Construction of Hydrocarbon Stapled Cell Penetrating Peptides for in vitro and in vivo Delivery of siRNAs

Soonsil Hyun,[a] Yoonhwa Choi,[b] Ha Neul Lee,[c] Changki Lee,[c] Donghoon Oh,[c] Dong-Ki Lee,[d] Changjin Lee,*[c] Yan Lee,*[e] and Jaehoon Yu*[ab]

[a]Institute of Molecular Biology and Genetics, Seoul National University, Seoul 08826, [b]Department of Chemistry & Education, Seoul National University, Seoul 08826, [c]Hugel, Inc., Chuncheon 24206, Korea, [d]Department of Chemistry, Sungkyunkwan University, Suwon 16419, Korea, [e]Department of Chemistry, Seoul National University, Seoul 08826,

Corresponding authors:

Jaehoon Yu

E-mail address: jhoonyu@snu.ac.kr

Yan Lee

E-mail address: gacn@snu.ac.kr

Changjin Lee

E-mail address: senovia@hugel.co.kr

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EXPERIMENTAL SECTION

Peptide synthesis. All peptides were synthesized by using the 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) method using rink amide MBHA resin (Merckmillipore) on SPS Microwave Peptide Synthesizer (Discover, CEM) as previously described.[1] Fmoc protected monomeric amino acids were purchased from Merckmillipore. 5-Carboxytetramethylrhodamine (5-TAMRA, Merckmillipore) fluorescence dye was conjugated to the *N*-terminal amino group using *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) activation. Hydrocarbon stapled peptides were synthesized following the previously published protocol.[2] Briefly, unnatural amino acids, (R)-2-(7′octenyl)alanine (R_8, Aldrich) and (S)-2-(4′pentenyl)-alanine (S_5, Aldrich), were introduced via standard Fmoc SPPS method. Olefin metathesis reaction was performed twice on solid support using 6 mM solution of Grubbs′ first-generation catalyst (Aldrich) in 1,2-dichloroethane (Aldrich). Unnatural aromatic amino acid containing peptides were synthesized using Fmoc amino acid derivatives, Fmoc β-(3-pyridyl)-Ala-OH (Aldrich) and N^{α}-Fmoc-N^{ε}-acridinyl Lysine.[3] HPLC chromatograms and MALDI-MS data are shown in Fig. S1 and Table S1.

Circular Dichroism. CD spectra of 20 μM of stapled peptide solutions in 10 mM sodium phosphate (pH 7.4) were measured as same as previously described.[4] Briefly, CD spectra were measured with a Chirascan plus circular dichroism detector (Applied Photophysics, Leatherhead, U.K.). Peptide solutions were transferred to a precision cell (Quartz Suprasil, 0.05 cm, Hellma analytics), and CD spectra were recorded from 190 to 260 nm with a bandwidth of 1.0 nm. The measurement was performed in triplicate, and the measured values were averaged. The graph of mean residue molar ellipticity was converted to the graph of Delta Epsilon, and analysis was performed using CDNN, version 2.1, authored by G. Böhm at the Institute for Biotechnology of Martin Luther University (Halle-Wittenberg, Germany). The α-helical content of peptides was determined by the calculated data in the range of 190−260 nm.

Cell culture. The human cervical cancer cell line HeLa (ATCC) were cultured in Dulbecco′s modified Eagle′s medium (DMEM) and high glucose (HyClone, Thermo Fisher Scientific)

supplemented with 10% (v/v) of fetal bovine serum (FBS) (HyClone, Thermo Fisher Scientific), 100 IU/mL penicillin, 100 μg/mL streptomycin (HyClone, Thermo Fisher Scientific) at 37 °C in presence of 5% CO_2.

Flow cytometry analysis. HeLa cells were seeded in 24-well plates at a density of 5×10^4 cells per well in DMEM containing 10% FBS. The experimental procedure used followed previously published methods.[5] Briefly, cells were incubated with 5-TAMRA labeled each sample in fresh complete growth medium for 24 h. The suspended cells detached by trypsin treatment were analyzed by using a flow cytometer (BD Accuri C6, BD Biosciences). Cellular uptake of peptides was determined by percentage of fluorescence positive cells treated with the TAMRA-labeled peptides. For mechanistic study, HeLa cells were pre-incubated with various endocytosis inhibitors (10 mM NaN_3 and 6 mM 2-deoxy-D-glucose in DMEM w/o glucose (Sigma) for ATP depletion, 15 μg/mL 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) to inhibit macropinocytosis, 5 mM methyl-β-cyclodextrin (MβCD) for lipid-raft-mediated endocytosis, and 80 mM sodium chlorate ($NaClO_3$) for disrupting proteoglycan synthesis in OptiMEM (Gibco)) for 30 min, and incubated further with final 100 nM of 5-TAMRA-labeled stEK or 100 nM of stEK complex with 50 nM siGLO Cyclophilin B Control siRNA (GE Healthcare Life Science, D-001610-01) for 2 h. All inhibitors are obtained from Sigma.

Confocal fluorescence microscopy. HeLa cells were seeded in 8 well chambered coverglasses (Thermo Fisher Scientific) at a density of 1.5×10^4 cells per well in DMEM containing 10% FBS. After 24 h at 37 °C, the cells were transfected with the complex of each TAMRA-labeled siRNA and the peptides. To prepare the complex of siRNA and CPP, the mixture of 2.5 μM of siGLO and designated molar excess of peptides were pre-incubated for 30 min at RT and diluted with OptiMEM (Gibco) media. The final concentrations of siGLO and CPP are shown in the text. The cells transfected with the complex of siRNA and CPP were incubated at 37 °C for 24 h. A Hoechst 33342 dye solution (Thermo Fisher Scientific) was added to the cells at a final concentration of 1 μg/mL and the cells were incubated for 10 min. After PBS washing, the cells were subjected to the analysis by using confocal fluorescence microscopy. Images

were acquired using LSM 700 laser scanning confocal microscope (Carl Zeiss, Germany) with a 40 × objective.

Transfection of siRNAs. HeLa cells were seeded in 24 well plates at a density of 5×10^4 cells per well in DMEM containing 10% FBS. Transfection, using a commercially available transfecting reagent, DharmaFECT™ 1, was performed using the protocol provided by the manufacturer. Ten nM of siRNA transfection using DharmaFECT™ 1 (final concentration is 0.4 μL/well) was used as a positive control. Otherwise, the peptide/siRNA complexes were added to the cells and incubated in a 37 °C incubator for 24 h as described above. SiGenome™ Cyclophilin B Control siRNA (GE Healthcare Life Science, D-001136-01-05), Bcl-2 siRNA (Santa Cruz Biotechnology, sc61899), LIN-28 siRNA (Santa Cruz Biotechnology, sc106829), GAPDH siRNA (ThermoFisher Scientific, 4390849), VEGF siRNA (Sigma NM_001025366, siRNA ID SASI_Hs01_0020117) were used as siRNAs targeting each gene.

Quantitative real-time reverse transcription-PCR. Total RNAs were extracted with TRIzol reagent (Life Technologies) according to the manufacturer′s instructions. One microgram of RNA was reverse transcribed with Superscripts III (Life Technologies) using OligodT primer and RNA was removed using RNase H (NEB). qRT-PCR was performed on a Rotor-Gene (Bio-Rad, USA) with gene-specific primers (Table S2) and Rotor-Gene SYBR® Green PCR kit (Qiagen). The relative amounts of mRNA were calculated by using the comparative C_T method [6]. GAPDH was used for normalization. For animal skin samples, RNA was transcribed using TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems) and Oligo dT primer (Invitrogen). Obtained cDNAs were subjected to qRT-PCR using TaqMan® Gene Expression Assay kit (Applied Biosystems) for CTGF and GAPDH.

Western blot analysis. Whole-cell Lysates of transfected HeLa cells were subjected to fractionation on a 15% SDS-PAGE and transferred to PVDF membrane (Millipore). After blocking for 30 min at room temperature with 5% Bovine Serum Albumin (BSA) in PBS containing 0.1 % Tween 20, blots were incubated overnight incubation at 4°C with mouse monoclonal anti-CypB (sc130626, Santa Cruz Biotechnology, 1:2000 diluted in PBS containing 0.1% Tween 20 and 5% BSA) as primary antibody followed by horseradish

peroxidase-linked goat anti-mouse secondary antibody (sc-2055, Santa Cruz Biotechnology, 1:5000 in PBS containing 0.1% Tween 20 and 1% BSA) for 1 h at room temperature. Beta-actin was used as a loading control. For beta actin control, blots were incubated for 1 h at room temperature with mouse monoclonal anti-beta actin (#3700, Cell Signaling, 1:5000 diluted in PBS containing 0.1% Tween 20 and 5% BSA,) as primary antibody. The immunoreactive proteins were detected by Luminata^{TM} crescendo western HRP substrate (Millipore), using X-ray film exposure.

Cytotoxicity studies. Cytotoxicities were determined using the colorimetric reagent, WST-1 (EZ-CYTOX, Dogen). Briefly, cells were seeded on 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h. Medium was replaced with fresh medium containing each peptide and incubated for an additional 24 h. The absorbance was measured using a plate reader at 450 nm after 30 min incubation with the WST-1 reagent.

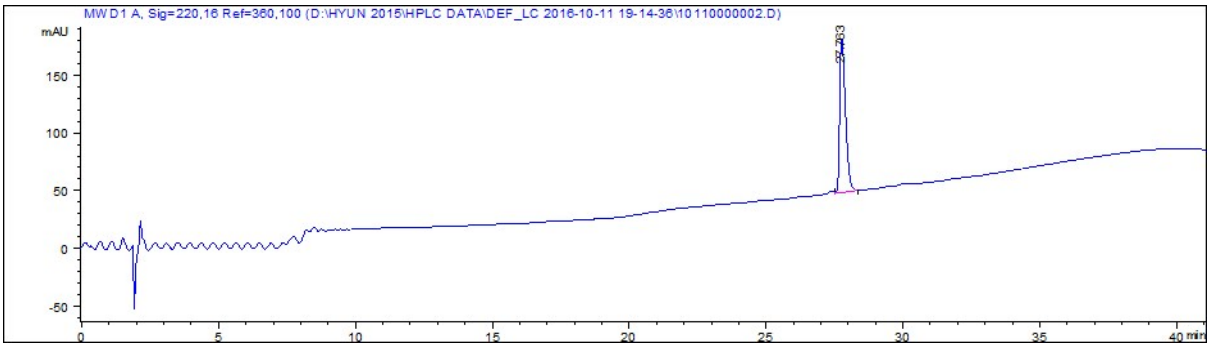
***In vivo* studies.** All animal studies were performed in compliance with the policies of the Hugel Animal Care and Use Committee. C57BL/6 mouse (female, 5 weeks old) were purchased from DBL (Chungcheongbuk-do, Korea). The mice were anaesthetized before asiCTGF treatment with 3 vol% of isoflurane (JW Pharmaceutical, Korea) funnel fill vaporizer (SurgiVet). A total dose of 10 nM or 100 nM siRNA dissolved in saline solution per injection was intradermally injected in a total volume of 40 μL containing 500 nM or 5 μM LKH-stEK at two points. For mRNA level analysis, injection site skin samples at 1 day and 5 day after treatment were obtained and cut into fragments. The total RNAs were extracted using TRIzol according to the manufacturer′s protocol. Briefly, skin samples were placed in 1 mL of TRIzol, then homogenized using a homogenizer. Two hundred microliters of chloroform was added and centrifuged at 12,000 rpm for 15 min at 4 °C to obtain the clarified sample supernatants. The total RNAs were EtOH precipitated and 1 μg of each RNA was subjected to RT-qPCR.

Immunohistochemistry and Masson′s trichrome staining. An immunohistochemeistry accessaory kit (Bethyl Laboratories, Inc.) was used and experiments were performed according to the manufacturer′s protocol. Briefly, after deparaffinization and rehydration, the slides were washed with PBS (1 min, twice). Then, the slide was incubated with anti-rabbit CTGF antibody

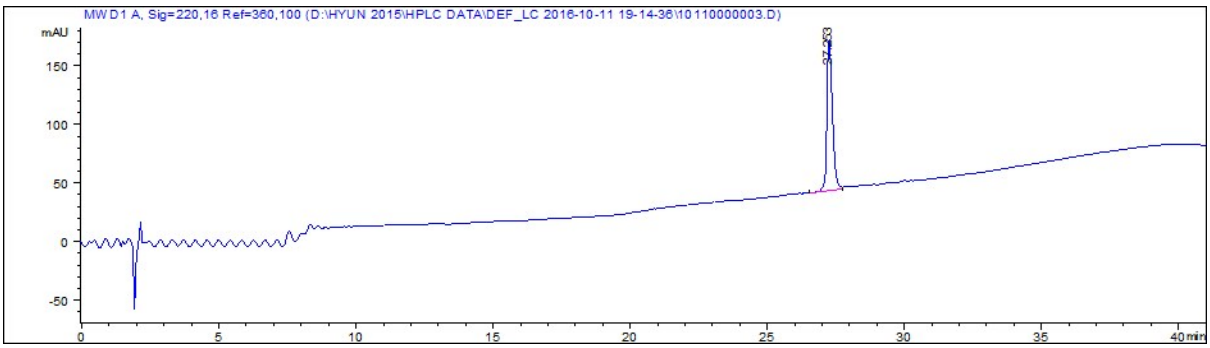
(NBP2-16025, Novus, 1:100 diliuted) overnight. Washing, incubation with Rabbit specific HRP/DAB (ABC) Detection IHC Kit (ab64261, Abeam), hematoxylin staining were performed. Masson′s trichrome staining was performed using Trichrome stain kit (ab150686, Abcam) according to the manufacturer′s protocol.

Figure S1. HPLC Chromatograms of the purified peptides. All peptides were confirmed with using RP- HPLC (Agilent HPLC 1100 series instrument). A Zorbax C18 column (3.5 μm, 4.6 × 150 mm) was used as the stationary phase. For the mobile phase, buffer A (water with 0.1% v/v TFA) and buffer B (acetonitrile with 0.1% v/v TFA) were used as a gradient. For stFK and 5-TAMRA-stFK, methanol with 0.1% v/v TFA was used as buffer B, due to the solubility. The gradient conditions are as follows: 5 min, 5% B followed by linear gradient 5-70% B over 25 min. The flow rate was 1.0 mL/min.

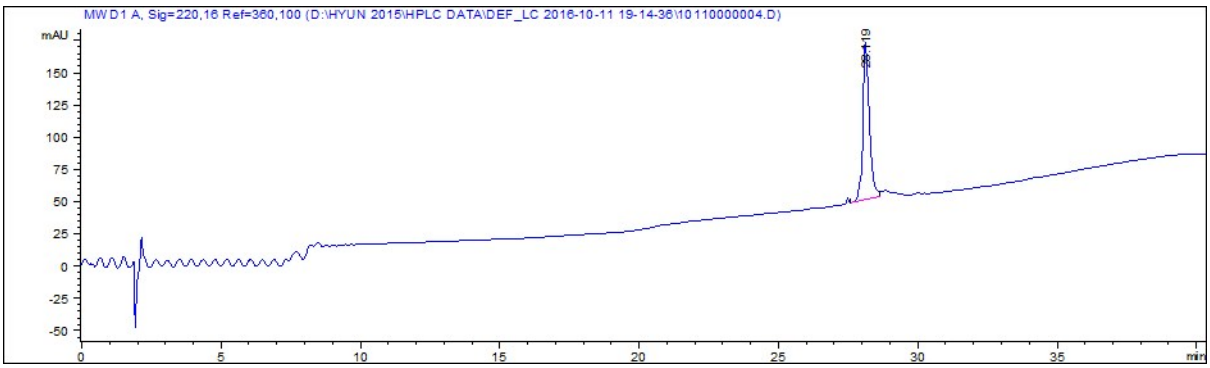
stAK (>95 % purity)



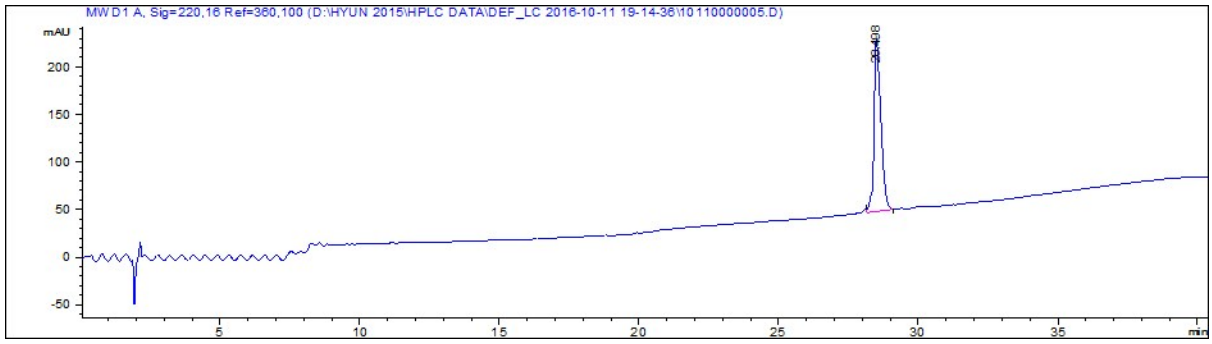
stBK (>95 % purity)



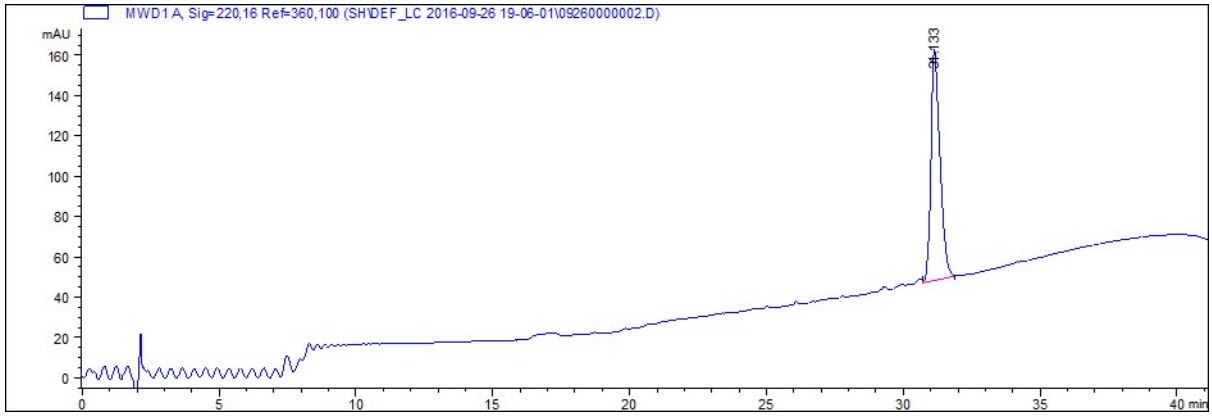
stCK (>95 % purity)



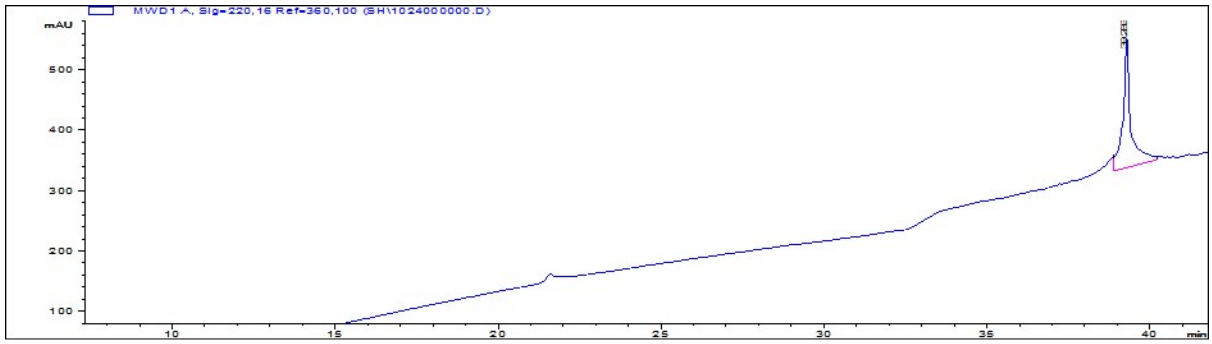
stDK (>95 % purity)



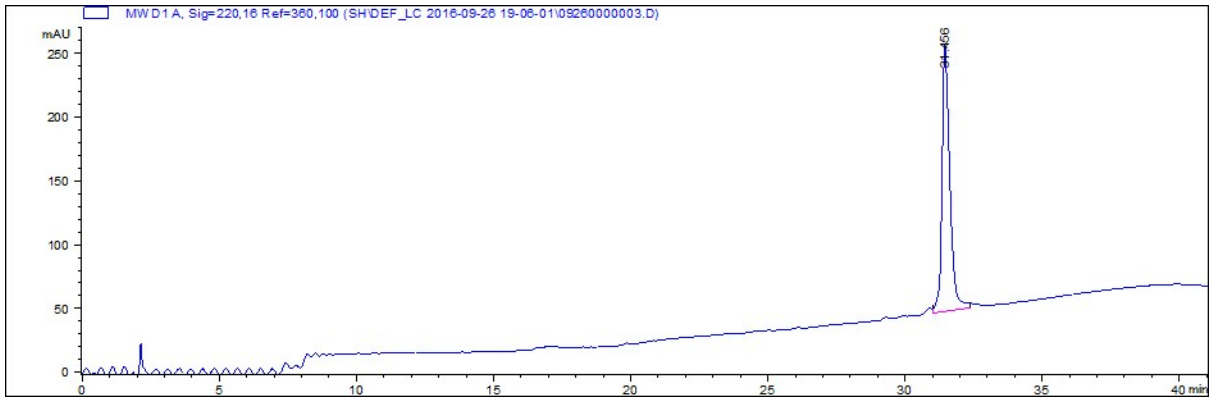
stEK (>95 % purity)



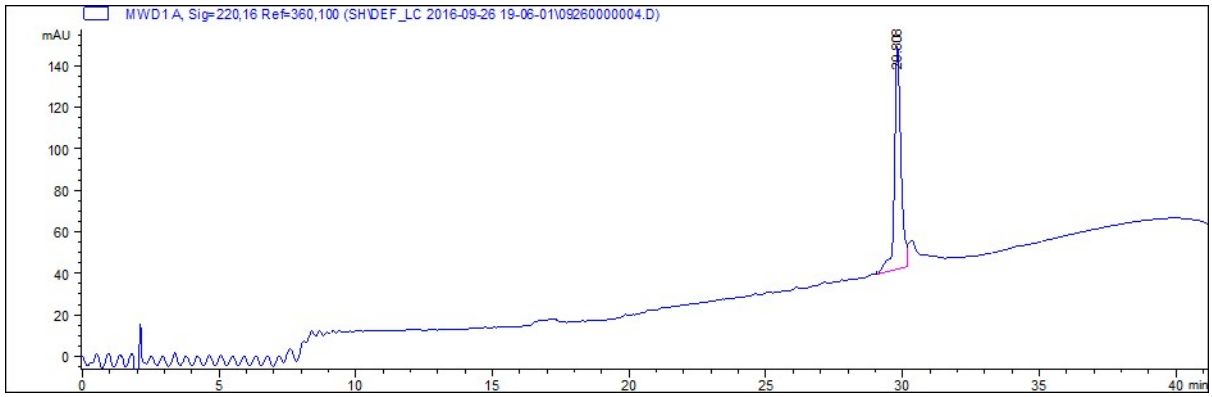
stFK (>95 % purity)



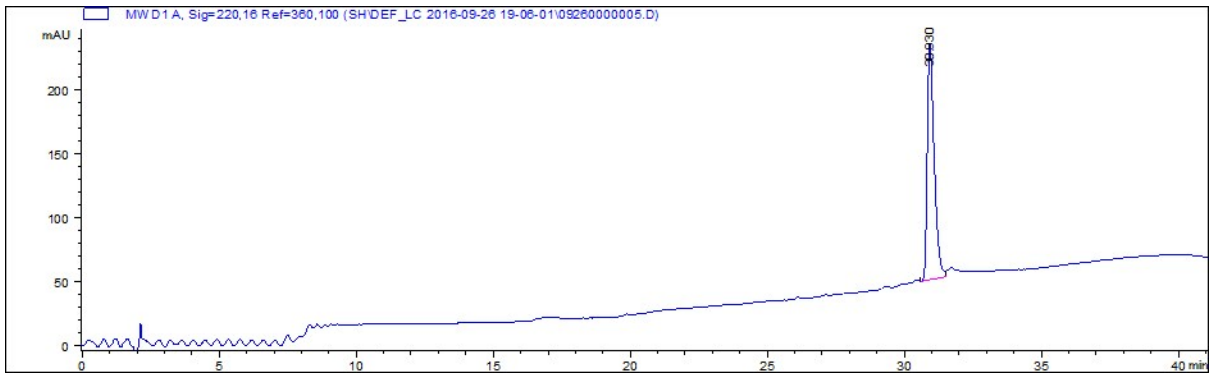
LKH-stEK (>95 % purity)



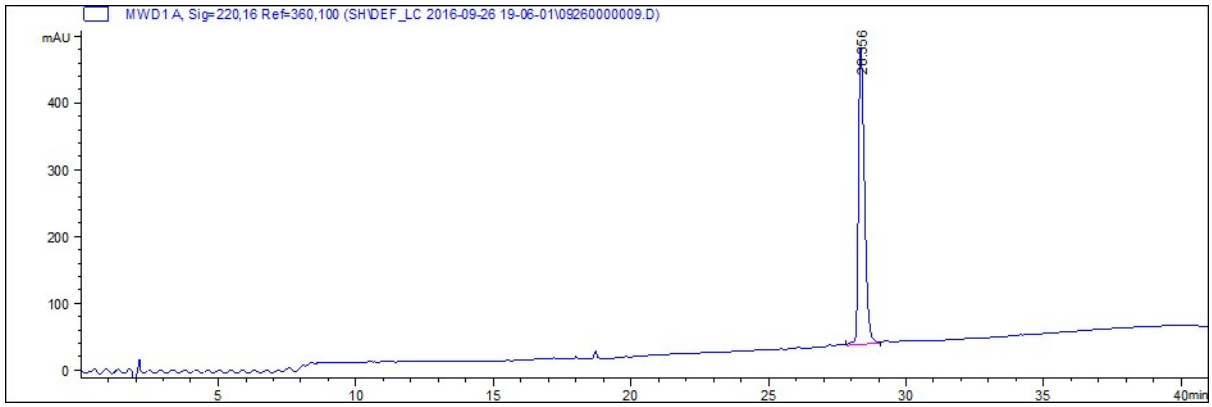
LKH-stEK-1 (>90 % purity)



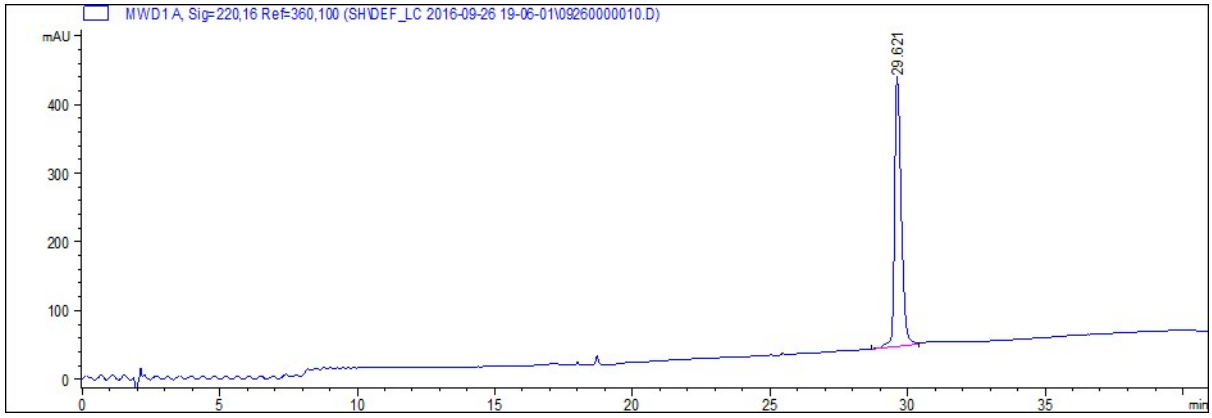
LKH-stEK-2(>95 % purity)



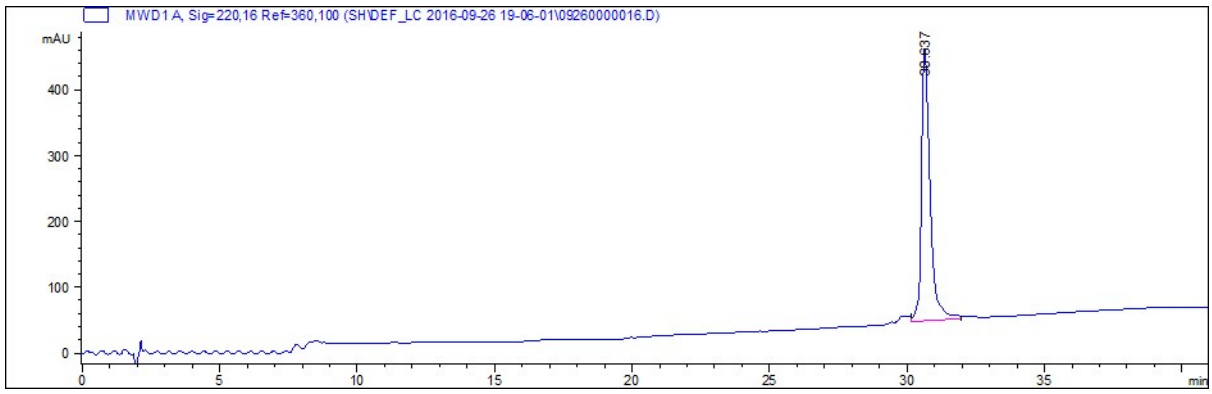
5-TAMRA-stAK (>95 % purity)



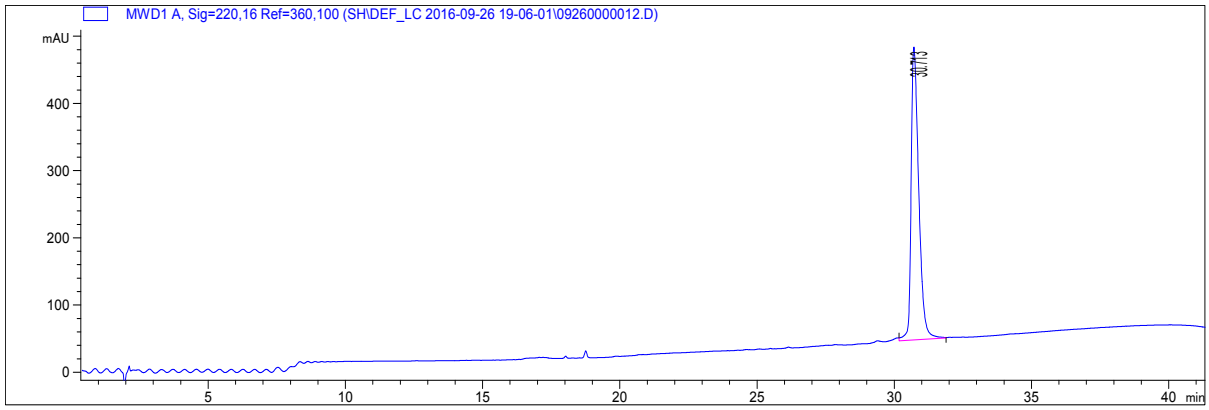
5-TAMRA-stBK (>95 % purity)



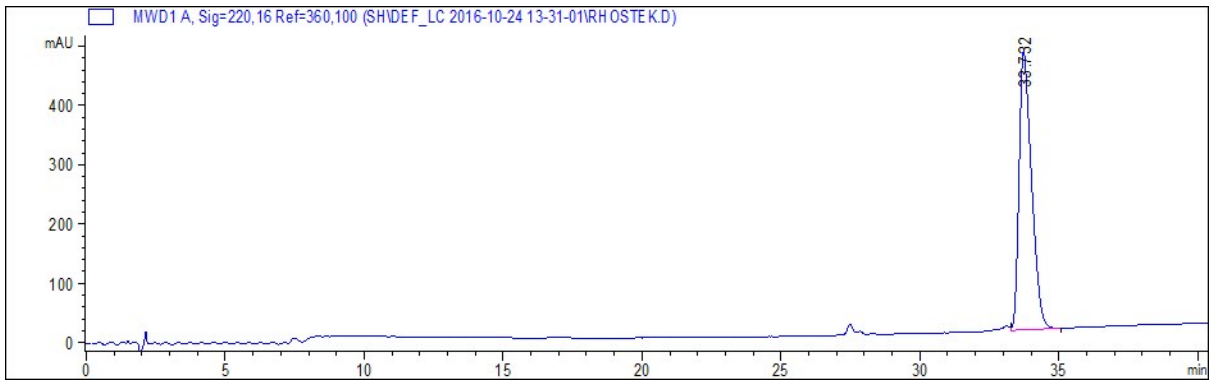
5-TAMRA-stCK (>95 % purity)



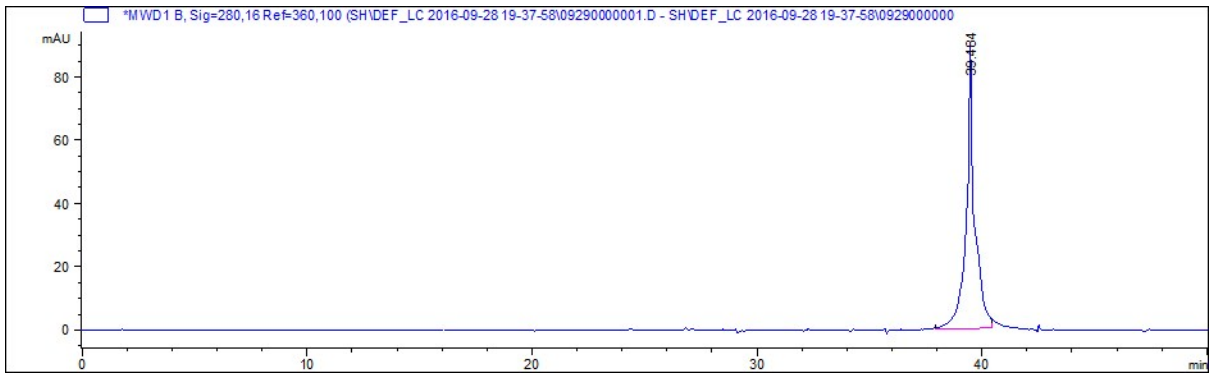
5-TAMRA-stDK (>95 % purity)



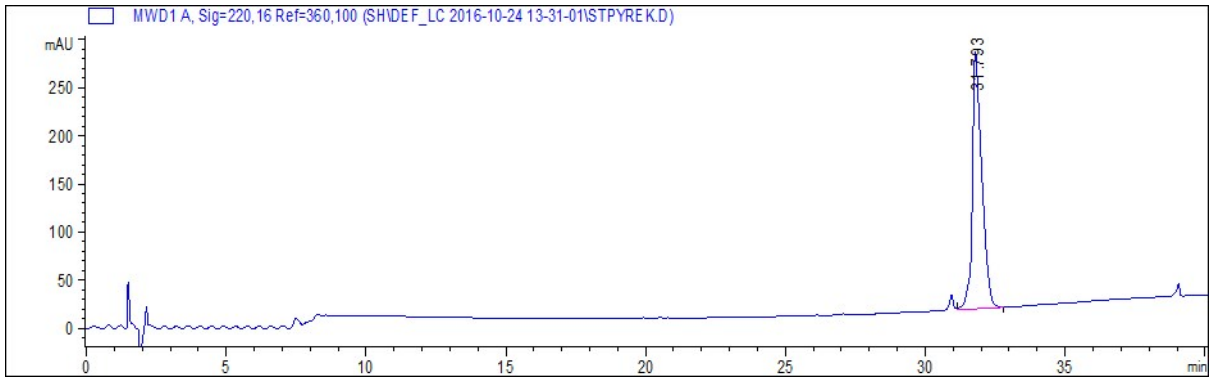
5-TAMRA-stEK (>90 % purity)



5-TAMRA-stFK (>90 % purity)



LKpyr-stEK (>95 % purity)



LKacr-stEK (>95 % purity)

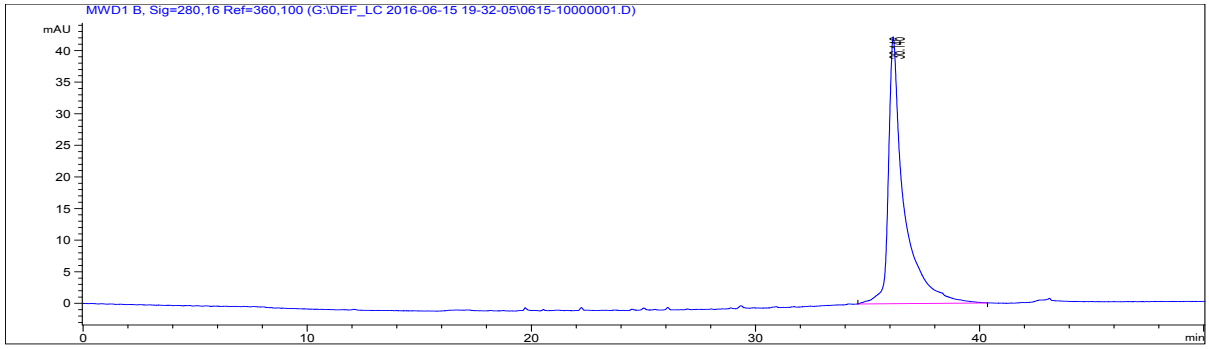


Figure S2. (a) Sequence and the schematic structure of LK-3. Two helices are connected by bis-disulfide bonds. (b) siRNA delivery efficiency of LK-3. Confocal laser scanning microscopy (CLSM) images of HeLa cells transfected with 50 nM of DY-547 labeled siGLO for 24 h using 100 nM of dimeric peptides. The fluorescence from DY-547 is colored red. Fluorescence images and differential interference contrast (DIC) images are overlaid (scale bar, 100 μm). (c) *In vitro* gene silencing efficiency of siRNA-dimeric peptide complexes in HeLa cells. Cells were incubated with 50 nM of siRNA and 500 nM of LK-3 for 24 h. DharmaFECT^{TM} 1 was used a positive (+) transfecting agent with 10 nM of siRNA (mean ± s.d., n = 4).

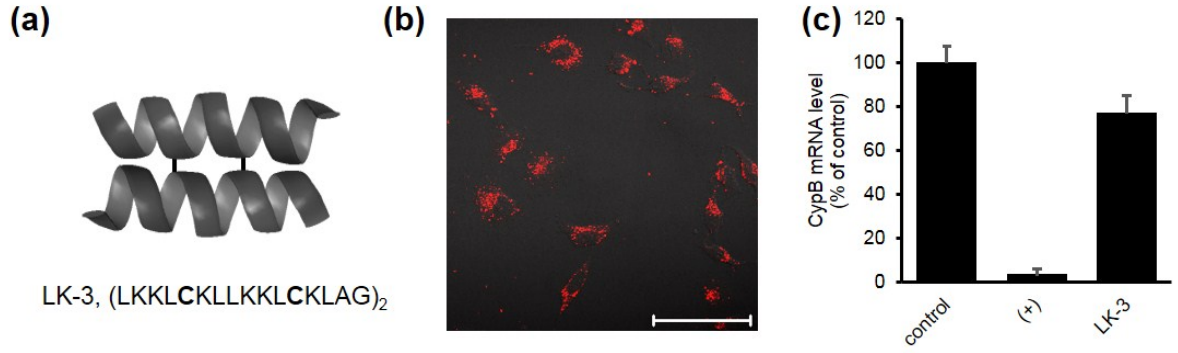


Figure S3. Helical wheel diagram of stapled peptides. Lowercase letters indicate positions in the heptad repeat (abcdefg), with hydrophobic residues at a, d, e, and g sites. Polar Lys, nonpolar Leu residues, alkene-bearing non-natural amino acids are shown in black, white, gray, and dot filled circles, respectively. Cationic hydrophilic face and hydrophobic face are marked as blue and pale blue.

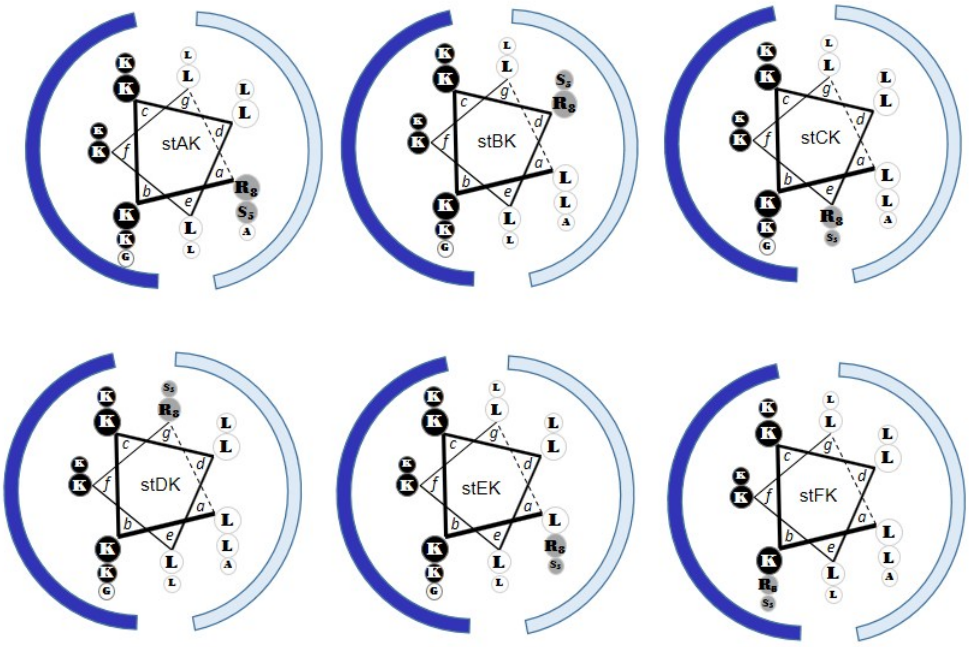


Figure S4. (a) Sequences of His containing stapled peptides. (b) Gel retardation assay of siRNA/His containing peptide derivatives complexes. The preformed siRNA/CPP complexes were analyzed by electrophoresis on 1% of agarose gel stained with ethidium bromide. SiRNA was pre-incubated with peptides for 30 min in 0.5 × PBS buffer. Relative complex formation is shown in a graph below (n = 4). (c) Relative mRNA expression of target gene transfected using siRNA/other stapled peptide complexes formed with 1:10 and 1:50 molar ratio (50 nM of siRNA, mean ± s.d., n = 4).

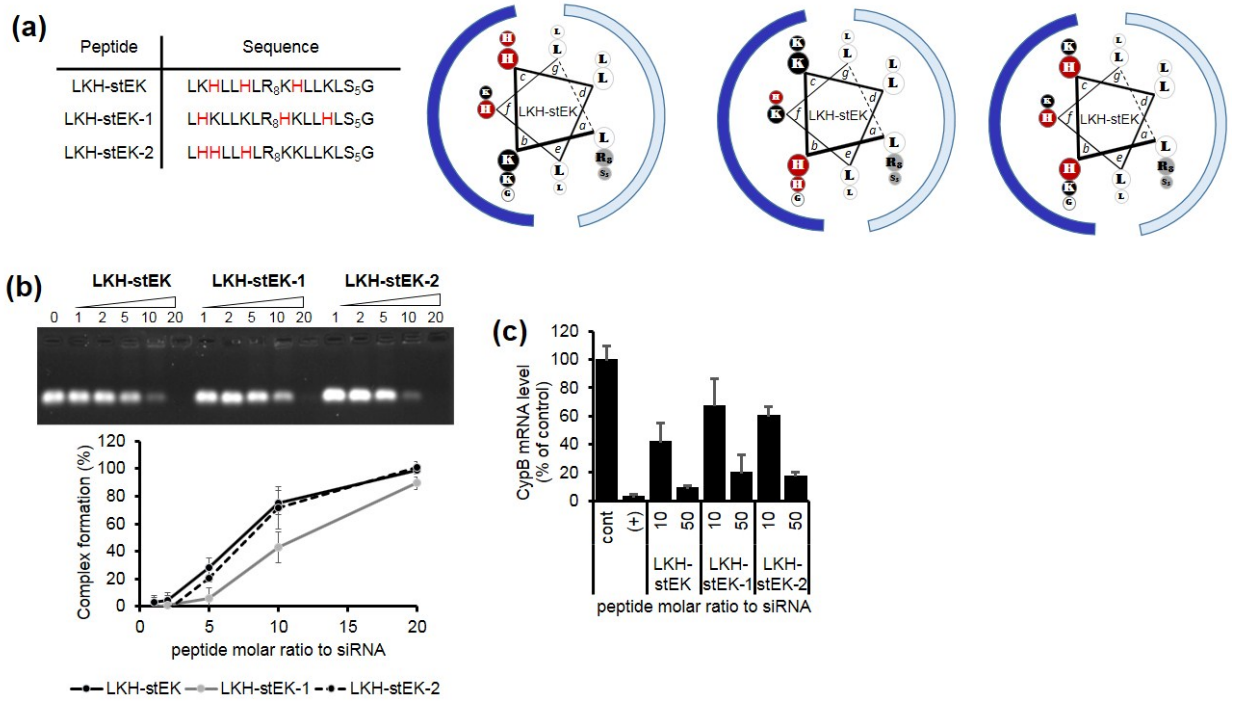


Figure S5. In vitro gene silencing efficiency of (a) siRNA/LKacr-stEK or (b) LKpyr-stEK complex as a function of molar ratio. HeLa cells were transfected using siRNA complexes formed with different molar ratios from 1:2 to 1:100 (50 nM of siRNA) and incubated for 24 h. (mean ± s.d., n = 3).

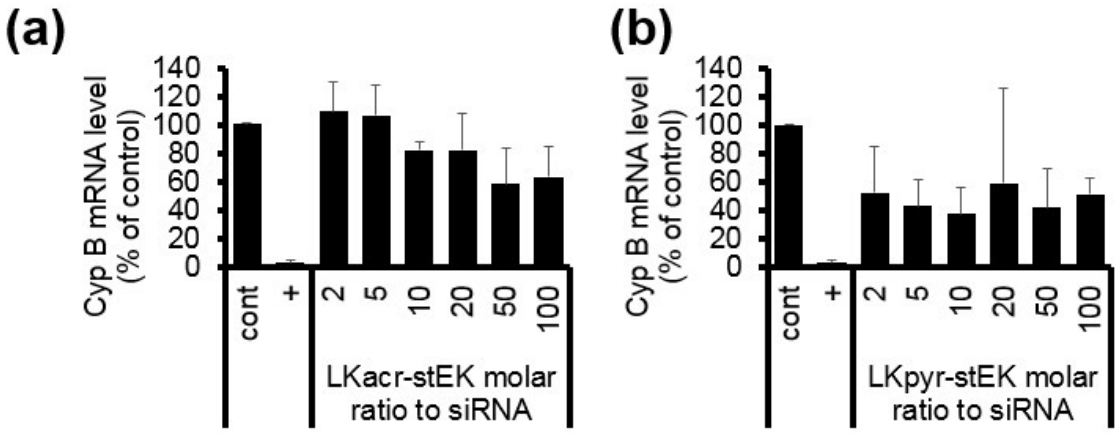


Figure S6. Cytotoxicity of LKH-stEK. HeLa cells were incubated with various concentrations of LKH-stEK for 24 h.

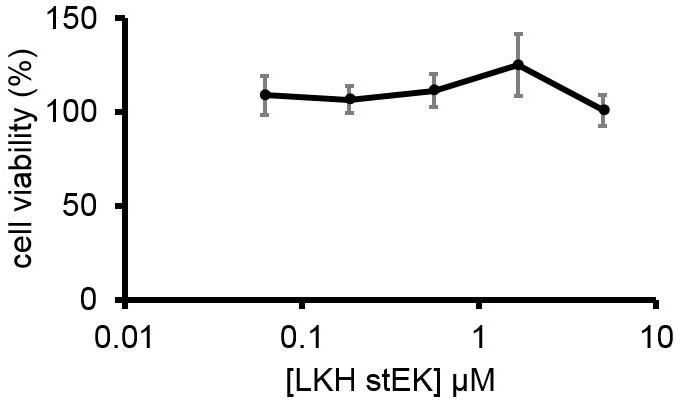


Figure S7. In vitro gene silencing efficiency of various siRNA/LKH-stEK. HeLa cells were transfected using siRNA complexes formed with a molar ratio 1:50 (50 nM of siRNA) and incubated for 24 h. (mean ± s.d., n = 4). Commercially available transfecting reagent, DharmaFECT™ 1 was used as positive controls.

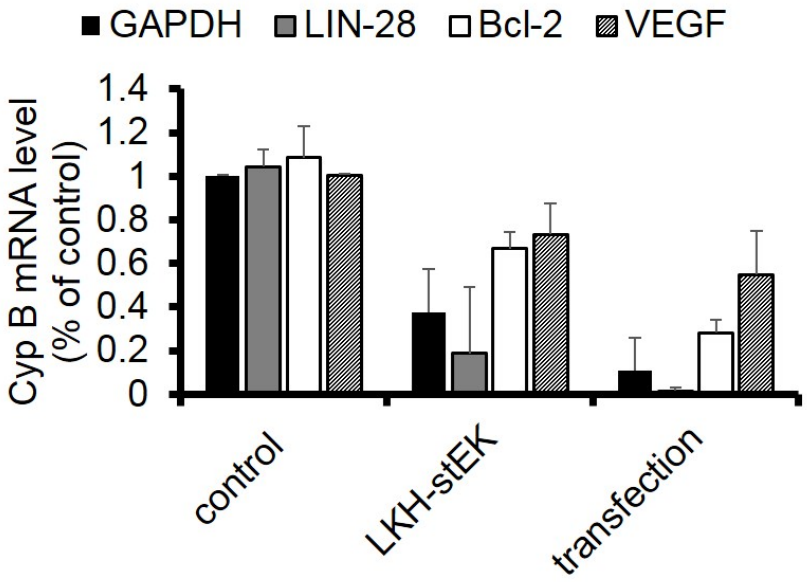


Table S1. Mass spectrometry data for the peptides.[a]

Peptide	equence	MS (calcd)	MS (obsd)
stAK	R_8KKLLKLS_5KKLLKLAG	1927.38 (2297.52)	1928.01 (2298.37)
stBK	LKKR_8LKLLKKS_5LKLAG	1927.38 (2297.52)	1928.37 (2298.42)
stCK	LKKLR_8KLLKKLS_5KLAG	1927.38 (2297.52)	1928.46 (2298.33)
stDK	LKKLLKR_8LKKLLKS_5AG	1927.38 (2297.52)	1928.18 (2298.36)
stEK	LKKLLKLR_8KKLLKLS_5G	1969.43 (2339.56)	1970.15 (2340.42)
stFK	LKKLLKLLR_8KLLKLAS_5	1968.44 (2338.57)	1969.21 (2339.53)
LKH-stEK	LKHLLHLR_8KHLLKLS_5G	1996.32	1997.18
LKH-stEK-1	LHKLLKLR_8HKLLHLS_5G	1996.32	1996.79
LKH-stEK-2	LHHLLHLR_8KKLLKLS_5G	1996.32	1996.91
LKpyr-stEK	LKpyrLLpyrLR_8KpyrLLKLS_5G	2029.34	2029.63
LKacr-stEK	LKacrLLacrLR_8KacrLLKLS_5G	2500.61	2501.42

[a]All mass spectrometric data correspond to $[M+H]^+$ peaks. 5-TAMRA labeled peptides are shown in parentheses

Table S2. Primers used for real time PCR

Class	Sequence of peptide
Cyclophilin B forward	5'-GGTGATCTTTGGTCTCTTCGG-3'
Cyclophilin B reverse	5'-TAGATGCTCTTTCCTCCTGTG-3'
Bcl-2 forward	5'- CTTGGACAATCATGAAATATGCATC -3'
Bcl-2 reverse	5'- GCAGAACAACCTTGTTGTTGATAG -3'
LIN-28 forward	5'- AGCCCCTTGGATATTCCAGTC -3'
LIN-28 reverse	5'- AATGTGAATTCCACTGGTTCTCCT -3'
VEGF forward	5'- GAGCCGGGCAGGAGGAA -3'
VEGF reverse	5'- GGTGATGGTGTGGTGGCG -3'
GAPDH forward[a]	5'-TCGCTCTCTGCTCCTCCTGTTC-3'
GAPDH reverse[a]	5'-CGCCCAATACGACCAAATCC-3'
18S rRNA forward[b]	5'- CGGCGACGACCCATTCGAAC -3'
18S rRNA forward[b]	5'- GAATCGAACCCTGATTCCCCGTC -3'

[a]GAPDH is normally used as a control. [b]18S rRNA is used as a control for GAPDH expression.

References

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