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ORIGINAL ARTICLE

Enhancing tumor-specific intracellular delivering efficiency of cell-penetrating peptide by fusion with a peptide targeting to EGFR

Long The Nguyen · Xu-Zhong Yang · Xuan Du · Jia-Wei Wang · Rui Zhang · Jian Zhao · Fu-Jun Wang · Yang Dong · Peng-Fei Li

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Abstract Cell-penetrating peptides (CPPs) are well known as intracellular delivery vectors. However, unsatisfactory delivery efficiency and poor specificity are challenging barriers to CPP applications at the clinical trial stage. Here, we showed that S3, an EGFR-binding domain derived from vaccinia virus growth factor, when fused to a CPP such as HBD or TAT can substantially enhance its internalization efficiency and tumor selectivity. The uptake of S3-HBD (S3H) recombinant molecule by tumor cells was nearly 80 folds increased compared to HBD alone. By contrast, the uptake of S3H by non-neoplastic cells still remained at a low level. The specific recognition between S3 and its receptor, EGFR, as well as between HBD and heparan sulfate proteoglycans on the cell surface was essential for these improvements, suggesting a syngeneic effect between the two functional domains in conjugation. This syngeneic effect is likely similar to that of the heparin-binding epidermal growth factor, which is highly abundant particularly in metastatic tumors. The process that S3H entered cells was dependent on time, dosage, and energy, via macropinocytosis pathway. With excellent cell-penetrating efficacy and a novel tumor-targeting ability, S3H appears as a promising candidate vector for targeted anti-cancer drug delivery.

Keywords Cell-penetrating peptide · Tumor-homing peptide · Targeted drug delivery · Growth factor

Abbreviations

CPP	Cell-penetrating peptide
VGF	Vaccinia virus growth factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ET	EGFP-TAT fusion protein
ES3T	EGFP-S3-TAT fusion protein
EH	EGFP-HBD fusion protein
ES3H	EGFP-S3-HBD fusion protein
ES3	EGFP-S3 fusion protein

Introduction

Cell-penetrating peptides (CPPs) are short peptides defined by the unique ability of intracellular translocation. Due to this effect, CPPs have been preferentially employed as the intracellular delivery vectors for a variety of therapeutic agents, including DNA, RNA, and proteins (Bolhassani 2011). The transactivator of transcription (TAT) protein of the HIV-1 was the first cell-penetrating peptide discovered and its ability to autonomously enter cells has been widely

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L. T. Nguyen and X.-Z. Yang are contributed equally.

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L. T. Nguyen · X.-Z. Yang · X. Du · J.-W. Wang · R. Zhang · J. Zhao (✉) · Y. Dong · P.-F. Li
State Key Laboratory of Bioreactor Engineering, Department of Applied Biology, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
e-mail: zhaojian@ecust.edu.cn

X.-Z. Yang · F.-J. Wang
Zhejiang Reachall Pharmaceutical Co. Ltd., 209 West Hulian Road, Dongyang 322100, Zhejiang, China

F.-J. Wang (✉)
Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, 1200 Cailun Road, Shanghai 201203, Shanghai, China
e-mail: wfj@shutcm.edu.cn

studied and applied (Fawell et al. 1994; Wadia and Dowdy 2005). Among the CPPs discovered so far, human-derived cell-penetrating peptides (hCPPs) attracted more attentions than those from non-human origin for the immunogenicity and safety considerations when they were used for in vivo drug delivery (Alexander et al. 2012; De Coupade et al. 2005; Zhao et al. 2011).

The main bottleneck for most CPP-based drug delivery system to be applied in clinic, especially for cancer treatment, is the lack of cell type selectivity that results in the diminishment of therapeutic efficiency and increased side effects (Vives 2005). Therefore, reengineering CPPs to improve tumor-targeting ability is obviously imperative. Tumor-homing peptides (HPs) are peptides that have special affinity to the cancer cell surface, hence have been used extensively as vectors for selective anti-cancer drug delivery (Arap et al. 1998; Cao et al. 2008; Laakkonen et al. 2002). So far, by means of conjugating HPs with CPPs, several tumor-homing CPPs (HP-CPPs) have been successfully developed (Svensen et al. 2012). Despite showing promising anti-cancer effects in vitro (Mäe et al. 2009), none of the HP-CPP drug deliver systems have really succeeded in in vivo and especially clinic. Among multiple factors that could contribute to the ineffectiveness, the impact of the incompatibility between HP and CPP in the conjugate appears to be significant, as it has been shown that HP integration can interfere with cellular uptake and intracellular activity of the reactive cargos (Mäe et al. 2009; Saleh et al. 2008). Therefore, it is essential to select an appropriate couple of HP and CPP for conjugating to optimize both tumor-targeting and cell-penetrating ability of the HP-CPP.

The overexpression of epidermal growth factor receptors (EGFR family) and their ligands (EGF) have been consistently implicated in the development of various types of cancer, especially malignant phenotypes (Hynes and Lane 2005). Therapeutic intervention of EGFR signaling pathway by antagonists remains the most successful approach among only few targeted cancer therapies applicable in human (Ciardiello and Tortora 2008). By means of conjugating EGFR family-targeting domains with nanoparticles or CPPs, several tumor-targeting drug delivery systems have been established (Tan et al. 2006). All EGFR agonists, such as EGF and TGF-α, shared a similar structure that is constituted by three disulfide loops (McFadden and Moyer 2000); among which, the third one has been used independently for targeting EGF-sensitive tumor (Lu et al. 2005). Vaccinia virus growth factor (VGF) is also a member of the EGF family (Tzahar et al. 1998), of which the third disulfide domain (S3) has been suggested to possess the same binding ability to EGFR as in other EGF-like factors (Purchio et al. 1987). Importantly, the synthetic unglycosylated form of VGF(S3) has only limited mitogenicity and tumorigenicity (Lin et al. 1990). Therefore, the recruitment of S3 is likely to improve tumor-targeting ability and intracellular delivering efficacy of the CPP conjugate, while barely inducing any significant tumor growth promotion.

Recently, our research group has discovered and successfully employed hC-SOD3 (HBD), a 29-amino acid CPP derived from the heparin-binding domain of human extracellular superoxide dismutase (EC-SOD), for in vitro/vivo delivery of apoptin, an anti-cancer protein (Zhao et al. 2011). Supposedly, the cell-penetrating mechanism of HBD can be referred to its interaction with heparan sulfate proteoglycans (HSPGs) on the cell surface. By conjugating HBD and S3, it is possible to create a bifunctional peptide capable of binding to both HSPGs and EGFRs. Interestingly, this closely imitates the unique ability of a naturally occurred protein, the heparin-binding EGF-like growth factor (HB-EGF) (Das et al. 1994), which plays an essential role in tumor progression through promoting cell growth, angiogenesis and metastasis (Nolan et al. 2004). The existence and high expression of HB-EGF in various cancer types (Miyamoto et al. 2006) imply a coherent cooperation of the heparin and EGFR-binding domains (HBD and S3) in adhesion, association and perhaps also transduction mechanisms to/into cancer-related cells. Therefore, we predicted S3-HBD fusion peptide could become an ideal vector for specific intracellular drug delivery to cancer cells.

In this study, we evaluated the uptake efficiency of S3H into various neoplastic and non-neoplastic cells and investigated the internalization characteristics of this novel HP-CPP.

Materials and methods

Bacterial strains and plasmids

E. coli BL21 (DE3) strain and pET28a (+) plasmid, both purchased from Novagen (USA), were selected as the expression system of the recombinant proteins in this study. Enhanced green fluorescence protein (EGFP) was fused to the recombinant peptides for visualization and cargo resembling purposes. The DNA sequences of EGFP, HBD and TAT, were cloned from previously constructed vectors (Zhao et al. 2011). The sequence of S3 was chemically synthesized (Generay Tech Co., Ltd, China) according to the corresponding amino acid residues $VGF_{52\text{-}67}$ (RCSHGYTGIRCQAVVL), and cloned in between Bam HI and Sal I restriction sites on the previously constructed pET28-EGFP-CPP plasmids, making it clipped by EGFP and CPP sequences (either HBD or TAT). Accordingly, six recombinant vectors were successfully established for expressions of both control and reactive proteins (Fig. 1a).

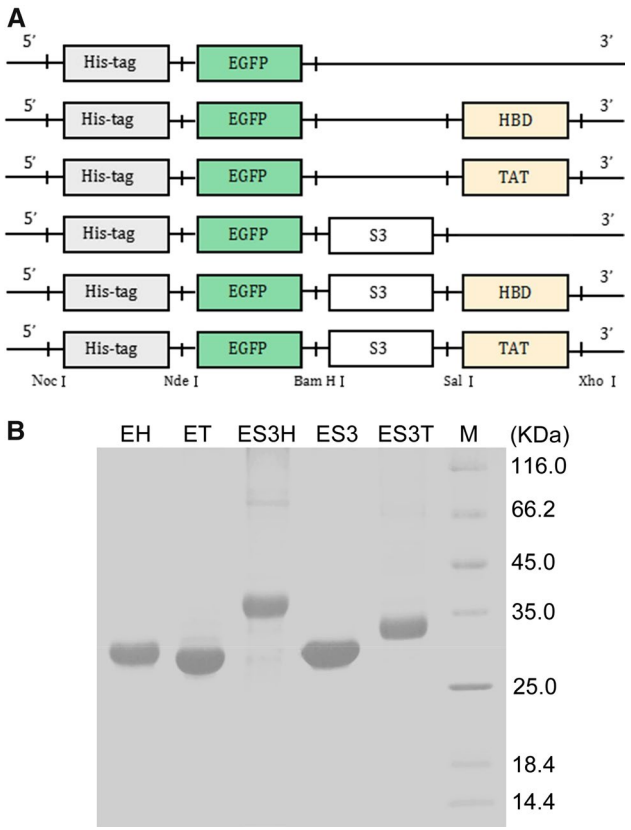

Fig. 1 Expression vectors and purified recombinant proteins. **a** Cloning of EH, ET, ES3H, ES3, ES3T to pET28a plasmid. **b** SDS-PAGE of the recombinant proteins following expression and purification

Cells lines and cell culture

Three cancer and two normal cell lines were tested for tumor specificity of the recombinant proteins, including HeLa (Human Cervical cancer cell), T24 (Human Bladder cancer cell), Bcap-37 (Human breast cancer cell), HEK293 (Human Embryonic kidney cell), and MRC-5 (Human Embryonic lung cell), were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The culture mediums used include DMEM (Hyclone, USA) for HeLa and HEK293 cells; MEM (Hyclone, USA) for MRC-5 cells; and RPMI-1640 (Hyclone, USA) for Bcap-37 and HEK293 cells. In most cases, the mediums were spiked with 10 % fetal calf serum (Gibco, USA), 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma, USA), if not indicated otherwise. The cells were raised in 5 % (v/v) CO_2 at 37 °C.

Protein expression and purification

Recombinant plasmids were transduced into *E. coli* BL21 (DE3) cells, which were subsequently raised on Kanamycin-contained Petri dish, allowing only transformed colonies to be developed; PCR was carried out to identify target gene-bearing clones. Once selected, the bacterial clone was amplified through a two-stage procedure, respectively in 30 then 200 ml of LB medium at 37 °C, followed by an overnight induction by 0.15 mM IPTG at 20 °C. Cells were harvested by centrifugation at 3,000×*g* for 20 min, resuspended into lysis buffer [20 mM Tris–HCl, pH 8.5, 5 % (v/v) glycerol] and sonicated in an ice-water bath until the solution becomes relatively clear. The supernatant was collected after centrifugation at 10,000×*g* for 15 min, and directly applied to Ni–NTA column at 1 ml/min for affinity chromatography. The column was washed with cold 20 mM Tris–HCl buffer, and then serially eluted at the rate of 0.5 ml/min with the elution buffers containing 20 mM, 200 mM or 1 M imidazole. The eluted solutions were gathered and dialyzed against cold 20 mM Tris–HCl (pH 7.4) overnight. Specially, as being expressed in the insoluble form (inclusion bodies) and thus precipitated together with the cell debris during centrifugation, ES3H was treated with 0.5 % Triton X-100 (Sinopharm Chemical Reagent Co., Ltd, China) and then 8 M urea for 0.5 h for denaturation. The solution was centrifuged and the supernatant was collected for renaturation of the protein by gradient dialysis. The protein was then purified through the same procedure that had been used for the soluble counterparts. Finally, protein concentration was measured by Bradford method. The purified protein samples can be applied directly to the cells or kept at −20 °C for latter usage.

Cellular uptake analysis

Cellular uptake was analyzed by either confocal laser scanning microscope (CLSM) or fluorescence-activated cell sorting (FACS) technique. In microscope, equivalent numbers of cells (~1.0 × 10^4 cells) were seeded in each well of a 24-well plate. After 12–16 h of growth, the cells were treated with the EGFP fusion proteins at 37 °C at defined conditions to investigate their kinetics (including dose and time dependency), penetrating efficiency and internalization mechanism. Cells were washed three times with PBS and then either applied directly to confocal microscope (Nikon A1R, Nikon Co. Ltd, Tokyo, Japan) for live cell observation, or fixed with 4 % paraformaldehyde (PFA) for staining and long-term storing purposes. If fixed, the cells were then rinsed twice in ddH_2O and incubated with DAPI, a nucleus-staining blue dye, for 1 h at room temperature followed by CLSM observation of cellular uptake. ImageJ (National Institutes of Health) and NIS-viewer (Laboratory Imaging) were used for image processing and analysis. In FACS, cells (~1.0 × 10^6/well) were seeded in 6-well plates to approximately 70 % confluency and incubated overnight at 37 °C. The following day, the medium was changed to serum-free medium and the protein samples were added to

the cells. After 10 h of incubation, the cells were washed three times with precooled PBS, digested with trypsin at 37 °C for 10 min, collected by centrifugation at 1,000 rpm for 8 min, resuspended into 2 ml of PBS, and finally applied to flow cytometer (Becton, Dickinson and Company, USA) for FACS analysis.

Internalization mechanism analysis

Different types of inhibitors were applied to HeLa cells before the treatment with ES3H to investigate the internalization mechanism of the recombinant proteins. These included anti-EGFR antibody (Sino Biological Inc. China); heparin sodium (Sinopharm Chemical Reagent Co., Ltd, China) as an antagonist of cell surficial HSPG for attracting HBD; cytochalasin D (cytoD; Cayman Chemical Company, USA) as a F-actin inhibitor; amiloride (EIPA, Cayman Chemical Company, USA), which blocks Na^+/H^+ exchange channel; genistein (GNT, Cayman Chemical Company, USA) and chlorpromazine (CPM, Cayman Chemical Company, USA), which interfere caveola and clathrin induced endocytosis respectively. Cellular uptake process was then detected by microscope and FACS according to the procedures previously described.

MTT Assay

HeLa and MRC-5 cells were seeded in 96-well plates respectively and incubated overnight. The next day, ES3H protein was added to cells at different concentrations (0.2, 0.5, 1 and 2 μM) and incubated for 24 h; 25 μl of 5 mg/ml MTT solution was then added to each well. After incubation for 4 h at 37 °C, the MTT solution was removed and 150 μl of dimethylsulfoxide was added to each well. The absorbance at 492 nm of each well was measured using a microplate reader.

Statistical analysis

Student's *t* test was performed to determine the significance of quantitative difference in cellular uptake between two fusion proteins, two cell types, or two conditions. $p < 0.05$ is considered significance.

Results and discussion

Expression and purification of fluorescence recombinant proteins

All EGFP fusion proteins were expressed at high levels in BL21 (DE3) cells. Most of these proteins were soluble and relatively stable in cold pH 8.5, 20 mM Tris–HCl buffer. Only ES3H, as being expressed in insoluble inclusion bodies, had to undergo denaturation by 0.5 % Triton X-100 and 8 M urea, and renaturation by gradual dialysis. As can be seen in Fig. 1b, all recombinant proteins were successfully purified by Ni–NTA affinity chromatography (purity >95 % according to densitometry assay). The result suggests the same expression system and purification techniques can be applied for future production of S3H-conjugated drugs.

Cellular uptake analysis

To investigate whether the intracellular delivery efficiency of HBD can be improved by conjugation with S3, three recombinant proteins including EH, ES3 and ES3H were tested for cellular uptake in HeLa cells. Each of the proteins was incubated with HeLa cells for 10 h respectively, which was then subjected to CLSM or flow cytometer for fluorescence detection. As could be seen from Fig. 2a, at the concentration of 25 μM, both EH and ES3 demonstrated obvious adhesion to HeLa cells, suggesting either proteins could effectively recognize and bind to corresponding receptors on the cancer cell surface. However, their capacity for intracellular delivery was rather limited, especially in comparison to ES3H. The ES3H significantly accumulated inside the cells even though its incubated concentration was merely 0.5 μM (50 times lower than that of EH and ES3), indicating a high efficiency of S3-HBD fusion peptide for transporting reactive proteins across the cell membrane. Similarly, according to FACS assay, the fluorescence intensity of HeLa cells incubated with 0.5 μM ES3H was equivalent to that with 40 μM EH (Fig. 2b). These results demonstrate a significant improvement (~80 times) in cell-penetrating efficacy of HBD after linking with S3.

To test if the same effect could be introduced to other CPPs such as TAT, the classic virus-derived CPP, HeLa cells were treated with 10 μM of either ET or ES3T. Figure 2c shows that cellular uptake of ES3T was substantially higher than that of ET.

Evaluation of tumor cell-penetrating selectivity

A major purpose of S3-HBD combination is to generate a tumor-homing cell-penetrating peptide. Therefore, ES3H was tested for cellular uptake in different cancer and normal cell types. As can be seen from Fig. 3a, ES3H (1 μM) showed a strong adhesive and penetrating ability to the three tested tumor cell lines (HeLa, Bcap-37 and T24) after 10 h of incubation. In contrast, there was no significant uptake by the two noncancerous cell lines (293 and MRC-5). Further quantitation of cellular uptake by FACS at different protein concentrations showed that ES3H accumulated in HeLa cells was 4–10 folds of the level measured in MRC-5 cells (Fig. 3b). Importantly, increasing

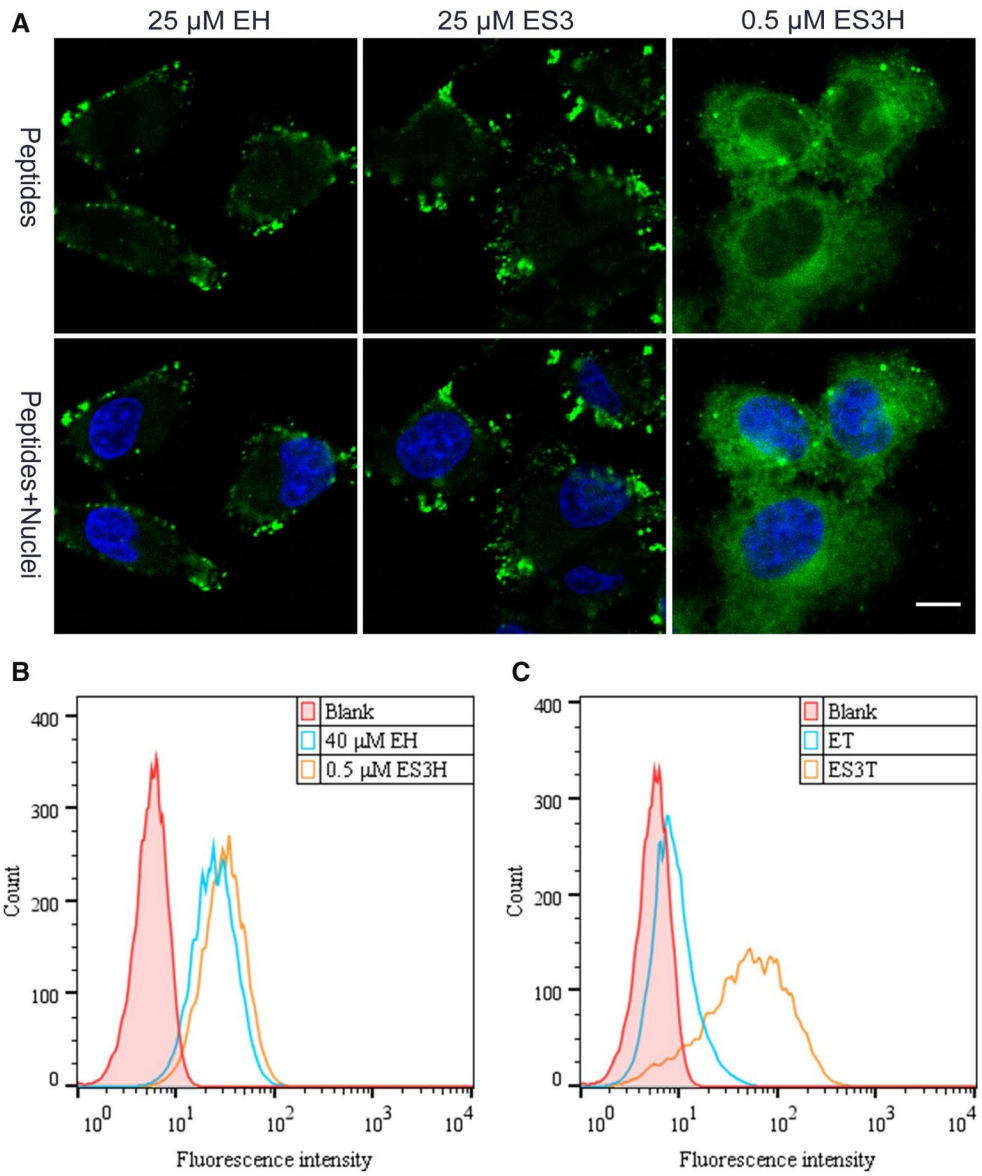

Fig. 2 Cellular uptake of S3-modifed CPPs. HeLa cells were incubated with different concentrations of EH, ES3, and ES3H for 10 h respectively. Cellular uptake (*green fluorescence*) was measured by **a** CLSM at the same optical settings following cell fixation and nuclear staining with DAPI (*blue fluorescence*), or **b** FACS following digestion by trypsin. **c** HeLa cells were incubated with 10 μM ET or ES3T for 10 h respectively, and cellular uptake was detected by FACS after trypsin administration. *Scale bar* 10 μm

ES3H concentration exponentially improved intracellular delivery into HeLa cells yet had no significant impact on MRC-5 uptake, suggesting an excellent cell selectivity of S3-HBD that could be utilized for anti-cancer drug delivery.

Supposedly, S3-HBD achieved such excellent tumor-homing ability through mimicking the dual binding effect to EGFR and HSPG of HB-EGF, a growth factor extensively expressed and received by cancer cells (Miyamoto et al. 2004; Naef et al. 1996). To confirm this speculation, we pretreated HeLa cells with anti-EGFR antibody or heparin sodium to see if the translocation would be affected. As can be seen from Fig. 4a, cellular uptake of ES3H (0.5 μM) was significantly reduced following the antibody treatment. As such, without S3 conjugated, EH entered both normal and cancerous cell lines equally at a more limited level (data not shown). The translocation of ES3H (1 μM) into HeLa cells was also completely inhibited in the presence of heparin (Fig. 4b). Without HBD conjugated, ES3 (1 μM) showed none of the adhesive or penetration activity that had been presented by ES3H (Fig. 4c).

Protein internalization kinetics and mechanism

The internalization kinetics of ES3H was assessed regarding time, dose and energy dependence. HeLa cells were incubated with 2 μM ES3H for different time periods from 15 min to 14 h. According to FACS assay, ES3H molecules started penetrating and accumulating inside the cell at less than l5 min post-treatment (Fig. 5a). The amount of cellular uptake increased dramatically and reached saturation after 10 h of incubation, suggesting S3H mediated cargo

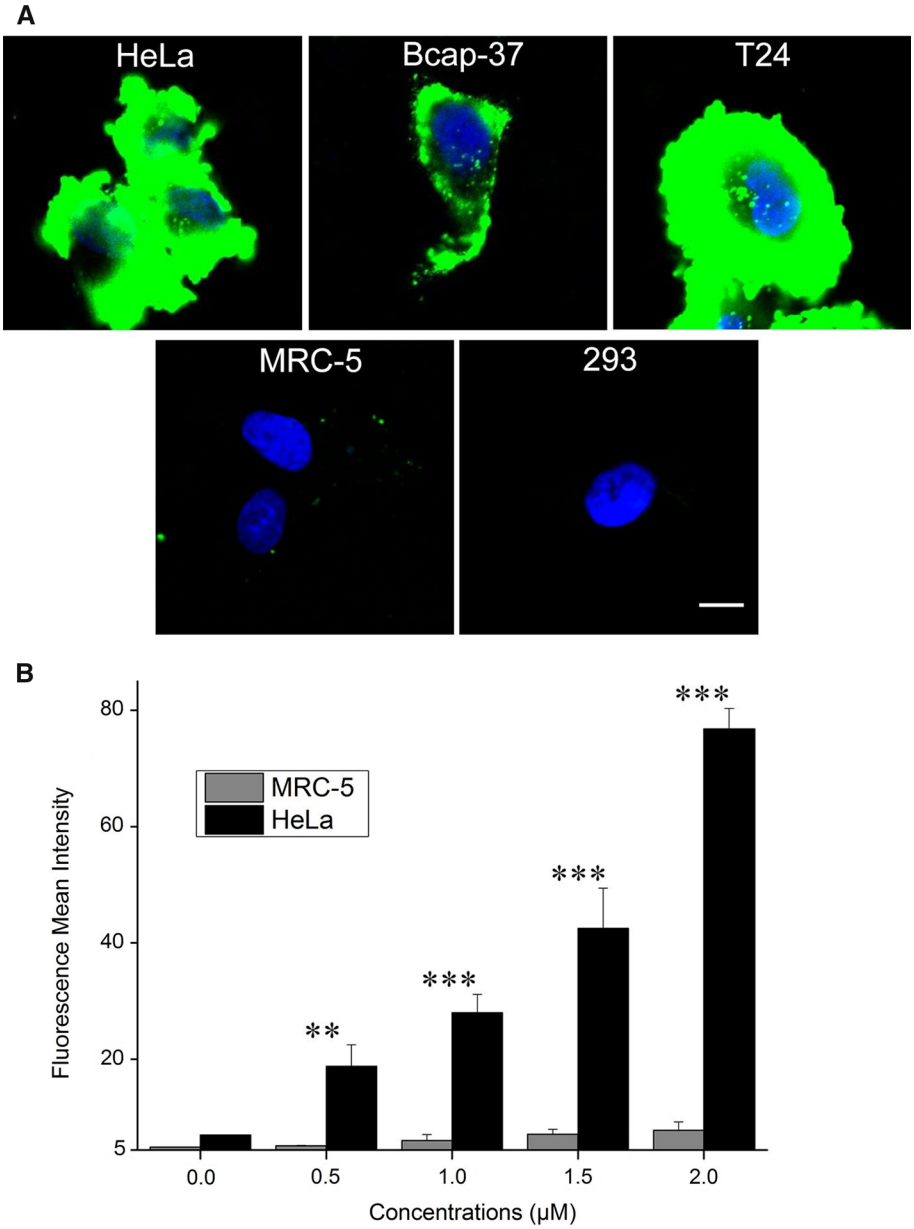

Fig. 3 Cell selectivity of S3-HBD conjugate. **a** HeLa, Bcap-37, T24, MRC5 and 293 cells were incubated with 1 μM ES3H for 10 h. Cells were fixed with 4 % PFA and stained with DAPI, then observed by CLSM at identical optical settings. **b** Cellular uptake of ES3H by HeLa and MRC-5 cells at various concentrations. Cells were digested and the fluorescence was detected by flow cytometry. *Scale bar* 10 μm. *Error bars* represent the mean ± SD, $n = 3$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

internalization in a time-dependent manner, and that 10 h was the optimal duration for cellular uptake.

The cells were also incubated for 10 h at different concentrations of ES3H, from 0.2 to 2 μM. The result (Fig. 5b) demonstrates a gradual increase of cellular uptake by increasing ES3H concentrations. The highest level of fluorescence intensity was also detected at the highest used dosage (2 μM). Interestingly, at lower concentrations (0.2–0.5 μM), the majority of fluorescence proteins were evenly distributed within the cytoplasm; while at higher concentrations (1–2 μM), they were greatly aggregated into high-density clusters (Fig. 5c).

Treating cells with NaN_3, an energy blockade, did not alter cellular uptake level of ES3H at 0.2 μM (Supplementary data, Fig. S1C), yet significantly reduced its penetrating capacity at 1 μM (Fig. 5d). HeLa cells were also pretreated with inhibitors of several endocytosis pathways, including cytoD (F-actin inhibitor), EIPA (Na^+/H^+ exchange inhibitor), GNT (caveolar inhibitor), and CPM (clathrin inhibitor). The result indicates that cellular uptake of ES3H was inhibited by cytoD and EIPA (5E), but not GNT and CPM.

Discussion

In this study, by means of fusing a human-derived heparin-binding CPP (HBD) with a vaccinia virus-derived

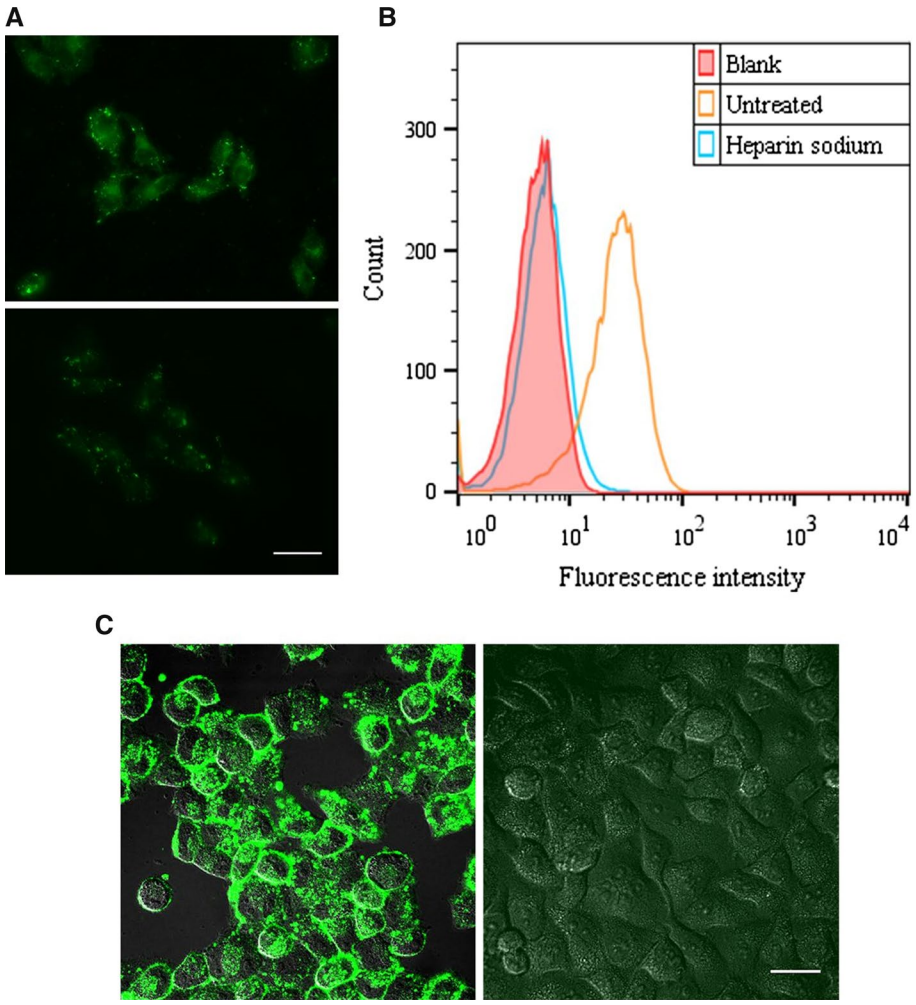

Fig. 4 The role of binding domains of S3H for targeting and intracellular delivery. **a** Anti-EGFR antibody pretreatment hinders intracellular transduction of ES3H. HeLa cells were incubated in 0.5 μM ES3H for 10 h with 10 μg/ml human IgG (*top*) or anti-EGFR antibody (*bottom*) for 2 h, and then observed by an epifluorescence microscope at identical optical settings. *Scale bar* 30 μm. **b** Competitive inhibition of ES3H cellular uptake by heparin. HeLa cells were pretreated with 10 μg/ml heparin sodium for 1 h, and then incubated for 10 h with 1.0 μM ES3H in the presence of heparin. Cells were digested and the fluorescence was detected by FACS. **c** HeLa cells were incubated with 1 μM ES3H (*left*) or ES3 (*right*) for 4 h, and observed by CLSM without fixation. *Scale bar* 30 μm

EGFR-binding domain (S3), we have successfully developed a novel targeted drug delivery vector S3-HBD with an excellent cell-penetrating and tumor cell-homing effect over a number of neoplastic cell types. The penetrating efficiency of S3H recombinant peptide into cancer cells depends critically on time, concentration, cellular energy status, and the co-binding of the chimeric molecule to both HSPG and EGFR. At the optimal dosage and with normal energy supply, S3H fusion proteins mainly enter cells by macropinocytosis.

It is important to notice that during FACS analysis, all cells were thoroughly treated with trypsin before the fluorescence intensity measurement, therefore all fluorescence protein molecules attached to the cell surface should have been cleaned, leaving only intracellular fluorescence to be detected (Supplementary data, Fig. S1A). Hence, our FACS result could reliably reflect cellular uptake. The same methodology has been used in previous studies (Rüter et al. 2010).

One common concern when developing a tumor-targeting CPP by fusion with a homing peptide is their incompatibility, or in another words, their mutual interference (Mäe et al. 2009; Saleh et al. 2008). In this study, we have shown that separately, S3 and HBD showed no obvious targeting or penetrating effect at low concentrations, but together, they mutually enhance the other's performance. The fact that S3H cellular uptake by HeLa cells was significantly suppressed under the effect of EGFR and HSPG inhibitors indicates that there was high compatibility and a synergetic effect between the two componential peptides during the binding and penetrating processes. Apparently, this closely resembled the unique characteristic of HB-EGF, an desirable growth factor in tumor microenvironment that can simultaneously bind to EGFR and HSPG on cancer or cancer-supporting cell surface (Das et al. 1994). However, unlike HB-EGF, which promotes angiogenesis and metastasis (Nolan et al. 2004), our recombinant peptide only preserves the key binding domains and as the result, it showed no growth promotion effect in either cancerous (HeLa) or noncancerous (MRC-5) cell types (Supplementary data, Fig. S1B). Obviously, its safety needs to be tested in higher scales such as in vivo and clinic, but at

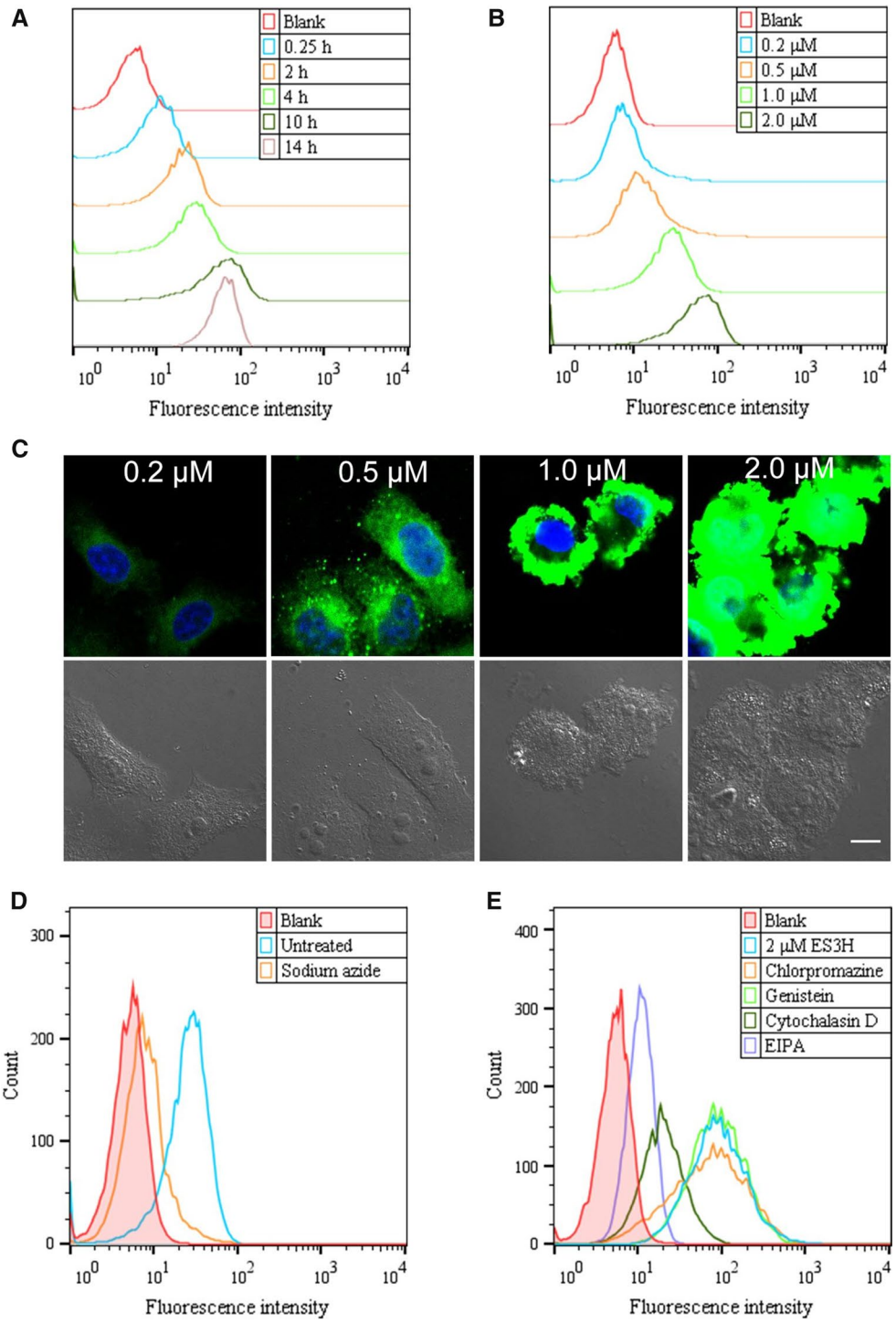

Fig. 5 Internalization kinetics and pathway of ES3H. **a** Time dependency of ES3H. HeLa cells were incubated with 2 μM of ES3H for different durations, followed by cellular uptake analysis by FACS. **b** Dose dependence of ES3H measured by FACS. **c** Dose dependency of ES3H measured by CLSM. In **b** and **c**, HeLa cells were incubated with increasing concentrations of ES3H for 10 h. *Scale bar* 10 μm. **d** Energy dependency of ES3H internalization at a high concentration of 2 μM. **e** Cellular uptake of ES3H under different inhibitors. HeLa cells were pretreated with 25 μg/ml EIPA, 10 μg/ml genistein, 10 μg/ml chlorpromazine and 10 μg/ml cytochalasin D for 1 h respectively, and then incubated with 2 μM of ES3H for 10 h, followed by cellular uptake analysis by FACS

least we have established a safety foundation to its future application in human.

The result also suggests that varying the concentrations of S3-HBD fusion proteins can alter the way they were imported and distributed inside the cells, which might in turn have significant impacts on the intracellular activity of the delivered drug. At lower concentrations (0.2–0.5 μM), ES3H was likely to enter the cells through a direct, energy-free mechanism; but at higher concentrations (0.5–2 μM), its translocation was an energy-dependent process, likely endocytosis induced by a specific agonist-receptor binding. In one previous study, stimulation by EGF in A431 cells was able to induce macropinocytosis (Hamasaki et al. 2004), a special mode of endocytosis that can be inhibited by actin and Na^+/H^+ exchange inhibitors (Koivusalo et al. 2010). The translocation of S3-HBD

fusion proteins might have been conducted according to the same mechanism. Indeed, as ES3H intracellular transport was inhibited by cytoD and EIPA (5E), but not GNT and CPM, it is likely that the internalization process of S3-HBD fusion proteins is driven by actin-dependent macropinocytosis, particularly when incubated at high concentrations. Accordingly, it is possible to speculate that S3-HBD fusion proteins translocate into cells through two mechanisms, HBD-dependent direct penetration and S3-stimulated macropinocytosis.

In this study, we showed that conjugation of S3 with not only HBD but also TAT is beneficial to cellular uptake. Although the mechanism for the enhanced penetration is unclear, we supposed it is through a very pathway by which S3H entered tumor cells. Indeed, there was convincing evidence suggesting that the ubiquitous translocation of TAT across the cell membrane relies on its interaction with HSPG (Tyagi et al. 2001), which is similar to our HBD. Further investigations need to be done to confirm this hypothesis, as well as to see if this modification approach can be universally applied to other CPPs.

Finally, despite showing a great potential for targeted cancer therapy, this S3-HBD-based drug delivery system needs to be assessed for actual therapeutic effectiveness in both in vitro and in vivo models.

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Conflict of interest The authors declare no conflict of interest.

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