lead clone)	2000 L (lead clone)
UP-SEC (%Monomer)	99.2	98.3	98.8
UP-SEC (%High Molecular Weight)	0.7	1.3	0.9
UP-SEC (%Low Molecular Weight)	0.1	0.4	0.2
CE-SDS non reduced (%Intact IgG)	98.4	97.2	97.0
CE-SDS non reduced (%Low Molecular Weight)	1.6	1.8	1.6
Binding ELISA (% relative potency)	103	109	108

across batches (Table 1). As expected, process related impurities such as host cell proteins, DNA, and endotoxin were within specification in all batches (data not shown). Lastly, we performed forced degradation studies using the same three MsAb-A drug substance batches. The results demonstrated that these materials underwent similar degradation pathways in various stress conditions including light, oxidation, high and low pH, and elevated temperature (Fig. 5). Taken together, the quality of the drug substance produced using pool of clones was highly comparable to that of the lead clone. In addition, the toxicity profiles of MsAb-A in non-human primates and humans were consistent, further supporting the feasibility of using non-clonal material for preclinical Tox study (data not shown).

Discussion

In biologics development, acceleration of the CMC development timeline is a major enabler of faster delivery of new biotherapeutics to patients in need. The utilization of representative non-clonally derived material from earlier stages of cell line development for toxicology (Tox) or other non-clinical studies can significantly accelerate FIH development. By using this material for Tox studies, it removes Tox from the critical path to FIH, ultimately shortening the overall development timeline. It also allows for an earlier readout of toxicology assessment, providing more time for clinical study design, regulatory interactions, and adjustments if necessary.

It is crucial to ensure that the non-clonal material used in preclinical Tox studies is representative of the clinical material to ensure a more translatable safety assessment. Previous works by others have demonstrated the feasibility and successful implementation of the "pool for Tox" approach for mAb molecules by establishing analytical comparability [5, 22]. This is mainly attributed to the wealth of mAb process and analytical knowledge accumulated in the past few decades. However, companies are hesitant in applying the same strategy to complex molecules due to their unique product quality attributes that may jeopardize the "representativeness" of Tox material.

In this study, we demonstrated consistent cell culture performance and product quality across stable pool, pool of clones, and lead clone, for mAbs and complex molecules. Furthermore, material generated using a pool of clones for preclinical Tox study and material generated using the lead clone for clinical study were analytically highly comparable for two multi-specific antibody programs. Thus, material made using a pool of top clones was suitable for preclinical studies such as analytical assay development and validation, reference standard generation, formulation development, and Tox study. It is important to have a good understanding of the molecule's critical quality attributes for this strategy to be effective. This can be challenging for some complex molecules, especially at the early development stage, when only limited data are available on the impact of quality attributes to drug efficacy and safety. Another essential element of this approach relies on the selection of a lead clone with similar product quality as the pool of top clones. This ensures that the non-clonal material generated for preclinical Tox study is representative of the clonal clinical material.

While analytical comparability is the most crucial element in the "pool for tox" approach, process performance comparability between the pool and the clone also needs to be considered. The risk of process or analytical

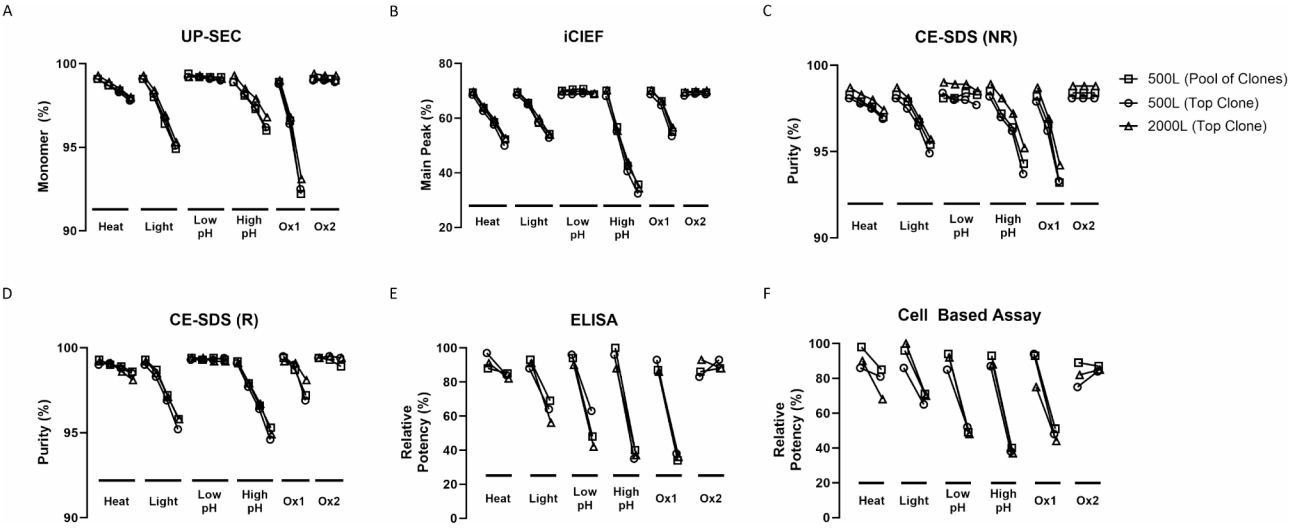

Fig. 5 Examining degradation pathways for MsAb-A. Each batch of MsAb-A was subjected to controlled chemical and physical stress according to ICH guidelines. Measurement of monomeric antibody structure and presence of aggregates was determined by (**A**) Ultra-High Performance Size Exclusion Chromatography (UP-SEC). Charge variant analysis was determined by (**B**) imaged capillary isoelectric focusing (iCIEF). Formation of low molecular weight mAb species was determined under native (**C**) Non-Reduced Sodium Dodecyl Sulfate Capillary Electrophoresis (CE-SDS (NR)) and denatured (**D**) Reduced Sodium Dodecyl Sulfate Capillary Electrophoresis (CE-SDS (R)). Relative potency was determined for only initial and maximum-stressed samples using (**E**) ELISA and (**F**) Cell-Based Assay

inconsistency during scale up is traditionally mitigated by using the same lead clone from small-scale to pilot scale to clinical manufacturing scale production. In the "pool for Tox" approach, the growth rates among the clones in the pool can be different, which may shift the clone composition during scale up and pose a higher risk of process and analytical inconsistency. One way to mitigate this population drift is by pooling the clones just before bioreactor inoculation. However, our data suggested pooling the clones early in the seed train or late pooling just before inoculation had minimal growth, productivity, or product quality differences, possibly due to the growth rate of the top clones being very similar (data not shown).

It is also important to distinguish between the product quality of the molecule in cell culture harvest and in the purified drug substance. Some quality attributes such as N-glycan species may not be significantly modified by the downstream process. However, the levels of product impurities such as homodimers of multi-specific antibodies in cell culture harvest can be significantly reduced through the purification process. Thus, the differences in the product impurity levels in cell culture harvest between the pool and the lead clone may be mitigated by the downstream process to yield comparable impurity levels between the preclinical and clinical batches in the final drug substance. Recent developments in predictive process modeling may further improve the outcome in this situation. The performance of the lead clone in clinical manufacturing can potentially be predicted using a model built from the small-scale process development data of the same program and other programs' historical data. This can significantly increase our confidence of applying the "pool for Tox" approach especially for complex molecules.

The method employed for developing production cell lines plays a critical role in the "pool for Tox" strategy. Traditional random integration methods typically result in the non-specific incorporation of transgenes into the CHO genome, leading to significant variability in the growth, productivity, and product quality of the resulting cell lines [14]. This variability is further exacerbated by the common occurrences of concatemerization and/or amplification during transgene integration, which can introduce phenotypic or genetic instability in clones due to mechanisms such as gene copy loss or gene silencing [23]. In contrast, the transposon technology employed in our study allows for the scar-free, single-copy integration of transgenes at transcriptionally active sites [14]. Stable pools generated through this semi-targeted method show significantly enhanced productivity compared to those derived from random integration approaches. Additionally, these pools result in greater homogeneity in growth, productivity, and product quality among the derived clones, along with a markedly improved stability rate (unpublished data) [14]. These factors substantially increase the likelihood of identifying a stable lead clone with productivity and product quality that are comparable to both the stable pool and the pool of top clones. Targeted integration can further increase the homogeneity of stable pools by ensuring that all clones share the same integration site. This consistency can significantly

enhance the suitability of stable pools for preclinical studies [24].

While previous work by Rajendra et al. [6, 22] have evaluated the feasibility of using the transposon technology to enable "pool for Tox" for mAbs, we leveraged our transposon technology platform to confirm the feasibility and implementation of the strategy for complex molecules. In addition, to enable a robust "pool for Tox" strategy that applies to complex molecules such as multispecific antibodies or Fc-fusion proteins, we optimized the expression vectors to enhance the assembly of multisubunit molecules by balancing the relative expression levels of the various polypeptide chains (manuscript in preparation). The semi-targeted integration capability of the transposon technology in combination with vector optimization significantly increase the likelihood of identifying stable pools and top clones with good product quality.

Notably, as a rapid response to the COVID-19 pandemic, several companies utilized CHO pools for GMP manufacturing of COVID-19 neutralizing antibodies for early phase clinical trials [25–29]. Using the same approach for non-COVID-19 programs will face significant regulatory hurdles. However, since even a clonal cell line is not truly homogenous at the genomic level because of the highly plastic nature of the CHO genome, one could argue that genetically homogenous stable pools derived from targeted integration should be suitable for early phase clinical manufacturing.

Conclusions

Our study demonstrated the "pool for Tox" approach can be successfully applied to accelerate the development of non-mAb complex molecules. It is important to ensure analytical comparability and consider process performance consistency when implementing this strategy.

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Author contributions
JP, JM, AD, DB, BG, PR, XW, KS performed the studies, analyzed, and interpreted the data. RL, JP, JM, NP, SL, YH, GL designed the work. All authors contributed to writing and editing the manuscript and JP, JM, AD, DB, BG, XW prepared the figures and tables. All authors read and approved the final manuscript.

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Data availability
The datasets used during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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