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ORIGINAL RESEARCH PAPER

Expression and characterization of recombinant humanized anti-HER2 single-chain antibody in *Pichia pastoris* for targeted cancer therapy

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Abstract
Objectives The availability of self-targeting and low immunogenic therapeutic agents is critical to efficient cancer therapy. Therefore, the development of humanized therapeutic antibodies is particularly appealing.
Results A humanized single-chain variable fragment (scFv) antibody that can target human epidermal growth factor receptor-2 (HER2)-overexpressing cancer cells was designed and produced via expression in *Pichia pastoris*. The expression gave a high yield of 8 mg protein/l (with a purity of 92 %) using shake-flask cultures. Functional studies also revealed that the purified recombinant anti-HER2 scFv exhibited anti-proliferative activity and could bind efficiently to HER2-overexpressing human breast cancer cell line SKBR3.
Conclusion The recombinant scFv offers promising therapeutic and binding efficiencies that are desirable for targeted cancer therapies.

Keywords Anti-HER2 · Breast cancer cell line SKBR3 · Cancer therapy · Heterogeneous expression · Humanized single-chain variable fragment · *Pichia pastoris*

Introduction

Cancer is a major cause of death in the world. For pharmacotherapy, targeted therapy has become increasingly critical for efficient cancer treatments since it minimizes side effects and enhances the availability of active agents to the cancer sites (Gerber 2008). Among the variety of targeting agents examined, humanized monoclonal antibodies are especially desired. One important cancer-targeting antibody is trastuzumab (herceptin), which has been widely examined for treatment of cancer cells overexpressing human epidermal growth factor receptor-2 (HER2) (Colombo et al. 2010).

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Compared to typical production of antibodies via higher animals or mammalian cell cultures, microbial expression of single-chain variable fragments (scFv) consisting of the variable regions of both the heavy chain (V_H) and light chain (V_L) provides several advantages. In addition to the convenience of production with the well-developed microbial processes (Spadiut et al. 2014), scFv are generally of low molecular weights that improve their penetrability into tumors while retaining specific affinity and low immunogenicity (Liu et al. 2011). In particular, anti-HER2 scFv can enable the efficient targeting of therapeutical proteins, drugs, and nanoparticles toward cancer cells (Afshar et al. 2009; Reynolds et al. 2012; Zdobnova et al. 2012).

Murine instead of humanized anti-HER2 scFv has been expressed in systems such as *Escherichia coli*, *Pichia pastoris*, etc. (Cheng et al. 2003; Galeffi et al. 2005, 2006; Hu et al. 2006; Lombardi et al. 2005; Sommaruga et al. 2011). Although these studies produced scFv with the desired targeting affinity, the yield was low (0.1–0.3 mg/l) (Cheng et al. 2003; Lombardi et al. 2005). Alternative production via transgenic plants has also been reported, yet that suffered from similar limitations (Galeffi et al. 2005). Improved yields could be obtained with cell-free translational systems; however, the increase in production costs can be daunting (Galeffi et al. 2006). Murine anti-HER2 scFv (scFv800E6) produced in *P. pastoris* KM71H offered higher yields, yet the product appeared to suffer from impurities (Sommaruga et al. 2011). In addition, the immunogenicity of the murine antibody fragment was a major obstacle for its further development as a therapeutic agent (Muzard et al. 2009; Pavlinkova et al. 2001).

To achieve efficient production of humanized anti-HER2 scFv, the *P. pastoris* expression system is appealing because it offers several advantages including mature protein processing technologies and favorable post-translational modifications (e.g. glycosylation) (Ahmad et al. 2014). Accordingly, we explore in this work the expression and production of humanized anti-HER2 scFv, other than murine scFv variants, in *P. pastoris*. Results showed that this approach gave yields in the order of 8 mg/l, and the product displayed the desired specificity and activity in recognizing and binding to HER2-overexpressed human breast cancer cells.

Materials and methods

Materials

Pichia pastoris GS115 (*his4*) and plasmid pPIC9 K were kindly provided by t Professor Xiangshan Zhou. Human breast cancer cell line SKBR3 was purchased from Cell Bank of Committee on Type Culture Collection of Chinese Academy of Sciences and was grown in McCoy's 5A (modified) medium (Gibco) with 10 % (v/v) fetal bovine serum, 100 U penicillin/ml, and 100 μg streptomycin/ml. A similar cultivation was applied to normal breast cell Hs 578Bst (ATCC) except the replacement of McCoy's 5A (modified) medium with MEM medium (Gibco). Cells were maintained at 37 °C with 5 % (v/v) CO_2. Geneticin (G418) was purchased from Majorbio Biotech (Shanghai, China). All other reagents and chemicals were of analytical grade.

Construction of expression plasmid and transformation of *P. pastoris*

The sequence of anti-HER2 scFv was customer synthesized by Generay Biotech (Shanghai, China) and cloned into the *Xho*I/*Eco*RI sites of the pPIC9 vector. From the resulted recombinant plasmid, product obtained by digesting with *Bam*HI/*Eco*RI was then inserted into the *Bam*HI/*Eco*RI sites of pPIC9 K to generate plasmid pXDC2. About 5 μg pXDC2 DNA was linearized with *Sal*I and electrotransformed into *P. pastoris* strain GS115 using a micropulser following the standard procedure as suggested by the supplier. The transformants were then plated onto minimal glycerol (MGY) agar plates.

Identification of high-level expression transformants

In order to screen for overproducers with multiple inserts, $\sim 10^5$ cells of selected transformants were grown on YPD (yeast extract peptone dextrose) agar plates containing 3 mg G418 l/ml and 4 mg G418/ml at 28 °C for 2–5 days. Ten colonies were chosen from the G418-resistant colonies and were grown in 50 ml buffered complex glycerol (BMGY) medium in baffled flasks and were grown at 30 °C until OD_{600} reached 2–6. The cells were then collected and

resuspended in 100 ml BMMY medium for continued cultivation till OD_{600} reached ~1. For induction, pure methanol was added every 24 h to give 0.5 % (v/v). Samples were taken every 24 h for SDS-PAGE analysis after being precipitated with trichloroacetic acid to determine the expression level. The genomic DNA of these colonies were isolated using TIANamp Yeast DNA Kit (Tiangen Biotech Beijing, China) and the integration of gene encoding anti-HER2 scFv was confirmed by PCR with primers specific to 5′ AOX1/3′ AOX1: 5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′.

Purification of humanized anti-HER2 scFv

The culture of the best transformant was centrifuged (10,000×*g*, 5 min, 4 °C) for collection. The supernatant was dialyzed against binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH7.2) using an Ultracel PL membrane (Millipore, cutoff 5 kDa) in a stirred cell module (Merck Millipore) and mixed with 1 ml Ni–NTA resin (Yeli, Shanghai, China) for 1 h. The supernatant-Ni–NTA mixture was loaded into a column and the column flow-through was collected. The column was then washed with a binding buffer containing 10 mM imidazole and the protein was eluted with a binding buffer with 100 mM imidazole. Ten µl of each fraction was analyzed by using SDS-PAGE (12 %). The scFv was then dialyzed against PBS in dialysis tubing (Mw cutoff 8–14 kDa) and detected by western blot analysis. The membrane was blocked by using 5 % (w/v) skimmed milk in PBST solution (0.1 % v/v Tween-20 in PBS) for 1 h at room temperature and incubated overnight with the rabbit anti-His tag antibody (Beyotime, Jiangsu, China) at 4 °C. After washing with PBST for several times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Merck) at room temperature for 1 h and was detected by using a Pro-light HRP Chemiluminescent Kit (Tiangen Biotech, Beijing, China).

Cell proliferation assay

SKBR3 cells (5×10^5/ml) were grown with different concentrations of anti-HER2 scFv overnight in 96-well plates. Cells without the addition of the antibody were used as controls. Cell viability was determined by using the MTT assay. The absorbance of the suspension was measured at 490 nm using a plate reader.

Binding of anti-HER2 scFv to SKBR3 cell

Cells of cancer cell line SKBR3 and normal cell Hs 578Bst were grown in 12-well plates to reach confluency, and were then treated with 20 µg anti-HER2 scFv/ml at 37 °C for 2 h. The same amount of PBS buffer was added to cell cultures to be used as controls. The cells were subsequently washed with ice cold PBS three times, fixed in 4 % (w/v) paraformaldehyde at room temperature for 10 min, and were permeabilized with 0.2 % (v/v) Triton X-100 for 10 min. The cells were then incubated in a blocking buffer containing (1 % (w/v) BSA, 0.05 % (v/v) Tween 20, PBS) at 37 °C for 1 h and washed again with PBS. Rabbit anti-histag antibody (1:50) (Affinity BioSciences, USA) was added to each of the cell samples and incubated at 37 °C for 1 h followed by the addition of FITC-conjugated goat anti-rabbit IgG (1:200) (Beyotime). The cells were imaged by using an inverted fluorescence microscope. Labeled cells were also analyzed using flow cytometry.

Results and discussion

Design of humanized anti-HER2 scFv

Anti-HER2 scFv can target many HER2-overexpressing cancer cells, such as bladder, pancreatic, breast, and gastric carcinoma (Ménard et al. 2001; Mitri et al. 2012; Qiu and Xu 2013). To obtain humanized anti-HER2 scFv for in vivo applications, the sequence of anti-HER2 scFv was designed according to that of the humanized monoclonal antibody trastuzumab (IMGT/mAb-DB database Chain ID INN 7637_H and 7637_L) and was constructed in the V_H-$(G_4S)_3$-V_L mode (Fig. 1). Codon optimization of the gene encoding the expressed protein can dramatically increase the expression level in *P. pastoris* (Hu et al. 2006; Sinclair and Choy 2002; Woo et al. 2002). For instance, the expression level of codon optimized human glucocerebrosidase was increased 10-fold (Sinclair and Choy 2002) when compared to that of the native enzyme. Accordingly, the gene of humanized anti-HER2 scFv (NCBI accession number: KM016462) was synthesized based on the codon

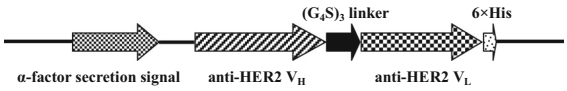

Fig. 1 Design and construction of anti-HER2 scFv. The sequence of anti-HER2 scFv was designed based on the amino acid sequence of trastuzumab and was constructed in the V_H-Linker-V_L mode. The linker sequence is $(GGGGS)_3$. A polyhistidine sequence (6× His) was introduced to *C*-terminus of scFv for antibody detection and purification. The α-factor signal sequence was designed to facilitate the anti-HER2 scFv secretion to the medium

preference of *P. pastoris* (Sinclair and Choy 2002; Zhao et al. 2000).

Screening for higher expresser

After pXDC2 was transferred into *P. pastoris* GS115, multi-copy inserts of the target gene that promise high level expression of anti-HER2 scFv, were selected from plates containing G418 (Nordén et al. 2011). PCR showed that the gene encoding anti-HER2 scFv (744 bp) has been successfully inserted into the genome of *P. pastoris* GS115 in all the selected G418-resistant colonies.

The expression of anti-HER2 scFv was carried out in baffled flasks and analyzed by SDS-PAGE. A band that showed a molecular weight of approx. 29 kDa was detected in the supernatant, although the calculated molecular weight of anti-HER2 scFv was 26.6 kDa. The difference might be attributed to the post-translational modification. The expression level increased initially with time; however, the expression level at 72 h was similar to that at 96 h, suggesting 72 h-induction was sufficient for anti-HER2 scFv production (Fig. 2). No target protein was detected at 0 h. Moreover, the transformant No. 6 displayed the highest expression level of anti-HER2 scFv among the ten selected transformants (Fig. 2b).

The relative gene copy number of the ten selected strains was then analyzed. As a result, all the strains possessed approximately the same relative copy number (Supplementary Table 1), suggesting that the copy number had no obvious effect on the expression of anti-HER2 scFv. Increased gene dosage may have positive effect on protein expression in some cases (Athmaram et al. 2012; Mansur et al. 2005; Vassileva et al. 2001; Yu et al. 2009), although negative effects have also been reported (Hohenblum et al. 2004; Inan et al. 2006; Zhu et al. 2009). Other

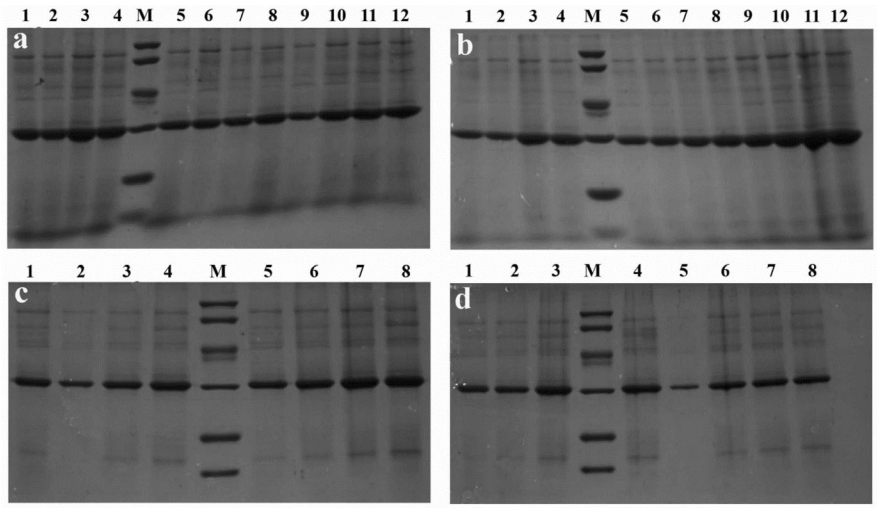

Fig. 2 Expression of humanized anti-HER2 scFv in *P. pastoris*. Transformants resistant to 4 mg/ml G418 were grown in BMMY medium. The equal amount of culture supernatants of various transformants at different times were examined by using 12 % SDS-PAGE. Afterwards, the gel was stained with Coomassie blue. Lane M: protein molecular weight marker (from the top: 97.2, 66.4, 44.3, 29.0, 20.1, 14.3 kDa). **a** Lanes 1–4: supernatant of transformant no. 1 at 24, 48, 72, 96 h; Lanes 5–8: supernatant of transformant no. 2 at 24, 48, 72, 96 h; Lanes 9–12: supernatant of transformant no. 3 at 24, 48, 72, 96 h. **b** Lanes 1–4: supernatant of transformant no. 4 at 24, 48, 72, 96 h; Lanes 5–8: supernatant of transformant no. 5 at 24, 48, 72, 96 h; Lanes 9–12: supernatant of transformant no. 6 at 24, 48, 72, 96 h. **c** Lanes 1–4: supernatant of transformant no. 7 at 24, 48, 72, 96 h; Lanes 5–8: supernatant of transformant no. 8 at 24, 48, 72, 96 h; **d** Lanes 1–4: supernatant of transformant no. 9 at 24, 48, 72, 96 h; Lanes 5–8: supernatant of transformant no. 10 at 24, 48, 72, 96 h

studies, in contrast, have demonstrated that there is no apparent correlation between the two factors for cases such as murine anti-HER2 scFv, NK1-fragment of human hepatocyte growth factor, and extracellular domain of mouse tissue factor (Hu et al. 2006; Mack et al. 2009), which is consistent with the results obtained in this work.

Purification of humanized anti-HER2 scFv

The transformant No. 6 that afforded the highest expression level was used for the production of anti-HER2 scFv. The 6× his-tagged scFv could be readily purified by using Ni–NTA resin. Mixing the supernatant with the Ni–NTA resin before loading onto the column apparently increased the binding efficiency, as indicated by the small amount of scFv remained in the flow-through fraction (Fig. 3a, lane 1). After washing with 4 ml binding buffer containing 10 mM imidazole to remove non-specific binding proteins (Fig. 3a, lane 2), the anti-HER2 scFv was eluted with binding buffer containing 100 mM imidazole (Fig. 3a, lanes 3–6). One clear band with a molecular weight (29 kDa) comparable to the theoretical value of anti-HER2 scFv

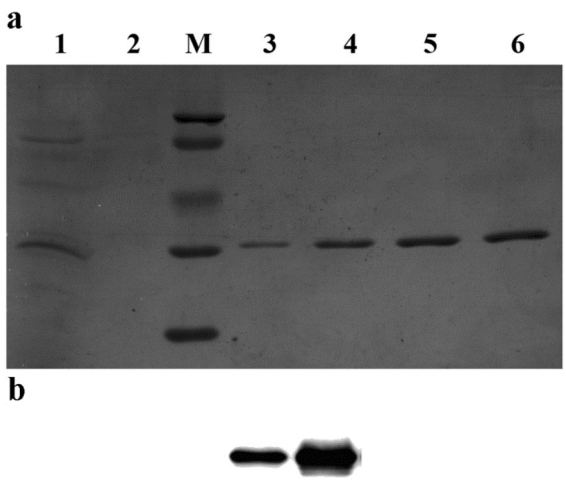

Fig. 3 Purification of humanized anti-HER2 scFv product. **a** The secreted scFv was purified by Ni^{2+}-affinity chromatography, subjected to 12 % SDS-PAGE analysis, and visualized by using Coomassie staining. Lane 1, flow-through; Lane 2, washing buffer; Lane M, protein molecular weight marker (from the top: 97.2, 66.4, 44.3, 29.0, 20.1 kDa); Lanes 3–6, eluted with 100 mM imidazole. **b** Purified scFv was subjected to 12 % SDS-PAGE and then transferred onto PVDF membrane (Millipore) using semi-dry electroblotter (Bio-Rad)

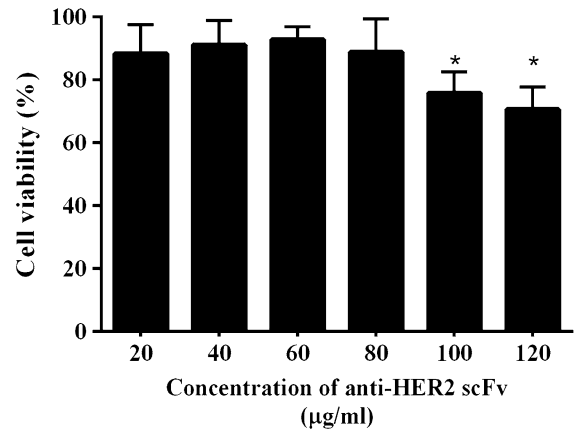

Fig. 4 Cell viability of SKBR3 cells against anti-HER2 scFv. Cells were plated in 96-well plates and treated with 20, 40, 60, 80, 100, 120 μg/ml anti-HER2 scFv for 48 h at 37 °C. After the treatment, 10 μl MTT (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The medium was then removed and 150 μl DMSO was added following incubation for 10 min. Cell viability was calculated by comparing the absorbance value at 490 nm of treated cells to the absorbance value of untreated cells. The asterisk indicates significant difference ($P < 0.05$) in cell viability between treated cells and untreated cells [analysis of variance (ANOVA)]

Table 1 Purity and yield of humanized anti-HER2 scFv produced via expression in *Pichia pastoris* GS115

Purification step	Total protein (mg)[a]	scFv (mg)	Purity (%)[b]	Yield (%)
Culture supernatant	21	2.31	11	100
Dialyzed supernatant	10.4	2.08	20	90
Ni^{2+}-affinity chromatography	1.54	1.39	90	60
Dialysis	0.87	0.8	92	35

[a] Protein content was determined for 100 ml culture, with protein concentration determined by using Bradford protein assay kit

[b] The purity of protein was analyzed by using SDS-PAGE, stained with silver according to the instruction of the supplier (Beyotime, Jiangsu, China), and estimated by Image J (http://rsb.info.nih.gov/ij/)

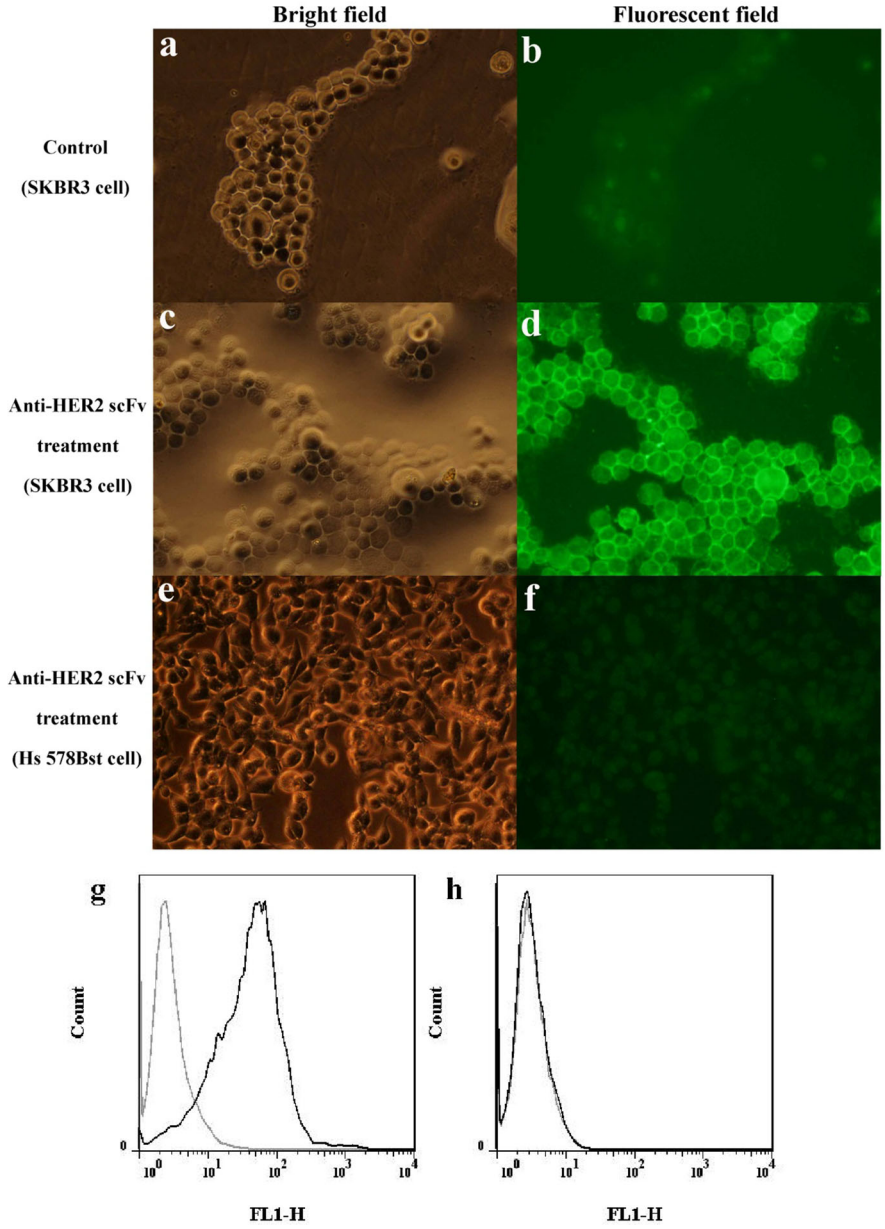

Fig. 5 Binding activity of humanized anti-HER2 scFv to SKBR3 cells. SKBR3 cells were incubated with anti-HER2 scFv at 37 °C for 2 h (**c**, **d**, **g**). After being washed with PBS buffer, the cells were detected with rabbit anti-histag antibody and FITC-conjugated goat anti-rabbit IgG with fluorescence microscope. SKBR3 cells incubated with PBS buffer (**a**, **b**) and human normal breast cell Hs 578Bst incubated with scFv (**e**, **f**) were used as control. The shifted fluorescent signal was evident for SKBR3 cells treated with anti-HER2 scFv [*solid line* in (**g**)], while not detected for SKBR3 cells treated with PBS [*gray line* in (**g**)] and normal cells (Hs 578Bst cells) treated with anti-HER2 scFv (**h**)

(26.6 kDa) was detected in the eluted fractions by both SDS-PAGE and western blot analysis (Fig. 3b). The amount, purity, and yield of scFv from each purification step are listed in Table 1. About 800 µg anti-HER2 scFv with the purity of 92 % was obtained from 0.1 L culture. The yield (8 mg/l) was comparable to murine anti-HER2 scFv (scFv800E6) produced in *P. pastoris* KM71H (10 mg/l) (Sommaruga et al. 2011). However, the latter appeared to suffer from impurities, as two major protein bands were reported for the SDS-PAGE analysis of the product, while the current product is of high purity and shows a single protein band. It was suspected by the authors of the previous study that different isoforms of scFv800E6 were probably formed due to inefficient cleavage by Ste13 aminopeptidase during the in vivo precursor processing. That is apparently not an issue for the current work considering the high purity observed.

Anti-proliferative effect of anti-HER2 scFv

To test the anti-proliferative effect of anti-HER2 scFv on the breast cancer cell line SKBR3, different concentrations of scFv were applied to incubation culture of the cells. The results showed that the application of 100 and 120 μg anti-HER2 scFv/ml could significantly inhibit cell growth ($P < 0.05$), while no significant effect was observed for SKBR3 cells treated with 20, 40, 60, 80 μg anti-HER2 scFv/ml ($P > 0.05$) (Fig. 4). Monnier et al. (2013) suggested that scFv lacking the Fc fragment had a limited therapeutic potential because it could only induce cytotoxicity by binding to the target without Fc-mediated cytotoxicity. Nejatollahi et al. (2014) also found individual anti-HER2 scFv inhibited cell proliferation to a less extent than a cocktail of anti-HER2 scFv antibodies. Our results agreed well with these previous findings. Although 100 and 120 μg anti-HER2 scFv/ml had a significant effect on cell proliferation, cell viability was kept to 76 and 71 %, respectively.

Binding efficiency of anti-HER2 scFv to SKBR3 cells

The binding activity of the anti-HER2 scFv product was further tested with the HER2-overexpressing breast cancer cell line SKBR3. After the treatment with scFv, SKBR3 cells apparently showed green fluorescence from FITC-labeled second antibody as detected with fluorescence microscopy (Fig. 5c, 5d). As a comparison, no fluorescence was detected on the control, the PBS-treated cells (Fig. 5a, 5b). Moreover, when human normal breast cell line Hs 578Bst (lacking HER2 expression) was incubated with scFv, no significant FITC fluorescence was detected either (Fig. 5e, 5f). Binding of anti-HER2 scFv to SKBR3 cells was also quantitatively evaluated by using flow cytometry. The shift of the FITC intensity peak was indicative of the binding of anti-HER2 scFv to SKBR3 cells (Fig. 5g). The mean fluorescent intensity of SKBR3 cells obtained by flow cytometry studies was 3.2-times higher (Fig. 5g) in comparison to that of Hs 578Bst cells (Fig. 5h). These findings demonstrated anti-HER2 scFv could specifically bind to HER2-overexpressing cells, but not to the normal cells.

In Conclusion, we demonstrate a simple but effective design and production protocol for the production of humanized single-chain antibodies for cancer therapy. A soluble form of the humanized anti-HER2 scFv was expressed in *P. pastoris*. The process enabled the production of a high purity product at 8 mg protein/l. An anti-proliferative assay and binding studies with HER2-overexpressing cancerous cells confirmed that the recombinant scFv offered promising therapeutic and binding efficiencies that are desired for efficient targeted cancer therapies.

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Supporting information Supplementary Table 1—Comparison of relative copy numbers of gene encoding anti-HER2 scFv in different strains using real-time PCR.

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