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Autophagy and Akt-Stimulated Cellular Proliferation Synergistically Improve Antibody Production in CHO Cells

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ABSTRACT

Over the past decade, engineered producer cell lines have led 10-fold increases in antibody yield, based on an improved understanding of the cellular machinery influencing cell health and protein production. With prospects for further production improvements, increased antibody production would enable a significant cost reduction for life-saving therapies. In this study, we strategized methods to increase cell viability and the resulting cell culture duration to improve production lifetimes. By overexpressing the cell surface adenosine A_{2A} receptor ($A_{2A}R$), the Akt pathway was activated, resulting in improved cellular proliferation. Alternatively, by inducing autophagy through temperature downshift, we were able to significantly enhance cellular-specific productivity, with up to a three-fold increase in total antibody production as well as three-fold higher cell-specific productivity. Interestingly, the expression levels of the autophagy pathway protein Beclin-1 appeared to correlate best with the total antibody production, of autophagy-related proteins examined. Thus, during cell clonal development Beclin-1 levels may serve as a marker to screen for conditions that optimize antibody titer.

1 | Introduction

Chinese hamster ovary (CHO) cells are one of the most robust and widely utilized workhorses for therapeutic protein production in the bioprocessing industry, meeting over 10 g/L monoclonal antibody production yields [1], resulting in a hundred-billion-dollar global market. Despite these successes, the cost of innovative antibody therapeutics remains prohibitive [2], leading to a need for even higher productivity.

Previously, we identified mTOR/Akt pathway activation as a plausible driver of a growth-proliferative metabolic phenotype in antibody-producing CHO cells [3]. We also determined through computational docking studies of rosmarinic acid (RA) that the pathway activation might be associated with cell-surface-receptor interactions, and we hypothesized that heterologous expression of the adenosine A_{2A} receptor ($A_{2A}R$) could serve to increase Akt signaling and to enhance cellular proliferation. $A_{2A}R$ overexpression offers a new genetic approach toward protein production enhancement.

2 | Materials and Methods

2.1 | Details in Supporting Information

3 | Results and Discussion

To test our hypothesis, we first transiently transfected IgG_1 antibody-expressing CHOZN cells (gift of Millipore-Sigma) with

Summary

- Adenosine receptor overexpression stimulated downstream phosphorylation in CHOZN cells and improved culture longevity and antibody production.
- Autophagy unveiled as an underlying mechanism in temperature-downshifted cultures.
- Akt stimulation increases cellular proliferation but reduces cell-specific antibody production.
- Autophagy increases specific productivity but does not increase the cumulative cell yield.
- Culture longevity-prolonging strategies (Akt stimulation) combined with autophagy induction (at 34°C) generated strong synergism toward improved antibody production with the final antibody titer correlating to Beclin-1 protein expression.

pCEP4-$A_{2A}R$ [4] and observed both $A_{2A}R$ increased mRNA levels (Figure S1) and an elevated level of cAMP production as a hallmark of constitutive $A_{2A}R$ signaling [5] in fed-batch cultures (71.8% ± 0.2% higher than empty vector controls; p value <0.1). As expected from earlier reports (e.g., see [6]), $A_{2A}R$ overexpression resulted in enhanced Akt phosphorylation (pAKT) 4 days after inoculation compared to mock-transfected cultures, as determined by Western blot analysis (Figure 1A). Quantitation of pAKT to total levels showed 3.4-fold higher levels of phosphorylated species relative to the vector control after normalizing by total protein levels (Figure S2).

Most importantly, we observed increased cell viability and culture longevity through transient transfection of $A_{2A}R$ in both fed-batch (Figure 1B) and batch (Figure S3) operation modes. Higher $A_{2A}R$ expression levels also had a direct effect on nutrient uptake and cellular metabolism (Figure 1C; Figure S3), where uptake and production rate calculations are described in the Supporting Information. On a cellular basis, $A_{2A}R$ expression resulted in a reduction in cell-specific glucose uptake (331 ± 132 μg/10^6 cells/day compared to 453 ± 2 μg/10^6 cells/day in control cells) and a slightly reduced level of lactate production (211 ± 7 μg/10^6 cells/day compared to 240 ± 63 μg/10^6 cells/day in control cells) during fed-batch growth, resulting in improved cell health and proliferation.

As a result of the prolonged culture period, the amount of antibody produced throughout the culture increased (Figure 1D). These observations corresponded to a growth-promoting phenotype similar to what we observed in RA-supplemented cultures due to pAKT [3]. Since endogenous $A_{2A}R$ expression level was found to be low in CHO cells based on unsuccessful qPCR transcript amplification (data not shown), we surmise that $A_{2A}R$ expression may greatly increase the cellular sensitivity to various stimuli and media nutrients. The current finding thus presents an exciting opportunity to engineer cell lines with overexpressed cell-surface receptors and proteins that are minimally expressed in CHO cells [7] to improve the overall culture performance.

Temperature downshifting and autophagy induction are classic antiaging approaches that have increased antibody production [8] and have been associated with reduced cell growth rates and

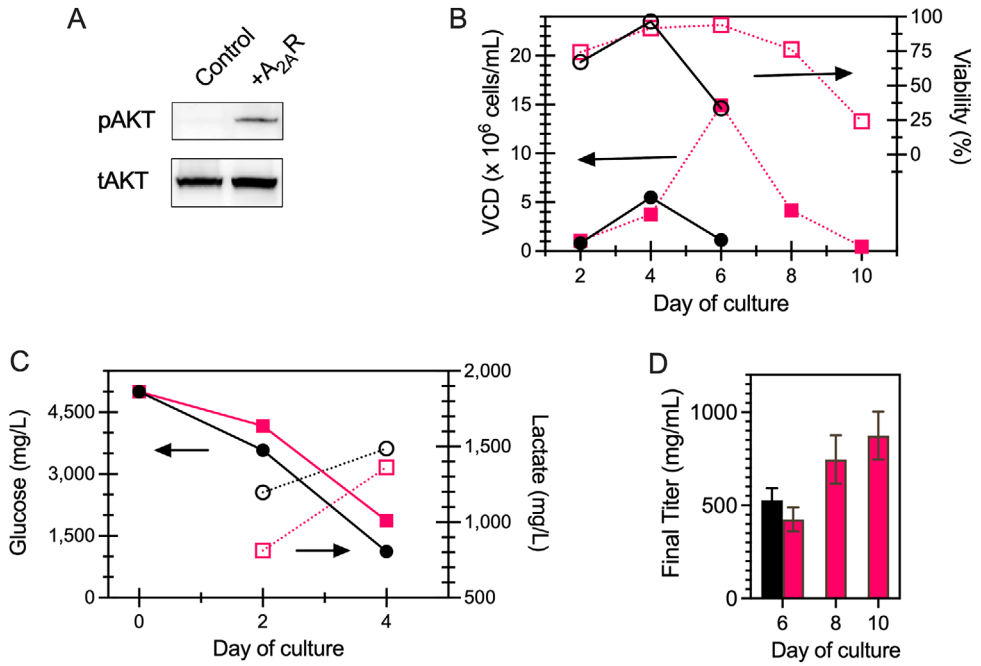

FIGURE 1 | The effect of A_{2A} receptor ($A_{2A}R$) overexpression on CHOZN IgG_1-expressing fed-batch culture. (A) Phosphorylated-Akt and total-Akt protein levels were measured 4 days post-inoculation in CHOZN cell lysates via Western blot. Following transient transfection of empty pCEP4 vector (control, black) or pCEP4-$A_{2A}R$ (red) in CHOZN cells, (B) viable cell density (VCD; filled symbols) and cell viability (open symbols), and (C) glucose (filled symbols) and lactate levels (open symbols) were measured during the fed-batch growth. Arrows serve as a visual guide to the figure axes. Results are representative of independent transient transfections. (D) Cumulative antibody produced over the duration of the culture. Samples as in (B) and (C). Note that control cultures were terminated after 8 days due to low viability. 0.5×10^6 cells were inoculated on Day 0. The results and error bars shown are representative of duplicate trials.

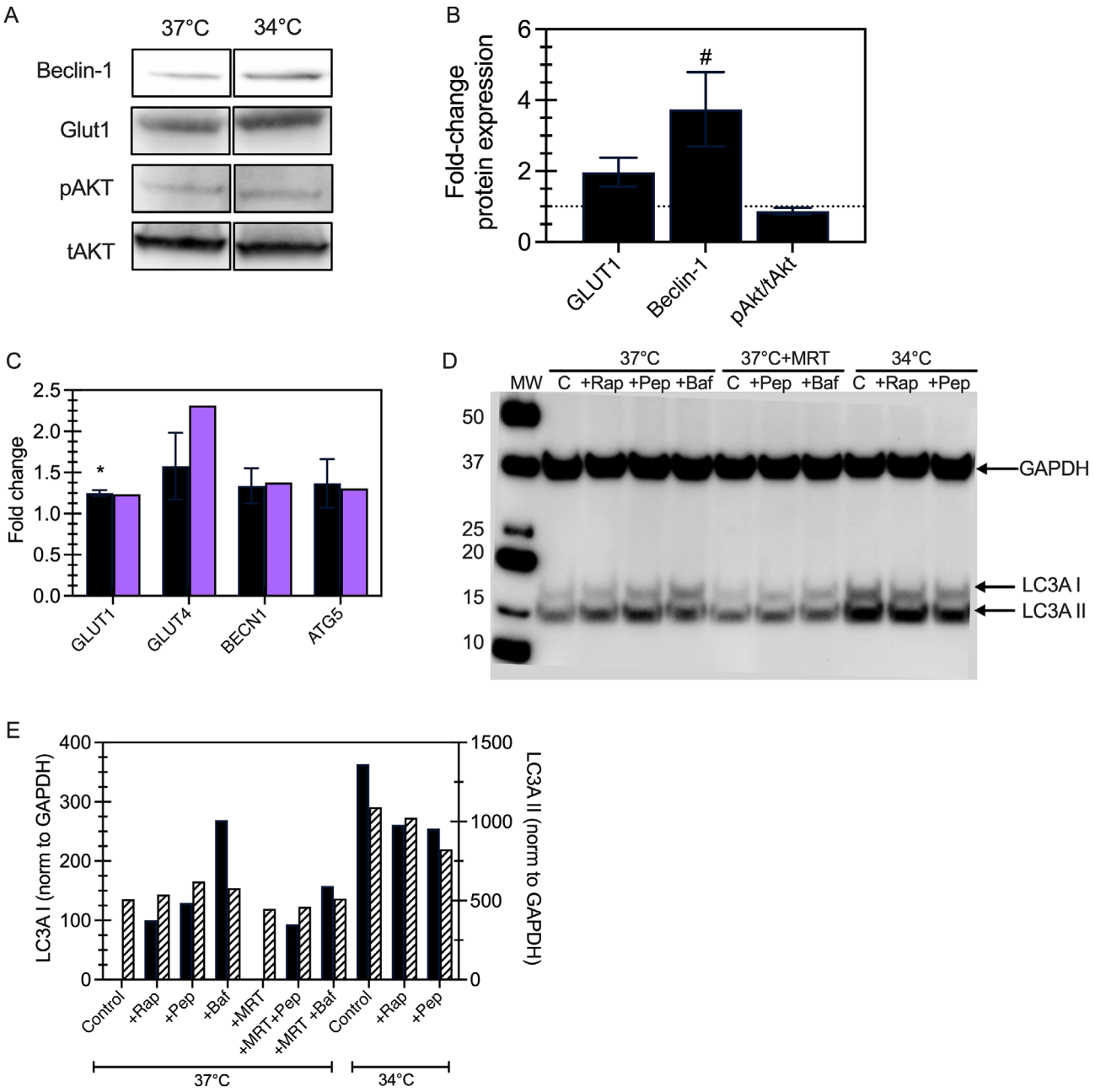

FIGURE 2 | Expression of autophagy markers during temperature-downshifted (A–C, E) cultures, or those grown with 1 μM MRT (D, E). (A) Western blot and (B) quantified protein expression levels of temperature downshifted cultures (34°C) relative to control cultures (37°C). Quantification for each blot was normalized to the amount of total protein by Coomassie Blue R-250 staining (not shown). (C) RT-qPCR was used to measure the expression of indicated genes for temperature downshifted cultures (black bars), normalized by the levels of genes in control cultures. 500 nM rapamycin and 60 μM chloroquine were used as a positive control (purple bars, $n = 1$) to activate autophagy. Error bars are representative of the standard error of three independent cultures, except for the positive control. (D) Autophagosome production was monitored by examining LC3A protein expression via Western blot. MW indicates Bio-Rad Precision Plus Protein WesternC ladder, with values shown in kDa. C, DMSO control; Rap, 500 nM rapamycin, Pep, 10 μg/mL pepstatin A; Baf, 50 nM bafilomycin A. Image is representative of three biological replicates. (E) Quantitation of LC3A II (hatched bars) and LC3A I (solid bars) levels from (D), normalized to GAPDH loading control. LC3A I in control samples was too low to be determined, and thus no bars are shown. The complete data associated with temperature-shifted and MRT addition is shown in Figure S4.

prolonged cell viability [9]. Studies in nonproducer cell lines have identified linkages between hypothermia and autophagy induction [10, 11]. Considering the two processes frequently yield similar outcomes in cell culture (increased productivity and culture longevity, and shifts in cellular metabolism, see Figure S4A), we hypothesized that autophagy is activated in the temperature-downshift process in our producer cell line (CHOZN), contributing to a favorable role in antibody production. Beclin-1 is a protein that participates in both phagophore nucleation and autophagosome maturation [12] and serves as a representative regulator of autophagy. Through both Western blot and RT-qPCR analysis (Figure 2A–C), we confirmed that Beclin-1

protein expression, but not pAKT, increased in the temperature downshifted process, alongside a transcript level increase of Beclin-1 (BECN1) and Atg5, which regulates autophagophore elongation [12]. This result validates the hypothesis that temperature downshift in CHOZN cells is associated with enhanced cell-specific productivity via cellular autophagy but not via Akt-stimulated proliferation (Figure 1).

Two glucose transporters, GLUT1 (SLC2A1) and GLUT4 (SLC2A4), responsible for basal and insulin-regulated glucose transport, were also upregulated with temperature downshift (Figure 2A–C). Upregulation of glucose transporters has been related to an increased cell-specific glucose uptake [13], which is consistent with increases observed in our study (535 ± 35 μg/10^6 cells/day in downshifted cells as compared to 495 ± 34 μg/10^6 cells/day for control), and reflects an altered glucose metabolism associated with autophagy [14]. The enhanced substrate consumption may be used to support crucial pathways such as enhanced flux through the TCA cycle or mitochondrial activities [13] manifesting as an increased oxygen consumption rate, which we also observed (Figure S5).

To identify autophagic processes that might occur downstream, we analyzed autophagosome formation through LC3A protein expression [15, 16]. As in previous results for CHO cells (e.g., [17]), we see that the majority of LC3A was in the membrane-bound (LC3A II) state at 37°C under nutrient-rich conditions. The addition of rapamycin to activate autophagy showed a slight increase in LC3A II levels, and a significant increase in soluble LC3A I levels, as determined by densitometry analysis (Figure 2D,E). At 34°C, the levels of LC3A II increased relative to that at 37°C, which indicates a potential increase in autophagic flux.

During autophagy, lysosomal hydrolases are expected to degrade LC3A [17], so pepstatin A, an inhibitor of cathepsins D and E, was added to investigate lysosomal turnover. Here, we see that the addition of pepstatin A increased the levels of LC3A II at 37°C (Figure 2D,E), consistent with an increase in autophagic flux, while the approximately constant levels of LC3A II upon pepstatin A addition at 34°C indicate a lysosomal degradation block. Similarly, disrupting autophagosome-lysosome fusion with bafilomycin A1 addition [16] increased the levels of LC3AII (Figure 2D,E) at 37°C but not at 34°C. The upregulation of the upstream autophagic markers (Figure 2A,C), combined with the increase in LC3A II levels at 34°C, confirm that early-stage autophagy was activated in low-temperature cultures.

We also used the previously reported autophagy inhibitor, MRT [18], to disrupt autophagic flux. Upon addition of 1 μM MRT, LC3A II levels were decreased relative to 37°C control cultures, as determined by Western blotting (Figure 2D). Similarly to control cultures, the addition of pepstatin A or bafilomycin A1 increased the levels of LC3A in cultures treated with MRT (Figure 2D,E). As shown in Figure 3A, B, the addition of 1 μM MRT improved cell viability, prolonged the culture duration, and improved antibody production, consistent with autophagy inhibition.

Synergistic applications of productivity-enhancing methods are often beneficial but underutilized, with a few exceptions using combined chemical supplementation [19, 20] and process variations [13, 21, 22]. Therefore, we tested whether several culture longevity-prolonging processes could be combined to yield a synergistic improvement in cell proliferation and antibody production. By combining temperature downshifts, MRT addition, and RA addition (which we previously identified to stimulate the Akt pathway [3]), we then examined effects on cell viability (Figure 3A), culture duration (Figure 3A), and antibody productivity (Figure 3B). Noticeably, processes related to autophagy strongly enhanced cell-specific productivity (qP), whereas processes related to Akt stimulation increased cellular proliferation. With the combined use of temperature downshift and MRT addition, the overall antibody titer and qP both increased threefold compared to control cultures (Figure 3B). However, increases per cell productivity alone may result in cell stress, reduced cell density, and cell death, resulting in lower culture productivity (end of run titer). Proliferation control via Akt pathway stimulation may therefore serve as another approach to improve cell health. Complete growth data for MRT addition in cultures is shown in Figure S4.

Beclin-1 protein levels were measured for all combinations tested, and we found that the measured antibody end-day titer showed a correlation with Beclin-1 expression levels (Figure 3C). We believe that Beclin-1 regulates both productive and proliferative processes by balancing autophagy with Akt-activation. Thus, while autophagy and Akt pathways negatively regulate each other [23, 24], we note that the interplay does not lead to the annihilation of one or the other pathway (Figure S6). Although we did not find a direct correlation between Beclin-1 expression and qP — likely since the effect of Akt stimulation on increasing cell density overweighed the effect of autophagy on qP — we demonstrate here that a balance of increased Akt activity and autophagy may be critical to the observed increase in viable cells and culture longevity, extending the antibody production phase, and resulting in increased antibody quality and production. Indeed, process methods and genetic engineering approaches that lead to increased Beclin-1 levels could serve to guide strategies to improve antibody production.

4 | Conclusion

Overall, we show cell surface receptors may be engineered to enhance cell culture duration and protein production via Akt pathway stimulation. This approach in combination with autophagy stimulation via low-temperature shift results in balanced improvements in cell proliferation (via Akt) and productivity (via autophagy) control, where Beclin-1 protein expression serves as a tangible marker to assess the overall effect of protein production enhancement.

Author Contributions

Leran Mao: investigation (lead), methodology and formal analysis (lead), writing–original draft (lead), and editing (equal). **Sarah Michelle Sonbati:** methodology and formal analysis (supporting), writing–editing (supporting). **James W. Schneider:** supervision (supporting), writing–editing (supporting). **Anne S. Robinson:** supervision and project management (lead), writing–original draft (supporting), and editing (equal).

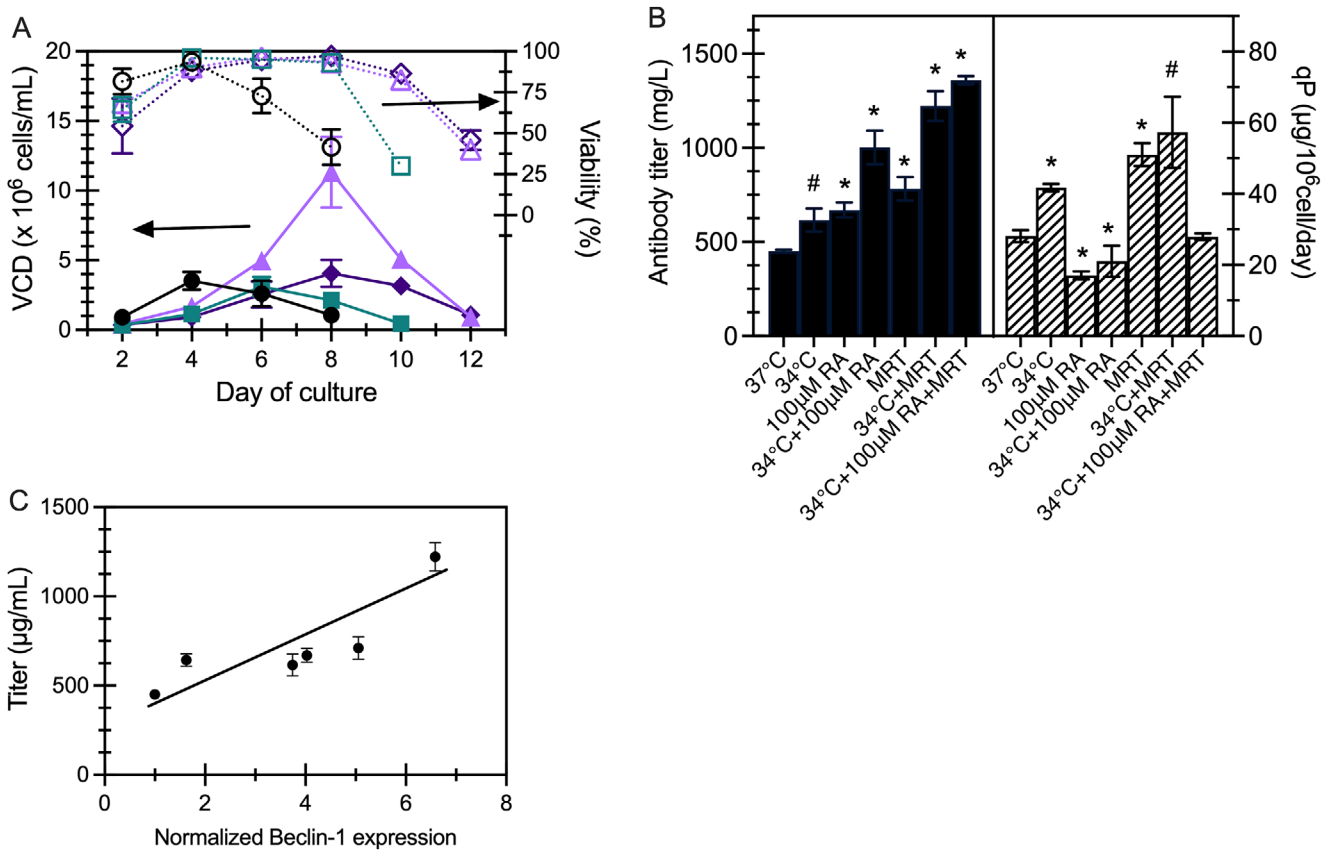

FIGURE 3 | Synergism is observed with multiple proliferation-control strategies. (A) Comparison of viable cell density (VCD) (filled symbols, solid lines) and viability (open symbols, dashed lines) of fed-batch cultures when temperature-downshift to 34°C is applied alone (teal squares), or with 1 µM MRT (dark purple diamonds) or 100 µM RA (light purple triangles). Data points are shown as symbols for the average of at least three independent cultures, with error bars showing standard error. Arrows serve as a visual guide to the figure axes. (B) Antibody titer and qP at culture termination (viability below 70%). Statistical significance indicated by * or # is relative to control cultures (37°C). C) Beclin-1 expression level (determined by Western analysis 4 days postinoculation) and final-day antibody titer levels are correlated. Data points shown are the average values of Beclin-1 expression and antibody titer for three biological replicates. Error bars that are not visible are smaller than the symbol. The line indicates a best fit of the data (Titer = 332 + 105*Beclin-1; $R^2 = 0.7$). (A) shows only representative culture progression data; a complete data set can be seen in Figure S4.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. J.-H. Zhang, L.-L. Shan, F. Liang, C.-Y. Du, and J.-J. Li, "Strategies and Considerations for Improving Recombinant Antibody Production and Quality in Chinese Hamster Ovary Cells," *Frontiers in Bioengineering and Biotechnology* 10 (2022): 856049, https://doi.org/10.3389/fbioe.2022.856049.

2. C. Chung, S. B. Kudchodkar, C. N. Chung, et al., "Expanding the Reach of Monoclonal Antibodies: A Review of Synthetic Nucleic Acid Delivery in Immunotherapy," *Antibodies* 12 (2023): 46, https://doi.org/10.3390/antib12030046.

3. L. Mao, J. W. Schneider, and A. S. Robinson, "Rosmarinic Acid Enhances CHO Cell Productivity and Proliferation Through Activation of the Unfolded Protein Response and the mTOR Pathway," *Biotechnology Journal* 19 (2024): 2300397, https://doi.org/10.1002/biot.202300397.

4. A. N. Naranjo, A. Chevalier, G. D. Cousins, et al., "Conserved Disulfide Bond Is Not Essential for the Adenosine A2A Receptor: Extracellular Cysteines Influence Receptor Distribution Within the Cell and Ligand-Binding Recognition," *Biochimica et Biophysica Acta (BBA)—Biomembranes* 1848 (2015): 603–614, https://doi.org/10.1016/j.bbamem.2014.11.010.

5. A. R. Jain, C. McGraw, and A. S. Robinson, "The Specificity of Downstream Signaling for A1 and A2AR Does Not Depend on the C-Terminus, Despite the Importance of this Domain in Downstream Signaling Strength," *Biomedicines* 8 (2020): 603, https://doi.org/10.3390/biomedicines8120603.

6. S. Borhani, C. Corciulo, A. Larranaga-Vera, and B. N. Cronstein, "Adenosine A 2A Receptor (A2AR) Activation Triggers Akt Signaling and Enhances Nuclear Localization of β-Catenin in Osteoblasts," *The FASEB Journal* 33 (2019): 7555, https://doi.org/10.1096/fj.201900014R.

7. S. H. Kim, M. Baek, S. Park, S. Shin, J. S. Lee, and G. M. Lee, "Improving the Secretory Capacity of CHO Producer Cells: The Effect of Controlled

Blimp1 Expression, a Master Transcription Factor for Plasma Cells," *Metabolic Engineering* 69 (2022): 73–86, https://doi.org/10.1016/j.ymben.2021.11.001.

8. E. Baek, C. L. Kim, M. G. Kim, J. S. Lee, and G. M. Lee, "Chemical Inhibition of Autophagy: Examining Its Potential to Increase the Specific Productivity of Recombinant CHO Cell Lines," *Biotechnology and Bioengineering* 113 (2016): 1953–1961, https://doi.org/10.1002/bit.25962.

9. J. Xu, P. Tang, A. Yongky, et al., "Systematic Development of Temperature Shift Strategies for Chinese Hamster Ovary Cells Based on Short Duration Cultures and Kinetic Modeling," *mAbs* 11 (2019): 191–204, https://doi.org/10.1080/19420862.2018.1525262.

10. S. Chung, J. Tian, Z. Tan, et al., "Modulating Cell Culture Oxidative Stress Reduces Protein Glycation and Acidic Charge Variant Formation," *mAbs* 11 (2019): 205–216, https://doi.org/10.1080/19420862.2018.1537533.

11. Y. Jin, Y. Lin, J. Feng, F. Jia, G. Gao, and J. Jiang, "Moderate Hypothermia Significantly Decreases Hippocampal Cell Death Involving Autophagy Pathway After Moderate Traumatic Brain Injury," *Journal of Neurotrauma* 32 (2015): 1090–1100, https://doi.org/10.1089/neu.2014.3649.

12. R. Kang, H. J. Zeh, M. T. Lotze, and D. Tang, "The Beclin 1 Network Regulates Autophagy and Apoptosis," *Cell Death and Differentiation* 18 (2011): 571–580, https://doi.org/10.1038/cdd.2010.191.

13. V. Avello, B. Tapia, M. Vergara, et al., "Impact of Sodium Butyrate and Mild Hypothermia on Metabolic and Physiological Behaviour of CHO TF 70R Cells," *Electronic Journal of Biotechnology* 27 (2017): 55–62, https://doi.org/10.1016/j.ejbt.2017.03.008.

14. S. Roy, A. M. Leidal, J. Ye, S. M. Ronen, and J. Debnath, "Autophagy-Dependent Shuttling of TBC1D5 Controls Plasma Membrane Translocation of GLUT1 and Glucose Uptake," *Molecular Cell* 67 (2017): 84–95, https://doi.org/10.1016/j.molcel.2017.05.020.

15. Y. Kabeya, N. Mizushima, T. Ueno, et al., "LC3, a Mammalian Homologue of Yeast Apg8p, Is Localized in Autophagosome Membranes After Processing," *The EMBO Journal* 19 (2000): 5720–5728, https://doi.org/10.1093/emboj/19.21.5720.

16. D. J. Klionsky, Z. Elazar, P. O. Seglen, and D. C. Rubinsztein, "Does Bafilomycin A1 Block the Fusion of Autophagosomes With Lysosomes?" *Autophagy* 4 (2008): 849–850, https://doi.org/10.4161/auto.6845.

17. I. Tanida, N. Minematsu-Ikeguchi, T. Ueno, and E. Kominami, "Lysosomal Turnover, but Not a Cellular Level, of Endogenous LC3 Is a Marker for Autophagy," *Autophagy* 1 (2005): 84–91, https://doi.org/10.4161/auto.1.2.1697.

18. K. J. Petherick, O. J. L. Conway, C. Mpamhanga, et al., "Pharmacological Inhibition of ULK1 Kinase Blocks Mammalian Target of Rapamycin (mTOR)-dependent Autophagy," *Journal of Biological Chemistry* 290 (2015): 11376–11383, https://doi.org/10.1074/jbc.C114.627778.

19. H.-H. Lin, T.-Y. Lee, T.-W. Liu, and C.-P. Tseng, "High Glucose Enhances cAMP Level and Extracellular Signal-Regulated Kinase Phosphorylation in Chinese Hamster Ovary Cell: Usage of Br-cAMP in Foreign Protein β-Galactosidase Expression," *Journal of Bioscience and Bioengineering* 124 (2017): 108–114, https://doi.org/10.1016/j.jbiosc.2017.02.010.

20. L. Montégut, P. C. Martínez-Basilio, J. Moreira V. da, L. Schwartz, and M. Jolicoeur, "Combining Lipoic Acid to Methylene Blue Reduces the Warburg Effect in CHO Cells: From TCA Cycle Activation to Enhancing Monoclonal Antibody Production," *PLoS ONE* 15 (2020): e0231770, https://doi.org/10.1371/journal.pone.0231770.

21. J. Coronel, C. Heinrich, S. Klausing, T. Noll, A. Figueredo-Cardero, and L. R. Castilho, "Perfusion Process Combining Low Temperature and Valeric Acid for Enhanced Recombinant Factor VIII Production," *Biotechnology Progress* 36 (2020): e2915, https://doi.org/10.1002/btpr.2915.

22. S. S. Nasseri, N. Ghaffari, K. Braasch, et al., "Increased CHO Cell Fed-Batch Monoclonal Antibody Production Using the Autophagy Inhibitor 3-MA or Gradually Increasing Osmolality," *Biochemical Engineering Journal* 91 (2014): 37–45, https://doi.org/10.1016/j.bej.2014.06.027.

23. L. Qin, Z. Wang, L. Tao, and Y. Wang, "ER Stress Negatively Regulates AKT/TSC/mTOR Pathway to Enhance Autophagy," *Autophagy* 6 (2010): 239–247, https://doi.org/10.4161/auto.6.2.11062.

24. R. C. Wang, Y. Wei, Z. An, et al., "Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation," *Science* 338 (2012): 956–959, https://doi.org/10.1126/science.1225967.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.