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Original Article

An innovative cell selection approach in developing human cells overexpressing aspartyl/asparaginyl β-hydroxylase

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Abstract

Background and purpose: Aspartyl/asparaginyl β-hydroxylase (ASPH) is abundantly expressed in malignant neoplastic cells. The establishment of a human cell line overexpressing ASPH could provide the native-like recombinant protein needed for developing theranostic probes. In the process of transfection, the obtained cells normally contain a range of cells expressing the different levels of the target of interest. In this paper, we report on our simple innovative approach in the selection of best-transfected cells with the highest expression of ASPH using subclone selection, quantitative real-time polymerase chain reaction, and gradual increment of hygromycin concentration.
Experimental approach: To achieve this goal, human embryonic kidney (HEK 293T) cells were transfected with an ASPH-bearing pcDNA3.1/Hygro(+) vector. During antibiotic selection, single accumulations of the resistant cells were separately cultured and the ASPH mRNA levels of each flask were evaluated. The best subclones were treated with a gradually increasing amount of hygromycin. The ASPH protein expression of the obtained cells was finally evaluated using flow cytometry and immunocytochemistry.
Findings / Results: The results showed that different selected subclones expressed different levels of ASPH. Furthermore, the gradual increment of hygromycin (up to 400mg/mL) improved the expression of ASPH. The best relative fold change in mRNA levels was 57.59 ± 4.11. Approximately 90.2% of HEK^{ASPH} cells overexpressed ASPH on their surface.
Conclusion and implications: The experiments indicated that we have successfully constructed and evaluated a recombinant human cell line overexpressing ASPH on the surface. Moreover, our innovative selection approach provided an effective procedure for enriching highly expressing recombinant cells.

Keywords: Cancer; Aspartyl/asparaginyl β-hydroxylase; Human embryonic kidney (HEK293T) cell line; Transfection; Overexpression.

INTRODUCTION

Aspartyl/asparaginyl β-hydroxylase (ASPH) is a type II transmembrane α-ketoglutarate-dependent dioxygenase that plays an important role in promoting cell migration, invasion, and metastasis (1,2). Recent findings show that ASPH is abundantly expressed in various types of neoplastic cells (2,3). Therefore, ASPH is considered as a tumor biomarker for detecting cancer, predicting prognosis, and observing the response to treatment (4). Recently, ASPH has also attracted attention as a potent tumor therapeutic target (5,6).

Regarding its role and overexpression in cancer cells, developing probes including antibodies and aptamers against ASPH would be valuable in both clinical cancer diagnostics and therapeutics. To study in this area, it is necessary to produce recombinant ASPH in the laboratory.

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Currently, the most frequent recombinant protein production platforms include prokaryotes, yeasts, baculovirus cell systems, and mammalian cell systems (7,8). Among them, mammalian cell hosts are more likely to produce properly folded mammalian proteins with native-like posttranslational modifications (9). Therefore, constructing an ASPH overexpressing mammalian cell line can serve as the target protein needed to produce and characterize identifying probes in molecular diagnostic studies.

In the selection step of the mammalian cell transfection, the cells that remained resistant mainly consist of various diversity of cells with different protein expression levels. Each cell in the population may produce a large or low amount of the intended protein (10,11). This problem may reduce production efficiency. Therefore, we need a strategy to decrease this expression diversity. Traditionally, researchers try to deposit one cell per well into 96-well plates to obtain exactly similar cells in each well (10,11). However, this strategy is extremely time-consuming and labor-intensive. Many studies use fluorescence-activated cell sorting to detect and separate highly expressing recombinant cells based on the fluorescence signal (10,12,13). To that end, various types of second selectable markers are needed (14,15). These techniques require expensive and special equipment (fluorescence-activated cell sorter). Moreover, the second selectable marker may interfere with normal protein expression.

In this paper, we report on our innovative approach in the selection of best-transfected cells with the highest expression of ASPH using subclone selection, quantitative real time polymerase chain reaction (qRT-PCR) and gradual increment of hygromycin B concentration. We finally report on our best subclone of human embryonic kidney (HEK293T) cell stably displaying ASPH on their cell surface.

The recombinant cells could be used in aptamer selection *via* the cell-based selection procedure. Moreover, the cells could be further used in monoclonal and polyclonal antibody production (16). Meantime, the expressed recombinant ASPH could be easily purified for further advanced studies.

MATERIALS AND METHODS

Chemicals and reagents

The cell culture medium, high glucose Dulbecco's modified eagle's medium (DMEM), and other culture-related reagents like the heat-inactivated fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were all purchased from Gibco (Germany). Hygromycin B was purchased from Solarbio Life Science (Beijing, China). The TBE electrophoresis buffer (10×) contained 1 M tris base, 1 M boric acid, and 0.02 M EDTA (disodium salt). GeneRuler 50 bp and GeneRuler 1K DNA ladder were both provided from Thermo Fisher Scientific (Waltham, USA). Restriction enzymes *XhoI*, *BglII*, and *FspI* were all obtained from either New England Biolabs (Beverly, USA) or Thermo Fisher Scientific (Waltham, MA, USA). The FB-50 biotinylated anti-ASPH monoclonal antibody was kindly provided by Behvazan Biopharma Company (I.R. Iran). Phycoerythrin (PE)-conjugated streptavidin was purchased from BioLegend (San Diego, USA).

Construct preparation

To achieve a mammalian system overexpressing recombinant ASPH, the pcDNA3.1/Hygro(+) plasmid (Thermo Fisher Scientific, USA) containing a mammalian promoter, known as cytomegalovirus, was utilized. This plasmid also includes hygromycin-B-phosphotransferase (hygromycin resistance gene) as a known selectable marker of stable mammalian transfectants and β-lactamase (the ampicillin resistance gene). To obtain the ASPH expressing vector, a DNA segment (2310 bp) containing Kozak consensus sequence (ACCATGG), cDNA of ASPH (NCBI accession No. NM-004318), and the C-terminal 6-His tag coding sequence was designed and cloned into the vector by General Biosystems Inc. (Morrisville, USA).

Cell culture and transfection

HEK293T cells provided from the Iranian Biological Resource Center (Tehran, I.R. Iran) were cultured in the DMEM medium with 10% FBS at 37 °C and under the humidified atmosphere of 5% CO2.

HEK293T cells were transfected using pcDNA3.1/Hygro(+)-ASPH expression plasmid and TurboFect^{TM} transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions to establish cells with overexpression of ASPH on the surface (HEK^{ASPH}). In brief, the plasmid DNA was isolated from the TOP10 F' *Escherichia coli* containing pcDNA3.1 /Hygro (+)-ASPH using GenElute™ plasmid miniprep kit (Sigma-Aldrich) according to the manufacturer's protocol. The plasmid was then linearized using *FspI* (Thermo Fisher Scientific, USA) digestion. HEK293T cells (2×10^5 cells/well) were seeded in 6-well cell culture plates to give optimal confluency (70%) at the time of transfection. Transfection was carried out using 4 μg of plasmid DNA and 8 μL of TurboFect^{TM} transfection reagent. After three days, the medium was replaced with a fresh medium containing 100 mg/mL hygromycin B (Solarbio Science & Technology, China). The plate was fed with fresh selective medium every 4 days and was screened for resistant cells remained in different parts of the plate. The detected resistant cells were monitored for 4 weeks to spot separated subclones with the ASPH expression potential. Five selected subclones were carefully scraped off the plate using sterile scrapers and transferred to separate flasks. After 2 days, the cells were trypsinized and redistributed in the flask. The mRNA expression level of each flask was evaluated as described below (subsection qRT-PCR) to select the subclones with the highest ASPH expression. Then, the concentration of hygromycin B was gradually increased in the selected flasks to reach 400 mg/mL after 4 weeks of culture.

Quantitative real time polymerase chain reaction

To evaluate the ASPH mRNA expression in the transfected cells (HEK^{ASPH}), a SYBR Green qRT-PCR assay was accomplished using $2^{-\Delta\Delta Ct}$ analysis method (17) and specific primers for ASPH and internal control gene, β-actin (18), as described here: forward primer (β-actin): 5′-GTTGTCGACGACGA-GCG-3′; reverse primer (β-actin): 5′-GCACAGAGCCTCGC-CTT-3′; forward primer (ASPH): TTGGCGTGGGATACCT-CTTG; reverse-primer (ASPH): GTCACAC-TCAGCACCTCTTC. To achieve this goal, total RNA wa-s first extracted using RNX plus (Cinagen, I.R. Iran) according to the manufacturer's protocol and then treated with DNase I enzyme (Thermo Scientific, USA) to remove the annoying unwanted genomic DNA. After the evaluation of total RNA quality and concentration *via* a Take3™ micro-volume plate (BioTek Instruments, USA) used in the microplate spectrophotometer Synergy™ HT (BioTek® instruments, USA), cDNA synthesis was performed using BioFACT RT kit (BioFACT, Korea). SYBR Green qRT-PCR assay was accomplished using BioFACT qRT-PCR master mix plus (BioFACT, Korea) on a StepOne Plus RT-PCR system (Applied Biosystems, USA) by the following reaction program: one cycle at 95 °C for 15 min as step 1, 40 cycles of iterative denaturation (95 °C, 15 s), annealing (58 °C, 30 s), and extension (72 °C, 30 s) as step 2, continued by melt curve stage as the last step. The experiment was accomplished for every sample in three replicates. Non-template control was also used as the negative control.

Polymerase chain reaction

To confirm the plasmid integration (pcDNA3.1/Hygro(+)-ASPH) into the genome of selected cells, a PCR was accomplished on the extracted genomic DNA of the HEK^{ASPH} cells using the protocol described in our previous study (19) and a pair of specific primers, as described here: forward primer: CCACTGCTTACTGGCTTATCG; reverse primer: GGGTCCATCTTCTTGTTGC. One of these primers binds to a specific site in the plasmid sequence and the other identifies a definite region of the ASPH gene sequence. Therefore, the appearance of an amplicon (591 bp) in the agarose gel electrophoresis assay would signify successful plasmid integration into the genome. Genomic DNA extraction was done utilizing the Genetbio DNA extraction kit (Genetbio, Korea) following the manufacturer's protocol.

The PCR assay was accomplished on both transfected and untransfected HEK293Ts. Furthermore, a negative control was run

using the same procedure but omitting the DNA template.

Flow cytometry

The ASPH protein display on the cell surface of both transfected and untransfected HEK293Ts was evaluated using an anti-ASPH monoclonal antibody and flow cytometry analysis.

The cells were initially detached by a sterile scraper and then washed twice with phosphate-buffered saline (PBS). In each separated tube, approximately 5×10^5 cells in 500 µL PBS were incubated with the biotinylated FB50 anti-ASPH antibody for 30 min at 4 °C. After being washed twice with PBS, the cells were resuspended in 500 µL PBS to be incubated with PE-streptavidin for 30 min at 4 °C in a dark place. After being washed twice with PBS , the cells were finally resuspended in 200 µL PBS and the fluorescence signal was evaluated using flow cytometer (FACSCalibur, BD Biosciences) and visualized by the FlowJo software (Tree Star, USA). The PE-fluorescence signals were measured at 580 nm using the FL2 channel.

Immunocytochemistry

To determine and visualize the overexpression of ASPH on the surface of HEK^{ASPH}, an immunocytochemistry assay was accomplished using a biotinylated FB50 anti-ASPH antibody and PE-streptavidin. A day before the experiment, the cells were seeded in a 96-well plate. Fixation was performed using 4% formaldehyde in PBS (fixation buffer, pH 7.4) for 30 min at room temperature at the time of the experiment.

The fixed cells were washed three times by PBS and then the wells were blocked using 5% bovine serum albumin for 60 min. After washing again with PBS (three times), the cells (transfected and untransfected HEK293Ts) were treated with biotinylated FB50 anti-ASPH antibody for 2 h. After the washing step, the cells were stained using PE-streptavidin. At the time of the assay, the stained cells were treated with 4',6-diamidino-2 phenylindole (DAPI; Invitrogen, USA) for 2 min.

The fluorescence signals were finally captured using a Nikon Microphot-5A inverted fluorescent microscope.

Statistical analysis

Statistical analysis was performed using the SPSS 21 (IBM Corporation) and one way ANOVA, Bonferroni post-hoc test between various groups. Data values were presented as mean ± standard deviation.

RESULTS

Construct confirmation and preparation

In addition to the Sanger sequencing of the construct, performed by General Biosystems Inc., a further experiment was done using digestion with *BglI* and *XhoI* restriction enzymes to confirm the proper insertion of cDNA of interest in the vector (Fig. 1A). *XhoI* and *BglI* were initially cut the construct at one and two points, respectively, as expected (Fig. 1A). The results obtained from agarose gel electrophoresis showed that the pcDNA3.1-ASPH vector was properly generated. The pcDNA3.1-ASPH vector was initially linearized using *Fsp I* (Thermo Fisher Scientific, USA) digestion, as visualized with agarose electrophoresis (Fig. 1B).

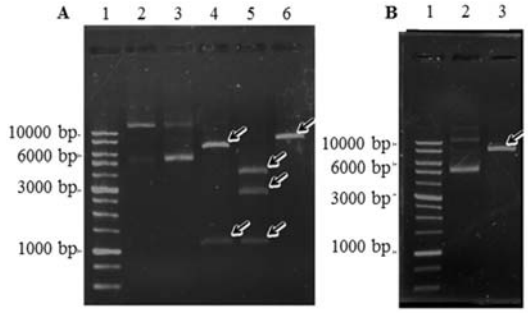

Fig. 1. Enzyme digestion on the plasmid. (A) the enzyme digestion was performed using *XhoI* and *BglI*. As expected, *XhoI* and *BglI* cut the construct at one and two points, respectively indicating proper insertion of aspartyl/asparaginyl β-hydroxylase. Lane 1: DNA ladder 1Kb; lanes 2 and 3: circular plasmid before transformation; lane 4: single digestion with *BglI*; lane 5: double digestion with *BglI* and *XhoI*; lane 6: single digestion with *XhoI*. (B) To linearize the vector before transfection, the enzyme digestion was performed using *FspI*. Lane 1: DNA ladder 1Kb; lane 2: circular plasmid; lane 3: single digestion with *FspI*.

Transfection, cell selection, and evaluation of ASPH expression

In the transfection procedure, cell treatment with hygromycin B (100 mg/mL) caused a gradual elimination of untransfected cells (Fig. 2). After transfection and treatment by hygromycin B for 4 weeks, the selected subclones (Fig. 2) in each flask were examined for ASPH mRNA expression level. Our findings from the quantitative measuring of mRNA (qRT-PCR) using the $2^{-\Delta\Delta Ct}$ analysis method demonstrated that the ASPH expression in different subclones is significantly diverse (Fig. 3A). Subclones 1 and 2 showed the greatest quantities of ASPH mRNA (around 34.15 and 25.22 times greater than the controls, respectively). Any increment in mRNA levels in various hygromycin B concentrations was also monitored, as depicted in Fig. 3B and C. The gradual increment of hygromycin B (up to 400 mg/mL) improved the expression of ASPH. Subclone 1 at 300 mg/mL of hygromycin B showed the best mRNA level (fold change: 57.59) and was selected for the plasmid integration test using the PCR analysis. The data of agarose electrophoresis stained with ethidium bromide showed a single sharp band (591 bp) verifying the insertion of the ASPH-bearing vector (pcDNA3.1-ASPH) into the HEK293T genome, as depicted in Fig. 3D.

Flow cytometry and immunocytochemistry

Using flow cytometry, it was confirmed that the protein of interest was displayed on the surface of the HEK^{ASPH}, while the control untransfected cells show no expression on their surface (Fig. 4). These results indicated that approximately 90.2% of HEK^{ASPH} cells overexpressed ASPH on their surface.

Using immunocytochemistry, the cell surface display of ASPH in HEK^{ASPH} was shown through the strong red fluorescence signal around the cells, as illustrated in Fig. 5. The control untransfected HEK293T cells showed no signal indicating the lack of ASPH on the membrane.

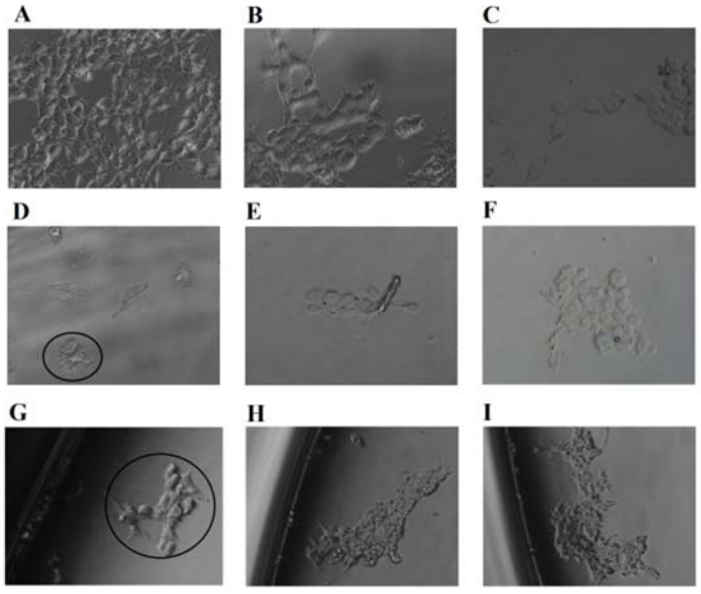

Fig. 2. Transfection, selection procedure, and verification of (A) HEK293T cells on day of transfection; (B) transfected cells: 5 days after transfection; (C) Transfected cells: 2 weeks after transfection; (D) A small detectable subclone (subclone 1): 3 weeks after transfection; (E) subclone 1: 4 days after detection (25 days after transfection); (F) subclone 1: a week after detection (4 weeks after transfection); (G) A small detectable subclone (subclone 2): 3 weeks after transfection; (H) subclone 2: 4 days after detection (25 days after transfection); (I) subclone 2: a week after detection (4 weeks after transfection).

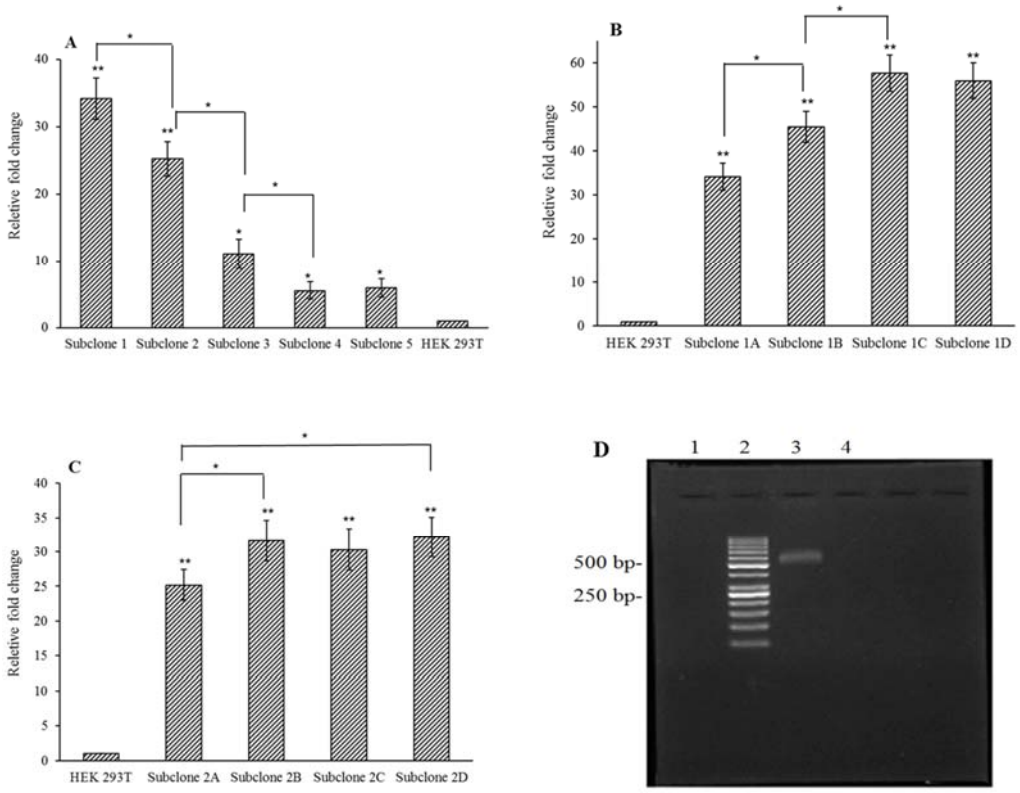

Fig. 3. Aspartyl/asparaginyl β-hydroxylase expression was evaluated using qRT-PCR and simple PCR analysis. The results of the qRT-PCR evaluation of (A) selected subclones (4 weeks after hygromycin treatment at 100 mg/mL); (B and C) subclone1A-D and subclone2A-D treated with different concentrations of hygromycin at 100, 200, 300, and 400 mg/mL, respectively; (D) the PCR product transfected cells genomic DNA (591 bp fragment) on the agarose gel. Lane 1: negative control, lane 2: DNA ladder 50 bp, lane 3, genomic DNA of transfected cells, lane 4: genomic DNA of untransfected cells. Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ indicate significant differences against the control group; $^{\#}P < 0.05$ shows significant differences between indicated groups. qRT-PCR, Quantitative real-time polymerase chain reaction.

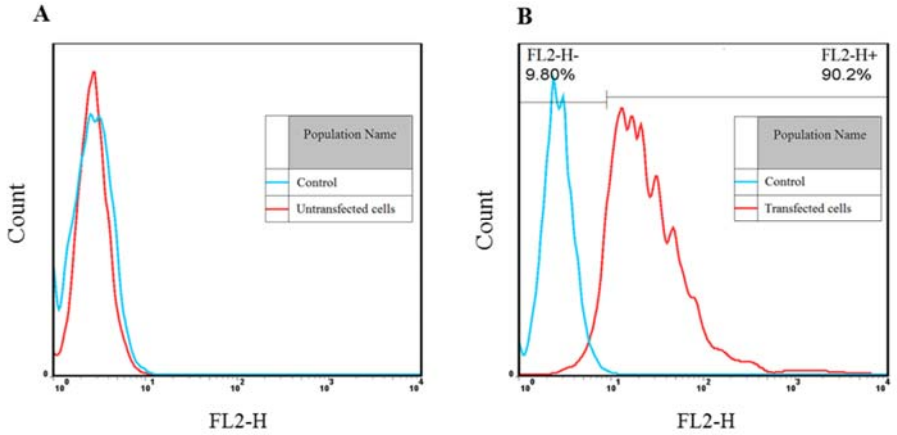

Fig. 4. Flow cytometry analysis of ASPH expression on the surface. The PI-fluorescence signal of the anti-ASPH antibody in the flow cytometry analysis verified that ASPH was displayed on the cell surface of the pcDNA3.1-ASPH-transfected HEK cells (HEK^{ASPH}), while the control untransfected cells show no expression on their surface. These results indicated that around 90.2% of HEK^{ASPH} cells overexpressed ASPH on their surface. (A) Untransfected cells and control isotype; (B) Transfected cells and control isotype. ASPH, Aspartyl/asparaginyl β-hydroxylase.

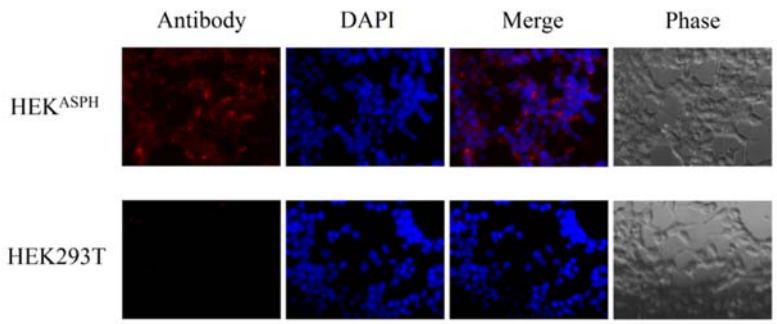

Fig. 5. Immunocytochemistry of transfected (HEK^{ASPH}) anduntransfected (HEK293T) cells. The cells were stained with the ASPH-specific antibody and nucleus-specific die (DAPI). The merge and optical (phase) figures are used to determine the position of expressed ASPH in the cells. Transfected cells (HEK^{ASPH}) showed red fluorescence signal of phycoerythrin, indicating overexpression of ASPH on the cell surface, while the control cells showed no detectable signal. ASPH, Aspartyl/asparaginyl β-hydroxylase; DAPI, 4',6-diamidino-2 phenylindole.

DISCUSSION

The role of ASPH in the pathogenesis of cancer (1,20), as well as its overexpression in a couple of malignancies (4,6), has been approved, thus the blockade or recognition of this molecule has been considered as a promising theranostic approach. Aptamers along with antibodies have been introduced as proper probes to achieve this purpose (21). In the present study, we constructed a permanent mammalian cell line overexpressing ASPH to serve as the target in developing theranostic probes. To achieve this aim, we first chose a suitable expression host.

Utilizing mammalian hosts to generate human protein targets is preferable to use other available hosts including prokaryotes and yeasts (7,9). The proteins displayed on the surface of mammalian cells would maintain their native conformation due to the proper post-translational modifications (9,22). Therefore, our recombinant ASPH produced by HEK^{ASPH} would be more similar to those available in nature.

The most important step in recombinant protein production is the establishment of a cell line with a high expression of the target. Other than antibiotic selection, some studies use no additional strategies to select higher expression cells. They collect all the antibiotic-resistant cells together in a single flask (16,23,24). In this traditional process of transfection and selection, the remained cells usually consist of various cells with different protein expression levels. Each cell in the population may produce a large or low quantity of the intended protein. Our results demonstrated that the expression levels in various subclones are different (Fig. 3). To deal with this problem, researchers implemented a variety of strategies, in most of them, flowcytometry-based cell sorting (fluorescence-activated cell sorting) is used to enhance the power of selection (10,12,13). Some studies have used a second selectable marker for the development of high producing subclones (14,15). These techniques require expensive and special laboratory equipment (fluorescence-activated cell sorter). Various types of second selectable markers like GFP-fusion proteins (14, 15) and internal ribosome entry site-dependent second gene expression (11,25) may interfere with the normal protein expression in addition to the extra cost of special materials. Using 96-well plates, some researchers try to deposit one cell per well to obtain exactly similar cells in each well (10,11). This procedure is extremely time-consuming and labor-intensive. Moreover, it would be very costly to check the expression level of each of these subclones. When antibiotic-resistant cells became stable and ready to be transferred from the bottom of the plate, they are usually dissociated several times and form small colonies. Therefore, when the cells are seeded at a dilution of one cell per well, many wells may have the same cells. In our approach, we chose and separately cultured each detectable subclone containing a group of cells that may not necessarily be derived from a single cell.

This strategy drastically reduces the number of samples needed for further testing at the cost of a small increase in cell diversity. We also used qRT-PCR assay as the initial monitoring approach which is substantially less expensive than flow cytometry and second fluorescence selectable reporters. Also, our findings are in agreement with other studies on the effect of increasing hygromycin concentrations, which suggest that higher doses of antibiotics would eliminate less resistant cells (16,23,24).

Recombinant cell lines overexpressing a predefined surface protein could be used as a positive target in the cell-based strategies for aptamer selection (21,26). In the case of ASPH, HEK^{ASPH} could be directly implemented in developing appropriate sensitive and specific anti-ASPH aptamers. The obtained recombinant HEK^{ASPH} cells could further be used in nanobody development and antibody production against ASPH. Meantime, if the purified ASPH protein is needed for further studies, the protein could be purified using the C-terminal 6-His tag. Moreover, large-scale cell culture of HEK293Ts is easily achievable because they simply adjust from adherent culture to suspension (27). Therefore, the obtained recombinant HEK^{ASPH} cells could be used for the massive production of ASPH.

CONCLUSION

In the present study, a recombinant human cell line overexpressing surface ASPH was constructed and successfully evaluated. Our cell selection strategy simply increased the mean of ASPH expression in the transfected cells in a short period time and at a low cost.

It would be of further interest to purify recombinant ASPH from HEK^{ASPH} on a large-scale for future studies. Furthermore, evaluation of the activity of obtained ASPH would be an attractive purpose for the studies focused on inhibiting the activity of this protein.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no conflict of interest.

AUTHORS' CONTRIBUTION

All authors contributed equally to this work.

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