REPORT

An efficient route to bispecific antibody production using single-reactor mammalian co-culture

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ABSTRACT

Bispecific antibodies have shown promise in the clinic as medicines with novel mechanisms of action. Lack of efficient production of bispecific IgGs, however, has limited their rapid advancement. Here, we describe a single-reactor process using mammalian cell co-culture production to efficiently produce a bispecific IgG with 4 distinct polypeptide chains without the need for parallel processing of each half-antibody or additional framework mutations. This method resembles a conventional process, and the quality and yield of the monoclonal antibodies are equal to those produced using parallel processing methods. We demonstrate the application of the approach to diverse bispecific antibodies, and its suitability for production of a tissue specific molecule targeting fibroblast growth factor receptor 1 and klotho β that is being developed for type 2 diabetes and other obesity-linked disorders.

The number of bispecific antibodies in clinical development and the variety of diseases being treated continue to increase, as exemplified by blinatumomab, solitomab, duligotuzumab, MGD006, AFM11, and MM-111. Bispecific antibodies offer various mechanistic advantages over monospecific antibodies, such as the ability to cross the blood-brain barrier, recruit immune cells for tumor cell killing or simultaneously aggregate 2 receptors for selective pathway activation. For example, Kolumam et al. reported the generation of a bispecific anti-fibroblast growth factor receptor 1 (FGFR1)/klotho β (βKL) antibody that can activate FGFR1 only in the presence of the coreceptor βKL. Selective activation of FGFR1/βKL receptor complex by this bispecific antibody enables reversal of obesity and insulin resistance in mice via induction of brown adipose tissue mediated thermogenesis, while sparing phosphate-related side effects of broad-FGFR1 activation.

For the Kolumam et al. study, anti-FGFR1/βKL bispecific antibody was produced using knobs-into-holes technology in which complementary non-covalent mutations in the fragment crystallizable (Fc) favor the association of 2 different heavy chains (HC) with distinct complementarity-determining regions (CDRs) (Fig. 1A). Since this bispecific antibody also contains distinct light chains (LC), a separate expression and fermentation strategy was employed to ensure cognate HC-LC pairing, followed by separate purifications of each arm, termed here the heavy-light (H-L) fragment. Once isolated, the 2 distinct H-L pools were mixed together at the desired ratio, followed by a redox step to enable HC heterodimerization and formation of interchain disulfides. The result is a stable, fully functional bispecific.

Currently, knobs-into-holes technology is one of many successful production techniques invoking HC heterodimerization to drive bispecific antibody formation. Many of these approaches involve separate expression and purification of each H-L fragment, followed by in vitro HC heterodimerization. Though effective, these strategies can be cumbersome because they require 2 expressions and 2 purifications. In addition, from a process development viewpoint, such parallel cell cultures and purifications can lead to increased risk of contamination and cost. Recently, Spiess et al. described an alternative approach using knobs-into-holes technology for E.coli single fed-batch production of bispecific antibodies with 2 distinct LCs. This method involves transformation of each heavy and light chain pair separately, followed by co-culture of the 2 different transformed cell types in a single vessel (Fig. 1B). As the H-L fragments are produced, they are secreted as half-antibody monomers into the periplasms of their respective cells. Upon harvest and cell lysis, the knob and hole H-L fragments are released into the shared vessel, where they are free to heterodimerize. Moreover, the oxidative environment created by the lysis enables proper disulfide formation between the 2 H-L fragments. The result is an E.coli-derived, stable, bispecific antibody with distinct LCs and natural IgG architecture, produced using fewer cell culture and purification steps. Hence, E.coli co-culture presents a powerful option for producing bispecific antibodies that contain minimal engineering changes, and no CH2 domain glycosylation.
Previously, we established that high-quality bispecific antibodies can also be produced from mammalian hosts, such as Chinese hamster ovary (CHO) cells. In contrast to E. coli, production in mammalian hosts enables posttranslational modification, a component often required for biological activity of antibody therapeutics. Moreover, mammalian-host cell culture offers unique process advantages such as the capacity for higher cell density, as well as proper folding and assembly. Mammalian-host, especially CHO, cell culture is also the dominant system for the production of antibody therapeutics with clinical applications.

A common strategy for the production of recombinant bispecific antibodies in a mammalian host involves transient expression in CHO cells. In contrast to E. coli, production in mammalian hosts enables posttranslational modification, a component often required for biological activity of antibody therapeutics. Moreover, mammalian-host cell culture offers unique process advantages such as the capacity for higher cell density, as well as proper folding and assembly. Mammalian-host, especially CHO, cell culture is also the dominant system for the production of antibody therapeutics with clinical applications.

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Figure 1. Comparison of different methods for producing bispecific antibodies (A) With transient transfection, the heavy chain (HC) and light chain (LC) plasmids for each H-L fragment are transfected into Chinese hamster ovary (CHO) cells and cultured separately. Antibodies are captured from the secreted media as a mixture of half-antibodies and homodimers. Bispecific assembly is performed by combining both pools together in vitro at a one-to-one molar ratio followed by addition of reduced glutathione (GSH) to catalyze disulfide formation. The result is fully oxidized bispecific antibody with very little homodimer impurities. (B) Using co-culturing in E. coli, cells are transfected with either plasmid and grown together in the same bioreactor, targeting a one-to-one molar ratio of H-L fragments based on historical data. During cell lysis, the H-L fragments dimerize and interchain disulfides are formed. Assembled bispecific antibodies are then captured from the supernatant after cell lysis with very little homodimer impurities. (C) With co-culturing of 2 stable cell lines, both cell types are grown in the same production flask targeting a one-to-one molar ratio of H-L fragments based on historical data. During cell culture, protein is secreted from each cell line as a mixture of half-antibody and homodimer. Addition of GSH favors correct pairing and disulfide formation in the co-culture media. Assembled bispecific antibodies are then captured from the secreted media with very little homodimer impurities.
2 different H-L fragments can easily be expressed in a shared culture vessel, and thus there is no need for separate HC-LC DNA transfections prior to cell mixing, as is the case with transient expression.

Accordingly, our hypothesis centered on the idea that, as distinct H-L fragments were secreted from each cell line into a shared culture vessel, knobs-into-holes mutations would thermodynamically favor assembly, while the oxidative environment in the cell culture media would drive proper interchain disulfide formation (Fig. 1C). Although it is known that H-L fragments secreted from CHO cells can be found as both homodimer and monomer, our expectation was that assembly would still be feasible because we had previously shown that homodimers had little bearing on bispecific assembly done in vitro, even with content greater than 50%, as seen by Shatz et al. Thus, it seemed likely that formation of intact bispecific antibody with cognate HC-LC pairing and proper disulfide formation in the co-culture media was feasible, and that, like in described.7,19,20 This approach, termed single-cell expression, is where all 4 chains are co-expressed in one cell have been as a fully formed IgG with native architecture, and very little antibody with cognate HC-LC pairing and proper disulfide bond were formed.

In the last few years, an increasing number of strategies where all 4 chains are co-expressed in one cell have been described.7,19,20 This approach, termed single-cell expression, is also a streamlined process with fewer steps, and thus it shares many advantages with co-culture. The main advantage of the single-cell production strategy is that singular steps are adopted further upstream along the production path, beginning with single cell line development. However, these processes require substantial upfront mutagenesis work, and can often have low yields.21-23 In addition, single-cell expression can be problematic when trying to balance antibodies with very different expression levels, whereas this is easily achievable with co-culturing.

As we demonstrate here, mammalian-host co-culture technology has many unique and desirable attributes, e.g., process efficiency, robustness, scalability, compatibility with HC dimerization techniques. It also offers the freedom to balance and change antibodies with fewer mutations compared to a single-cell approach. Overall, this novel technology has the potential to save substantial cell culture and purification resources by streamlining bispecific antibody production while retaining useful properties inherent in mammalian expression.

Results

Experimental workflow

Development of the co-culture process began with implementation of 2 analytical assays. A workflow was designed involving separation and quantification of the different antibody moieties (half-antibody, homodimer, bispecific) based on either charge using a cation exchange method (CEX) or hydrophobicity using a reverse phase method (RP). Both methods were coupled to ultraviolet (UV) detection at 280 nm whereas mass spectrometry (MS) was in-line with the RP column only. The RP method was used primarily for investigation of the total amounts of HC and LC produced by the co-cultured cells without taking into account the dynamics of bispecific assembly and disulfide formation. Each protein mixture was captured from the HCCF using Protein A; eluates were denatured and reduced, then analyzed using the RP method in-line with electrospray ionization time of flight (ESI/TOF) MS. Absorbance at 280 nm was used to calculate molar ratios of total HC and LC in each capture pool. However, the RP method was not able to resolve individual HC and LCs from each other (Fig. S1A). For those evaluations, intensities of deconvoluted ionization signals were compared. Though deconvoluted peak intensities are not strictly correlated with mass, the assumption was made that each LC would ionize very similarly to one another, and thus the intensities could be used to quantitate approximate relative abundance of each H-L fragment (Fig. S1B).

The CEX method proved suitable for investigating the dynamics of assembly in the co-culture media. Many different resins may be used to purify excess H-L and homodimers, including anion-exchange (AIX), hydrophobic interaction chromatography (HIC), or a mixed mode resin. Here, the assay was run using a sepharose-based resin (SPHP), not typically used for analytical work due to its lower resolution compared to traditional analytical CEX resins. However, for these bispecific pairs, we found that SPHP resin under high pressure proved efficient in resolving each bispecific antibody pair from its impurities (e.g., H-L fragments, homodimers, host cell proteins). Although these results may not be easily predicted based on the H-L sequences and theoretical isoelectric points (Table 1), we believe that differences in CDRs may be driving these results. In addition, use of this resin enabled simple adaptation of the CEX method for bispecific antibody purifications on a preparative scale.

Five distinct test cases were examined (Table S2), where all 5 pairs belonged to the immunoglobulin G1 (IgG1) super family and contained knobs and holes mutations. Each pair contained a unique CDR sequence, 2 different frameworks were represented and 2 pairs contained glycosylation. Once the characterization methods were in place, an analysis of expression levels was performed. This analysis was designed to assess the feasibility of achieving balanced expression of both knob and hole H-L fragments, despite variable expression titers among different cell lines. H-L fragments were expressed from stable pools or clonal cell lines and co-cultured in 0.04 L using the shake flask procedure described in the methods section. After validation of expression control, efficiency of bispecific assembly and hinge disulfide formation was examined, followed by scalability. Finally, anti-FGFR1/βKL was used for a head-to-head comparison of bispecific antibody production from separate

| Table 1. Theoretical isoelectric points (pI) for each H-L fragment and combined bispecific antibody pair. |
|-------------------------------------------------|
| | Knobs | Holes | Bispecific |
| anti-A/B | 9.0 | 9.0 | 9.0 |
| anti-C/D | 8.8 | 7.8 | 8.5 |
| anti-C2/D | 8.8 | 7.8 | 8.5 |
| anti-E/F | 8.8 | 9.0 | 8.9 |
| anti-FGFR1/βKL | 6.7 | 6.6 | 6.7 |
cultures and co-culture, as well as to functionally validate the produced bispecific molecules.

**Expression ratio of each H-L fragment can be controlled on a cellular level**

Since there were known differences in expression titers of individual cell lines (or pools), initial experiments focused on determining whether relative expression of each H-L fragment could be modulated on a cellular level. Anti-A/B pair was chosen as an initial test case due to historical knowledge of disparities in expression levels. Various cell culture ratios were prepared by mixing the 2 cell lines together and successively increasing the ratio of the lower expressing arm (anti-B). Relative abundances of each H-L pair in the co-cultured mixtures were determined by comparing the intensities of the distinct LC signals derived from the MS deconvolution spectra (Fig. S1B). As shown in Table 2, titration of cells expressing anti-B led to increases in production of anti-B. A cellular ratio of 1:7 proved best for targeting balanced expression of H-L fragments, demonstrating that balanced expression was achievable, even with dissimilar expression titers. Comparisons of expression levels while varying cell-to-cell ratios were also done for anti-C/D, anti-C2/D and anti-FGFR1/bKL. These pairs similarly demonstrated modulation of relative expression levels based on cellular ratio (Table 2). Overall, adjusting cell-to-cell ratios based on individual expression titers proved an effective means of targeting balanced expression of each H-L fragment in all 5 cases (Table 2).

### Table 2. Table summarizing the relative abundance of each H-L fragment with varying cell-to-cell ratios. Reverse phase (RP) under reducing conditions was used to characterize the relative abundance of each arm in the co-culture mixtures, as described in Methods. For each pair, bolded values denote the selected ratio. N/A denotes not applicable, for those ratios that were not done in duplicates and where no % error could be calculated.

| Cell to Cell Ratio | Knob | Hole | % Knob | % Hole | % Error (n = 2) |
|--------------------|------|------|--------|--------|----------------|
| anti-A/B           | 1 3  |      | 83.0   | 17.0   | ±1.1          |
|                    | 1 5  |      | 67.1   | 32.9   | ±1.5          |
| anti-C/D           | 1 7  |      | 49.6   | 50.4   | ±5.4          |
| anti-C2/D          | 1:2  | 1    | 40.0   | 60.0   |               |
|                    | 1:1  | 1    | 45.0   | 55.0   |               |
| anti-E/F           | 1 1  |      | 47.4   | 52.6   |               |
| anti-FGFR1/bKL     | 1 2  |      | 49.1   | 50.9   |               |
|                    | 1 1  |      | 50.0   | 50.0   |               |
|                    | 1    |      | 41.2   | 58.8   |               |
|                    | 2 3  |      | 49.3   | 50.7   |               |

#### Heterodimerization can be tuned using GSH

Once it was established that balanced expression could be controlled independently of Fab arms, our focus turned to tuning bispecific assembly. Akin to our strategy using separate cultures and in vitro bispecific assembly, production in co-culture media took advantage of knobs-into-holes Fc mutations to enable preferential heterodimerization of H-L fragments, although most any HC dimerization technique could be used instead. Previously, we have shown that GSH effectively enables proper disulfide formation between 2 H-L fragments. Examination of the effect of GSH addition on bispecific assembly from co-culture media was thus our next step. We initially added GSH to the cell cultures from a 0.5 M stock solution dissolved in water; however, a discernable effect on cell culture viability was observed early on (data not shown), which was perhaps due to the influence of GSH on the pH. Subsequent experiments were done using a...
0.5 M GSH stock solution dissolved in 1 M arginine (pH 9.0). With the exception of this finding, cell culture parameters such as titer and cell viability remained unaltered by GSH addition, even with concentrations as high as 20 mM (Fig. 2A).

Bispecific pairs were treated with GSH 15 h prior to harvest and assayed for bispecific content using the CEX method (Fig. 2). In this study, percent covalent bispecific antibody represented the abundance of assembled and oxidized bispecific molecules in each pool relative to the total amount of protein expressed and captured. When no GSH was added, differences in efficiency of bispecific assembly from co-culture media were observed. For example, without GSH addition, anti-C/D co-culture pool was captured with nearly 40% bispecific content (Fig. 2A top panel), whereas anti-E/F co-culture pool was captured with 10% covalent bispecific content (Fig. 2A bottom panel). These results confirmed that knobs-into-holes bispecific assembly is achievable without the addition of GSH. Although an explanation for the variability in assembly efficiency without GSH is not clear, we postulate these differences may be a result of culture variability, or possibly endogenous fluctuations in redox potential during cell culture. Nonetheless, adding GSH to the co-cultures significantly increased assembly efficiencies. For all 5 pairs, using optimized cell-to-cell ratios and 15 mM GSH enabled efficient formation of covalent bispecific antibody, with the percentage bispecific of total captured IgG 68–88% (Fig. 2B).

**Co-culturing of CHO stable cell lines can be scaled up in bioreactors**

During early assessment of manufacturing scalability, stable pools were used for some co-culture case studies while stable cell lines were in development. For the early co-culture experiments, titers of H-L pools were being routinely checked prior to cell mixing to confirm in-process expression levels (Unknown Titer) (Fig. 3). Once stable cell lines were chosen, the process was simplified to rely on historical titer information (Known Titer) to target one-to-one molar expression of each H-L fragment (Fig. 3). Importantly, an essential criterion for choosing lead stable cell lines was demonstration of high stability of titer and productivity over a period of time exceeding the manufacturing window.

Manufacturing processes for CHO cell culture are typically executed in bioreactors. To assess the feasibility of scaling up the co-culture process, we compared bispecific antibody originating from a 40 L bioreactor with controlled pH, temperature and dissolved oxygen to that from 0.04 L shake flask cell cultures (Fig. 4). Differences in routine production methods between 0.04 L and 40 L include vessel type and associated process parameter set points and controls, and differences in feed volumes and cell seeding densities. For comparative studies, the 0.04 L shake flask process from early co-culture development was modified slightly (see Methods) to more closely resemble the bioreactor process. Most notable are the day 3 and day 6

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**Figure 3. Schematic of CHO stable cell co-culture processes**

Overview of the different steps involved in co-culturing 2 stable cell lines. With unknown titers, scale up of seed train flask production of each cell line to 1 L and individual 35 mL shake flask cell cultures are done concurrently. At both day 7 and day 10 day, titers are measured from each 35 mL culture and the averages of these titers inform the cell-to-cell ratio used for mixing the 2 seed train flask productions in the third step. The cell mixture is then successively scaled up from 3 L to 40 L over 18 d. With known titers, individual seed train flask production is scaled up to 1 L shake flask as with unknown titers, however 35 mL shake flasks cultures are not grown concurrently. At day 10, mixing of both 1 L seed trains is done using historical titers.
splits, which were included in the shake flask process to mirror the splits required to scale up for bioreactor production.

Our assessment of scalability was predominantly focused on determining whether bispecific antibodies from the bioreactor co-culture media could be produced in similar quality and yields as those from shake flask experiments. Expressions of 3 of the 5 pairs, anti-A/B, anti-E/F and anti-FGFR1/bKL, were scaled up to 40 L and compared with expression from 0.04 L shake flask (Table 2). The feasibility of using the cell-to-cell ratio determined from shake flask for co-culture in the bioreactor was examined first. Expression ratios were compared using the RP method at the 2 culture scales. Despite an imbalance in expression levels, anti-A/B expression trends were well conserved across both culture volumes (Table S1). This data, along with results from the other 2 pairs (results not shown), suggested the cell-to-cell ratios determined in shake flask could be applied to the bioreactor co-culture process and the optimized ratios were used for subsequent analyses.

Since pH, agitation and oxygen consumption are controlled in the bioreactor but not in shake flask, process development is typically required when scaling up cell culture production. In examining scalability of bispecific assembly using co-culture, the focus was thus primarily on quality of the bispecific antibody product. In one example, the cation-exchange based method was used to compare anti-E/F bispecific antibodies isolated from the 2 different co-culture volumes (Fig. 4). Fig. 4A shows the chromatographic traces for both lots of anti-E/F, where absorbance at 280 nm was used to quantitate the relative abundance of the 3 peaks present in each pool. This comparison revealed minimal differences between the 2 traces, where bispecific antibody (Peak 2) accounted for ~63% from the 0.04 L culture (top panel) and 58% of the capture pool from the 40 L culture (bottom panel). In both cases, Peak 3 contained a mixture of covalent and non-covalent homodimer from both H-L fragments, as determined by ESI/TOF MS. The main observed difference between the chromatographic traces was Peak 1, which was more abundant in the pool coming from the 40 L culture than from the 0.04 L culture. Moreover as shown in Fig. S2, CEX results for anti-FGFR1/bKL demonstrate overall agreement between culture volumes. In this case, differences were more pronounced, with Peak 3 (anti-bKL homodimer) being the most obviously different. In addition, SEC and ESI/TOF analysis of anti E/F Peak 2 from both scales revealed minimal aggregate or homodimer impurities (Fig. 4B), highlighting that both culture scales were able to produce high-quality covalent bispecific antibody. Hence, although some differences were observed, overall results supported the notion that efficient bispecific antibody production from co-culture was scalable from 0.04 L to 40 L.

**Overall recoveries of bispecific assembly from separate cultures and co-culture yield comparable results**

Forty Liter bispecific antibody production from co-culture was also compared to our standard assembly process using separate cultures, where each H-L fragment is grown in a separate bioreactor (20 L each). The two processes were compared quantitatively by looking at assembly efficiencies and purification step
recoveries (Table 3). Assemblies from both separate cultures and co-culture yielded very similar overall recoveries, although individual step recoveries differed between assembly methods. For example, mixing H-L fragments from separate cultures in vitro yielded an assembly recovery that was less than 100%, whereas the recovery from the co-culture process was 100% (Table 3). This inequality was normalized during the cation-exchange polishing step, where a higher amount of bispecific antibody was recovered from the pool assembled in vitro relative to that from the co-culture pool (Table 3). Hence, in comparing overall recoveries, a nominal difference between bispecific production strategies was observed, where the final recovery was 0.31 g of bispecific antibody per liter of harvested cell culture using in vitro assembly, and 0.35 g per liter of harvested cell culture from assembly during co-culture. Moreover, although lower than for monoclonal antibody production, these yields are standard process yields for bispecific antibody production. In addition, assessment of protein quality revealed well behaved bispecific antibodies, with no detectable aggregate or homodimer content (Fig. 5, Table 4). These findings were consistent across the 5 different pairs (data not shown), suggesting robustness in the CHO cell co-culture production technology.

Lastly, we compared the functionality of anti-FGFR1/βKL bispecific antibodies produced from separate cultures and co-culture by employing a cell-based GAL-ELK luciferase assay, as described in Methods. Briefly, HEK293 cells expressing appropriate luciferase reporter constructs and target receptors (FGFR1 with or without βKL) were incubated with an agonist antibody, and activation of the receptor was assessed by induction of cellular luciferase activity. As shown in Fig. 6A, both assembly techniques produced bispecific antibodies with comparable dose response for FGFR1/βKL receptor complex activation. Bispecific antibodies were also assayed for βKL-independent FGFR1 agonism (Fig. 6B), a mechanism that requires bivalent anti-FGFR1 for activation, in cells expressing FGFR1 but not βKL. The lack of signaling activity in such cells indicated the paucity of anti-FGFR1-related impurities such as anti-FGFR1 homodimers or aggregated bispecific antibodies.

**Discussion**

Co-culturing of knob and hole mammalian cell lines offers a simple, robust means for the production of bispecific antibodies with natural IgG architecture. Our results indicate that assembly from co-culture media can produce therapeutically relevant bispecific antibodies that are not only functional, but also devoid of undesirable impurities. In its early conception, adapting the E.coli co-culture model for use in mammalian cell culture appeared to be limited by differences in expression and cell culture processes between the 2 organisms. When using E. coli, both HC and LC DNA are integrated into a single plasmid.

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**Table 3.** Comparison of bispecific antibody recoveries (% relative abundance) from each purification step. Limit of detection (LoD) indicates the value was below assay detection limit.

| Production Method | Assembly Recovery (%) | CEX Recovery (%) | Total Recovery (%) | Monomer by SEC (%) | Homodimer (%) |
|-------------------|-----------------------|-----------------|-------------------|--------------------|--------------|
| Separate Culturing| 62.8                  | 57.8            | 21.2              | 98                 | <LoD         |
| Co-Culturing      | 100                   | 40.5            | 29.9              | 99                 | <LoD         |

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**Figure 5.** Characterization of anti-FGFR1/βKL bispecific antibody pairs assembled in vitro and captured directly from co-culture media (A) SEC chromatograms of bispecific antibodies produced from separate cultures and assembly in vitro (top panel), and from assembly in co-culture media (bottom panel). The insets show a zoomed-in view indicating both assembly methods produce bispecific antibody with less than 1% aggregate and very minimal residual H-L fragments. (B) Bispecific antibodies from both assembly methods were analyzed by ESI-TOF MS as described in Methods. The top panel shows the deconvoluted spectra over a mass range of 25,000–160,000 amu, indicating the overall purity of the bispecific antibody. The bottom panel shows a zoomed in view around the bispecific antibody and the arrows point to the theoretical locations of each knob/knob (K/K) and hole/hole (H/H) homodimer.
Cells are transformed individually, with each HC-LC plasmid and mixed together targeting one-to-one expression of each H-L fragment. This is typically a straightforward process if expression titers are known and reproducible, and H-L fragments are produced as monomers, with very little homodimer impurities. In contrast, early stage mammalian host antibody production generally relies on transient expression, where HC and LC genes are expressed non-chromosomally. When CHO cells are used, it is common for cells to be simultaneously co-transfected with separate HC and LC DNA. In principle, transient expression is compatible with bispecific antibody production using co-culture, and a transient expression strategy was considered for this study. However, in order to avoid non-cognate HC/LC pairing, at least one extra step involving transient transfection of each HC/LC pair into separate cells and combination of the cells only after gene uptake has occurred would likely have been necessary. Furthermore, transient expression is often an unreliable process, with relatively low and inconsistent titers. For these reasons, we excluded transient expression as a strategy to be used in conjunction with co-culturing for the production of bispecific antibodies from mammalian host cells.

The development of stable cell lines (and stable pools) enabled a more straightforward approach for the production of knobs-into-holes bispecific antibodies from mammalian-host co-culture, akin to use of E.coli. Since HC and LC gene integration into the genome is inherent to a stable cell line, routine co-culturing of 2 stable cell lines can originate in a shared culture vessel, without the possibility of DNA cross contamination. In addition, stable cell lines provide consistent, high titers, permitting greater control over expression of each H-L fragment. Importantly, in earlier experiments where stable pools were being used, titer stability was not known. For this reason, the original workflow involved measurement of individual titers and comparison to historical data prior to cell mixing. Nevertheless, in all cases historical titers and newly measured titers differed only marginally (≤ 10%), and thus, when experimental work moved to the bioreactor, titer analysis was removed. It is important to note that, for commercial application of co-culture, a more long-term examination of cell-line expression titer stability would need to be performed.

Although efforts described here focus on stable cell co-culture for the production of bispecific antibodies, mammalian-host cell co-culture may in principle also be compatible with transient expression. An advantage of transient expression is the avoidance of any stable pool or cell line development, which can be costly and time consuming. As delineated previously, transient transfection is currently not amenable to expression of 2 different H-L fragments in the same culture vessel. However, as mentioned an extra step could be added to the co-culturing process, in which cells are transfected separately with each HC-LC pair and mixed together only once gene uptake into the cell is complete. This type of strategy could potentially offer advantages for small-scale bispecific antibody production, where several pairs or affinity variants could be rapidly assessed before investment in stable cell line development is made. Nonetheless, adding extra steps could pose additional production challenges, such as prolonging timelines, additional cost of goods or introduction of contaminants.

We observed that the addition of GSH to cell culture media prior to harvest greatly increased bispecific formation. In E.coli fermentation, H-L fragments are secreted as half-antibodies only and GSH is endogenous to E.coli cells. Thus, at the time of harvest and cell lysis, H-L fragments are assembled and oxidized without addition of a reducing agent. However, mammalian-host cell culture does not have the same set of metabolites

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Table 4. Comparison of theoretical masses with those measured by ESI-TOF. Limit of detection (LoD) indicates the values were below the assay detection limit.

| Functional Mass (Da) | Measured Mass (Da) |
|----------------------|--------------------|
| anti-KL/\( \text{KL} \) | 143,488.6          |
| anti-FGFR1/FGFR1     | <LoD               |
| anti-FGFR1/\( \text{KL} \) | 144,477.7          |
| anti-FGFR1/\( \text{KL} \) | 144,483.7          |
| anti-FGFR1/\( \text{KL} \) | 144,486.8          |

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Figure 6. Comparison of anti-FGFR1/\( \text{KL} \) bispecific antibody activity derived from different cell culture processes reveals no functional differences. (A) Anti-FGFR1/\( \text{KL} \) bispecific antibodies produced from separate cultures with in vitro assembly and assembly in co-culture media were tested for activation of FGFR1 receptor in the absence of \( \text{KL} \) expression. The lack of signaling activity is an indication of non-detectable anti-FGFR1-related impurities in the bispecific antibody pools using both strategies. (B) In cells expressing both FGFR1 and \( \text{KL} \), cell-specific activation of FGFR1/\( \text{KL} \) complex with the bispecific antibodies produced from both methods was tested. Equivalent activity was observed with antibodies from both production methods, where the error bars indicate standard error of the mean. Included in both assays is a reference standard (Ref. STD) of anti-FGFR1/\( \text{KL} \), where the bispecific antibody was produced from assembly in vitro and polished to a high purity, confirming expected activity of the assembled bispecifics, in addition to full length, parental anti-FGFR1 (R1Mab3)\textsuperscript{a} and anti-\( \text{KL} \) (KLBMab1)\textsuperscript{a} antibodies.
present. Moreover, CHO H-L fragments are secreted as both homodimers and half-antibodies. Thus, addition of a reducing agent, in this case GSH, greatly increased homodimer disruption and disulfide formation. However, the addition of a reducing agent is not absolutely required; when examining the effects of GSH, some pairs readily formed covalent bispecific without the addition of GSH. Nonetheless, adding GSH prior to harvest increased bispecific antibody content, and ensured robustness of assembly from co-culture media in all 5 cases.

Translatability of results from experiments done in shake flask was demonstrated by comparison with large scale bioreactor production using anti-FGFR1/βKL. As outlined in the Results section, expression ratios yielded generally equivalent results across scales. Small differences in expression levels of different moieties within each co-culture pool were observed, but this was expected. Since differences in cell culture environments exist between the 2 scales, it was not unexpected to observe some small fluctuations in levels of impurities. Yet, overall, the same cell-to-cell ratio was successfully applied to the 2 different expression scales using very little process development, and yielded similar bispecific antibody recoveries. A more complete understanding of the implications of co-culture in a manufacturing setting will require process development work, akin to process development for the production of any biologic.

Upon evaluation of the 2 different bispecific antibody production strategies, a comparison of overall titer, quality and functionality revealed minimal differences, though individual step recoveries differed between assembly methods. This discrepancy in step recoveries was expected and attributed to inherent differences in expression and assembly steps between the 2 processes. Moreover, observation of these differences provided insight into comparability of product-related impurities emerging from each production method. Since the same downstream purification techniques were used, overall agreement in final recoveries suggests impurities from expression and assembly may be similar in characteristics.

In summary, the development of mammalian-host co-culture has enabled a rapid and cost effective method for the production of bispecific antibodies. Akin to use in E.coli, this novel mammalian-host technology has numerous advantages over conventional production methods. It saves time by removing the need for parallel cell culture and purification steps. It is robust and straightforward, enabling a “plug and play” approach. It is designed to be compatible with any bispecific technology using HC heterodimerization, e.g., controlled Fab arm exchange (cFAE),13 strand-exchange engineered domain (SEED),10 charge pair residues,9 HC hinge point mutation technology.12 And finally, it shows promise for use with manufacturing scale production.

Preliminary work has suggested that, even when H-L expression is imbalanced, the limiting H-L arm will nonetheless participate in bispecific antibody formation. Thus, it is likely that mammalian-host co-culture can be compatible with transient expression even though expression titers are typically unknown or unpredictable. It may also prove well suited for high throughput screens or proof of concept experiments where only a small amount of bispecific antibody is needed. Overall, mammalian-host co-culture is well matched for a growing pipeline as it offers versatility, while remaining cost efficient by saving on time and resources.

Materials and methods

Cloning of half-antibodies

Each antibody sequence was inserted in the Genentech proprietary expression vector cell line. The knob and hole mutation sequences used were those cited in Ridgway et al., 1996.6 In addition, all but 2 pairs had the N-linked glycosylation sites removed (Table S2).

Stable cell line development

CHO-K1 cells were transfected using Lipofectamine 2000 CD according to the manufacturer’s recommendation (Invitrogen, Carlsbad, CA). Transfected cells were selected at various concentrations of MSX (25 and 50 uM). Picked clones were evaluated for antibody production by sampling supernatant from clones and analyzing by ELISA. To make stable pools, on the basis of ELISA titers, the top 48 clones were combined and scaled up. Individual clones were also expanded and scaled up for antibody productivity evaluation to determine the top clones.25,26,18

Fed-batch shake flask antibody productivity evaluation and sample analysis

Shake flask antibody production was a 14 day fed-batch culture process in proprietary production medium with 3 bolus feeds on day 3, 7 and 10, and a temperature shift from 37°C to 33°C on day 2. Individual shake flask production for the knob and hole pools or cell lines was carried out prior to co-culture study so as to determine the antibody titer of the individual pool or cell line. Antibody titers were determined using conventional protein A affinity chromatography with UV detection. Culture samples were also analyzed for viable cell concentration and viability by a Vi-Cell AS cell counter (Beckman Coulter, Fullerton, CA). The production process of co-culture was similar to that of fed-batch shake flask production of individual cell lines. The two pools or cell lines were mixed either in seed train culture prior to setting up shake flask experiments or mixed directly into production medium on day 0 of the shake flask production. Mixing ratio was based on titer and cell growth differences between the 2 knob and hole pools or cell lines. The aim was to target a molar ratio of 1:1 of the half antibody at the end of the production. Fifteen hours prior to harvest, reduced GSH (Sigma Life Science, St Louis, MO) dissolved in either water or 1 M arginine, 400 mM succinic acid pH 9.0 was added to the production as a reducing agent to facilitate bispecific antibody formation.

Shake flask/bioreactor cell culture comparison

Evaluation of differences between small scale and large scale cell culture was accomplished by comparing 40 mL shake flask co-culture to 40 L bioreactor co-culture. Both culture volumes began with CHO cells being cultured in 40 mL of a proprietary
DMEM/F12-based medium in 125 mL shake flask vessels at 150 rpm, 37°C and 5% CO₂. For small scale, 3 feeds at 10% volume were used and cells were passaged with a seeding density of 4 × 10⁶ cells/mL every 3 to 4 d. Cells were split on day 3 and day 6 and following addition of 15 mM GSH 1 day prior, cells were harvested on day 12.

For bioreactor cultures, a single feed at 20% volume was used and cells were seeded at 1.2 × 10⁶ cells/mL in 2.8 L of complete media. After 72 h, the culture was shifted from 37°C to 35°C, and 250 mL/L batch feed was added. Cells were split on day 3 for scale up to 10 L wavebag (N-1), followed by another split on day 6 for scale up to 40 L (N) (Fig. 4). The 40 L glass bioreactor was agitated at 90 rpm and maintained at a pH set point of 7.0 ± 0.03 and 30% dissolved oxygen (dO₂). Fifteen mM GSH was added on day 11, followed by cell harvest on day 12.

**Purification**

Assemblies done in vitro and subsequent purifications were done following the method described in Shatz et al. For bispecific antibodies assembled using co-culture in shake flasks, HCCFs underwent purification using 1 mL PhyNexsus Phytips containing 160 μL protein A resin. Samples underwent 2 sets of captures. During each capture, IgG was bound to the resin during a 4-cycle binding step, eluted with acetic acid and then immediately neutralized with 1 M HEPES.

For 40 L bioreactor co-cultures, 1 L of HCCF was passed over a 100 mL GE Healthcare MabSURE SELECT column. The column was then washed with 10 column volumes (CV) of an equilibration buffer consisting of 50 mM TRIS pH 7.5, 150 mM NaCl, followed by a wash buffer consisting of 25 mM sodium citrate, pH 6.0 and eluted with 0.15 M sodium acetate, pH 2.7. Following protein A capture, each eluate was loaded onto a 5 mL GE Healthcare strong sulfopropyl (SP) high performance (HP) cation exchanger (CEX) column. The column was washed with 5 CVs of 25 mM sodium acetate pH 5.0, then eluted with 1 M NaCl over 30 CVs.

**Characterization**

Protein A elution pools were analyzed by one of 2 methods. In the RP method, protein A eluates were diluted one-to-one in 25 mM NaOAc pH 5.0 and loaded onto a prepacked 1 mL GE Healthcare SP HP cation-exchanger column, operated under high pressure. This resin contains 6% cross-linked agarose, has an average particle size of 34 μm and a dynamic binding capacity of 80 mg/mL (lysozyme). The CEX step used standard strong cation exchanger methodology, where each eluted pool was firstly washed with 5 CVs of 25 mM sodium acetate pH 5.0 followed by elution based on net charge, using a gradient of 1 M NaCl over 30 CVs, previously optimized for each pair.

Identities of peaks of interest were determined using ESI/TOF operating in positive ion mode with deconvolution using the maximum entropy algorithm. In addition, the peaks were collected and assessed for aggregate levels and native conformation by SEC using a Sepax ZENIX SEC-300 column (7.8 × 300 mm), run under isocratic conditions using phosphate-buffered saline pH 7.2, with 150 mM NaCl spiked in.

**Luciferase activity assay**

The activity of bispecific antibody from separate cultures and bispecific antibody assembled from co-culture media toward the fibroblast growth factor receptor 1c (FGFR1c)/βKL complex was determined using a luciferase-based reporter assay in cells expressing GAL4ELK1 fusion protein and a reporter gene that encodes firefly luciferase under the control of GAL4 binding sites. In this assay, HEK293T cells were transiently transfected with expression vectors encoding human FGFR1c/βKL or human FGFR1c alone, as well as plasmids containing a firefly luciferase reporter driven by GAL4 binding sites (pFR-luc, Agilent), a GAL4-ELK1 transcriptional activator fusion (pFA2-ELK1, Agilent) and a reference Renilla luciferase reporter (pRL-SV40, Promega/GAL-ELK1).

The FGFR1c/βKL or FGFR1c expressing cells were incubated with various concentrations of ligand starting with a concentration of 100 ng/mL for 7.5 h. FGFR activation leads to phosphorylation of GAL4-ELK1 fusion protein and expression of firefly luciferase protein. The cellular luciferase activity was determined using the DualGlo® Luciferase Assay System (Promega) and EnVision® Multilabel Reader (PerkinElmer). Relative light units (RLUs) represent the ratio between firefly (test) and Renilla (reference) luciferase. Included in both assays was a control version of anti-FGFR1/βKL, which was assembled in vitro and polished to a high purity, confirming expected activity of the assembled bispecifics, in addition to full length, parental anti-FGFR1 (R1Mab3) and anti-βKL (KLBMab1) antibodies. Data analysis was performed using GraphPad Prism 6 software.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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