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Human epidermal growth factor coupled to different structural classes of cell penetrating peptides: A comparative study

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ABSTRACT

Human epidermal growth factor (hEGF) plays important roles in wound healing. Due to large molecular weight and hydrophilic nature, cellular uptake and skin permeation of hEGF are very poor, significantly limiting its efficacy. By using recombinant technology, four structural classes of cell penetrating peptides (CPPs) were fused at the C-terminus of hEGF, expressed and purified into homogeneity. Comparative studies were conducted to evaluate their activity, cytotoxicity, cellular uptake and skin permeation. Cell viability assay and *in vitro* scratch wound-healing assay showed that all four fusion proteins had similar activities with commercial rhEGF. Obvious cytotoxicity was not detected for EGF-TAT, EGF-Pep-1 and EGF-AA3H. However, EGF-MAP was cytotoxic at both moderate and high concentrations. Confocal microscopy indicated that the cellular uptake of the fusion proteins was markedly improved compared with rhEGF, with EGF-TAT and EGF-Pep-1 showing the most abundant presence within cells at incubation concentration of 25 μM. Permeation across the excised mouse skin followed the order of EGF-Pep-1 > EGF-TAT > EGF-AA3H > rhEGF. These findings demonstrated that there were great gaps between the abilities of different structural types of CPPs to deliver EGF across cell membrane and the skin. EGF coupled with a well–chosen CPP will become a more promising pharmaceutical agent than rhEGF.

1. Introduction

The process of wound healing is a complex, dynamic and interactive process,involving hemostasis, inflammation, proliferation and wound remodeling [1]. Various growth factors have been identified in this process, among which epidermal growth factor (EGF) is the most broadly studied. Human EGF (hEGF) is a single polypeptide chain of 53 amino acid residues with a molecular weight of ~6.4 kDa and three intramolecular disulfide bonds. Many studies have shown that EGF can stimulate the proliferation and migration of epidermal cell, fibroblasts, and endothelial cells, the generation of other growth factors such as transforming growth factor and accelerate epithelialization. The efficacy of EGF in wound healing has been reported experimentally and clinically in acute wounds [2–4], chronic wounds [5–7] and burn wound [8,9]. EGF is now commercially available as a wound-healing agent in some Asian and South American countries.

In most cases, EGF is delivered dermally and thus the efficacy heavily depends on its ability to permeate the skin with sufficient quantities. Skin is a very effective barrier to prevent the entry of external pathogens and toxins, presenting great difficulties for drug delivery to pathological sites within the skin or into the systemic circulation. The outmost layer of skin is stratum corneum (SC) which is typically 10–20 μm in thickness and consists primarily of free fatty acids (15–25%), long-chain ceramides 35–50%), cholesterol (15–25%), and cholesterol sulfate [10]. It is generally thought that only small hydrophobic drugs can be efficiently delivered into or across the skin. With large molecular size and hydrophilic nature, EGF is difficult to be delivered across SC and into the epidermis layer and dermal stem cell layer, which is critical for tissue repair.

Twenty years ago, the first cell penetrating peptide (CPP), the Trans-Activator of Transcription (TAT) protein of the human immunodeficiency virus, was identified [11]. Since then, the field of CPPs evolved rapidly and now a large number of CPPs are under investigation for the delivery of various biological drugs, with some of them already in clinical trials [12]. According to their structures, CPPs are classified into polycationic, amphipathic (primary and secondary) and hydrophobic sequences. They are generally composed of less than 30 amino acid residues and have the capacity to enhance cellular uptake of cargoes electrostatically or covalently bound to them. Several attempts have been made for CPP-mediated dermal and transdermal delivery of proteins and peptides for skin diseases and for the improvement in skin properties (in case of cosmet-

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ics). Examples include cyclosporin A for cutaneous inflammation [13], superoxide dismutase for protection of skin damage from ultraviolet light [14] and lipolyticpeptide, glycine-lysine-histiding (GKH), as a new cosmetic ingredient in slimming products [15]. Compared with other routes of administration, topical delivery is non-invasive, practically painless, easy for in long-term or repetitive dosing, thus providing better patient compliance.

As a wound healing agent, more efficient topical delivery of EGF will maximize its clinical efficacy. Current investigations on this issue mainly focused on delivery technologies, including sustained release of EGF from gelatin gel sheets [16], controlled delivery of EGF from copolymer formed by vinylpyrrolidone and 2-hydroxyethyl methacrylate [17], loading of EGF on heparin-based hydrogel sheet [18], *etc*. Although these strategies did provide some improvement on wound healing by prolonging retention of EGF around the wound area, the poor permeation of EGF across cell membrane and skin has not been radically changed. It's a pity that very few studies have been conducted for the role of CPPs in topical delivery of EGF. In this work, we constructed fusion proteins of EGF with four different structural classes of CPPs, purified them to homogeneity and made comparative studies on their bioactivity, cytotoxicity, cellular uptake and skin permeation.

2. Materials and methods

2.1. Materials

Host strain *E.coli* BL 21 Star^{TM}(DE3) and vector pET-22b(+) were from Invitrogen. DNA marker, T4 DNA ligase, restriction endonucleases *Nde* I and *Xho* I were from Fermentas. FITC of analytical grade was purchased from Klamar (Shanghai, China). Sephadex G50 was supplied by GE Healthcare. Commercial rhEGF was a generous gift from PangogeneBioScience co., LTD (Nanjing, China).

2.2. Cell line and maintenance

BALB/c 3T3 cells were purchased from the American Type Culture Collection (ATCC number: CCL-92). The cells were grown in Dulbecco's modified Eagle's medium (DEME) (Gibco, Paisley,UK) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml) at 37 °C in an atmosphere of 5% CO_2. Stock cultures were preserved in liquid nitrogen. Cells were sub-cultured when the confluency reached 70–80%. When necessary, cells were maintained in maintenance solution with 0.4% fetal bovine serum.

2.3. Construction, expression and purification of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

Four structure types of cell penetrating peptides were selected to be fused at the C-terminus of EGF by recombinant expression (Table 1). Coding sequences of the four fusion proteins were designed, codon optimized and chemically synthesized by GenScript Corporation (Nanjing, China). Restriction sites *Nde* I and *Xho* I were introduced at the 5′-and 3′-terminus respectively. After digestion and ligation, the sequence of interest was inserted into the multiple cloning site of pET-22b(+) vector which was then transformed into competent *E. coli* BL21(DE3). Positive clones harboring the recombinant plasmids were screened on LB plate supplemented with 100 μg/ml ampicillin.

For the expression of each fusion protein, seed culture was prepared by overnight cultivation in LB medium containing 100 μg/ml ampicillin and then inoculated into fermentation medium containing 10.0 g/L yeast extract, 20.0 g/L tryptone, 1.6 g/L potassium dihydrogen phosphate and 20.0 g/L glucose. Flask cultivation was conducted at 37 °C and 220 rpm 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) was added when OD_{600} reached 0.8–1.0. After induction for 16 h, fermentation broth was collected and the expression of each fusion protein was determined by 15% SDS-PAGE.

Cells were harvested after centrifugation at 8000×*g* for 30 min at 4 °Cand then suspended in chilled PBS buffer (pH 7.4). The cell suspension was sonicated for 15 min (sonication power, 40%; 3 s pulse, 3 s pause). The cell lysate was centrifuged at 12,000×*g* for 20 min to collect cell debris, from which the fusion proteins were then purified. A washing solution (50 mMTris, 200 mM NaCl, 2 M urea, 0.1% Triton X-100, 1 mM EDTA,pH7.4) was used to wash the pellet twice to remove contaminating proteins. A solubilization buffer (50 mMTris, 200 mM NaCl, 8 M urea, 0.3 mM β-mercaptoethanol, adjusted to the pH that is 2–3 points away from the isoelectric point of specific fusion protein) was then used to dissolve the cell debris at room temperature and under constant stirring. The solubilization step was repeated for three times. The supernatant containing the solubilized fusion protein was collected and dropped into refolding solution (50 mMTris, 10% glycerol, 0.5 M arginine, 0.4 mM GSSG, 4 mM GSH, 0.5 M urea, adjusted to the pH that is 2–3 points away from the isoelectric point of specific fusion protein) at a rate of a one drop per minute. The refolding solution was kept at 4 °C and under constant stirring. Afterwards, the refolding solution was filtered to remove any precipitation and concentrated by ultrafiltration using Millipore Amicon® Ultra (molecular weight cutoff, 3 kDa). The concentrated refolding solution was loaded onto a Sephadex-G50 column(1.6 × 70) pre-equilibrated with PBS(pH 7.4). After elution with the same buffer, fractions showing a single band of the target protein on 15% SDS-PAGE were collected and stored at −20 °C for further analysis. Protein concentration was determined by Pierce BCA^{TM} assay. Molecular weights of purified proteins were determined by MALDI-TOF mass spectrometry (AB Sciex TOF/TOF^{TM} 5800 System).

2.4. FITC labeling of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

All the four fusion proteins were labeled with FITC using the same protocol previously described [19]. FITC was dissolved into DMSO to a concentration of 1 mg/ml. The prepared proteins were dialyzed against 0.1 M sodium carbonate buffer (pH 9.0). FITC and the protein of interest were incubated with a molar ratio of 15–20:1. Conjugation reaction was conducted overnight in the dark at 4 °C. Afterwards, the mixture was loaded onto a Sephadex G-50 column (1 × 30 cm) to remove free FITC. The molars of protein and FITC in the labeled protein were determined by measuring absorption at 280 nm and 488 nm respectively. The labeling ratio (F/P) was calculated according to the following equation: F/P = ($M_{[FITC]}$)/(M

Table 1
Four structure types of CPPs.

Fusion protein (EGF-CPP)	Predicted molecular weight(Da)	CPP sequence	CPP structural class
EGF-AA3H	7317.30	MASIWVGHRG	Cationic
EGF-MAP	7968.31	KLALKALKALKAALKLA	Secondary amphipathic
EGF-TAT	7923.03	GRKKRRQRRRPPQ	hydrophobic
EGF-Pep-1	9052.26	KETWWETWWTEWSQPKKRKV	primary amphipathic

$_{[Protein]}$).The molar ratio between FITC and the protein in the reaction mixture was adjusted so that approximately one FITC molecule was bound to one molecule of the target protein.

2.5. Growth-promoting activity and cytotoxicity of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

Both the growth-promoting activity and cytotoxicity of the four fusion proteins were determined by MTT assay which measures the metabolic activity of cells. BALB/c 3T3 cells were inoculated into 96-well plates at a density of 1×10^4 cells per well in DEME containing 10% fetal bovine serum. After overnight incubation at 37 °C, the cultivation medium was aspirated and cells were washed with PBS (pH 7.4). Fresh maintenance medium containing different concentrations of the fusion protein was added into the wells. For all the four fusion proteins, concentrations of 1.0 ng/ml, 5.0 ng/ml and10.0 ng/ml were used for the determination of growth-promoting activity. As to determination of cytotoxicity, cells were allowed to grow into 100% confluency and then incubated with 1.5 μM, 3.0 μM, 6.0 μM, 12.0 μM, 24.0 μM and 48.0 μM of each fusion protein. Each concentration had three duplicate wells. Maintenance medium without any fusion protein was added into control wells. The cells were incubated for an additional 12 h, 20 μL MTT solution (5 mg/ml) was then added into each well. Following incubation for 2 h at 37 °C, supernatant was removed and 100 μL dimethylsulfoxide (DMSO) was added into each well to dissolve formazan. After gentle agitation for 15 min, the absorbance at 570 nm was measured with a microplate reader.

2.6. Scratch wound healing assay

The effects of the four fusion proteins on the migration of BALB/c 3T3 cells were determined by Scratch wound healing assay. Cells were inoculated into 6 well-plate and cultivated until 100% confluency. A wound-like scratch was created in the center of each well by using a cell scraper. The presence of the scratch was confirmed by inverted light microscopy (Olympus CKX41) and detached cells were washed away with sterile PBS (pH 7.4). Maintenance medium containing 5 ng/ml of the fusion protein was added into the well. Cells without any treatment were used as the control. Photos were taken every 4 h after scratching.

2.7. Delivery of EGF across cell membrane

The abilities of different CPPs to deliver EGF across cell membrane were analyzed by confocal laser scanning microscopy. BALB/c 3T3 cells were inoculated into a 6-well plate with a coverslip (18 mm × 18 mm) at the bottom of each well. When confluency reached 60–70%, cells were washed with PBS (pH7.4) twice. Fresh maintenance medium with different concentrations of FITC-labeled fusion proteins was added into the wells and incubated at 37 °C for 3 h. Subsequently, cells on the coverslip were rinsed with PBS for three times and fixed by 4% PFA. The coverslip was taken out of the 6-well plate and sealed on a slide using ProLong® Gold antifade reagent with DAPI (Invitrogen™; Thermofisher Scientific). The image acquisition was performed in a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). For quantitation of fluorescence intensity, three replicate coverslips were used for each treatment condition and images of 10 random fields were taken from each coverslip with a 60X silicone immersion objective lens and the same exposure time. All the images were then analyzed by Image-Pro Plus 7.0. Within each field, at least three cells were chosen as the "area of interest", the sum fluorescence intensity of all the selected cells were calculated and then divided by the total area of the selected cells to obtain mean fluorescence intensity. Cellular uptake of EGF and EGF fusion proteins was estimated by mean fluorescence intensity.

2.8. Skin permeability study

BALB/c mice were euthanized via urethane injection. The skin from the dorsal surface was excised, and the hairs on the skin and subcutaneous fat were carefully removed. The skin was then thoroughly washed with physiological saline and mounted between the donor and receptor compartments of the Franz diffusion cells (Permegear Inc., Riegelsville, PA) with the stratum corneum facing the donor compartment. 300 μL of each FITC-labeled fusion protein was added into the donor compartment after the skin was equilibrated in PBS for at least 30 min. The receptor compartment was filled with PBS (pH7.4) and stirred at 200 rpm. Water bath was used to maintain the temperature of the receptor compartment at 37 °C. The surface area for diffusion is 0.64 cm^2. After 12 h' permeation in the dark, the skin was taken out and washed with physiological saline. Skin sections of 8 μm thickness were obtained by using a cryostat (CM3050S) and visualized with a fluorescence microscope (Leica, USA) using a 10× objective. All the procedures and experiments involving animals were approved by the Animal Experiment Ethics Committee of China pharmaceutical University.

2.9. Statistical analysis

All the data are expressed as means ± standard deviation from at least three independent experiments. Student's *t*-test was carried out for the comparison between two groups. Differences were considered significant when P value <0.05.

3. Results and discussion

3.1. Construction, expression and purification of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

After double digestion with restriction enzymes *Nde* I and *Xho* I, chemically synthesized DNA sequences encoding the four

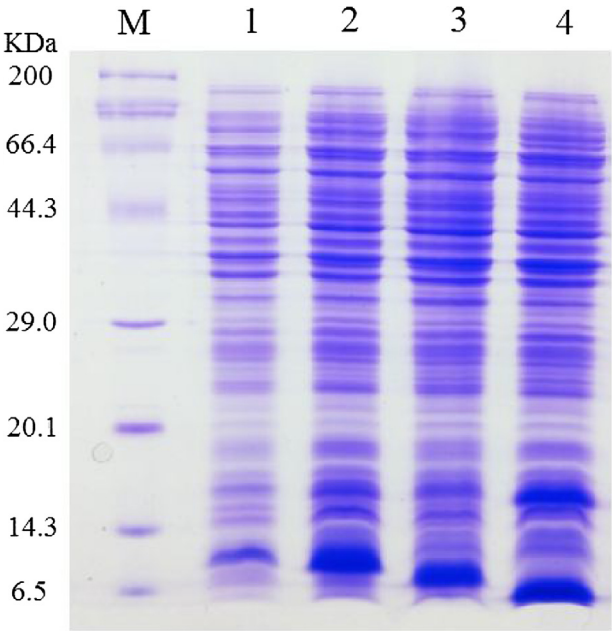

Fig. 1. SDS–PAGE of cell pellets obtained after fermentation of the constructed EGF-CPPs in *E.coli* BL21. Lane M: molecular weight markers; Lane 1:EGF-TAT; Lane 2: EGF-Pep-1; Lane 3: EGF-MAP; Lane 4: EGF-AA3H.

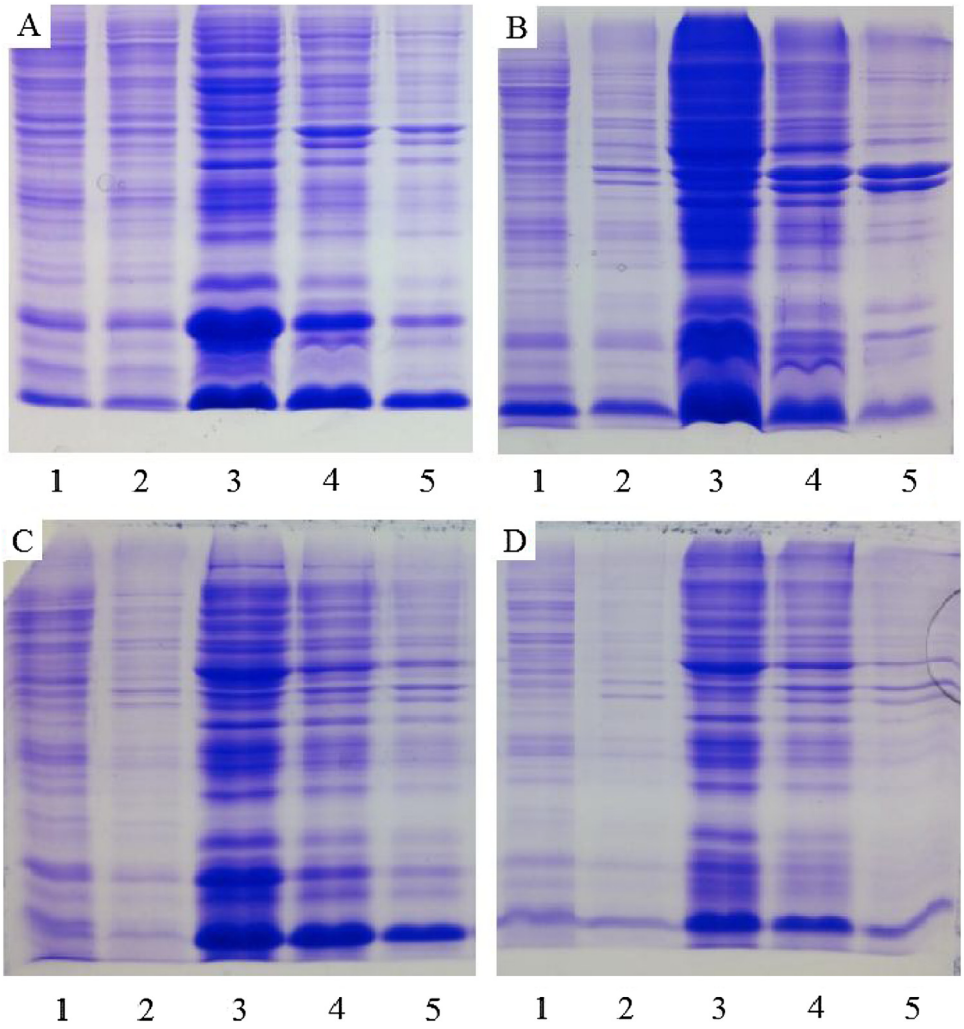

Fig. 2. Washing and solubilizing processes in the purification of four EGF-CPPs(A, EGF-AA3H; B, EGF-MAP; C, EGF-Pep-1; D, EGF-TAT) from inclusion bodies. Lane 1: supernatant of 1st washing; Lane 2: supernatant of 2^{ed} washing; Lane 3: supernatant of 1st solubilization; Lane 4: supernatant of 2^{ed} solubilization; Lane 5: supernatant of 3rd solubilization;.

fusion proteins were inserted into pET-22b(+) vector. The vectors were transformed into competent *E.coli* BL21, and positive clones harboring the recombinant plasmids were screened on LB plate supplemented with 100 μg/ml ampicillin. Flask fermentation was conducted at 37 °C and 220 rpm with IPTG induction for 16 h. SDS-PAGE analysis of the cell pellets after fermentation showed very obvious overexpression of target proteins around 8 kDa (Fig. 1), which was consistent with the expected size of the fusion proteins. Further analysis of the supernatant and debris after sonication showed that all the four fusion proteins were mainly accumulated as insoluble inclusion bodies within the host cells (data not shown).

Washing solution containing a low concentration of urea was used to remove some contaminating proteins (mainly membrane proteins) from the inclusion body with minimum loss of the target proteins (Fig. 2). Solubilization buffer containing 8 M urea successfully solubilized the target protein. The supernatant obtained after each time of solubilization was pooled and dropped into the refolding solution. Due to incorrect folding and aggregation, some precipitation was formed during the dropping process although violent agitation was applied. After removal of the precipitation by centrifugation, the refolding solution was concentrated and loaded onto a Sephadex-G50 column. Compared with contaminating proteins, the target protein has the smallest molecular weight and thus was eluted from the column after impurities. As shown in Fig. 3,

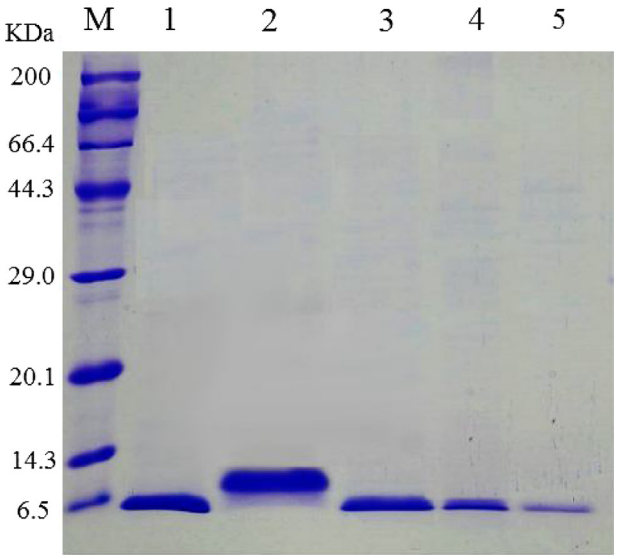

Fig. 3. SDS-PAGE of purified fusion proteins and commercial rhEGF. Lane M: marker; Lane 1: EGF-AA3H; Lane 2: EGF-Pep-1; Lane 3: EGF-TAT; Lane 4:EGF-MAP; Lane 5: commercial rhEGF.

all four fusion proteins were purified into homogeneity. Molecular weight determined by MALDI-TOF mass spectrometry was consistent with the theoretical value (Fig.S1-S4).

3.2. Growth promoting activity of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

It has been well known that fibroblasts are essential for cutaneous wound repair, and wound healing is associated with fibroblast proliferation [20]. The growth promoting activity of the four fusion proteins on BALB/c 3T3 cells was determined in MTT assay. As shown in Table 2, after 12 h' incubation, the four fusion proteins all significantly stimulated cell growth compared with the control group. Their growth promoting activities were dose-dependent and similar to that of rhEGF, suggesting that the CPPs connected to the C-terminus of EGF didn't cause significant loss in EGF activity.

EGF binds to the EGF receptor family of proteins and gives rise to a cascade of intracellular mitogenic signaling and other cellular activities [21]. The three disulfide bonds formed within EGF molecule largely decide its tertiary structure and thus critical to its activity. The study of Alewood*et al.* showed that although the disulfide bond between residues 6 and 20 could be removed with significant retention of biological activity, the removal of any other disulfide bond resulted in a large loss of activity [22].The four fusion proteins were all purified from inclusion bodies that were composed of densely packed denatured protein molecules. Elaborate solubilization, refolding and purification procedures were carried out to cover functionally active products. The concentrations of both oxidizing and reducing agents for the formation of disulfide bonds were optimized. Low molecular weight additive arginine was added into the refolding buffer to reduce protein aggregation and thus improves the refolding yield of solubilized protein. The result of MTT assay in this study indicated that the purified fusion proteins had similar activity with rhEGF, suggesting correct refolding and good retention of biological activity.

3.3. Effects of fusion proteins and rhEGF on cell migration

The bioactivities of the four fusion proteins were further confirmed by promotion on cell migration in *in vitro* scratch wound-healing assay. An important step of epithelial wound healing is cell migration to cover the damaged area [23]. One of the mechanisms for EGF to accelerate wound healing is its ability to stimulate fibroblast motility [24]. After scratching confluent monolayers of BALA/c 3TA cells to create a wound-like gap, cells were treated with fusion protein or rhEGF. In Fig. 4, compared with untreated cells, a larger number of cells migrated into the gap in the fusion protein treated groups when observed at 4 h after scratching, similar with the commercial rhEGF. At 8 h, the wounded areas nearly closed in the treated groups, while the improvement in the control group was very limited. This significantly accelerated "wound closure" in the treated groups indicated that the fusion protein had comparable activities with rhEGF and that covalently bond CPPs didn't cause significant loss of EGF activity.

3.4. Cytotoxicity of EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1

Cell viability was determined by MTT assay after 12-h exposure to different concentrations of fusion proteins. In the range of 1–48 μM, EGF-TAT and EGF-Pep-1 didn't affect cell viability (Table 3). Very mild cytotoxicity was observed for EGF-AA3H at 48 μM, which was consistent with previous study [25]. However, the cytotoxicity of EGF-MAP was more obvious. Although cell viability was not affected at low EGF-MAP concentrations (1–6 μM), slight decrease in cell viability was observed after incubation with 12 μM EGF-MAP. When further high concentrations were used, cell viability decreased dramatically, indicating pronounced cytotoxicity. rhEGF showed a remarkable low toxic potential, probably because its structure is very close to that of β-urogastrone, a human protein known to inhibit gastric secretion [26]. Its good tolerability and safety have been demonstrated in various experiments [27]. It is reasonable to attribute the toxicity of EGF-MAP to the CPP moiety. CPPs are generally considered as non-toxic. However, some studies showed that the entry mechanisms of some CPPs were not as low toxic as we considered before. Several independent studies reported that amphipathic CPPs were more toxic than cationic ones, demonstrating that even 5 μM of transportan 10 affected cellular metabolism [28] and 10 μM MAP caused significant LDH leakage inseveral cell lines [29], whereas the cationic peptides such as TAT were not cytotoxic *in vitro* even at concentrations up to 0.4 mM [30]. It was speculated that there might be a correlation between amphipathicity and toxicity of CPPs.

3.5. Cellular uptake of FITC-labeled fusion proteins

The cell penetrating abilities of FITC-labeled fusion proteins were determined in BALA/c 3T3 cells by confocal laser scanning microscopy. Cells were grown into 60–80% confluency and incubated with fusion proteins of various concentrations. As shown in Fig. 5, EGF-AA3H, EGF-TAT, EGF-Pep-1 were added into the concentrations of 5 μM, 15 μM and 25 μM. Because of the obvious cytotoxicity of EGF-MAP, only a low concentration of 5 μM was assessed. Membranes of live cells are permeable only by small hydrophobic compounds. Hydrophilic compounds like proteins are very difficult for cells to internalize. Because of this, very weak fluorescence of FITC-rhEGF was detected within the cells even at the highest concentration. However, cell permeability of EGF was significantly improved when covalently bond with CPP, as shown by the obvious fluorescence detected within the cells after incubation with the fusion proteins. The translocation of EGF-Pep-1 and EGF-TAT were comparable at 25 μM, but EGF-TAT was more potent at 5 μM and 15 μM. Compared with EGF-TAT, weaker fluorescence was detected for EGF-AA3H across all concentrations. EGF-MAP showed minimum presence within the cells at 5 μM. When internalized into the cell, CPP/cargo complex could be redirected out of the cell intact and further permeates into deep layers of cells [31]. There has been a wealth of evidence from human and animal tests which indicates that EGF is very valuable in the treatment of gastrointestinal tract ulcers [32,33] and epithelium damage after eye surgery [34] in addition to skin wound. Compared with rhEGF, EGF coupled with CPP is expected to be more potent in clinical efficacy since enhanced cellular uptake could increase drug permeation into deep layers of epithelial cells.

The four CPPs selected in this study belong to different structure types, and their translocation mechanisms are not the same. TAT is the first discovered and also the most studied CPP. As a typical example of polycationic CPP, TAT is rich in arginine residues whose interaction with the negatively charged polysaccharides and lipids on cell membrane is a critical step before internalization occurs. Studies have shown that truncation or alanine substitution of any charged residue in the basic region of TAT peptide significantly reduce the rate of uptake [35]. The uptake efficiency is attributed to guanidinium head group of the arginine side chain rather than to the positive charge alone since substitution of lysine residue with arginine increased the rate of uptake [36,37]. TAT has shown advantages over many other CPPs in delivering of both small and large cargos across cell membrane, including but not limited to doxorubicin [38], anti-rpoA peptide nucleic acid [39] and α-crystallin [40]. In this study, TAT also demonstrated the best ability to translocate

Table 2
Growth-promoting activity of the four fusion proteins and rhEGF.

Concentration (ng/ml)	Cell viability compared with control (%)				
	EGF-AA3H	EGF-MAP	EGF-TAT	EGF-Pep-1	rhEGF
1	118.3 ± 3.7	115.2 ± 1.9	103.9 ± 2.4	107.8 ± 4.4	112.5 ± 5.9
5	121.7 ± 2.8	128.1 ± 3.4	119.6 ± 4.0	129.4 ± 2.2	123.3 ± 4.3
10	143.3 ± 6.1	140.5 ± 4.6	131.7 ± 3.8	132.1 ± 4.7	138.0 ± 5.1

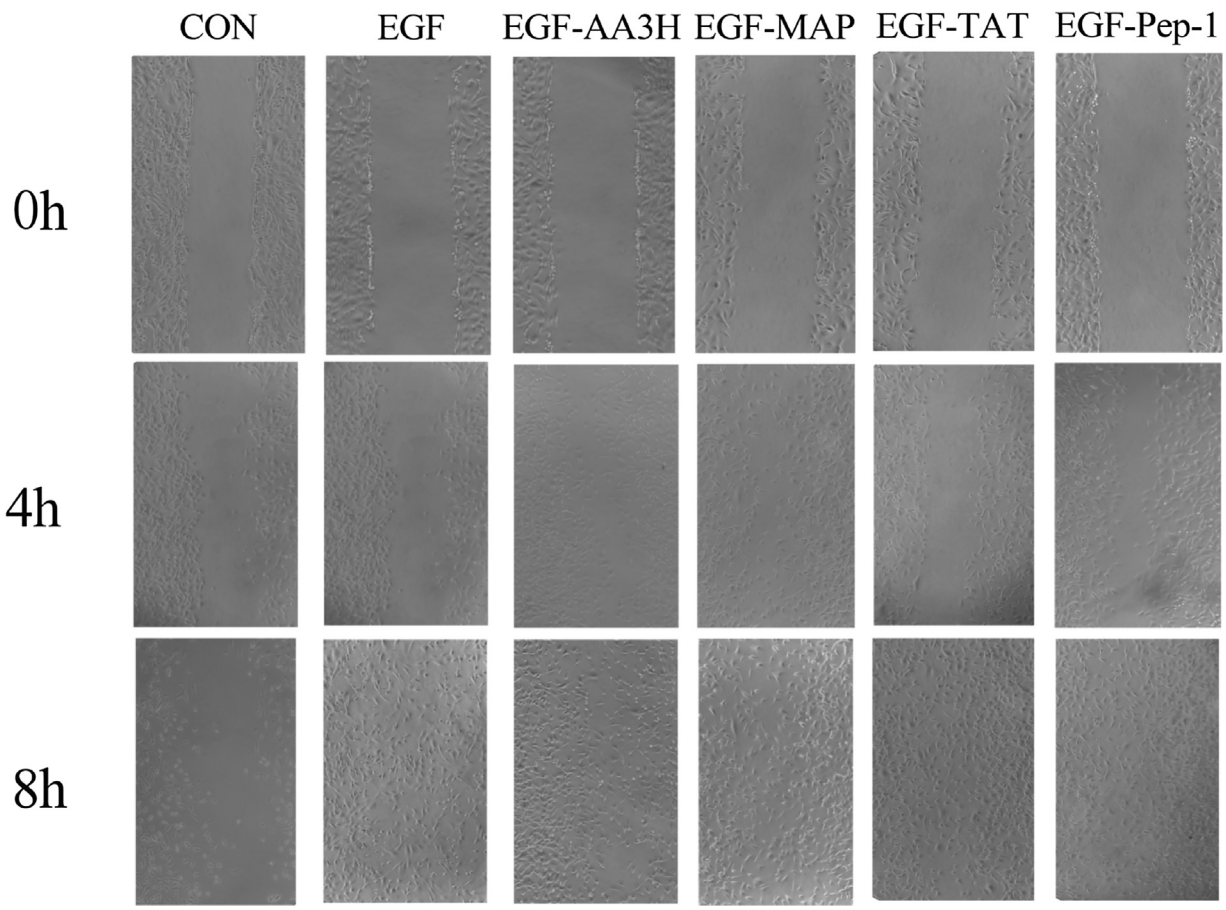

Fig. 4. The effect of four fusion proteins and EGF on the migration of BALA/c 3T3 cells. Cells were cultured to 100% confluent in 6-well plate, and a scrape wound was made across the culture dish. Maintenance solution containing 5 ng/ml EGF or the fusion protein was added. Cells without any treatment were used as the control (CON). Photos of wound closure were taken at 4 and 8 h under microscope.

Table 3
Cytotoxicity of the four fusion proteins.

Concentration (μM)	Cell viability compared with control (%)				
	EGF-AA3H	EGF-MAP	EGF-TAT	EGF-Pep-1	rhEGF
1.5	98.4 ± 2.2	99.1 ± 3.1	104.4 ± 3.9	100.8 ± 3.7	94.3 ± 2.6
3	102.7 ± 3.8	96.8 ± 2.3	101.0 ± 5.0	98.1 ± 4.4	98.9 ± 3.5
6	97.6 ± 3.3	90.4 ± 2.6	97.6 ± 3.6	99.4 ± 2.1	103.7 ± 2.9
12	104.9 ± 2.9	78.3 ± 4.5	99.7 ± 2.5	105.7 ± 3.2	96.2 ± 2.8
24	93.2 ± 5.3	36.1 ± 5.1	102.8 ± 2.9	103.5 ± 2.4	100.1 ± 3.1
48	90.1 ± 4.6	22.4 ± 3.5	96.9 ± 2.8	97.4 ± 4.1	106.0 ± 4.0

EGF across cell membrane. The delivery was much more efficient than other CPPs at lower concentrations.

Pep-1 is a primary amphipathic CPP containing the nuclear localization sequence PKKRKV and a hydrophobic tryptophan-rich cluster (KETWWETWWTEW). MAP is a secondary amphipathic a-helical CPP that has a highly hydrophobic patch on one face and a cationic patch on the other face. Although most amphipathic CPPs are cationic, evidence suggests that membrane translocation is a consequence of amphiphilicity and not of positive charges [41].When lysines in MAP are substituted with other polar residues to ensure the amphipathicity is conserved, the neutral MAP17 [42] and anionic MAP12 [43] are still cell-penetrating. Besides forming electrostatic interactions with negatively charged groups on cell membrane, strong interaction between hydrophobic residues and the hydrophobic part of cell membrane such as membrane lipids also contribute to cell penetration. NMR, circular dichroism, and FTIR analysis have revealed that the interaction of Pep-1 with phospholipids mediates their folding into a-helix, leading to pore-like

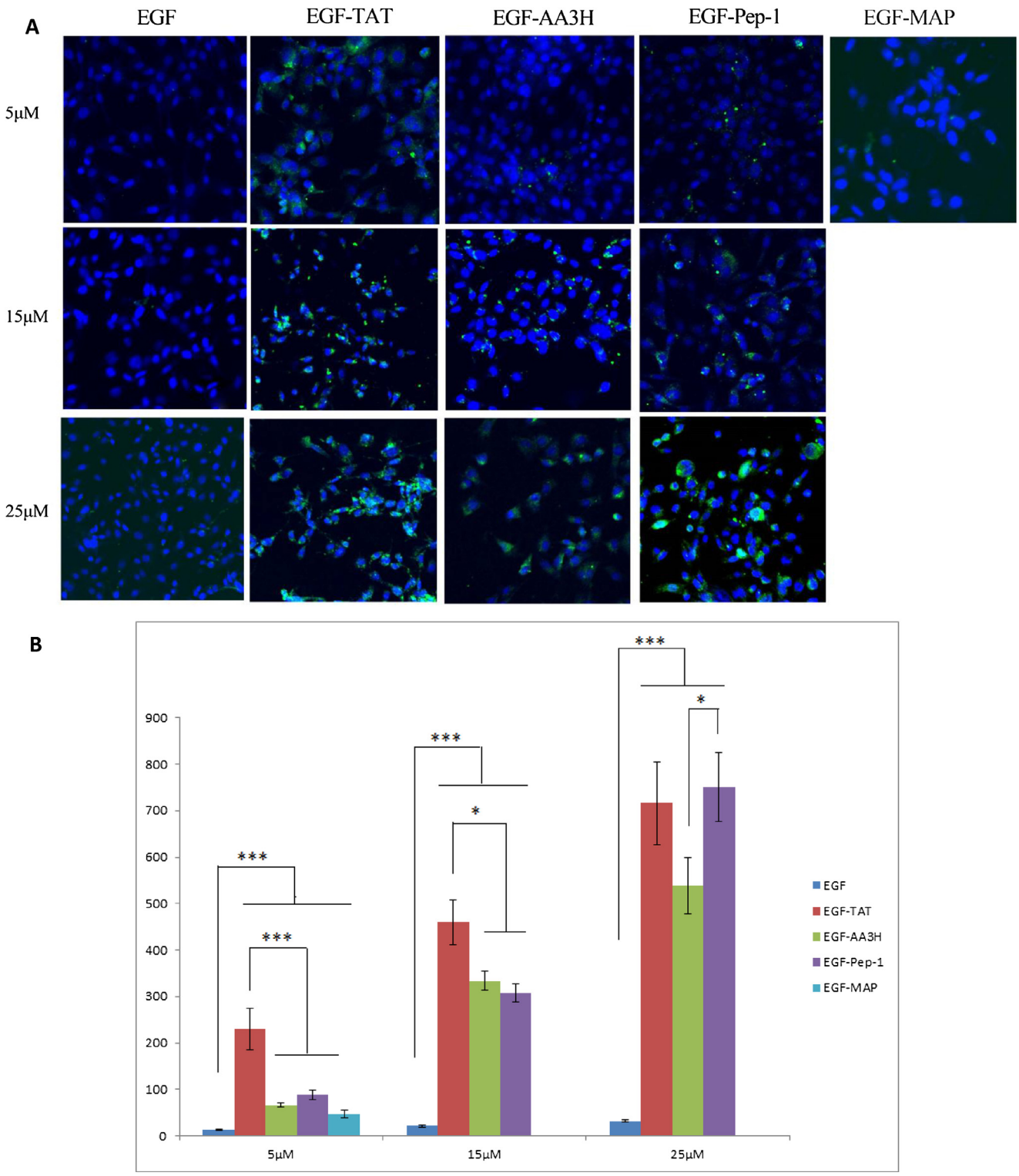

Fig. 5. Cellular uptake and mean fluorescence intensity of FITC-labeled EGF, EGF-AA3H, EGF-MAP, EGF-TAT and EGF-Pep-1 incubated with BALA/c 3T3 cells. (A) After incubation for 3 h, cells were washed three times with PBS, and then fixed with 4% paraformaldehyde. The blue color represents nuclei stained with DAPI, while green represents FITC-labeled recombinant proteins. (B) Mean fluorescence intensity was analyzed by Image-Pro Plus (mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure formation in cell membrane [44]. In addition, direct penetration is most likely to occur for primary amphipathic CPPs at high peptide concentrations [45]. This may account for the comparable translocation of EGF-Pep-1 at 25 μM with EGF-TAT although less translocation was detected at 15 and 5 μM.

Compared with cationic and amphipathic peptides, only a few hydrophobic CPPs have been discovered. AA3H is derived from annexin family and rich in hydrophobic residues. Despite the presence of only one arginine in its sequence, AA3H still appeared to have electrostatic interaction with heparan sulfate proteoglycan during the initial step of penetration. In addition, interaction with

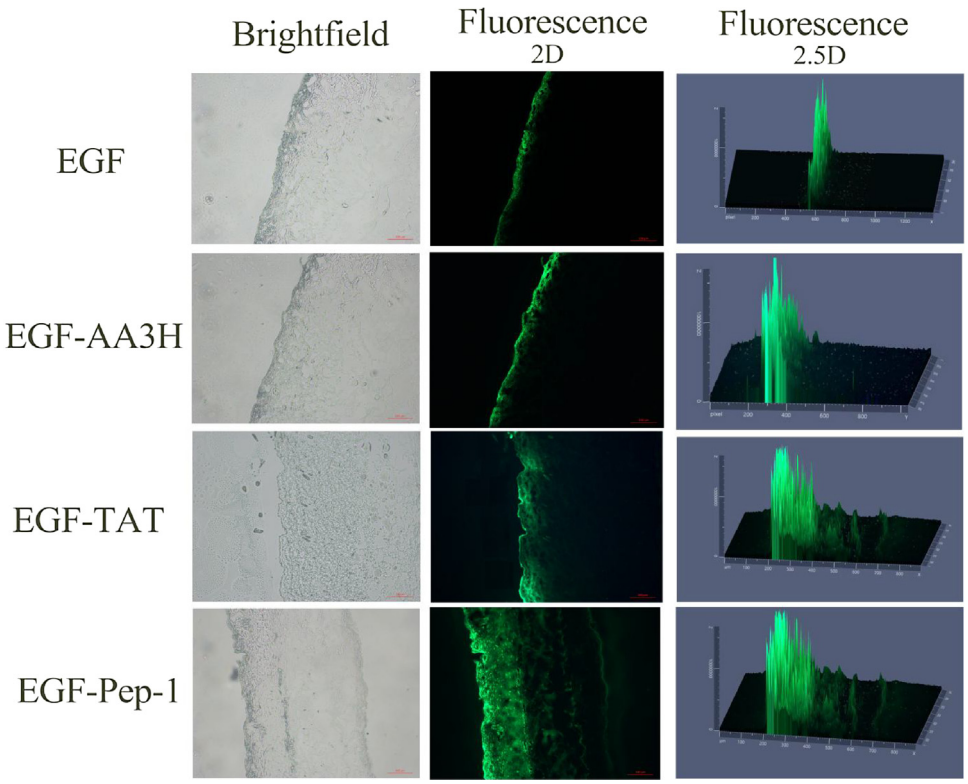

Fig. 6. Bright field and fluorescence microscopic images (2D and 2.5D) of mouse skin after 12-h permeation of 25 μM FITC-labeled EGF, EGF-AA3H, EGF-TAT and EGF-Pep-1.

phospholipid also contributed to the cell-penetrating property [25]. It is suggested that the entry of CPP complex into the cell could be influenced by variety of factors, including the nature ofthe cargo (such as size and charge), the properties of CPP, the cell line, and the CPP concentration. As a result, the delivery efficiency of CPP is case-dependent, and thus it is not surprise to find that AA3H which could deliverβ-galactosidase into fibroblast cells with a 6-fold higher efficiency than TAT lost its advantage in our case of EGF delivery.

3.6. Delivery of EGF into skin

The four fusion proteins as well as rhEGF were labeled by FITC so that fluorescence could be observed to monitor the penetration into skin. In Fig. 6, 8 μm sections of mouse skin after 12 h permeation by 15 μM FITC-EGF-CPP were photographed by fluorescencemicroscope. FITC labeled EGF was used as the control. Without covalent binding with any CPP, very small amount of EGF permeated into the skin and mainly accumulated in SC (Fig. 6). When fused with CPP by recombinant expression, EGF was delivered across the SC layer and into the viable epidermis and dermis. The most excellent delivering ability was observed with Pep-1, followed by TAT and AA3H. Although TAT and Pep-1 were comparable in delivering EGF across cell membrane at high concentrations, the latter is more potent in delivery across skin.

SC is the outermost layer of skin and is comprised of a 10–15 μm thick matrix of dehydrated and dead keratinocytes that are embedded in highly ordered lipidlayers.The SC serves as a cover to protect our body from the entry of external materials into the skin. The underlying epidermis is approximately 100–150 μm thick and composed of multiple layers of keratinocytes and fibroblasts which are target cells of EGF and whose proliferation and migration to resurface the wound are critical for wound healing. However, large molecular weight and the hydrophilic nature limited the skin permeation of EGF. Thus, it is difficult to maintain high concentrations of EGF in the epidermal and dermis region around the wound area. Previous study has shown that EGF penetrated only slightly into the upper granulating layers of full-thickness excisional wounds in rats; applications of generous amounts of growth factors will be required before they may penetrate into deeper layers of wounds [46]. In the present study, significantly increased skin permeability of EGF is achieved by coupling to CPP especially to Pep-1. This enhanced dermal delivery of EGF is expected to maximize its wound healing effect and reduce the cost of treatment.

Due to the importance in treating skin diseases, topical vaccination and in cosmetics to improve skin conditions, more and more attention has been focused on enhanced topical delivery by using CPPs. Rothbard *et al.* (2000) were first to report the application of CPP for the delivery of peptides into the skin [47]. Afterwards, antioxidant enzymes [48], antigenic peptide (OVA257–264) [49], P20 (Heat shock protein 20) [50] and several other proteins/peptides have been delivered into skin by using CPPs. However, the exact mechanism of translocation is still not defined. The routes for topical delivery of CPP/cargo complexes include transcellular, appendage and intercellular (extracellular matrix) routes [51]. It is reasonable to predict that mechanisms for skin penetration of CPPs are different from those for cell membrane crossing, which is supported by the inconsistence between results of cell permeation assay and skin permeation assay in this study. Historically, two major cellular uptake mechanisms, non-endocytotic/transduction pathways and the endocytotic pathways, have been proposed for cell penetration. Although endocytosis is believed to be the major mechanism for CPP/cargo complexes to be internalized by live cells, it is unlikely to occur in those metabolically active but non-viable cells of SC layer. As a result, the non-endocytotic pathway may take a lead. Some elaborate models have been proposed for this mechanism, including inverted micelle formation, pore-like structure formation, the carpet-like model and the membrane thinning model. It follows a 3-step process of internalization, namely, membrane interaction, membrane permeation

and release into the cytosol. Parameters such as charge, primary sequence, length, linearity and chirality of the peptidescan influence the translocation efficiency of CPPs. The intercellular route for topical delivery of CPP/cargo complexes should never be neglected. The intercellular lipid domain of the stratum corneum differs from cell membrane not only in lipid composition, but also in water content and lipid/protein ration [52].The interaction of CPPs with SC lipids may destabilize SC, thus increasing the membrane permeability. CPPs such as poly-arginine were reported to increase the permeability of mucosal tight junction [53]. A similar effect may also exit on the tight junctions in skin. The disruption of these structures by CPPs might be important for successful penetration of peptides into the viable layers of skin.

4. Conclusions

A comparative study was conducted to evaluate the abilities of different structural classes of cell penetrating peptide to deliver epidermal growth factor across cell membrane and the skin. The CPP moieties in the fusion proteins didn't cause significant loss of EGF activity, as illustrated by the activity to promote cell growth and *in vitro* "wound closure" in MTT assay. Although EGF-AA3H, EGF-TAT and EGF-Pep-1 didn't show obvious cytotoxicity, EGF-MAP dramatically decreased cell viability at moderate and high concentrations and was thus not subjected to further investigation. At 25 μM, cellular uptake of EGF-TAT and EGF-Pep-1 was comparable although less translocation was detected for EGF-Pep-1 at 15 and 5 μM. Permeation across the excised mouse skin followed the order of EGF- Pep-1 > EGF-TAT > EGF-AA3H > rhEGF. Altogether, these findings suggested that CPPs of different structural types showed very different abilities to translocate EGF across cell membrane and the skin. Coupling EGF with a well-chosen CPP was expected to increase its drug efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijbiomac.2017.07.041.

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