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Research Article

Heat shock protein 27 overexpression in CHO cells modulates apoptosis pathways and delays activation of caspases to improve recombinant monoclonal antibody titre in fed-batch bioreactors

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CHO cells are major production hosts for recombinant biologics including the rapidly expanding recombinant monoclonal antibodies (mAbs). Heat shock protein 27 (HSP27) expression was observed to be down-regulated towards the late-exponential and stationary phase of CHO fed-batch bioreactor cultures, whereas HSP27 was found to be highly expressed in human pathological cells and reported to have anti-apoptotic functions. These phenotypes suggest that overexpression of HSP27 is a potential cell line engineering strategy for improving robustness of CHO cells. In this work, HSP27 was stably overexpressed in CHO cells producing recombinant mAb and the effects of HSP27 on cell growth, volumetric production titer and product quality were assessed. Concomitantly, HSP27 anti-apoptosis functions in CHO cells were investigated. Stably transfected clones cultured in fed-batch bioreactors displayed 2.2-fold higher peak viable cell density, delayed loss of culture viability by two days and 2.3-fold increase in mAb titer without affecting the N-glycosylation profile, as compared to clones stably transfected with the vector backbone. Co-immunoprecipitation studies revealed HSP27 interactions with Akt, pro-caspase 3 and Daxx and caspase activity profiling showed delayed increase in caspase 2, 3, 8 and 9 activities. These results suggest that HSP27 modulates apoptosis signaling pathways and delays caspase activities to improve performance of CHO fed-batch bioreactor cultures.

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1 Introduction

In 1987, Activase, an anti-coagulant used for the treatment of acute myocardial infarction, became the first approved recombinant biologic produced from Chinese hamster ovary (CHO) cells. Since then, CHO cells have been used for production of many different classes of biologics such as blood factors, cytokines, fusion proteins, growth factors, hormones, monoclonal antibodies (mAbs) and therapeutic enzymes. In recent years, recombinant mAbs produced in CHO cells were among the top best-selling biologics and approximately 40 newly US Food and Drug Administration (FDA) approved recombinant mAbs were made available in the market [1]. As the demands for recombinant mAbs continue to grow, there is continual

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Abbreviations: CHO, Chinese hamster ovary; **Co-IP**, co-immunoprecipitation; **ECL**, enhanced chemiluminescent; **HRP**, horse-radish peroxide; **HSP**, heat shock protein; **IFN-γ**, interferon-gamma; **IRES**, internal ribosome entry site; **IVCD**, integrated viable cell density; **LDS**, lithium dodecyl sulphate; **mAb**, monoclonal antibody; **MALDI-TOF MS**, matrix-assisted laser desorption ionization-time of flight mass spectrometry; **qRT-PCR**, quantitative real-time polymerase chain reaction; **RIPA**, radio-immunoprecipitation assay; **RT-PCR**, reverse transcription polymerase chain reaction.

need for robust CHO production cell lines with maximized product titers. Furthermore, with the emergence of innovative biologics and biosimilars in the market [2], different CHO production cell lines are most likely to be used as production hosts of reference biologics are propriety knowledge [3]. To meet these needs, this current study presents the use of a cell line engineering strategy to improve recombinant mAbs production in CHO cells.

An in-house apoptosis transcriptome profile of CHO fed-batch bioreactor cultures established that the expression of heat shock protein (HSP) 27 was down-regulated towards the late-exponential and stationary growth phase. HSP27 is a small 27 kDa stress inducible HSP located mainly in the cytosol and functions primarily as a molecular chaperone where it aids in preventing aggregation of proteins and participates in the refolding of misfolded proteins with the help of other molecular chaperones [4]. Recent studies on human pathological tissues and cell lines such as U937, THP-1 and MCF-1 reported high expression of HSP27. In response to a variety of different apoptotic insults, the high expression level of HSP27 was demonstrated to modulate the apoptosis signaling pathways and resulted in increased resistance to apoptotic cell death [5, 6]. The link between high HSP27 expression and the observed apoptosis-resistant phenotypes was HSP27 interactions with targets along the apoptosis signaling pathways: Akt [7], cytochrome c released from dysfunctional mitochondria [8], inactive form of caspase 3, pro-caspase 3 [9] and Daxx [10].

Henceforth, it was hypothesized that engineering CHO production cell lines with overexpression of HSP27 can increase robustness of CHO fed-batch bioreactor processes by delaying apoptotic cell death and result in higher recombinant biologics yield. Earlier overexpression of HSP27 in CHO-DxB11 cells resulted in extension of fed-batch bioreactor culture duration and increased recombinant human interferon gamma (IFN-γ) yield [11]. This paper reports on the application of HSP27 overexpression strategy in CHO-DG44 cells producing a recombinant IgG mAb against Rhesus D antigen [12]. To date, besides the reported delayed increase in caspase activities in CHO-IFN-γ fed-batch bioreactor cultures [11], no other mechanistic studies on HSP27 anti-apoptotic roles in CHO fed-batch bioreactor cultures have been reported. Hence, an attempt to further expand this limited knowledge by validating HSP27-interacting apoptosis targets in CHO-DG44 cells producing recombinant mAb based on human and mouse cell line studies is also reported.

2 Materials and methods

2.1 Cell culture

CHO-mAb 2.11 subclone M250-9, a CHO-DG44-derived suspension cell line expressing a recombinant secreted humanized IgG monoclonal antibody against Rhesus D antigen [12] was used and referred to as CHO-mAb in this study. The cells were maintained in a protein-free media mixture consisting of a 1:1 ratio of HyQ-PF-CHO (HyClone-GE Healthcare, Sweden) and CD CHO (Life Technologies, USA), supplemented with 6 mM glutamine (Sigma-Aldrich, USA), 0.05% Pluronic F-68® (Life Technologies), 600 μg/mL Geneticin® (Life Technologies) and 250 nM methotrexate (Sigma-Aldrich). Cells were grown in shake flasks maintained at 37°C, 8% CO_2 atmosphere on an orbital shaker maintained at 110 rpm with shaking amplitude of 12.5 mm, and subcultured every three to four days with exponentially growing cells at seeding viable cell concentration of 3.0×10^5 cells/mL and starting culture volume of 20 mL.

2.2 Generation of expression vectors

To generate the control null vector pIRES2, the truncated internal ribosome entry site (IRES) fragment from mammalian expression vector, pIRES (Clontech Laboratories, USA), was replaced with that from mammalian expression vector, pIRES-DsRedExpress (Clontech Laboratories), using restriction enzymes, *EcoR I* and *Xba I* (Supporting information, Fig. S1A). The neomycin selection marker was then replaced with hygromycin selection marker from vector pcDNA 3.1-hyg (Life Technologies) using restriction enzymes, *Stu I* and *Bst BI*. To generate vector pIRES2-27 for overexpression of HSP27, the coding region of CHO-K1 HSP27 from pIRES-27 vector [11] was digested using restriction enzymes, *Nhe I* and *Xho I*, and inserted into multiple cloning site A of pIRES2 vector (Supporting information, Fig. S1B). To ensure efficient translation, a Kozak sequence, GCCGCC, was placed upstream of the HSP27 start codon.

2.3 Generation of stable HSP27-overexpressing CHO cells

CHO-mAb cells were stably transfected with pIRES2 and pIRES2-27 expression vectors to obtain stable p2.Blank and p2.27 CHO-mAb cells respectively. Briefly, transfection was performed using Lipofectamine® 2000 reagent (Life Technologies), as per manufacturer's instructions, in six-well culture plates under static condition and expanded into shake flasks, upon confluence, at conditions described in Section 2.2. Pools of stable transfectants were obtained after 14 days of culturing under 300 μg/mL hygromycin (Clontech Laboratories) selection pressure and expanded thereafter under 100 μg/mL hygromycin reduced selection pressure for banking, initial assessment in batch shake flasks and single cell cloning. For batch shake flasks assessment, the exponentially growing cells were seeded at viable cell concentration of 3.0×10^5 cells/mL with a starting culture volume of 130 mL. Viable cell concentration, culture viability, recom-

binant mAb titer and extracellular metabolite concentrations were monitored off-line daily till culture viability dropped below 50%. Concurrently, single cell clones were obtained from the respective stable transfected cell pools for evaluation in fed-batch bioreactors by seeding one cell per well in 96-well plates using limiting dilution. Wells containing more than one cell were excluded and half of the media in each of the remaining wells were replaced twice weekly. Surviving clones, upon reaching confluence, were expanded into shake flasks for banking and selected clones evaluated in fed-batch bioreactors.

2.4 Fed-batch bioreactor set-up and feeding strategy

Fed-batch bioreactor systems of 1.5-L working volume were set up and performed as previously described [11]. Two separate feeds were used; a glucose-free, protein-free feed formulated based on a fortified 10X DMEM/F12 (Sigma-Aldrich) supplemented with 10 g/L of soybean protein hydrolysate (Kerry, USA) and a 180 g/L glucose feed (Sigma-Aldrich). Feed-forward control strategy was employed to achieve discrete control of glutamine and glucose concentrations at set points of 0.04 g/L and 0.5 g/L respectively. Briefly, based on pre-determined glutamine and glucose specific consumption rates during exponential growth phase, projected amount of glutamine and glucose that will be consumed by the cells until the next forecasted sampling time was fed at pre-set intervals of 90 min.

2.5 Analysis of cell growth, extracellular metabolite concentrations and recombinant mAb titer

Viable cell concentration was determined by manual cell counting using an Improved Neubauer haemocytometer (Weber, England) and culture viability was estimated based on the trypan blue exclusion method. Integrated viable cell density (IVCD) was calculated using the formula: $IVCD = 0.5(t_2 - t_1)(x_1 + x_2)$, where t is the culture time (h) and x is the viable cell concentration (cells/mL). Concentrations of extracellular metabolites (glucose, glutamine, lactate and ammonium) were determined by analysing spent media offline using the YSI 7100 biochemical analyser (Yellow Spring Instruments, USA). Recombinant mAb titers were determined using an IMMAGE 800 immunochemistry system (Beckman Coulter, USA) based on nephelometry method, according to manufacturer's instructions.

2.6 Detection of caspase 2, 3, 8 and 9 activities

2.0×10^6 cells were collected at every 24 h intervals from the fed-batch bioreactor cultures until cultures viabilities dropped below 80%. Caspase 2, 3, 8 and 9 activities were detected as previously described [11].

2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

1.0×10^7 cells were collected on day 3 of the batch shake flask cultures and total RNA was extracted using Trizol™ reagent (Life Technologies) as per manufacturer's instructions. Total RNA extracted was quantified using GeneQuant™ Pro RNA/DNA Calculator (GE Healthcare), followed by reverse transcription using 1 μg of total RNA and the Improm-II™ Reverse Transcription System (Promega, USA) as per manufacturer's instructions. The resulting first strand cDNA was used in equal amount for qRT-PCR carried out using ABI PRISM 7000 Sequence Detection System (Life Technologies) with SYBR Green PCR Master Mix (Life Technologies). Gene expression levels of HSP27 and pIRES2-27 expression vector were evaluated. Duplicate runs were conducted per sample and normalized against β-actin (U20114). Fold change in gene expression was calculated using the delta-delta threshold cycle method as described in [13]. Primers used are listed in Supporting information, Table S1.

2.8 Semi-quantitative reverse-transcriptase PCR (RT-PCR)

1.0×10^7 cells were collected on day 3 of the batch shake flask cultures and total RNA was extracted using RNeasy™ mini kit (Qiagen, Netherlands) as per manufacturer's instructions. Total RNA extracted was quantified using NanoDrop™ 2000 UV-VIS spectrophotometer (Thermo Scientific, USA), followed by RT-PCR with 1 μg of total RNA using OneStep™ RT-PCR kit (Qiagen) as per manufacturer's instruction. Gene expression levels of HSP27 and pIRES2-27 expression vector were evaluated and normalized against β-actin. The RT-PCR exponential phase was determined at 26 cycles and the amplified RT-PCR products were subjected to electrophoresis for visualization on a 1.5% agarose gel containing 0.05 μg/mL of ethidium bromide, followed by UV analysis using the InGenius3 system (Syngene, UK). Primers used are listed in Supporting information, Table S1.

2.9 Western blots

1.0×10^7 cells were collected, washed once with ice-cold 1× phosphate buffered saline solution (Life Technologies) and lysed in radio-immunoprecipitation assay (RIPA) buffer (Pierce-Thermo Scientific, USA) supplemented with 1× Complete™ protease inhibitor cocktail (Roche, Switzerland) for 40 min on ice with intermittent vortexing. The complex mixture was centrifuged at 14 000 rpm for 20 min at 4°C to obtain total cellular proteins in suspension and quantified using BCA Protein Assay kit as per manufacturer's instructions (Pierce-Thermo Scientific). Equal amount of total cell lysate was treated with 1× lithium dodecyl sulphate (LDS) sample buffer (Life Technolo-

gies), denatured for 5 min at 95°C, separated on 10% Bis-Tris gel (Life Technologies) and transferred onto iBlot™ nitrocellulose membranes (Life Technologies). The membrane was then probed for HSP27 and β-actin using rabbit anti-HSP27 (#06-517, Upstate-Merck Millipore, Germany) and mouse anti-β actin (ab8226, Abcam, England) antibodies respectively. The primary antibodies were detected using horse radish peroxide (HRP)-conjugated anti-rabbit (ab6721, Abcam) and HRP-conjugated anti-mouse HRP (ab6728, Abcam) respectively, visualized using enhanced chemiluminescent (ECL) substrate (GE Healthcare) for HRP and captured on ImageQuant LAS 500 imager system (GE Healthcare). The antibodies were verified and shown to work for CHO cellular proteins prior to use.

2.10 Co-immunoprecipitation (Co-IP)

1.0×10^7 cells were collected, washed twice with ice-cold 1× phosphate buffered saline solution (Life Technologies) and lysed by mechanical pipetting in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 1× Complete™ protease inhibitor cocktail and 1× PhosSTOP™ phosphatase inhibitor cocktail (Roche). The mixture was then centrifuged at 13 000 rpm for 10 min at 4°C to collect the total cellular proteins containing the protein-protein interaction complexes in suspension and quantified using the BCA Protein Assay kit as per manufacturer's instructions (Pierce-Thermo Scientific). Protein samples were pre-cleared by incubating with 100 μL of Protein G agarose beads suspension (Roche), for 3 h at 4°C with constant mixing. Pre-cleared protein samples were collected after centrifugation at 13 000 rpm for 20 s before incubating overnight with mouse anti-HSP27 (sc13132, Santa Cruz, USA) or anti-mouse (sc2025, Santa Cruz) antibodies respectively in separate tubes at 4°C with constant mixing. 100 μL of Protein G agarose beads suspension was then added followed by further incubation for 3 h at 4°C with constant mixing. The bead complexes were washed sequentially twice with lysis buffer, followed by twice with buffer containing 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Nonidet P40 and 0.05% sodium deoxycholate and once with buffer containing 10 mM Tris-HCl pH 7.5, 0.1% Nonidet P40 and 0.05% sodium deoxycholate. Protein complexes retained on beads were eluted by incubating with 1× LDS sample buffer for 5 min at room temperature, followed by incubating at 95°C for 5 min. Eluted proteins were separated from the Protein G agarose beads by centrifuging at 13 000 rpm for 20 s. The eluted proteins and total cellular proteins were separated on 4–12% Bis-Tris gel and transferred onto iBlot™ nitrocellulose membranes. The membranes were then probed for Akt, cytochrome c, Daxx, HSP27 and pro-caspase 3 using rabbit anti-Akt (#9272, Cell Signalling Technology, USA), rabbit anti-cytochrome c (#4280, Cell Signalling Technology), rabbit anti-Daxx (#07-471, Upstate-Merck Millipore), rabbit anti-HSP27 (#06-517, Upstate-Merck Millipore) and rabbit anti-pro-caspase 3 (#9662, Cell Signalling Technology) antibodies respectively. The antibodies were diluted in blocking buffer (1× Tris Buffered Saline, 0.1% Tween20 and 3% BSA) except rabbit anti-cytochrome c was diluted in Signal Enhancer HIKARI Solution A (Nacalai Tesque, USA). The primary antibodies were detected using HRP conjugated anti-rabbit (ab6721, Abcam) diluted in blocking buffer or Signal Enhancer HIKARI Solution B (Nacalai Tesque) for detection of rabbit anti-cytochrome c, visualized using ECL substrate (GE Healthcare) for HRP and captured on ImageQuant LAS 500 imager system. The antibodies were verified and shown to work for CHO cellular proteins prior to use.

2.11 N-glycan profiling of purified recombinant monoclonal antibody

The recombinant mAb present in the fed-batch bioreactor cultures spent media were purified using a Tricorn 5/150 Protein A column packed with MabSelect SuRe (GE Healthcare). The N-glycan distribution of the purified recombinant mAb was analysed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) before categorization as described previously [14].

3 Results

3.1 Generation of stable HSP27-overexpressing CHO-mAb cell pools and evaluation in batch shake flask culture

CHO-mAb cells were maintained in culture for 12.5 weeks before being transfected either with control null vector pIRES2 or HSP27-overexpressing vector pIRES2-27 for the generation of p2.Blank cells and p2.27 CHO-mAb cells respectively. Stably transfected p2.Blank and p2.27 CHO-mAb cell pools were obtained after two weeks of selection in hygromycin-supplemented culture media and expanded for another two and a half weeks before banking and initial assessment in batch shake flasks. To verify that HSP27 was overexpressed in the p2.27 CHO-mAb cell pool, qRT-PCR and Western blot analyses were performed on day 3 of the batch shake flask cultures. Higher HSP27 gene and protein expression level was detected in p2.27 CHO-mAb cell pool compared to p2.Blank CHO-mAb cell pool, confirming that p2.27 CHO-mAb cell pool was overexpressing HSP27 (Supporting information, Fig. S2).

As an initial assessment of whether HSP27 overexpression can improve CHO-mAb batch shake flask culture performance, stably transfected CHO-mAb cell pools were evaluated under hygromycin selection pressure

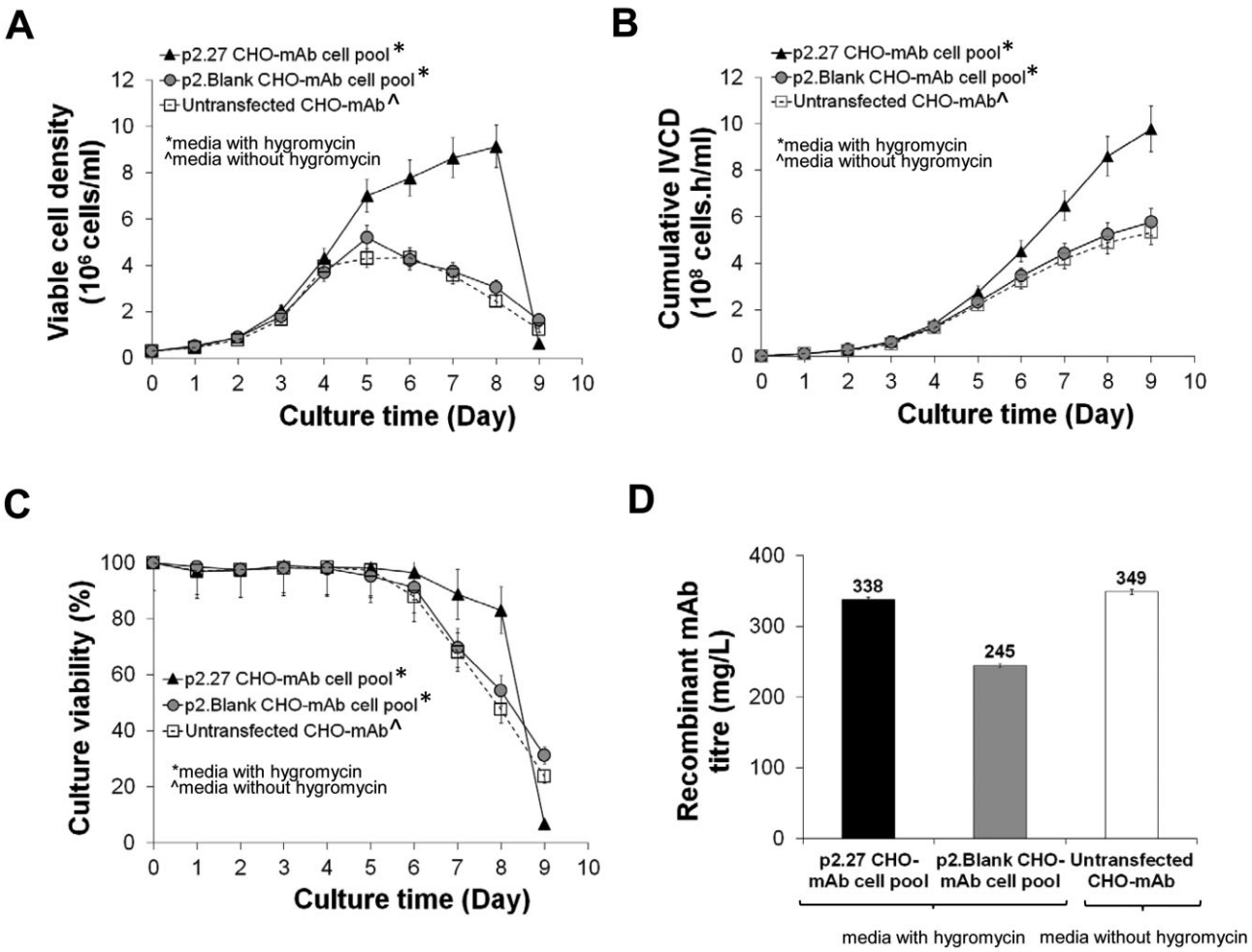

Figure 1. Characterization of stably transfected CHO-mAb cell pools and untransfected CHO-mAb cells in batch shake flasks. Stably transfected HSP27-overexpressing CHO-mAb cell pool (p2.27; black triangles) and stably transfected null vector-overexpressing CHO-mAb cell pool (p2.Blank; grey circles) were cultivated in hygromycin-containing selection media. The untransfected CHO-mAb cells (unfilled squares) were cultivated in hygromycin-free non-selecting media. Exponentially growing cells were seeded at viable cell concentration of 3.0×10^5 cells/mL with a starting culture volume of 130 mL. (**A**) Viable cell concentration, (**B**) culture viability, (**C**) cumulative IVCD were monitored off-line daily till culture viability dropped below 50%. (**D**) Recombinant mAb titers were quantified on day 9 of the batch shake flask cultures. N=1 and the error bars represents the SD from the mean of technical triplicates.

(Fig. 1). This assessment on the stably transfected cell pools also served to eliminate possible artefacts from clonal selection. To assess the effect of transfection and hygromycin selection on the transfected cell pools, the growth and production curves from the untransfected CHO-mAb cells cultivated without hygromycin selection was also determined.

Comparing the p2.Blank CHO-mAb cell pool with the untransfected CHO-mAb cells, it was observed that while these cells had similar growth profiles (Fig. 1A and B), the recombinant mAb titer of the p2.Blank CHO-mAb cell pool was 30% lower than that of the untransfected CHO-mAb cells (Fig. 1D), demonstrating that the transfection process and the presence of hygromycin in the culture medium have negatively affected the recombinant mAb production in the transfected cell pool. This may be due to the non-specific inhibitory effect of hygromycin on the p2.Blank CHO-mAb cells, and post-transfection chromosomal rearrangements in stably transfected cell pools that may have occurred during the stable transfection process, especially since CHO cells are prone to spontaneous mutations when under selection pressure or stressful conditions [15, 16]. Notably, the effect of stable selection can also be observed from the differences in extracellular metabolite profiles of the untransfected and transfected cell pools (Supporting information, Fig. S3).

On the other hand, the p2.27 CHO-mAb cell pool achieved 76 and 110% higher maximum viable cell concentration compared to p2.Blank CHO-mAb cell pool and untransfected CHO-mAb cells respectively (Fig. 1A) and sustained at high culture viability of above 80% for an additional one day (Fig. 1B). These growth improvements

lead to 70 and 84% higher maximum cumulative IVCD of 10.0 × 10^8 cells-h/mL compared to p2.Blank CHO-mAb cell pool and untransfected CHO-mAb cells respectively (Fig. 1C). It is postulated that the inhibition of apoptosis by HSP27 may be a likely mechanism for the observed growth improvements in p2.27 CHO-mAb cell pool batch shake flask cultures: while p2.Blank CHO-mAb cell pool and untransfected CHO-mAb cells entered death phase due to culture stress after day 5, overexpression of HSP27 (Supporting information, Fig. S2) may have prevented the same from happening to the p2.27 CHO-mAb cell pool to allow further proliferation of these cells. With the improvement in cumulative IVCD, the p2.27 CHO-mAb cell pool achieved a 38% higher recombinant mAb titer (Fig. 1D) that that of p2.Blank CHO-mAb cell pool although the recombinant mAb specific productivity of p2.27 CHO-mAb cell pool was also negatively affected by the transfection process and the presence of hygromycin in the culture medium.

3.2 Fed-batch bioreactor culture performance of HSP27-overexpressing CHO-mAb cell clones

Next, effects of HSP27 overexpression on cell growth and recombinant mAb production characteristics of CHO-mAb fed-batch bioreactor cultures were evaluated (Fig. 2). As the stably transfected cell pools are heterogeneous in population, single cell clones are preferred for better consistency in fed-batch bioreactor cultures. Limiting dilution was thus carried out and three p2.27 CHO-mAb clones (p2.27-1, p2.27-2 and p2.27-3) were selected out of 23 clones based on HSP27 expression, cell growth and recombinant mAb production profiles in batch shake flasks (Supporting information, Fig. S4). Three p2.Blank CHO-mAb clones (p2.Blank-1, p2.Blank-2 and p2.Blank-3) were selected out of 15 clones as controls in later fed-batch bioreactors evaluation. The selected p2.Blank CHO-mAb clones had lower HSP27 expression level compared to p2.27 CHO-mAb clones

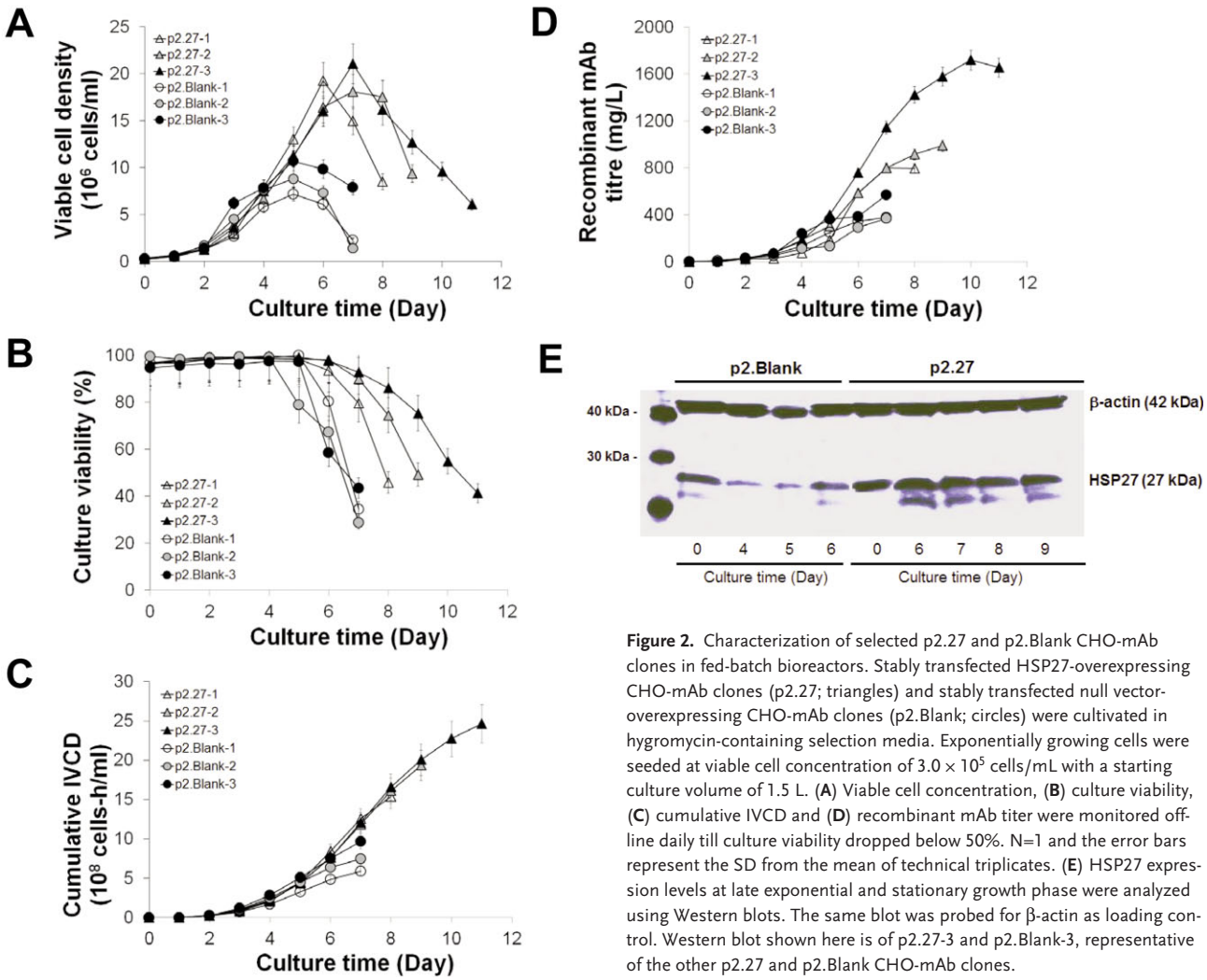

Figure 2. Characterization of selected p2.27 and p2.Blank CHO-mAb clones in fed-batch bioreactors. Stably transfected HSP27-overexpressing CHO-mAb clones (p2.27; triangles) and stably transfected null vector-overexpressing CHO-mAb clones (p2.Blank; circles) were cultivated in hygromycin-containing selection media. Exponentially growing cells were seeded at viable cell concentration of 3.0 × 10^5 cells/mL with a starting culture volume of 1.5 L. (**A**) Viable cell concentration, (**B**) culture viability, (**C**) cumulative IVCD and (**D**) recombinant mAb titer were monitored off-line daily till culture viability dropped below 50%. N=1 and the error bars represent the SD from the mean of technical triplicates. (**E**) HSP27 expression levels at late exponential and stationary growth phase were analyzed using Western blots. The same blot was probed for β-actin as loading control. Western blot shown here is of p2.27-3 and p2.Blank-3, representative of the other p2.27 and p2.Blank CHO-mAb clones.

(Supporting information, Fig, S4). Notably, p2.Blank-2 had similar maximum cumulative IVCD and recombinant mAb titer compared to the three selected p2.27 CHO-mAb clones in batch shake flasks (Supporting information, Fig. S4).

Compared to fed-batch bioreactor cultures of p2.Blank CHO-mAb clones, p2.27 CHO-mAb clones achieved 2.2-fold higher maximum viable cell concentration of $2.0 \pm 0.2 \times 10^7$ cells/mL (Fig. 2A) and sustained for an additional average of two days at culture viabilities above 80% (Fig. 2B). These growth improvements led to a 2.5-fold higher maximum cumulative IVCD of $2.0 \pm 0.5 \times 10^9$ cells-h/mL (Fig. 2C) and 2.3-fold higher recombinant mAb titer of 1146 ± 451 mg/L (Fig. 2D) compared to p2.Blank CHO-mAb clones fed-batch bioreactor cultures. All the clones in fed-batch bioreactor cultures had similar average specific recombinant mAb productivities. Figure 2E shows that HSP27 remained highly expressed in the late-exponential and stationary growth phase of p2.27 CHO-mAb clones cultured in fed-batch bioreactors as compared to p2.Blank CHO-mAb clones cultured in fed-batch bioreactors. This demonstrates that overexpression of HSP27 greatly increases recombinant mAb titer by improving cell growth and extending culture duration at above 80% viability of CHO-mAb fed-batch bioreactor cultures. The growth improvements observed in fed-batch bioreactor cultures was not a consequence of the feeding strategy as all cultures were not deprived of glucose and glutamine during culture (data not shown).

Additionally, the effect of HSP27 overexpression strategy on the quality of the recombinant mAbs produced by HSP27-overexpressing CHO-mAb cells cultured in fed-batch bioreactors were evaluated. p2.27-3 and p2.Blank-1, cultured in triplicated fed-batch bioreactors as described in Section 2.4, were used in this study (Supporting information, Fig. S5). There was no significant difference between the N-glycan distribution profiles of the recombinant mAbs produced in both p2.27-3 and

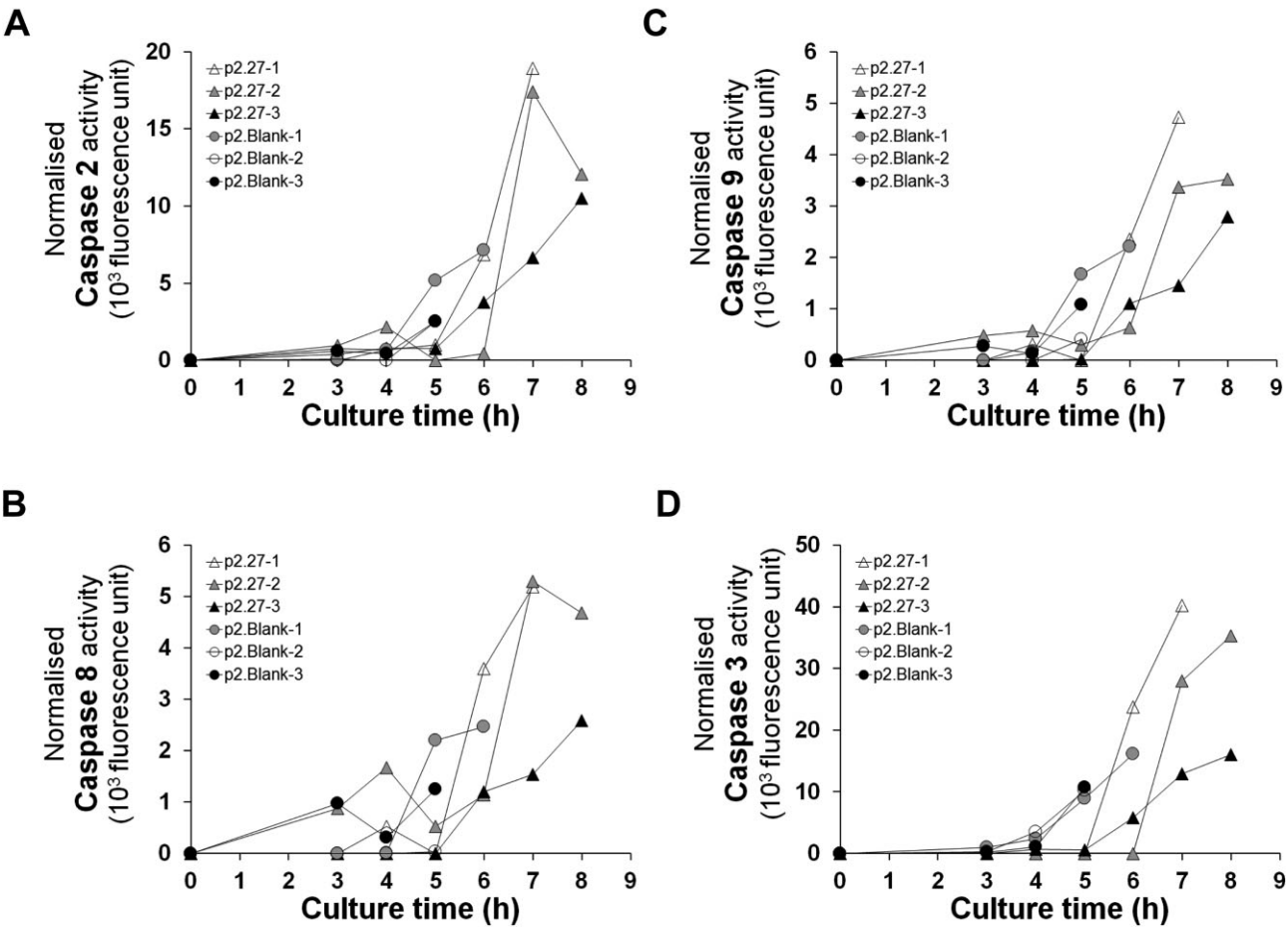

Figure 3. Caspase activity profiles of stably transfected p2.27 and p2.Blank CHO-mAb clones fed-batch bioreactor cultures. Stably transfected HSP27-overexpressing CHO-mAb clones (p2.27; triangles) and stably transfected null vector-overexpressing CHO-mAb clones (p2.Blank; circles) were cultivated in hygromycin-containing selection media before cultured in fed-batch bioreactors (Fig. 2). (**A**) Caspase 2, (**B**) caspase 8, (**C**) caspase 9 and (**D**) caspase 3 activities were monitored off-line daily in 24 h intervals until culture viabilities dropped below 80%. N=1 and the error bars represent the SD from the mean of technical triplicates.

p2.Blank-1 CHO-mAb fed-batch bioreactor cultures (Student t-test with p-Value < 0.05) despite p2.27-3 having 4.6-fold higher recombinant mAb titer than that of p2.Blank-1 (Supporting information, Fig. S6). This demonstrates that overexpression of HSP27 in CHO-mAb cells did not result in changes in N-glycan profiles or introduce abnormal glycan structures.

3.3 Caspase 2, 8, 3 and 9 activities of HSP27-overexpressing CHO-mAb cell clones in fed-batch bioreactor cultures

Current knowledge of HSP27 molecular roles in improving CHO fed-batch bioreactor cultures is limited to delayed increase of caspase 2, 3, 8 and 9 activities reported previously [11]. To verify whether HSP27 overexpression resulted in the same phenotype in CHO-mAb clones in fed-batch bioreactor cultures, activity profiles of the above-mentioned caspases were evaluated (Fig. 3).

Increases in caspase 2, 8 and 9 activities were delayed by an average of one day in p2.27 CHO-mAb clones cultured in fed-batch bioreactors as compared to p2.Blank CHO-mAb clones cultured in fed-batch bioreactors (Fig. 3A–C). Notably, caspase 8 activity of p2.Blank-1 was observed to be lower than that of other two p2.Blank and p2.27 CHO-mAb clones cultured in fed-batch bioreactors (Fig. 3B). This depicts clonal variability and inherent mutability of transfected CHO cells. Executioner caspase 3 activities were also delayed by an average of two days in p2.27 CHO-mAb clones cultured in fed-batch bioreactors as compared to p2.Blank CHO-mAb clones cultured in fed-batch bioreactors (Fig. 3D). These delays in caspase activities corroborated with the delayed loss of culture viabilities in p2.27 CHO-mAb clones as compared to p2.Blank CHO-mAb clones cultured in fed-batch bioreactors (Fig. 2B). This suggests that one of HSP27 anti-apoptotic roles in CHO-mAb fed-batch bioreactor cultures is modulating apoptosis by delaying increase in caspase 2, 3, 8 and 9 activities.

3.4 Interacting targets of HSP27 in CHO-mAb fed-batch bioreactor cultures

Studies done on human and mouse pathological cell lines reported that HSP27 interactions with Akt, cytochrome C, pro-caspase 3 and Daxx from the apoptosis signalling pathways resulted in increased resistance to apoptotic cell death [5]. As delays in caspase 2, 3, 8 and 9 activities were observed in p2.27 CHO-mAb clones cultured in fed-batch bioreactors compared to p2.Blank CHO-mAb clones cultured in fed-batch bioreactors (Fig. 3), it is postulated that HSP27 in CHO-mAb fed-batch bioreactor cultures interacts with similar apoptosis targets reported in human and mouse cells studies. Henceforth, the above-mentioned reported HSP27 interacting targets were examined in this study (Fig. 4). p2.27-3 cultured in triplicated fed-batch bioreactors (Supporting information, Fig. S5) was used.

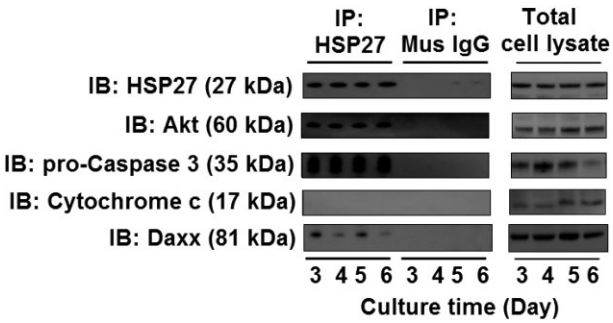

Figure 4. Western blots of total cellular lysates and eluted Co-IP complex of HSP27 interacting targets in p2.27-3 CHO-mAb clone fed-batch bioreactor cultures. Total cellular lysates were co-immunoprecipitated with anti-HSP27 and anti-mouse antibodies separately. Anti-mouse antibody serves as a negative control for isotype and species specificity. Both total cellular lysates and eluted Co-IP complex were immunoblotted with anti-Akt, anti-cytochrome c, anti-pro-caspase 3, anti-Daxx and anti-HSP27 antibodies. Figure shown here is representative of biological triplicates.

HSP27 was pulled down with anti-HSP27 antibody, but not with anti-mouse antibody, which served as a negative control for isotype and species specificity, demonstrating that the Co-IP method can pull down HSP27 specifically (Fig. 4). Akt, Daxx and pro-caspase 3 were also pulled down using anti-HSP27 antibody, but not with anti-mouse antibody (Fig. 4). HSP27-Akt interaction may enhance the activity of Akt and increase regulation of pro-survival and pro-proliferation signals and activities in CHO-mAb cells [17]. Akt has also been reported to inhibit activity of caspase 8 indirectly via a death-effector domain [18] and caspase 9 directly [19]. This corroborated with the delayed increased in caspase 8 and 9 activities of p2.27 CHO-mAb clones as compared to p2.Blank CHO-mAb clones, when cultured in fed-batch bioreactors (Fig. 3B–C). HSP27-Daxx interaction may inhibit caspase-independent cell death by preventing interaction of Ask1 with Fas-receptor, resulting in delayed loss of culture viabilities observed in p2.27 CHO-mAb clones cultured in fed-batch bioreactors as compared to p2.Blank clones cultured in fed-batch bioreactors (Fig. 2). The interaction of HSP27 with pro-caspase 3 may have prevented its maturation into active caspase 3, resulting in the delayed increased in caspase 3 activity (Fig. 3D). caspase 3 active proteolytic functions have been shown to activate pro-caspase 2 to active caspase 2 [20]. This corroborated with the delayed activation of caspase 2 in p2.27 CHO-mAb clones compared to p2.Blank CHO-mAb clones, when cultured in fed-batch bioreactors (Fig. 3A).

On the other hand, cytochrome c, was not pulled down using both anti-HSP27 and mouse IgG, although the protein was detectable in the cellular protein lysates of p2.27-3 cells (Fig. 4). This was despite the long exposure time of 2 min 30 s for the Co-IP complex blot versus 10 s

for the cellular protein lysates blot. While HSP27 has been shown to interact with cytochrome c in human cells [8], CHO HSP27 [11] and CHO cytochrome c (XP_003498601) protein sequences differ from those of human (NP_001531 and NP_061820 respectively) by 14% (data not shown). These variations may have resulted in different HSP27-cytochrome c interaction in human and CHO cells.

Hence, we demonstrated that HSP27 protein is able to interact with Akt, pro-caspase 3 and Daxx, but not cytochrome c, in CHO-mAb cells and these interactions may have contributed to better p2.27 CHO-mAb fed-batch bioreactor cultures performance as shown in Fig. 2.

4 Discussion

Differential HSP27 transcript expression during the late-exponential and stationary growth phase of CHO fed-batch bioreactor cultures has led to the hypothesis that overexpression of HSP27 in CHO cells can improve CHO fed-batch bioreactor culture performance. Consequent overexpression of HSP27 in a CHO-DxB11 cell line prolonged fed-batch bioreactor culture duration and increased recombinant human IFN-γ yield [11]. In this study, we wanted to apply the HSP27 overexpression strategy in fed-batch bioreactor cultures of a different CHO cell line (CHO-DG44), producing a different recombinant protein (recombinant mAb, a class of rapidly expanding recombinant biologics). Moreover, with the emerging introduction of innovative biologics and biosimilars in the market, there are opportunities to use new CHO production cell line engineered with increased culture robustness. Hence, it would be of importance to evaluate the effects of HSP27 overexpression in improving recombinant mAb production in CHO fed-batch bioreactor cultures.

Preliminary evaluation of HSP27 overexpression in improving CHO-mAb batch shake flask cultures was car-

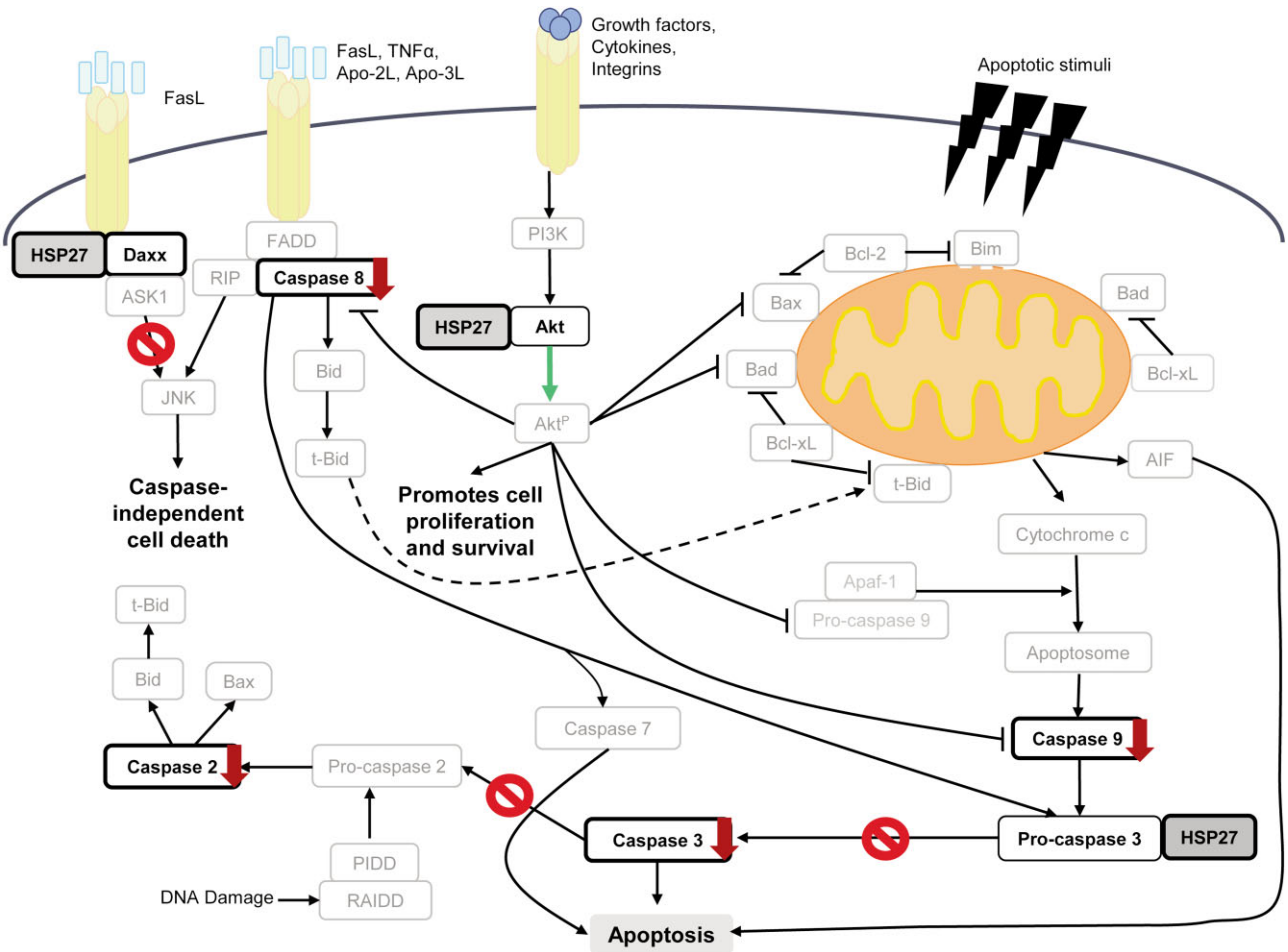

Figure 5. Proposed schematic illustration of HSP27 anti-apoptotic functions in HSP27-overexpressing CHO-mAb cells. HSP27 interactions with Akt, Daxx and pro-caspase 3 enhance survival and proliferation, inhibit caspase-independent cell death and inhibit caspase 3-induced apoptosis respectively. However, HSP27 does not interact with cytochrome c as reported in human cell lines studies. Overexpression of HSP27 also delays hike in caspase 2, 3, 8 and 9 activities.

ried out using stably transfected cell pools (Fig. 1). It was established that HSP27 overexpression can increase peak cell density and delay loss of culture viability but not improve recombinant mAb production in CHO-mAb batch shake flask cultures (Fig. 1). This was further verified during the selection of stably transfected clones under batch shake flask conditions. Stably overexpressing HSP27 p2.27 clones had slightly higher cumulative IVCD but similar recombinant mAb titer to p2.Blank clones stably transfected with the null vector (Supporting information, Fig. S4). Subsequently, the selected p2.27 and p2.Blank clones were evaluated in fed-batch bioreactors. The p2.27 CHO-mAb clones cultured in fed-batch bioreactors achieved higher maximum cumulative IVCD that greatly increased recombinant mAb titers (Fig. 2) without significantly altering the N-glycan distribution profile of purified recombinant mAb as compared to p2.Blank CHO-mAb clones in fed-batch bioreactor cultures (Supporting information, Fig. S6).

In addition, we wanted to identify anti-apoptotic functions of HSP27 that contributed to the improvements in p2.27 CHO-mAb fed-batch bioreactor cultures performance as compared to p2.Blank CHO-mAb fed-batch bioreactor cultures (Fig. 2). Figure 5 shows the proposed schematic illustration of HSP27 anti-apoptosis functions identified and validated in HSP27-overexpressing CHO-mAb cells: modulation of caspase activities (Fig. 3) and HSP27 interactions with Akt, pro-caspase 3 and Daxx along the apoptosis signaling pathways (Fig. 4).

Comparing the effect of HSP27 overexpression in CHO-mAb and in CHO cells producing recombinant human IFN-γ, both engineered cells showed improvement in IVCD and delayed hikes in caspase activities in fed-batch bioreactor cultures. However, while the peak viable cell density was improved in the HSP27-overexpressing CHO-mAb fed-batch bioreactor cultures, IVCD improvement in HSP27-overexpressing CHO-IFN-γ fed-batch bioreactor cultures was only due to extension of culture duration without improvement in peak viable cell density [11]. It would be interesting to elucidate whether the difference in parental host cell lines or recombinant proteins produced had any effects on the molecular functions of HSP27 that resulted in the different CHO fed-batch bioreactor culture phenotype. Furthermore, even though HSP27 overexpression has been demonstrated to delay loss of culture viability via its anti-apoptotic functions, culture viability of p2.27 CHO-mAb clones in fed-batch bioreactor cultures continued to drop after day 8 (Fig. 2). This indicates that there may be other signaling pathways leading to apoptosis that are not affected by overexpression of HSP27 and these would make interesting cell line engineering targets for further improving robustness of HSP27-overexpressing CHO-mAb fed-batch bioreactor cultures to achieve even higher recombinant mAb titers.

In conclusion, together with the earlier study [11], we have successfully demonstrated the applicability of HSP27 overexpression strategy in improving titers of two classes of recombinant biologics (recombinant cytokine and recombinant mAb) produced by two CHO production host cell lines (CHO-DxB11 and CHO-DG44 respectively) under fed-batch bioreactor culture mode. We have also validated the anti-apoptotic functions of HSP27 that contributed to better p2.27 CHO-mAb fed-batch bioreactor culture performances.

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The authors declare no commercial or financial conflict of interest.

5 References

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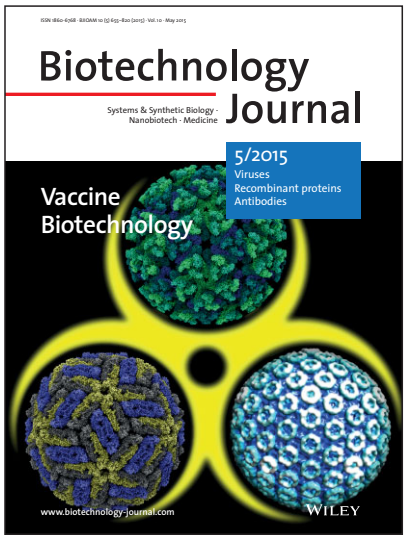

Cover illustration

Special issue: Vaccine Biotechnology. This Special issue edited by Reingard Grabherr and Udo Reichl includes articles on the design of cell lines for viral vaccine production, downstream processing of virus-like particles and plant-based production of vaccines. The cover shows particles of highly pathogenic viruses transmitted by mosquitoes: Chikungunya, dengue and Rift Valley fever virus. Image by Gorben Pijlman.

Biotechnology Journal – list of articles published in the May 2015 issue.

Research Article

Adjuvant poly(*N*-isopropylacrylamide) generates more efficient monoclonal antibodies against truncated recombinant histidine-rich protein2 of *Plasmodium falciparum* for malaria diagnosis

Reena Verma, Ramakrishnan Ravichandran, Naatamai S. Jayaprakash, Ashok Kumar, Mookambeswaran A.Vijayalakshmi, and Krishnan Venkataraman

http://dx.doi.org/10.1002/biot.201400386

Biotech Method

Bacterial cytoplasmic display platform Retained Display (ReD) identifies stable human germline antibody frameworks

Matthew D Beasley, Keith P Niven, Wendy R Winnall and Ben R Kiefel

http://dx.doi.org/10.1002/biot.201400560

Research Article

Heat shock protein 27 overexpression in CHO cells modulates apoptosis pathways and delays activation of caspases to improve recombinant monoclonal antibody titre in fed-batch bioreactors

Janice G.L. Tan, Yih Yean Lee, Tianhua Wang, Miranda G. S. Yap, Tin Wee Tan and Say Kong Ng

http://dx.doi.org/10.1002/biot.201400764

Research Article

Wheat enolase demonstrates potential as a non-toxic cryopreservation agent for liver and pancreatic cells

Mélanie Grondin, Mélanie Chow-Shi-Yée, François Ouellet and Diana A. Averill-Bates

http://dx.doi.org/10.1002/biot.201400562

Biotech Method

Purification and simultaneous immobilization of *Arabidopsis thaliana* hydroxynitrile lyase using a family 2 carbohydrate-binding module

Benita Kopka, Martin Diener, Astrid Wirtz, Martina Pohl, Karl-Erich Jaeger and Ulrich Krauss

http://dx.doi.org/10.1002/biot.201400786