Multi-omics profiling of a CHO cell culture system unravels the effect of culture pH on cell growth, antibody titer and product quality

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Abstract

A robust monoclonal antibody (mAb) bioprocess requires physiological parameters such as temperature, pH, or dissolved oxygen (DO) to be well-controlled as even small variations in them could potentially impact the final product quality. For instance, pH substantially affects N-glycosylation, protein aggregation and charge variant profiles, as well as mAb productivity. However, relatively less is known about how pH jointly influences product quality and titer. In this study, we investigated the effect of pH on culture performance, product titer and quality profiles by applying longitudinal multi-omics profiling, including transcriptomics, proteomics, metabolomics and glycomics, at three different culture pH set points. The subsequent systematic analysis of multi-omics data showed that pH set points differentially regulated various intracellular pathways including intracellular vesicular trafficking, cell cycle, and apoptosis, thereby resulting in differences in specific productivity, product titer and quality profiles. In addition, a time-dependent variation in mAb N-glycosylation profiles, independent of pH was identified to be mainly due to the accumulation of mAb proteins in the endoplasmic reticulum (ER) over culture time, disrupting cellular homeostasis. Overall, this multi-omics-based study provides an in-depth understanding of the intracellular processes in mAb-producing CHO cell line under varied pH conditions and could serve as a baseline for enabling the quality optimization and control of mAb production.

INTRODUCTION

Chinese hamster ovary (CHO) cells are one of the main host systems used by the biopharmaceutical industry to manufacture biologics such as monoclonal antibodies (mAbs). While advances in process development, cell line engineering and clonal selection have largely improved recombinant mAb yields, ensuring consistent product quality still remains a major challenge (Hong et al., 2018). A key product quality attribute is N-linked glycosylation, which has been reported to significantly impact the biological activity of mAbs

(Jennewein and Alter, 2017). For example, the absence of fucose in the terminal N-glycan of the fragment crystallizable (Fc) region of mAbs is reported to increase antibody-dependent cell-mediated cytotoxicity up to 50-fold (Shields et al., 2002), while N-glycan structures with high mannose content lead to decreased *in vivo* half-life (Goetze et al., 2011). In addition to N-glycosylation, protein aggregation is another key determinant of mAb quality (Paul et al., 2018). Particularly, high molecular weight (HMW) aggregates have been shown to enhance the risk of undesirable immunogenicity (Vázquez-Rey and Lang, 2011). Variations in protein charge can also adversely affect the pharmacokinetics of the mAb (Xie et al., 2016), especially decreases in net positive charge (leading to formation of acidic variants) can decrease tissue retention and increase whole body clearance of antibody (Boswell et al., 2010). Therefore, to ensure cells are in desired metabolic state for consistent quality profiles of mAbs, cell culture processes need to be well controlled by maintaining process parameters within an acceptable range.

Several cell culture process parameters including media components, feeding strategy, temperature, pH and osmolality are known to influence the quality profiles of mAbs. Among these, pH is a critical parameter which has been shown to greatly affect N-glycosylation profiles (Aghamohseni et al., 2014; Ivarsson et al., 2014; Jiang et al., 2018), aggregation levels (Paul et al., 2018) and charge variant species (Xie et al., 2016) of the mAb product. Current understanding of how process conditions affect quality profiles has stemmed from empirical understanding and experience within each organization without detailed scientific discussion in literature. With the greater emphasis placed by regulatory agencies on adopting the quality by design (QbD) approach in process understanding and product quality control, a more detailed characterization of cellular mechanisms arising from changes to process conditions, in part, facilitates the development of rational control strategies (Sommeregger et al., 2017). In this regard, a knowledge-driven approach based on the combined use of systems modeling and high-throughput "-omics" data profiling offers immense potential to link process conditions and/or clonal traits with product quality in a systematic manner (Lakshmanan et al., 2019; Yusufi et al., 2017). Only a handful of studies have adopted high-throughput "-omics" methodologies to understand the biological mechanisms in cell culture conditions that affect productivity and/or quality profiles.

Despite the enormous potential, significant resource demand has limited the ability of organizations to take on these omics approaches. Most efforts have utilized only a single omics or at most two different omics approaches for their investigations since substantial resources and knowledge are required to extend it further for multi-omics analyses. Such examples include the application of transcriptomics and proteomics profiling to identify the pathways that are associated with improved recombinant protein titers in low-temperature CHO cultures grown under batch or perfusion modes (Baik et al., 2006; Kaufmann et al., 1999; Tossolini et al., 2018). Similarly, a combination of metabolomics and proteomics detected higher reactive oxygen species (ROS) and glutathione peroxidase expression levels during the CHO inoculum scale-up step, indicating hypoxia as the cause of lower productivity (Gao et al., 2016). More recently, proteomics was used to assess the effect of pCO_2, media hold duration, and media manganese content on mAb N-glycosylation structures in CHO fed-batch cultures (Nguyen Dang et al., 2019). However, to the best of our knowledge, no in-depth investigation has combined more than two "-omics" datasets to discern the complex underlying mechanisms between changes in process conditions and their impact on the product titer and/or quality profiles.

In this study, we obtained comprehensive time-based transcriptomics, proteomics and metabolomics data from CHO fed-batch cell cultures as a result of varying pH set points to understand the cellular effect of pH controls on the biology of CHO cells. Relevant statistical analyses of multi-omics datasets were carried out to investigate the variations in both mAb titer and quality, including N-glycosylation, protein aggregation and charge variants, as a consequence of pH variation. Our study pinpointed the influences of pH and cell culture time on mAb quality attributes through varying responses of several pathways including N-glycosylation, intracellular vesicular trafficking, and response to ER and oxidative stress. Specifically, we noted culture pH set points impacted organellar homeostasis, N-glycosylation, and vesicular trafficking efficiency, thus resulting in differences in cell productivity and product quality.

MATERIALS AND METHODS

The materials and methods used in seed train process (cell line and media used); production process and bioreactor operations (14-day fed-batch culture); transcriptome, proteome and metabolome profiling of CHO cells; IgG quality attribute analyses (purification of harvest filtrate; N-glycome, charge variant and aggregation analyses), and computational analysis of multi-omics data, and the determination of specific growth and metabolite consumption/production rates, are presented in **Supplementary Methods** .

RESULTS

Impact of culture pH and time on cell performance and mAb quality attributes

14-day fed-batch 2 L bioreactor cultures of a mAb-producing GS-CHO cell line were performed at three defined pH set points of 6.7 (low), 6.9 (medium) and 7.1 (high) in biological triplicates. Growth profiles of cultures at medium and high pH were shown to be comparable achieving peak viable cell densities (VCD) at 8.68×10^6 – 1.10×10^7 cells/mL (**Figure 1A**) and cumulative viable cells (CVC) at 8.20×10^7 – 9.71×10^7 cells.days/mL (**Figure 1C**). The first 5 days of culture was considered as exponential growth phase with all cultures achieving highest growth rates at their respective culture pH conditions. Stationary phase occurred between days 5 – 10 during which the cultures maintained steady growth and cell viability. Death phase was assigned to cultures after day 10 when both viable cell density and viability began to decline.

Overall, cultures controlled at low pH exhibited lower VCD ranging from 5.40 – 6.85×10^6 cells/mL, suggesting that maintenance of low pH hindered cell growth as reflected in the specific growth rates during the first 6 days (**Figure 1E**). In agreement with the lower peak VCD, bioreactor cultures at low pH showed a significantly lower mAb titer compared to cultures controlled at medium pH and high pH (**Figure 1D** ; repeated measures two-way ANOVA with Tukey's post hoc tests, p <0.05) although specific productivity (qP) was slightly higher in low pH in later phases of the cell culture (**Figure 1F**). Daily glucose, lactate, glutamine, glutamate and ammonium concentrations were obtained throughout the 14-day bioreactor cultures (**Supplementary Figure 1A-E**) and used to calculate consumption or production rates across culture growth phases (**Supplementary Figure 1F-J**). Glutamine and glutamate concentrations (**Supplementary Figure 1C and 1D**) and specific consumption rates (**Supplementary Figure 1H and 1I**) were comparable regardless of culture pH set points. However, glucose consumption and lactate production rates under high-pH culture set point trended higher than those of their lower pH set points (**Supplementary Figure 1F and 1G**). Notably, such trend was also similar to a previous study where increased glucose consumption and lactate production were observed in mAb-producing GS-CHO cells under high pH cultures (Jiang et al., 2018). In addition, we noted that the low-pH cultures accumulated ˜3× higher concentrations of ammonium than their medium- and high-pH counterparts from day 8 (**Supplementary Figure 1E**) with the most dramatic differences in ammonium production rate occurring between days 2 – 6 (**Supplementary Figure 1J**). This time period coincided with slower growth (**Figure 1A, 1C**) and lower titer (**Figure 1D**) and lower glucose consumption (**Supplementary Figure1F**) in the low-pH cultures.

Due to the observed changes in extracellular biochemical profiling, we investigated whether the variation in mAb quality profiles are affected across different pH conditions. To do so, cell culture supernatant samples were obtained from the three pH set points over the 14-day runs and analyzed for N-glycosylation, charge variants and aggregation levels (quality attributes). Using hierarchical clustering (see**Methods**), we analyzed how the quality attributes vary with respect to pH and time and observed the clustering of the quality attributes into three groups (**Figure 2; Supplementary Figure 2**). Group 1 attributes were elevated in low-pH – high-mannose structures, G0, G0-GlcNAc, G0F-GlcNAc ("early glycans") and Total Basic; group 2 attributes increased with time – agalactosylated structures G0F, G0F+GlcNAc ("intermediate glycans"), Total Acidic and Total HMW; and group 3 attributes decreased with time – galactosylated structures G1', G1F/G1F', G2F and sialylated structures A1G1F and A1G2F ("complex glycans"), Main, Total LMW and

Monomer. Similar to earlier reports (Ivarsson et al., 2014; Jiang et al., 2018; Xie et al., 2016), we observed N-glycosylation and charge variant differences arising from cell culture pH. Among the three pH conditions, low pH culture exhibited the most dramatic effects on group 1 attributes compared to their medium and high pH counterparts (**Figure 2**). Common observations for groups 2 and 3 product quality attributes for the three pH conditions are that the changes are culture time dependent. Although the overall degree of changes varies, the directionality of change is consistent across the pH conditions (**Figure 2**).

We noticed that mAb N-glycosylation varied with respect to both culture pH set points and time. Early glycans (group 1) such as high-mannose structures and G0 were consistently elevated in low pH across most of the days in cell culture. On the other hand, intermediate glycans (group 2) tended to increase with time, with greater effect at medium and high pH than low pH. Complex glycans (group 3) generally decrease with time suggesting that none of the culture conditions were able to maintain the same levels of complex N-glycosylation over the entire cell culture. Previously, G1F+G2F was reported to increase with pH in the range of 6.9 – 7.1 but remained relatively unchanged outside of this range (Jiang et al., 2018). In this regard, while we found galactosylated structures to be most dramatically influenced by time, they decreased slightly as pH set point decreased (16.2%, 15.0%, 10.7% in high-, medium-, low-pH cultures respectively on day 14). We also observed increasing amounts of high-mannose (1.17%, 1.26%, 1.79% respectively) and agalactosylated structures (82.7%, 83.7%, 86.6% respectively). Similar to N-glycosylation, charge variants also varied with culture pH and time. Total basic variants (group 1) was higher in low-pH culture and conversely, total acidic variants (group 2) was higher in high-pH culture, while both increased with time (particularly in low-pH and high-pH respectively). The decrease of acidic variants in pH-downshift culture conditions (pH 6.95 to 6.75) and increase over time regardless of pH were similarly reported in 14-day fed-batch CHO cell cultures (Xie et al., 2016). Unlike N-glycosylation and charge variants, product aggregation levels do not appear to be affected by pH set points, similar to previous studies that found no significant impact of pH on protein aggregation in batch and fed-batch CHO cell cultures (Paul et al., 2018; Xie et al., 2016). It could be observed that HMW (high molecular weight) species (group 2) increases with culture time while LMW (low molecular weight) species and monomer levels (group 3) decrease with time, irrespective of pH set points.

Intracellular multi-omics profiling of key biological pathways impacted by culture pH

To understand how culture pH could alter the observed cell culture performance and product quality, multi-omics profiling including transcriptomic, proteomic and metabolomic (polar and lipid metabolites) approaches, was carried out on cell pellet samples obtained from the inoculum and at specific time-points (days 1, 5, 8, 10, 12, 14) throughout the bioreactor runs. The raw omics files were preprocessed and normalized for principal component analysis (PCA) and correlations with mAb quality profiles (**Figure 3**). PCA of the individual omics datasets (**Figure 3A**) showed that culture time (PC1) was the largest contributor of variation among the samples, followed by culture pH (PC2) which had more pronounced effects on the transcriptome and proteome than the metabolome. The metabolomic data showed a biphasic effect as a function of culture time (PC1) and this may indicate that the general cellular metabolic changes are governed by differences between exponential (up to day 5) and stationary (days 5 - 10) phases. The differences observed for transcriptomic and proteomic findings indicating that transcription and translation activities are impacted by culture pH conditions, are consistent with our hierarchical clustering analysis (**Figure 2**) of the product quality phenotypes.

In order to further elucidate the specific cellular transcripts and proteins that are responsible for these observed phenotypes, we first identified the differentially expressed features (FDR-adjusted$p$ -value < 0.01) in all three omics datasets across the different pH conditions (**Figure 3B**). This analysis uncovered 3,246 transcripts, 212 proteins and 364 metabolites that were differentially expressed upon pH variations. Enrichment analysis of the differentially expressed features further revealed 231, 126 and 40 biochemical pathways to be differentially regulated at transcript, protein and metabolite levels respectively (**Figure 3B**). Among the cellular pathways significantly enriched (p -value < 0.05) in at least two of the omics datasets (**Supplementary**

Table 1), pathways directly involved in the process of secretory protein expression, including generic transcription and translation processes as well as glycosylation, nucleotide sugar transport and vesicular trafficking in the endoplasmic reticulum (ER) and Golgi cisternae, are identified and subsequently found to correlate well with observed product quality differences in glycosylation and charge variant species. Additionally, differential expression analysis also indicated pH-specific effects on cell cycle and apoptosis in agreement with cell culture profiles (**Figure 1**), with potential involvement of the PI3K-Akt signaling pathway accounting for significantly lower viability observed at later stages of culture in high pH condition (see**Supplementary Results** for details). Culture pH also has a differential effect on the metabolism of various classes of biomolecules and the cellular response to culture stress, such as hypoxia, heat stress, cellular senescence and reactive oxygen species (**Supplementary Table 1**).

To gain further insights into specific biological pathways associated with variation in mAb quality attributes, we ranked the omics features by their average Pearson's correlation to each group of quality attributes with similar trends (**Figure 2**), and carried out gene set enrichment analyses using GSEA Preranked (Subramanian et al., 2005). Various biological pathways, many of which were already identified in the enrichment analysis of differentially expressed features, correlated with these three groups of quality attributes (**Figure 3B** ;**Supplementary Tables 2 - 4**). For example, ER-to-Golgi anterograde transport, intra-Golgi transport and Golgi-to-ER retrograde transport protein expression are positively correlated with early glycans (group 1), indicating that proteins involved in vesicle-mediated transport between ER and Golgi tend to be more highly expressed in low-pH conditions. In addition, proteins present in the pathways "IRE1α activates chaperones" and "detoxification of reactive oxygen species" are positively correlated with intermediate glycans (group 2) which tend to increase with time, indicating that there is an upregulation of ER stress and oxidative stress response genes as the culture progresses.

Culture time regulates ER and oxidative stress responses

Biosynthesis of all secretory proteins occurs through the intracellular secretory pathway that begins with co-translational translocation from the cytosol into the endoplasmic reticulum (ER) (**Figure 4**). N-linked glycosylation of secretory proteins is initiated in the ER where the glycan is added onto proteins at the consensus sequence Asn-X-Ser. The ER luminal pH (˜pH 7.2) is slightly more acidic than the cytosolic pH (˜pH 7.4), thus ensuring oligosaccharyltransferase efficiency in transferring precursor glycans to the co-translational translocated protein acceptors. In addition to being the site of glycosylation initiation, the ER is also the quality check center that monitors cellular stresses and triggers proper stress responses. As the glycoproteins move through the Golgi cisternae and secretory vesicles as they exit into the extracellular milieu, the luminal pH of different organelles in the secretory pathway maintain a gradient of increasing acidity (**Figure 4**) which is essential for proper post-translational processing and trafficking of secretory proteins (Paroutis et al., 2004).

Since each organelle has an optimal pH, we speculate that pH culture set point may alter the efficiency of each organelle and in turn affect mAb product quality profiles. GSEA Preranked analysis using the Pearson's correlation values of omics features with product quality attributes, revealed that intermediate glycans (group 2 attributes) were correlated with gene expression related to unfolded protein response and ER stress response ("IRE1α activates chaperones", proteomics $p< 0.001$, transcriptomics $p = 0.0026$)**(Supplementary Figure 3A; Supplementary Tables 2C and 3C**) and oxidative stress response ("detoxification of reactive oxygen species", proteomics $p = 0.022$) (**Supplementary Figure 3B**). These pathways included known markers of ER stress (e.g. HSPA5, HYOU1) and oxidative stress (e.g. SOD2, CAT) which showed increased expression as the culture progressed, indicating that CHO cells experienced increasing levels of ER and oxidative stress independently of culture pH. In addition, as levels of intermediate glycans are positively correlated with expression of proteins involved in fatty acid metabolism (**Supplementary Figure 4A; Supplementary Table 3C;** proteomics $p = 0.061$), we further inspected a key component of fatty acid metabolism - fatty acid β-oxidation, a catabolic process that is associated with formation of reactive oxygen species such as superoxide and hydrogen peroxide (Quijano et al., 2016). We found that the expression of several genes

(e.g. HADH, SLC25A20 at protein level; CD36, CRAT, MLYCD at transcript level) (**Supplementary Figure 4B**) and abundance of short-chain acylcarnitines statistically increased as the culture progressed (**Supplementary Figure 4C;** FDR-adjusted $p < 0.01$). Overall, during exponential growth phase, stress responses were low, but were elevated as the cultures entered stationary phase and into death phase, and these correlated with increase in intermediate glycans (concomitantly a decrease in complex glycans) towards the end of culture.

Total high molecular weight (HMW) species increased with culture time, similarly to intermediate glycans (**Figure 2** ;**Supplementary Figure 2**). The increase of HMW species at later stages of culture corresponded strongly with unfolded protein response (UPR), ER stress and oxidative stress, all of which suggest an accumulation of unfolded proteins in the ER and disruption to ER homeostasis. Aggregation of mAb can be induced by partial protein unfolding, leading to structural changes that expose hydrophobic stretches which can constitute aggregation nuclei (Li et al., 2016), and the protein unfolding and aggregation can be mediated by protein adsorption to bulk interfaces, chemical degradation (e.g. deamidation, oxidation) or fragmentation of the mAb (Roberts, 2014). Protein disulfide isomerases (PDIs) act as chaperone proteins to induce the refolding of misfolded or unfolded proteins, and the protein expression profiles of four PDIs – P4HB, PDIA3, PDIA4 and PDIA6 – corroborated the presence of unfolded proteins in our cell cultures; they were found to be highly correlated with HMW levels and significantly upregulated over culture time (FDR-adjusted $p < 0.01$;**Supplementary Figure 5**). Aggregation at late stage culture is also previously observed to arise from the lack of N-glycosylation (as a result of deglycosylation or aglycosylation), which promoted the formation of mAb dimers likewise through exposure of hydrophobic residues upon absence of glycosylation (Onitsuka et al., 2014). The dimer subsequently acts as an aggregation nucleus and promotes polymerization of the antibody into large aggregates. The cause of absence of glycosylation is not known but given that ER is the site of glycan precursor synthesis and attachment, disruption of ER homeostasis may potentially contribute to protein aglycosylation.

Culture pH- and time affect protein trafficking and glycosylation

When we further examined correlation analyses of mAb quality profiles, we found that protein and transcript expression of ER-to-Golgi anterograde transport genes (proteomics GSEA Preranked p=0.0037, transcriptomics p<0.001), intra-Golgi and Golgi-to-ER retrograde transport genes (proteomics and transcriptomics GSEA Preranked p<0.001) were influenced by culture pH set point (**Supplementary Figure 6; Supplementary Tables 2A and 3A**). Particularly, evaluation of the genes involved in protein trafficking in the secretory pathway revealed higher expression of certain ER and Golgi vesicular transport proteins in low-pH cultures, with a similar distribution to that of early glycans in these cultures (**Supplementary Figure 6**). These proteins include RAB1A and RAB1B, Rab GTPases which are localized to ER-Golgi intermediate compartment and Golgi, and are required for targeting and fusion of ER-derived vesicles to Golgi; YKT6 which is a component of v-SNARE (vesicular soluble NSF attachment protein receptor) complex that mediates vesicle docking to cis-Golgi; COPI coatomer complex subunits COPA and COPB1 which are involved in intra-Golgi transport as well as retrograde vesicle transport from the Golgi to the ER; and finally, ADP-ribosylation factor (Arf) ARF5 and guanine nucleotide exchange factor GBF1 which activates Arfs such as ARF5, in turn mediating COPI coatomer recruitment and vesicle formation at the ER-Golgi interface. Moreover, increased levels of lyso-phosphatidylcholine (LysoPC) suggest vesicle fusion with ER and Golgi membranes occurred more frequently in low-pH cultures (McIntyre and Sleight, 1994), while the trend of higher qP in low-pH cultures observed throughout culture duration (**Figure 1F**) further supports increased protein trafficking through these organelles, diminished residence time within these two compartments and reduced glycan processing in low-pH cultures. This also helps to account for the higher proportion of complex glycans observed in high-pH cultures, in which products have a relatively longer residence time within the ER and Golgi. As ER and Golgi pH homeostasis are important for the functions of these organelles such as protein glycosylation, membrane trafficking and protein sorting (Kellokumpu, 2019; Paroutis et al., 2004), we searched for pH-specific differences in the expression levels of vacuolar-type H+-ATPases (v-ATPases), which pump protons into intracellular organelles to reduce the luminal pH. We found that the expression lev-

els of v-ATPases tend to be higher in low-pH cultures than medium and high-pH (**Supplementary Figure 7**), suggesting that there is a reduction of luminal pH in the ER and Golgi in low-pH cultures.

In order to better understand the pH-specific N-glycosylation variations, differentially expressed features of the glycosylation pathway from all three omics datasets were further examined (**Figure 4**). The distribution of mAb N-glycan variants with corresponding differential expression of the N-glycosylation pathway omics features revealed possible perturbation to the pathway in a pH-dependent manner with a higher proportion of glycans derived from the more basic cis- to medial-Golgi compartments in the low-pH cultures, and from the more acidic trans-Golgi compartment in the high-pH cultures (**Figures 2 and 4**). Higher expression of omics features associated with GDP-mannose synthesis (MPI, GMPPB, PPM2), lipid-linked oligosaccharide (LLO) precursor synthesis (UDP-GlcNAc transferase ALG13) and mannose trimming (mannosidases MAN1A1, MAN2A1, MAN2A2) was associated with higher accumulation of high-mannose glycans in low-pH cultures. ALG13 not only catalyzes the beginning of the precursor glycan assembly but is also a possible regulator for flux control of the LLO pathway (Averbeck et al., 2008). Higher expression of UDP-GlcNAc synthesis enzyme (UAP1) and transporters (SLC35A3, SLC35A4, SLC35D2), and GlcNAc transferases (MGAT5) were also observed with increased G0-GlcNAc, G0F-GlcNAc and G0 in the low-pH cultures. While percentage composition of complex glycans with terminal galactose and sialic acid added was higher in high-pH cultures, no corresponding pH-dependent differential omics feature was found. However, the decrease of complex glycans over time co-occurred with decreasing levels of direct glycan precursors, UDP-Gal and CMP-Neu5Ac (sialic acid). These observations implied that there are cellular processes besides glycosyltransferase levels likely to be involved in the pH-dependent distribution of complex glycans among the cultures.

Culture pH- and time regulates genes associated with mAb charge variant distributions

Charge variants were significantly affected by both pH and time (**Figure 2B**). More basic species were observed in low-pH cultures and conversely, more acidic species in high-pH cultures. Acidic variants increased over time regardless of pH, with low-pH showing the least pronounced increase.

Chemical degradation pathways are known to contribute to the formation of either acidic or basic species (Hintersteiner et al., 2016; Khawli et al., 2010). For example, C-terminal α-amidation gives rise to basic species, while pyroglutamate formation through cyclization of N-terminal glutamine residues and C-terminal lysine clipping lead to acidic species. The peptidylglycine α-amidating monooxygenase enzyme PAM catalyzing the occurrence of C-terminal α-amidation on the mAb heavy chain (Hu et al., 2017) was found to be more highly expressed at the transcript level in low-pH cultures particularly on day 14 (**Supplementary Figure 8A**). PAM has an acidic optimum pH, and is active in the lumen of secretory granules (Vishwanatha et al., 2014) and extracellular region, suggesting that the activity of PAM increases with decreasing culture pH, in turn increasing basic species. In contrast to basic variants, acidic variants were most highly represented in high-pH cultures, and additionally at late stages of culture regardless of pH (**Figure 2B**). Pyroglutamate formation occurs through the cyclization of N-terminal glutamine and glutamic acid residues, which respectively lead to loss of an amine group (resulting in acidic variants) and simultaneous loss of amine and carboxylic acid groups (demonstrated to result in basic variants) (Liu et al., 2019). Pyroglutamate formation is catalyzed by the glutaminyl cyclase iso-enzymes QPCT and QPCTL (Perez-Garmendia and Gevorkian, 2013), and QPCTL transcript expression was found to increase with pH (**Supplementary Figure 8B**). Pyroglutamate formation can also occur through nonenzymatic processes by modifying the products in the culture media. The efficiency of nonenzymatic N-terminal glutamine cyclization is reported to be higher at pH 7.2 than 6.2, as a deprotonated N-terminal amino group will favor the nucleophilic reaction (Dick Jr. et al., 2007; Gazme et al., 2019). This may have contributed to the increased acidic species in high-pH cultures as well as its accumulation in late-stage cultures. Another possible cause for acidic species accumulation over time was the corresponding increased transcript expression of the carboxypeptidase D enzyme (CPD) that catalyzes C-terminal lysine clipping (Hu et al., 2016) (**Supplementary Figure 8C**).

When we compared the levels of charge variants with N-glycans, the total percentage of basic variants

trended similarly with early glycans, while total acidic variants were higher in high-pH than low-pH cultures similar to complex glycans (**Figure 2A**). High-mannose structures may contribute to charge differences by conferring subtle conformational differences that affect charge distribution (Du et al., 2012), and might have partially contributed to the increase in basic variants as they were demonstrated previously to be enriched in basic fractions of a mAb produced in CHO cells (Hintersteiner et al., 2016). Sialic acid is known to contribute to the formation of acidic charge variants (Khawli et al., 2010) and some increase in sialylated glycans was observed in high-pH cultures.

DISCUSSION

A multi-omics data-driven systems biotechnology approach is presented here, characterizing the effect of pH on titer and various quality profiles of mAbs produced in CHO cells. This approach is necessary for the dissection of underlying molecular mechanisms as the changes in specific rates of by-product production alone could not explain the variations in observed quality profiles. Specifically, our work clearly demonstrated that N-glycan structures are influenced by transcript and protein expression changes in various pathways including N-glycan biosynthesis, ER and Golgi vesicular transport, fatty acid β-oxidation, and responses to unfolded proteins (UPR), ER stress and oxidative stress. Similarly, temporal variations in gene expression profiles of UPR and response to ER stress were associated with high molecular weight (HMW) species as the culture progressed. The pH-dependent changes in charge variants were also positively correlated with the expression differences of relevant post-translational modification enzymes.

The relative abundance of early glycans in low-pH cultures may be explained by the pH specificity of intracellular organelles where the resting pH of the Golgi ranges from ˜pH 6.7 in the cis-Golgi to ˜pH 6.0 in the trans-Golgi (**Figure 4**). The maintenance of ER and Golgi pH homeostasis is important for ER and Golgi functions such as protein glycosylation, membrane trafficking and protein sorting (Kellokumpu, 2019; Paroutis et al., 2004). We postulate that the influx of H^+ into the cell in low-pH cultures decreases the pH of the cytoplasm and the luminal pH of the ER and Golgi via increased expression of v-ATPases (**Supplementary Figure 7**) and PAM (**Supplementary Figure 8A**), the latter a proposed v-ATPase associated pH sensor within the secretory pathway (Rao et al., 2019) (**Figure 5, left panel**). The resultant disruption of luminal pH gradient through acidification may constitute a significant perturbation in the more basic compartments of the early secretory pathway and is associated with an increased expression of the corresponding localized N-glycosylation enzymes. Coupled with higher expression of members of ER and Golgi vesicular trafficking pathways in low-pH cultures (**Supplementary Figure 6**), the implied increase in general secretory pathway trafficking and thus a shorter residence time for cargo proteins in ER and Golgi will result in higher abundance of early mAb glycoforms.

Cellular stress resulting from prolonged high production of mAb may account for the decreased relative abundance of late glycans, with culture time having a much more pronounced effect in comparison to culture pH. As the cell culture progresses, we hypothesize there is a disruption of ER homeostasis due to ER stress associated with increased levels of unfolded proteins (Gerlach et al., 2012) as well as oxidative stress associated with oxidative protein folding in the ER and increased fatty acid β-oxidation that generate reactive oxygen species (ROS) (Malhotra and Kaufman, 2007) (**Figure 5, right panel**); the increase in the bioenergetic fatty acid β-oxidation is possibly a response to higher energy demand for protein productivity by the cells. ER stress and oxidative stress are also known to exacerbate each other (Nakka et al., 2016), and their resultant disruption of ER homeostasis promotes accumulation of unfolded proteins which can lead to aggregation of mAb into HMW species (Li et al., 2016; Zhou et al., 2018). The decrease in complex (late) mAb glycoforms, resulting in hypoglycosylation and reduced glycodiversity, may arise from accumulated irreversible oxidative damage to glycosylation enzymes that causes increasing loss of functionality over time (Korovila et al., 2017). Such damage will naturally have a larger cumulative effect further down in a sequential chain of enzymatic reactions.

While previous studies have investigated the effects of pH on quality attributes such as N-glycosylation

(Aghamohseni et al., 2014; Ivarsson et al., 2014; Jiang et al., 2018), aggregation (Paul et al., 2018) and charge variants (Xie et al., 2016), to the best of our knowledge, no report has investigated the underlying causes of these pH-specific effects. In this regard, this study systematically characterizes the effects of pH on quality attributes at various levels of the cellular hierarchy, i.e. transcripts, proteins and metabolites. Such integrative analyses allow us to understand that some cellular processes emerge at transcriptional level whereas others emerge only at the post-transcriptional or metabolite levels. The increased expression of vesicular transport genes in low-pH cultures, and its potential contribution to higher levels of early glycans, was evidenced by transport, protein and metabolite datasets. Such concordant observations across multiple omics datasets strongly suggest the activation or suppression of certain pathways and its consequences at downstream layer, thereby allowing us to derive relevant hypotheses at the global level which may not be possible with only one of the omics datasets. However, certain observed changes in product quality were found to be linked to a specific omics dataset – for example, the variations in mAb aggregation levels were found to be associated with differential expression of several protein disulfide isomerases only at the protein, i.e. post-transcriptional, level. We also noted a decrease in glycodiversity as the cell culture progressed, irrespective of pH with concomitant increased expression of multiple ER stress and oxidative stress markers in all three omics datasets (**Supplementary Figures 3, 4**).

Overall, the integration of multi-omics data allowed us to unravel mechanisms involving multiple pathways and enzymes that are associated with variations in product quality, and also highlighted the complex and dynamic interactions between these quality profiles and pathways. Taken together, the systematic approach adopted in the current study augments our understanding of factors affecting mAb quality attributes, and we believe that the insights gained from this study can contribute towards the development of targeted approaches that result in more effective product quality control strategies.

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AUTHOR CONTRIBUTIONS

A.P.L., Y.J.K. and M.L. contributed equally to this work, carried out data analysis, data interpretation, and wrote the manuscript with contributions from all other authors. D.L. conducted the CHO bioreactor cultures. Y.J.K., L.Z., H.L.L., S.C. and S.Y.M. undertook sample processing, multiomics profiling and data acquisition. K.S.A. contributed to data analysis. N.T., S.K.N. and Y.S.H. conceived the study and coordinated the work. N.T., T.S., X.W., E.G., A.H-M.T., X.B., S.K.N., D-Y.L., W.L.W.L. and Y.S.H. contributed to interpretation of the data, and critical revision of the manuscript.

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FIGURE LEGENDS

Figure 1. Cell culture profiles of a mAb-expressing CHO cell line cultured at various pH set points. (A) Viable cell density, (B) viability, (C) cumulative viable cells, (D) titer, (E) specific growth rate, and (F) specific productivity (qP) at pH 6.7 (low), 6.9 (medium), 7.1 (high). Error bars denote mean ± SD (n=3).

Figure 2. Groups of correlated product quality attributes identified through hierarchical clustering. Heatmap of hierarchical clustering of product quality attributes (rows) across cell culture samples (columns). The product quality attributes include N-glycoforms, charge variants (Total Acidic, Main, Total Basic) and aggregation (Total LMW, Monomer, Total HMW). The samples are grouped by a horizontal bar with increasingly darker shades of red (low pH), green (medium pH) and blue (high pH) at increasing time-points.

Figure 3. Global analysis of multi-omics data. (A) PCA of individual omics datasets. Each data point is a single replicate sampled on a particular day of a pH set point. A polynomial spline trend line is added for each pH set point. The numbers in each plot denote culture day for the sample. (B) Overview of data analysis steps. Overlapping pathways across significantly enriched Reactome pathways are identified by individual omics analyses. The numbers of enriched pathways are shown in the Venn diagrams.

Figure 4. Localization of glycan variant profiles and associated differential omics features in the N-glycosylation pathway among the three different pH set-points. The process of N-glycosylation takes place in the ER and Golgi complex along a luminal pH gradient of increasing acidity. Accumulation of group 1 "early glycans" at the more basic cis- to medial-Golgi were observed with increased expression of features associated with precursor glycan assembly (at the ER), demannosylation and GlcNAc extension in the low-pH cultures. Culture pH had minimal effect on distribution of group 2 medial-Golgi "intermediate glycans" which include the dominant G0F variant. Group 3 "complex glycans" at the more acidic trans-Golgi mainly decreased with time but with a slightly higher proportion observed in high-pH cultures; associated omics features reflected the same trend over time but were not differentially expressed by culture pH. Omics features are differentially expressed at FDR < 0.01 (except for those marked with '*') between cultures of different pH.[1,2,3]Glycan: Group 1, 2 or 3 of mAb quality attributes in the samples that were clustered according to their distributions over pH and time. ER: endoplasmic reticulum; TGN: trans-Golgi network; T: transcriptomics, P: proteomics, M: metabolomics.

Figure 5. Hypotheses of culture pH and time effects on mAb glycosylation, charge variants and aggregation.

Left panel: At low $pH_{culture}$, increased v-ATPases and PAM (pH sensor) levels help remove excess H^+ which has entered the cytoplasm from the culture media. This leads to perturbation of the luminal $pH_{ER/Golgi}$ gradient, with a larger effect on the more basic compartments of the early secretory pathway as suggested by the increased expression of N-glycosylation enzymes in the ER and cis/medial Golgi. Concomitant increase in ER/Golgi trafficking not only increases the rate of excess H^+ removal but also reduces residence (and processing) time of cargo proteins, resulting in increased relative abundance of early mAb glycoforms. The increase in basic variants is a result of protein modification (distinct from pH sensing function) by elevated PAM level and possibly its activity too as PAM has an acidic pH optimum.

Right panel: With increasing culture time regardless of $pH_{culture}$, prolonged production of mAb results in increasing unfolded proteins and ER stress, as well as increasing exposure to reactive oxygen species (ROS) derived from oxidative protein folding and fatty acid β-oxidation. The consequent disruption of ER homeostasis leads to failure of ER quality control and further accumulation of unfolded proteins which can self-associate to form aggregation nuclei that promote HMW species. The concomitant increase in oxidative stress leads to the accumulation of oxidative damage to glycosylation enzymes which in turn decreases late mAb glycoforms.