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Induction of EGFP expression in *Pichia pastoris* during co-culture with human endothelial cell line

Der Thor*, Nan Xiao, Ryan Yu, Anita Jivan, Bomi Cha

Department of Biomedical Sciences, Arthur A. Dugoni School of Dentistry, University of the Pacific, 155 Fifth Street, San Francisco, CA 94103.

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ABSTRACT

While *Pichia pastoris* has been developed into a versatile recombinant protein expression system, there are only few studies that have investigated the efficacious use of this yeast with human cells. In this study, we demonstrated that *P. pastoris* can be cultured under mammalian cell culture conditions and co-cultured with human endothelial cells. Co-cultures did not affect endothelial cell morphology or viability. Additionally, *P. pastoris* was induced to express enhanced green fluorescence protein when co-cultured with human endothelial cell line EA.hy926 under mammalian cell culture conditions. Our study provides data to support the use of *P. pastoris* as a vehicle for direct delivery of recombinant proteins to mammalian cells during co-culture.

1. Introduction

The methylotrophic yeast *Pichia pastoris* has been engineered to produce over five thousand recombinant proteins according to pichia.com. Its popularity is due to several factors. *P. pastoris* has the ability to post-translationally modify proteins and can grow to high cell densities. Genetic modifications, which includes plasmid transformation and gene knockouts, to this yeast are also accomplished relatively easy. Furthermore, the yeast's promoter systems have been enhanced, and its secretion capabilities have been improved. Along with an increasing number of available vectors and strains (Ahmad et al., 2014; Juturu and Wu, 2018), the aforementioned factors help contribute to the versatility of *P. pastoris* as a protein expression system.

Recombinant proteins expressed in *P. pastoris* have been used to study structure and function or as therapeutic agents (Juturu and Wu, 2018). Such applications require that the heterologous protein be purified. Improvements to the secretory capability of *P. pastoris* combined with low secretion of its own endogenous proteins reduce the difficulty of purifying secreted recombinant peptides. However, purification can be time consuming, costly, and result in reduced protein yields (Potvin and Zhang, 2012). More recent developments in the use of whole cell yeasts as a vaccine delivering system (Ardiani et al., 2010; Kim et al., 2014) suggest a plausible use of recombinant proteins that do not require purification from the host expression system.

Because therapeutic proteins have been produced and secreted successfully by *P. pastoris* (Corchero et al., 2013; Meehl and Stadheim, 2014), we are interested to determine whether this yeast has the potential to serve as a carrier organism, delivering heterologous proteins directly to cultured cells without purification. In this role, the yeast is co-cultured with mammalian cells so that its recombinant proteins are secreted into a medium shared with the higher eukaryote. *P. pastoris* serves as a potentially good candidate because it is able to produce and secrete recombinant protein while secreting very little of its own endogenous proteins (Cereghino and Cregg, 2000). Certain proteins made in this system have been classified as generally regarded as safe (GRAS) for food ingredients by the Food and Drug Administration (Ciofalo et al., 2006; Thompson, 2010). While many microbial species have been investigated for their use as probiotics (Didari et al., 2014; Kerry et al., 2018), not much is known about the interactions between *P. pastoris* and human cells. Due to the plausible uses of *P. pastoris* as a protein delivery system in the human body, we believe that investigations into protocols that allow *P. pastoris* and mammalian cells to be co-cultured are warranted. The human endothelial cell line, EA.hy926 (Edgell et al., 1983), was chosen for these studies. We pursued a set of experiments to characterize the interactions between *P. pastoris* and human endothelial cells and developed a protocol to express enhanced green fluorescent protein (EGFP) in *P. pastoris* while cultured with human endothelial cells.

2. Methods

2.1. Cell culture

Wild type *P. pastoris* (JC100) and JC100 containing plasmid pKS1

* Corresponding author.
E-mail address: dthor@pacific.edu (D. Thor).

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(JC100::pKS1), which expresses EGFP under the control of the alcohol oxidase 1 (*AOX1*) promoter, were used in our work. The construction of pKS1 was described previously (Li et al., 2010). Cultures were grown in yeast peptone dextrose (YPD, 1% yeast extract, 2% peptone, 2% glucose) at 30 °C in a shaking incubator. Cells were counted by measuring the absorbance at 600 nm and calculated as an OD_{600} of 1.0 equal to 5×10^7 cells/mL (Asada et al., 2011).

The human endothelial cell line EA.hy926 (ATCC, Manassas, VA) was cultured using low glucose (1 g/mL) Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 µg/mL streptomycin in a 37 °C humidified incubator under 95% air and 5% CO_2. We will refer to this culture medium as complete DMEM throughout this manuscript. Stock cultures of EA.hy926 were grown in 100 mm tissue-culture coated petri dishes (Greiner Bio-One, Monroe, NC). Cells were subcultured in either 12-well or 96-well tissue-culture coated, flat-bottom plates for respective experiments. The 12-well plates have a growth area of 3.9 cm^2 per well (Greiner Bio-One, Monroe, NC) and 2 mL complete DMEM per well was used to culture EA.hy926. The 96-well plates have a growth area of 0.32 cm^2 per well (Greiner Bio-One, Monroe, NC) and 100 µL complete DMEM per well was used to culture EA.hy926.

Endothelial cells were counted with a hemocytometer using the following procedure. All solutions were warmed to 37 °C. After aspirating the culture medium, each well was washed with phosphate buffered saline (PBS). Cells were lifted with trypsin-EDTA solution (2.5 g/L porcine trypsin plus 0.2 g/L EDTA in Hanks' balanced salt solution with phenol red, MilliporeSigma, St. Louis, MO) and neutralized with complete DMEM. Gentle pipetting with a 10 mL serological pipette was used to create a homogenous mixture of suspended cells. The suspended cells (50 µL) were added to 50 µL of trypan blue (0.4% trypan blue prepared in 0.81% sodium chloride and 0.06% potassium phosphate, diabasic, MilliporeSigma, St. Louis, MO) and cells were counted with a hemocytometer. Suspensions were prepared in trypan blue two times, and each suspension of cells were counted twice.

2.2. Growing P. pastoris using mammalian cell culture conditions

A starter culture of *P. pastoris* was prepared in YPD at 30 °C overnight. This culture (5×10^4 cells) was resuspended in 3.0 mL of either YPD or complete DMEM. Cultures were incubated under non-shaking conditions at 30 °C or 37 °C. The cell population from each growth condition was counted by measuring the absorbance at 600 nm after 24, 48, 72, and 96 h.

2.3. P. pastoris and endothelial cell co-cultures

For co-culturing EA.hy926 with *P. pastoris*, the endothelial cells were first seeded into 12-well plates (1.0 mL complete DMEM containing 1×10^5 cells per well) and allowed to grow for 24 h. The endothelial cells were then co-cultured with 5×10^2, 5×10^3, or 5×10^4 yeast cells/mL in a final volume of 1 mL per well. Yeasts were prepared by harvesting an overnight culture grown in YPD at 30 °C. *P. pastoris* cells were centrifuged for five minutes at 2000 x g, and the pellet was resuspended in complete DMEM at concentrations mentioned above. The complete DMEM was aspirated from the 24 h EA.hy926 culture and replaced with the freshly made complete DMEM containing *P. pastoris* at the designated concentrations. The co-cultures were grown in a humidified CO_2 (5%) incubator at 37 °C. Endothelial cell morphology and proliferation were analyzed at 24, 48, and 72 h after co-incubation.

2.4. EA.hy926 morphology and actin

The morphology of EA.hy926 that was cultured with *P. pastoris* was analyzed using bright field microscopy (Leica DMi1). The co-culture was carried out in 12-well plates as described above. The complete DMEM was removed and cells were gently washed three times with PBS. Pictures were taken with cells in PBS.

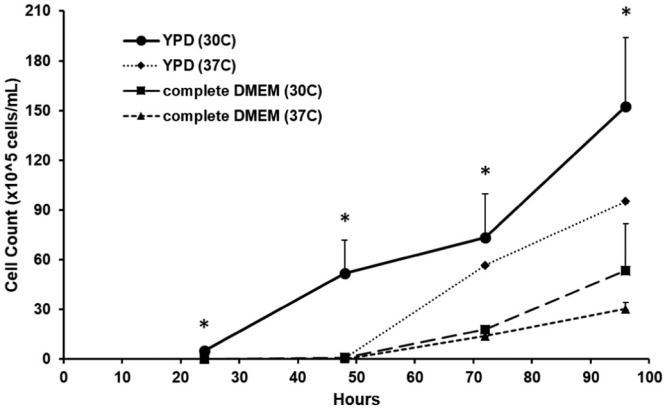

Fig. 1. Growth of *P. pastoris* cultured under various conditions. Temperature (30 °C and 37 °C) and culture media (YPD and complete DMEM) were analyzed. Cells cultured in YPD at 30 °C had significantly higher growth at 24, 48, 72 and 96 h compared to the other culture conditions. There was differences in growth under other conditions but the differences were not significant. Values are reported as mean ± standard deviation. Comparisons of growth at each collection time was analyzed using one-way ANOVA followed by a Bonferroni post hoc test (*, $p < 0.05$, $n = 6$).

The actin of EA.hy926 was analyzed using confocal microscopy after staining with Alexa Fluor® 647 phalloidin (Life Technologies, Eugene, OR). EA.hy926 (1×10^5 cells/mL, 500 mL complete DMEM) was subcultured into each chamber of a 35 mm, 4-chamber glass bottom dish (No.1.5 glass, Cellvis, Mountain View, CA). *P. pastoris* was prepared by resuspending an overnight culture in complete DMEM (37 °C) to a final concentration of 5×10^4 cells/mL. After 24 h of culture, the complete DMEM from the endothelial cells was aspirated and replaced with the prepared complete DMEM containing *P. pastoris* (500 mL). After 72 h of co-culture, cell medium was removed and cells washed three times with PBS. Cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and then washed three times with PBS. Triton X-100 (0.1%) was added for five minutes and cells were washed three times with PBS. Cells were then incubated (20 min at room temperature) with Alexa Fluor® 647 phalloidin (1 unit/mL), followed by a five minute incubation in 300 nM 4′, 6-diamidino-2-phenylindole (DAPI). Cells were washed three times with PBS and images were acquired using confocal microscopy (Leica TCS SPE confocal microscope). To demonstrate the disruption of actin, EA.hy926 was subcultured at 37 °C, humidified CO_2 (5%) incubator for 24 h in the four-chamber glass bottom dishes as mentioned above. Cells were then transferred to a 42 °C, humidified CO2 (5%) incubator for 2 h, modified from Tazawa et al. (Tazawa et al., 2015). Staining and imaging of actin was carried out as described above.

2.5. Endothelial cell proliferation

The proliferation of endothelial cells co-cultured with *P. pastoris* was assessed using a cell proliferation assay kit based on WST-1 (Abcam, San Francisco, CA). EA.hy926 (5×10^3 cells in 100 µL complete DMEM) were cultured overnight and then co-cultured with 20, 200, or 2000 *P. pastoris* cells for 72 h. To perform the assay, cells were washed with PBS to remove *P. pastoris*. Fresh complete DMEM (100 µL) containing WST-1 reagent (10 µL) was carefully added into each well. Complete DMEM (100 µL) plus WST-1 reagent (10 µL) was used as a blank. The plate was incubated at 37 °C in a humidified CO_2 (5%) incubator. Formazan formation was assayed at 1.5, 3.0, and 4.5 h. The reaction between mitochondrial dehydrogenase and the WST-1 reagent led to the formation of formazan dye. Absorbance of the formazan dye was measured using a plate reader set to 450 nm.

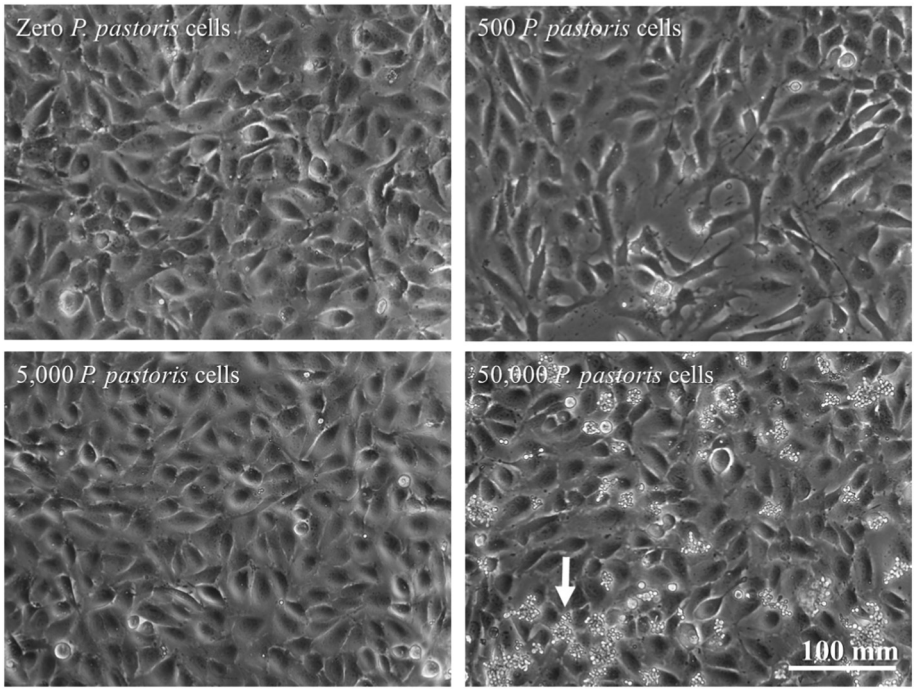

Fig. 2. Morphology of EA.hy926 co-cultured with *P. pastoris*. Pictures were taken 72 h after co-culture and are representative of the morphology observed for endothelial cells. Endothelial cells were washed three times with PBS. The arrow indicates clusters of residual *P. pastoris* that remained after washing.

2.6. GFP expression

To determine if *P. pastoris* can express EGFP while co-cultured with EA.hy926, JC100 expressing EGFP under the control of the *AOX1* promoter (JC100::pKS1) was cultured overnight in YPD at 30 °C in a shaking incubator. The overnight culture was then transferred to glucose-free DMEM (Gibco, Invitrogen, Carlsbad, CA) that was supplemented with 0.5% methanol to a final OD_{600} of 1.0. The suspension of yeast cells (4.0 mL) was added to cultured endothelial cells. EA.hy926 were prepared by seeding 2.0×10^5 cells/mL in complete DMEM (final volume of 4.0 mL) into 60 mm dishes (Greiner Bio-One, Monroe, NC). The dishes have a growth area of 21 cm^2. The endothelial cells were cultured for 24 h. After 24 h, the complete DMEM was aspirated and the endothelial cells were washed three times with 37 °C PBS. *P. pastoris* JC100::pKS1 in glucose-free DMEM plus 0.5% methanol (4.0 mL), prepared above, was warmed to 37 °C and added to the endothelial cells. Inductions of EGFP expression were carried out under nonshaking conditions at either 30 °C or 37 °C in a humidified 5% CO_2 incubator for 24 h. To collect *P. pastoris*, the 60 mm dishes were gently swirled to resuspend the yeast cells. The glucose-free DMEM was collected and centrifuged (2000 x *g* for 5 min) to pellet *P. pastoris*. Pellets were washed once with PBS and resuspended in 500 mL of PBS.

EGFP expression was detected using a Leica TCS SPE confocal microscope. *P. pastoris* suspended in PBS was briefly vortexed. This mixture (7.0 uL) was added a coverslip (24x50mm, No. 1.5, Globe Scientific, Mahwah, NJ) and covered with a glass coverslip (20x20mm, No. 2, Globe Scientific, Mahwah, NJ). Images were captured using 10× and 63× oil immersion objectives (Leica TCS SPE confocal microscope).

2.7. NF-κB activation assay

We used HEK-Blue Null one cells (Invivogen, San Diego, CA) to assay the ability of *P. pastoris* to elicit an immune response. These cells express secreted alkaline phosphatase (SEAP) under the control of the interferon-β (INF-β) minimal promoter fused to five nuclear factor-κB (NF-κB) and activator protein-1 binding sites (https://www.invivogen.com/hek-blue-null1). Stimulation with various peptides and nucleic acids may activate the INF-β promoter (Nourbakhsh et al., 1993), which in HEK-Blue Null one cells will induce the production of SEAP. SEAP can be detected using spectroscopy. HEK-Blue Null1 cells were maintained as per the manufacture's suggestions. For the colorimetric assay, 2.8×10^4 cells were seeded into each well of a 96-well plate. Cells were cultured overnight in a humidified 37 °C incubator supplemented with 5% CO_2 and then treated with extracted *P. pastoris* protein at final concentrations of 5.0, 20, or 150 μg/mL for 72 h. The medium was then aspirated without disturbing the HEK-Blue Null1 cells. HEK-Blue detection assay medium (200 μL) was added to each well and incubated for 15 h at HEK cell culture conditions. Tumor necrosis factor-α (TNF-α, 100 ng/mL) was used as a positive control. Colorimetric changes were detected with a plate reader at optical density of 620 nm.

2.8. P. pastoris protein extracts

An overnight culture of *P. pastoris* was washed twice and resuspended in 200 μL of cold PBS to varying cell concentrations. Glass beads (200 μL) were added to each cell suspension and vortexed for one minute and then placed back on ice for one minute. This cycle was repeated three times and the mixture was centrifuged (10,000 × *g* at 4 °C for 10 min). The supernatant was collected and protein concentrations were determined using a BCA assay kit (Invitrogen).

2.9. Statistics

Values in all figures were expressed as mean ± standard deviation. Multiple comparisons were made using one-way ANOVA. Individual differences from the one-way ANOVA were analyzed using Bonferroni post hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Mammalian cell culture condition decreased the growth of P. pastoris

We initially investigated whether *P. pastoris* can grow in mammalian

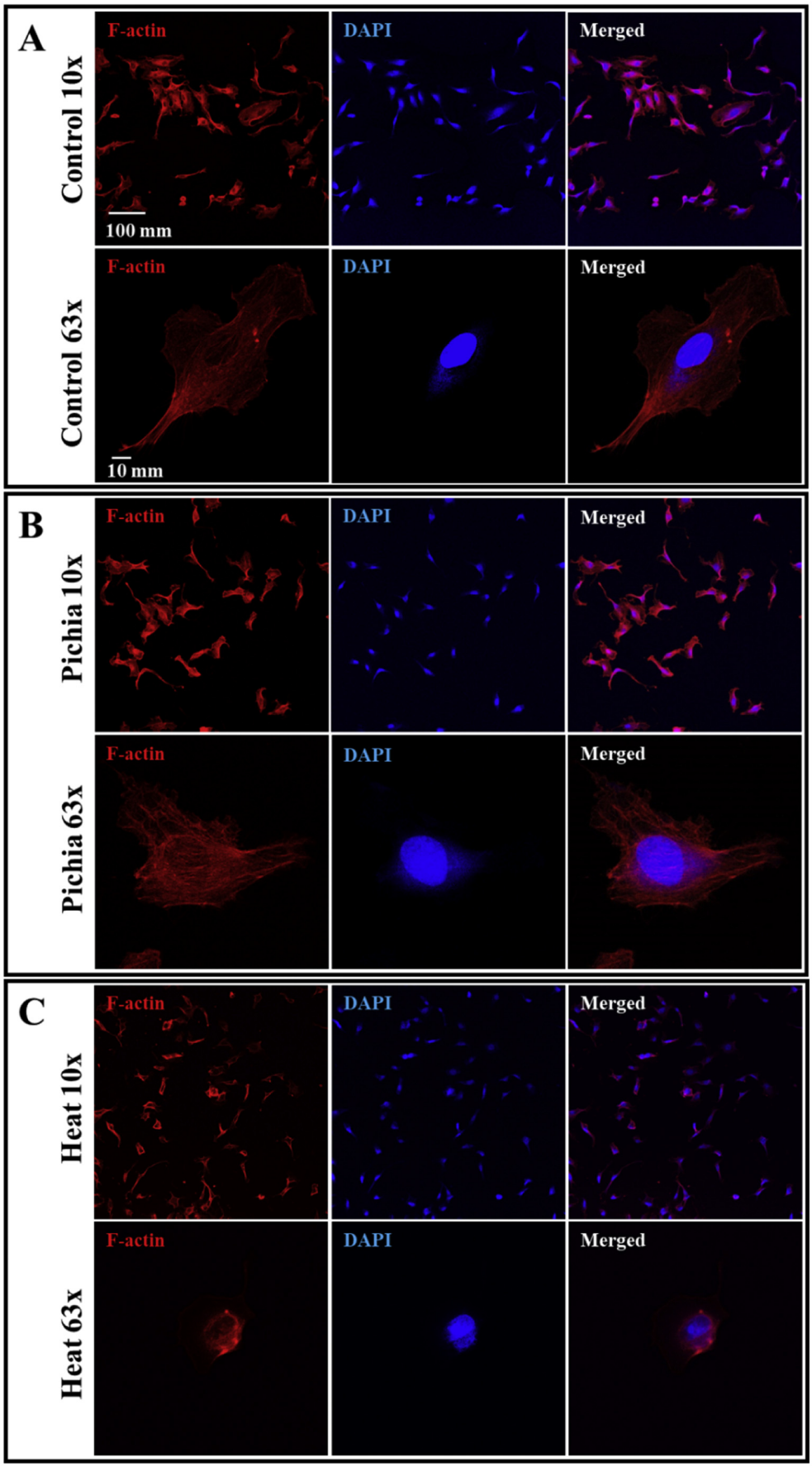

Fig. 3. Actin of EA.hy926 co-cultured with *P. pastoris*. The first column shows actin structures (F-actin, red fluorescence). Actin was stained with Alexa Fluor® 647 phalloidin. The second column shows the nucleus of cells, stained with DAPI. The third column is a merged image of the actin and nuclear stained cells. Panel A (Control) indicates endothelial cells cultured without *P. pastoris*. Panel B (Pichia) are endothelial cells cultured with *P. pastoris* for 72 h. *P. pastoris* was washed off during the multiple steps necessary to stain actin and were not visible. Panel C (Heat) are endothelial cells incubated at 42 °C to alter actin morphology. Images were taken with the 10× and 63× objectives to show population and individual cells, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

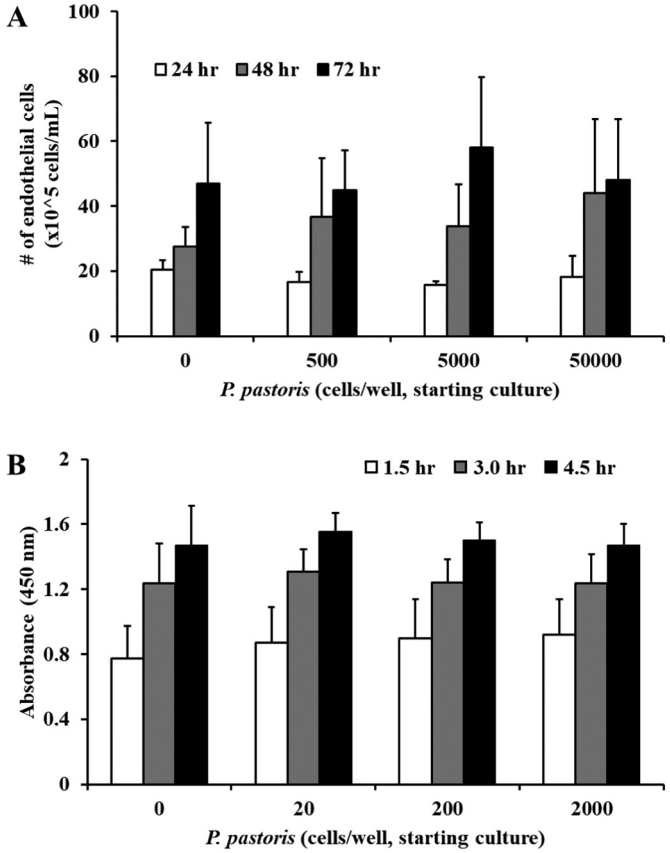

Fig. 4. Proliferation of EA.hy926 during co-cultures with *P. pastoris*. Fig. 4A shows EA.hy926 cell counts (cells/mL) after 24, 48, and 72 h of co-culture using different starting *P. pastoris* cell numbers. Proliferation of EA.hy926 co-cultured with *P. pastoris* was not significantly different from the control group, EAhy926 cultured in the absence of *P. pastoris* (one-way ANOVA, $n = 4$, $p = 0.49, 0.66,$ *and 0.81* for 24, 48, and 72 h, respectively). Fig. 4B shows the level of formazan formed during the WST-1 assay, which indicates the number of endothelial cells. After 72 h of co-culture there was no significant difference between EA.hy926 co-cultured with *P. pastoris* and cultured in the absence of *P. pastoris* when assayed at 1.5, 3.0, and 4.5 h (one-way ANOVA, $n = 10$, $p = 0.40, 0.70,$ *and 0.54* for 1.5, 3.0 and 4.5 h, respectively). Values for both figures were mean ± standard deviation.

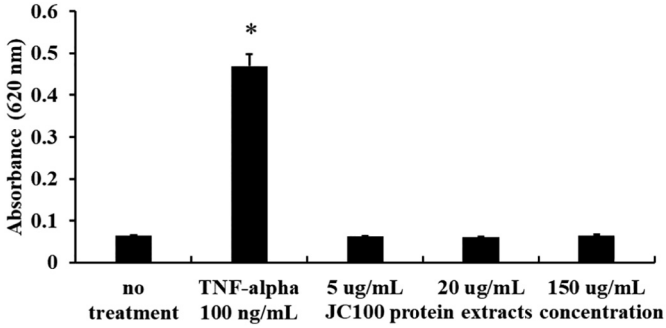

Fig. 5. Analysis of NF-κB activation. HEK-Blue Null1 cells treated with *P. pastoris* protein extracts (5, 20, and 50 μg/mL) showed no significant changes in NF-κB activation as detected by the formation of SEAP. Treatment with TNFα, known to active NF-κB signaling pathways, showed a significant increase in SEAP formation. Reported values were mean ± standard deviation. Significant difference among treatment groups was indicated (*, n = 6, $p < 0.01$), analyzed using one-way ANOVA, followed by a Bonferroni post hoc test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell culture conditions. The standard culture conditions for *P. pastoris* are growth in YPD medium at 30 °C. Wild type *P. pastoris* was able to grow in mammalian cell culture medium at 30 °C and a higher temperature of 37 °C. With YPD cultures, *P. pastoris* cell counts after 96 h were approximately 37.5% less at 37 °C than at the standard temperature of 30 °C (Fig. 1). Overall, growth was reduced when *P. pastoris* was cultured in complete DMEM (30 °C and 37 °C) compared to YPD (Fig. 1).

3.2. EA.hy926 co-cultured with P. pastoris showed no change in morphology and proliferation

Since *P. pastoris* can proliferate under mammalian cell culture conditions, this yeast was grown with the human endothelial cell line, EA.hy926. Co-culture with different concentrations of *P. pastoris* cells for 72 h did not alter the morphology of EA.hy926 as observed under bright field microscopy. EA.hy926 retained the appearance that is comparable to endothelial cells cultured in the absence of *P. pastoris* (Fig. 2). To further examine the morphology of the endothelial cells, we stained the actin structure of EA.hy926. Confocal microscopy showed no visible changes to the arrangement of actin for EA.hy926 that were cultured with *P. pastoris* (Fig. 3A and B). Fig. 3C showed altered actin arrangement due to heat (42 °C, 2 h), as a positive control.

We then examined how *P. pastoris* may affect the growth of EA.hy926. Endothelial cell were counted 24, 48 and 72 h following co-culture with different numbers of *P. pastoris*. There was no significant difference in growth between EA.hy926 cultured with or without *P. pastoris* (Fig. 4A). The endothelial cell counts were verified using cell proliferation assay. The assay measured the activity of mitochondrial dehydrogenases, which cleaved the tetrazolium salt WST-1 to formazan. Higher number of viable cells correlated with increased activity of mitochondrial dehydrogenases. The formation of formazan was not significantly different between EA.hy926 cultured with and without *P. pastoris*, 72 h after co-culture (Fig. 4B). This demonstrated that the activity of mitochondrial dehydrogenases was similar between EA.hy926 cultured in the presence and absence of *P. pastoris*.

3.3. Activation of NF-κB by P. pastoris extracts

Since co-cultures with *P. pastoris* did not appear to alter the morphology or the growth rate of EA.hy926, we were curious to determine if *P. pastoris* affected other cellular processes. We tested the ability of *P. pastoris* to activate NF-κB in HEK-Blue Null1 cells. In HEK-Blue Null1 cells, NF-κB activation correlated with abundance of SEAP. As a positive control, activation of NF-κB was demonstrated with TNFα (100 ng/mL) (Fig. 5). TNFα-treated cells induced a 7.5 fold increase of the enzymatic reaction compared to nontreated cells. HEK-Blue Null1 cells treated with *P. pastoris* protein extracts (5.0, 20, or 150 ng/mL, final concentration) showed no significant difference in the activation of NF-κB compared to nontreated HEK-Blue Null1 cells (Fig. 5).

3.4. Induction of GFP expression

P. pastoris JC100::pKS1 was used to determine whether expression of heterologous proteins during co-cultures with EA.hy926 was possible. EGFP expression in *P. pastoris* JC100::pKS1 was driven by the AOX1 promoter and induced by transferring cells to medium containing methanol as the only carbon source. Confocal microscopy showed that *P. pastoris* JC100::pKS1 expressed GFP when co-cultured with endothelial cells in glucose-free DMEM supplemented with 0.5% methanol at either 30 °C or 37 °C (Fig. 6).

4. Discussion

P. pastoris has been increasingly used to express recombinant proteins for human consumption but the interaction between *P. pastoris*

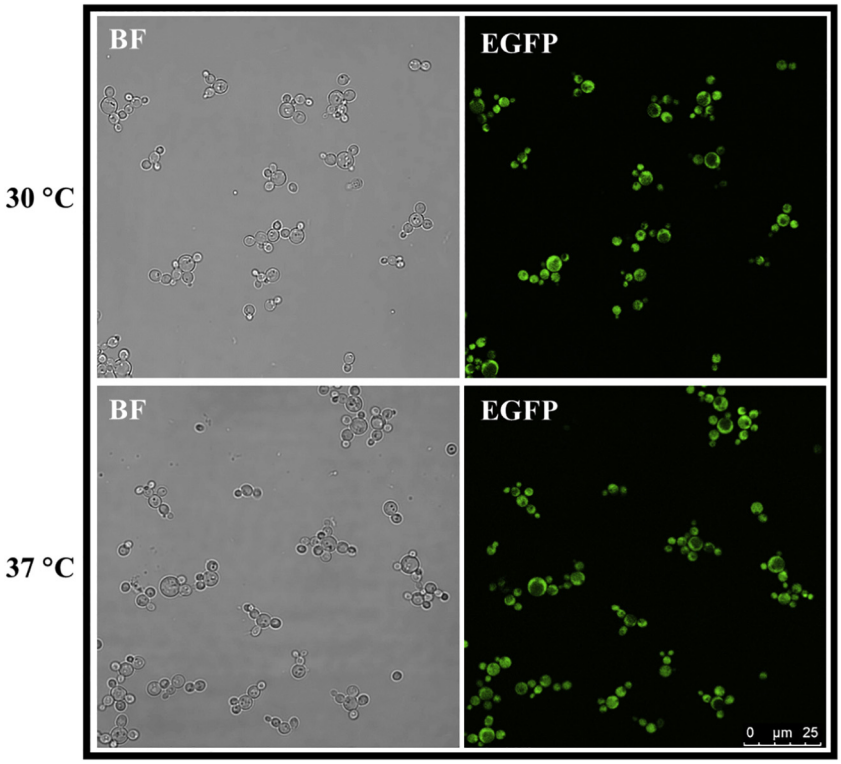

Fig. 6. Methanol induced intracellular expression of EGFP in *P. pastoris* cultured in glucose-free DMEM supplemented with 0.5% methanol. The induction was carried out during co-cultures with EA.hy926. Expression was induced at both 30 °C (top row) and 37 °C (bottom row). Images were captured approximately 24 h after induction using bright field microscopy (BF) and fluorescence (EGFP) 63× oil immersion objective.

and human cells have not been well characterized. Our study served as an initial investigation into the response of the human endothelial cell line, EA.hy926, to co-culturing with *P. pastoris* and provided a method to express recombinant protein in P. *pastoris* during cultures with human endothelial cell line. Under general mammalian tissue culture conditions, *P. pastoris* was viable and capable of expressing EGFP. Additionally, there was no changes in endothelial cell morphology or growth when cultured with *P. pastoris*.

Propagating *P. pastoris* under standard mammalian tissue culture conditions did affect the yeast's growth rate. Mammalian cells are generally grown at 37 °C in a buffered salt solution containing nutrients and supplemented with serum. While a higher than normal temperature may have been the cause of the curtailed growth, the slower reproduction in complete DMEM cultures may also be attributed to the lower concentration of glucose (1.0 g/L). In comparison, the concentration of glucose in YPD is 20 g/L. *P. pastoris* had more growth at 37 °C in YPD compared to complete DMEM at the ideal temperature of 30 °C, suggesting that glucose may be a limiting factor for growth. Antibiotics were also present in the DMEM we used; however, penicillin and streptomycin have been reported to have no effect on *P. pastoris* (Haon et al., 2015). FBS or other types of serum are standard supplement for mammalian cell maintenance and proliferation (Bauman et al., 2018). Investigations on how FBS or other serum may affect *P. pastoris* have not been reported. The overall slower growth of *P. pastoris* under our test conditions compared to that of standard conditions was most likely attributed to the non-shaking cultures. The non-shaking condition was chosen to mimic the non-shaking aspect of mammalian tissue culture. EA.hy926 are adhesive cells and would not survive the shaking conditions normally used to culture *P. pastoris*.

Endothelial cell proliferation was analyzed by counting cell and WST-1 assay. EA.hy926 was allowed to grow for 24 h before introducing *P. pastoris*. Starting the endothelial cell cultures first allowed for cell attachment to the bottom of the well, which is crucial for adherent mammalian cells to survive. We chose a lower starting culture concentration of *P. pastoris* since the doubling time of *P. pastoris* (Larsen et al., 2013) was greater than that of EA.hy926 (Lu et al., 2009). At 72 h after starting the co-culture, proliferation of EA.hy926 cultured with and without *P. pastoris* showed no significant difference. This data suggested that complete DMEM provided sufficient nutrients to support both cell types. We verified cell counting data using the WST-1 assay, which quantifies mitochondrial dehydrogenase activity. Mitochondrial dehydrogenase activity was not significantly different between EA.hy926 cultured alone and cultured with *P. pastoris,* indicating that the viability of EA.hy926 was similar under both conditions.

After 72 h of co-culture with *P. pastoris*, the endothelial cells showed no change in morphology or actin structure. Pathogenic yeasts have been shown to alter the actin structure, leading to the necrosis of cultured mammalian cells (Mendes-Giannini et al., 2004; Park et al., 2005). For example, Shintaku showed that human umbilical vein endothelial cells (HUVEC) rearranges actin to endocytosis *Candida parasilosis* (Shintaku et al., 2013).

Although *P. pastoris* did not alter EA.hy926 morphology or proliferation under the conditions in our study, other cellular processes may be affected. It was recently demonstrated that *P. pastoris* and other yeast, including *S. cerevisiae*, can cause differentiation of dendritic cells and inducing these phagocytic cells to release cytokines (Bazan et al., 2018). We were curious as to what cellular responses *P. pastoris* may trigger in nonphagocytic cells. HEK-Blue Null1 cells were used to assay the ability of *P. pastoris* cell lysates to activate NF-κB. NF-κB is an inducible transcription factor involved in many cellular signaling pathways including the inflammatory response (Zhang et al., 2017). Unlike the known stimulator TNFα, treatment with *P. pastoris* total soluble protein did not induce NF-κB activation in HEK-Blue Null1 cells. This preliminary work would suggest that *P. pastoris* may not elicit cellular pathways associated with NF-κB activation, such as inflammation. Further studies are necessary to fully characterize the effects of co-culturing *P. pastoris* with nonphagocytic mammalian cells. Protein lysates, rather than whole cells, were used for these studies because HEK-Blue Null1 cells are nonphagocytic and *P. pastoris* cells absorb wavelengths close to 600 nm, potentially interfering with the 620 nm assay. Positive tests could have been indicative of *P. pastoris* cell counts rather than NF-κB activation.

In this study, we demonstrated that *P. pastoris* can be cultured with the human endothelial cell line, EA.hy926, without significantly changing the growth capabilities of the endothelial cells. To our knowledge, this is the first demonstration of successful co-cultures between *P. pastoris* and mammalian cells. This study opens up the possibility that other mammalian cells can potentially be co-cultured with *P. pastoris*. Additionally, we induced the expression of EGFP in *P. pastoris* while co-cultured with human endothelial cell line, EA.hy926. Further studies that investigate the potential use of *P. pastoris* for directly delivering proteins to mammalian cells during co-culture are warranted. While our induction was driven by the *AOX1* promoter, the use of the *GAP* promoter would overcome challenges associated with methanol induction (Waterham et al., 1997; Vogl and Glieder, 2013). The use of secretion signals would allow heterologous proteins to be secreted directly into the tissue culture medium. Such procedures would provide cultured cells with direct and continuous access to proteins expressed by *P. pastoris*. Our finding opens up the potential for further studies into the safety of *P. pastoris* as a protein delivery system, which can increase the versatility of this yeast.

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References

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl. Microbiol. Biotechnol. 98, 5301–5317.

Ardiani, A., Higgins, J.P., Hodge, J.W., 2010. Vaccines based on whole recombinant Saccharomyces cerevisiae cells. FEMS Yeast Res. 10, 1060–1069.

Asada, H., Uemura, T., Yurugi-Kobayashi, T., et al., 2011. Evaluation of the *Pichia pastoris* expression system for the production of GPCRs for structural analysis. Microb. Cell Factories 10, 24.

Bauman, E., Granja, P.L., Barrias, C.C., 2018. Fetal bovine serum-free culture of endothelial progenitor cells-progress and challenges. J. Tissue Eng. Regen. Med. 12, 1567–1578.

Bazan, S.B., Walch-Ruckheim, B., Schmitt, M.J., Breinig, F., 2018. Maturation and cytokine pattern of human dendritic cells in response to different yeasts. Med. Microbiol. Immunol. 207, 75–81.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45–66.

Ciofalo, V., Barton, N., Kreps, J., Coats, I., Shanahan, D., 2006. Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. Regul. Toxicol. Pharmacol. 45, 1–8.

Corchero, J.L., Gasser, B., Resina, D., et al., 2013. Unconventional microbial systems for the cost-efficient production of high-quality protein therapeutics. Biotechnol. Adv. 31, 140–153.

Didari, T., Solki, S., Mozaffari, S., Nikfar, S., Abdollahi, M., 2014. A systematic review of the safety of probiotics. Expert Opin. Drug Saf. 13, 227–239.

Edgell, C.J., McDonald, C.C., Graham, J.B., 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc. Natl. Acad. Sci. U. S. A. 80, 3734–3737.

Haon, M., Grisel, S., Navarro, D., Gruet, A., Berrin, J.G., Bignon, C., 2015. Recombinant protein production facility for fungal biomass-degrading enzymes using the yeast *Pichia pastoris*. Front. Microbiol. 6, 1002.

Juturu, V., Wu, J.C., 2018. Heterologous protein expression in *Pichia pastoris*: latest research Progress and applications. Chembiochem 19, 7–21.

Kerry, G.R., Patra, J.K., Gouda, S., Park, Y., Shin, H.S., Das, G., 2018. Benefaction of probiotics for human health: a review. J. Food Drug Anal. 26, 927–939.

Kim, H.J., Lee, J.Y., Kang, H.A., Lee, Y., Park, E.J., Kim, H.J., 2014. Oral immunization with whole yeast producing viral capsid antigen provokes a stronger humoral immune response than purified viral capsid antigen. Lett. Appl. Microbiol. 58, 285–291.

Larsen, S., Weaver, J., de Sa Campos, K., et al., 2013. Mutant strains of *Pichia pastoris* with enhanced secretion of recombinant proteins. Biotechnol. Lett. 35, 1925–1935.

Li, Z., Leung, W., Yon, A., et al., 2010. Secretion and proteolysis of heterologous proteins fused to the Escherichia coli maltose binding protein in *Pichia pastoris*. Protein Expr. Purif. 72, 113–124.

Lu, Z.J., Ren, Y.Q., Wang, G.P., Song, Q., Li, M., Jiang, S.S., Ning, T., Guan, Y.S., Yang, J.L., Luo, F., 2009. Biological behaviors and proteomics analysis of hybrid cell line EAhy926 and its parent cell line A549. J. Exp. Clin. Cancer Res. 28, 16.

Meehl, M.A., Stadheim, T.A., 2014. Biopharmaceutical discovery and production in yeast. Curr. Opin. Biotechnol. 30, 120–127.

Mendes-Giannini, M.J., Hanna, S.A., da Silva, J.L., Andreotti, P.F., Vincenzi, L.R., Benard, G., Lenzi, H.L., Soares, C.P., 2004. Invasion of epithelial mammalian cells by Paracoccidioides brasiliensis leads to cytoskeletal rearrangement and apoptosis of the host cell. Microbes Infect. 6, 882–891.

Nourbakhsh, M., Hoffmann, K., Hauser, H., 1993. Interferon-beta promoters contain a DNA element that acts as a position-independent silencer on the NF-kappa B site. EMBO J. 12, 451–459.

Park, H., Myers, C.L., Sheppard, D.C., Phan, Q.T., Sanchez, A.A., EE, J., Filler, S.G., 2005. Role of the fungal Ras-protein kinase a pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell. Microbiol. 7, 499–510.

Potvin, G.A.A., Zhang, Z., 2012. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. Biochem. Eng. J. 64, 91–105.

Shintaku, T., Glass, K.A., Hirakawa, M.P., Longley, S.J., Bennett, R.J., Bliss, J.M., Shaw, S.K., 2013. Human endothelial cells internalize Candida parapsilosis via N-WASP-mediated endocytosis. Infect. Immun. 81, 2777–2787.

Tazawa, H., Sato, K., Tsutiya, A., Tokeshi, M., Ohtani-Kaneko, R., 2015. A microfluidic cell culture system for monitoring of sequential changes in endothelial cells after heat stress. Thromb. Res. 136, 328–334.

Thompson, C.A., 2010. FDA approves kallikrein inhibitor to treat hereditary angioedema. Am. J. Health Syst. Pharm. 67, 93.

Vogl, T., Glieder, A., 2013. Regulation of *Pichia pastoris* promoters and its consequences for protein production. New Biotechnol. 30, 385–404.

Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V., Cregg, J.M., 1997. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 186, 37–44.

Zhang, Q., Lenardo, M.J., Baltimore, D., 2017. 30 years of NF-kappaB: a blossoming of relevance to human pathobiology. Cell 168, 37–57.