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YAC tripeptide of epidermal growth factor promotes the proliferation of HaCaT keratinocytes through activation of EGFR

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Epidermal growth factor (EGF) is known to play key roles in skin regeneration and wound-healing. Here, we demonstrate that Pep2-YAC, a tripeptide covering residues 29-31 in the B loop of EGF, promotes the proliferation of HaCaT keratinocytes with activity comparable to EGF. The treatment of HaCaT cells with Pep2-YAC induced phosphorylation, internalization, and degradation of EGFR and organization of signaling complexes, which consist of Grb2, Gab1, SHP2, and PI3K. In addition, it stimulated the phosphorylation of ERK1/2 at Thr 202/Tyr 204 and of Akt1 at Ser 473 and the nuclear translocation of EGFR, STAT3, c-Jun, and c-Fos. These results suggest that Pep2-YAC may be useful as a therapeutic agent for skin regeneration and wound-healing as an EGFR agonist. [BMB Reports 2014; 47(10): 581-586]

INTRODUCTION

EGFR signaling plays key roles in skin regeneration and wound healing by stimulating epidermal keratinocyte proliferation (1). Additionally, EGFR is essential for epidermal and hair follicle homeostasis, and is important for the massive *ex vivo* expansion of human keratinocyte stem cells in patients with extensive burns and genetic disorders (2). EGFR is a 170-kDa transmembrane protein that belongs to ErbB family receptors, which includes three other members (ErbB2/HER-2, ErbB3/HER-3, and ErbB4/HER-4). Ligand binding to EGFR results in its dimerization and tyrosine autophosphorylation, and subsequent activation of several downstream pathways, such as the RAS-ERK, PI3K-Akt, and STAT3 pathways, which stimulate cell proliferation, survival, and migration (3, 4). Epidermal growth factor, a major ligand for the ErbB family, is a 53-residue polypeptide expressed in many tissues and body fluids of mammals. It has been shown to improve wound healing by increasing keratinocyte proliferation and the tensile strength of the healed dermis (5). All EGF family members contain one or more repeats of a conserved six-cysteine-containing motif (6). These six cysteine residues are contained within a sequence of 35-40 amino acids and have the potential to form three intra-molecular disulfide bond pairings to produce three loops that are essential for high-affinity binding to the EGFR receptor (7). The A loop contains a short alpha helix-like segment, while the B and C loops each contain a short antiparallel β sheet structures. Crystal structure analysis showed that the B loop of EGF interacts hydrophobically with domain I of EGFR (8). The B loop fragment of EGF produced mitogenic and angiogenic effects like its parent molecule (9).

Due to its mitogenic activity, EGF has been used as a therapeutic reagent for skin wound regeneration (10). However, poor skin penetration of macromolecules with a molecular weight of more than 500 Da limits the use of EGF in topical treatments for enhancing wound healing (11). Here, we demonstrate that a tripeptide derived from the EGF B-loop enhances keratinocyte proliferation through activation of EGFR. Using analyses of intracellular signal protein interaction, EGFR activation studies, and *in vitro* proliferation assays, we show that the EGF-derived peptide can activate EGFR signaling pathway in keratinocytes with activity comparable to its parent EGF protein.

RESULTS

Identification of an EGF-derived agonist peptide Pep2-YAC

Because previous studies showed that the B loop region of EGF plays an important role in the activation of the EGFR signaling pathway (6), we performed homologous sequence analysis of the B loop region to identify EGFR agonist peptides. Based on a protein sequence homology search, we synthesized three EGFR agonist peptide candidates with molecular weights below 500 Da to examine their agonistic effects on EGFR activation (Fig. 1A). First, we investigated whether the three peptide candidates induced a physical interaction between EGFR and Grb2 or Gab1 and SHP2. HaCaT keratino-

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cytes were treated with three EGF-derived peptide candidates for 15 min and then the physical interaction of the signaling molecules was examined by an *in situ* proximity ligation assay (PLA). EGF itself was used as a positive control. Among peptide candidates, Pep2-YAC, a tripeptide covering residues 29-31 of EGF, enhanced the association of EGFR with Grb2 or Gab1 with SHP2 (Fig. 1B). Consistently, it stimulated keratinocyte proliferation with mitogenic activity comparable to EGF (Fig. 1C). From these data, we identified Pep2-YAC as an effective EGFR agonist peptide.

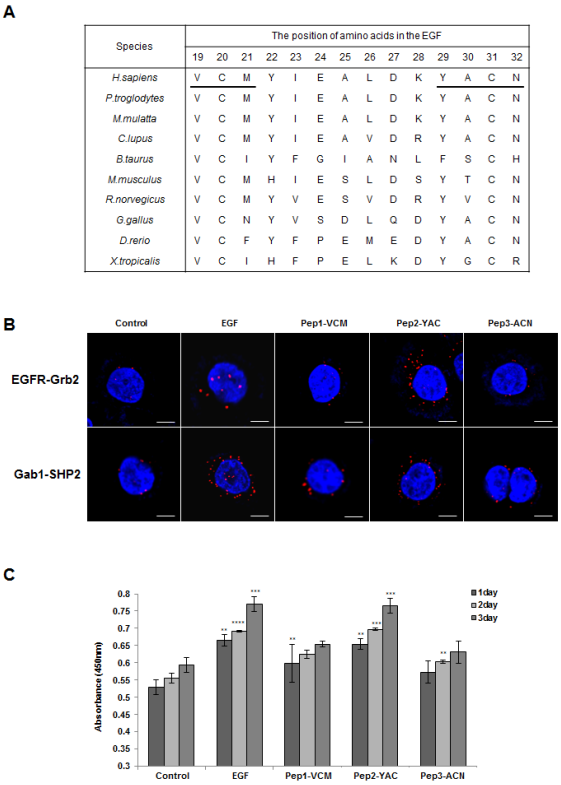

Fig. 1. Identification of an EGF-derived agonist peptide, Pep2-YAC. (A) The amino acid sequences covering residues 19-32 in EGF from 10 different species were aligned. The numbers indicate the position of a particular amino acid in the EGF. The underlined sequences represent conserved sequences used for the synthesis of agonist tripeptide candidates as follows: Pep1-VCM, Pep2-YAC, and Pep3-ACN. (B) *In situ* PLA reactions were performed to determine the agonistic effect of three peptides on the association of EGFR with Gab1 or Grb2 with SHP2 using specific antibodies. HaCaT cells were counterstained with DAPI (blue) to visualize nuclei. Red spots represent the interactions between the indicated proteins of interest. Scale bar, 5 μm. Original magnification, ×600. (C) Graph shows the effects of three EGF agonist peptide candidates on HaCaT cell proliferation, determined using the CCK-8 assay, as described in the Materials and Methods. The *y*-axis represents the absorbance values that were measured at a wavelength of 450 nm. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Pep2-YAC activated ERK and Akt signaling pathway and promoted cell proliferation through EGFR

EGF is known to promote the activation of ERK and Akt signaling pathway (12), which prompted us to examine whether Pep2-YAC induced phosphorylation of ERK1/2 and Akt1. As shown in Fig. 2A, treatment of HaCaT cells with Pep2-YAC showed phosphorylation of ERK1/2 and Akt1, dose-dependently. To confirm whether the effect of Pep2-YAC on phos-

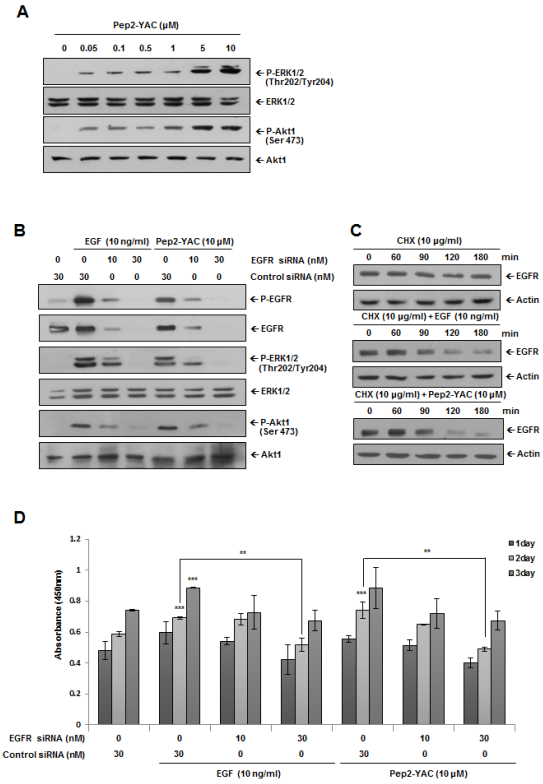

Fig. 2. Pep2-YAC promotes keratinocyte proliferation through EGFR activation. (A) HaCaT keratinocytes were treated with Pep2-YAC at the indicated concentrations for 15 min and then phosphorylation levels of ERK1/2 (pERK-T202/Y204) and Akt1 (pAkt-S473) were analyzed by Western blot analysis. (B) HaCaT cells were transfected with control siRNA (30 nM), or with EGFR siRNA (10 or 30 nM). Then, they were treated with EGF (10 ng/ml) or Pep2-YAC (10 μM) for 15 min. The total amount of EGFR, ERK1/2, or Akt1 protein and phosphorylation levels of EGFR, ERK1/2, or Akt1 were analyzed by Western blotting. (C) HaCaT cells were pretreated with cycloheximide (CHX; 10 μg/ml) for 1 h before being treated with EGF (10 ng/ml) or Pep2-YAC (10 μM) in the presence of CHX for the indicated times and cell lysates were subjected to immunoblotting with anti-EGFR mAb. (D) After HaCaT cells were treated as described in (B), the rates of cell growth for each sample were determined for 3 days using the CCK-8 assay. The y-axis represents the absorbance values that were measured at a wavelength of 450 nm. Values represent the means of three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$. Lines indicate additional statistical comparisons.

phorylation of ERK1/2 and Akt1 was mediated by EGFR molecules on HaCaT cells, we transiently transfected HaCaT cells with siRNA for the EGFR gene and then treated them with EGF or Pep2-YAC. Western blot analysis revealed a dose-dependent decrease in EGFR expression in cells transiently transfected with EGFR siRNA, compared with control cells transfected with control siRNA (Fig. 2B). Comparable to 10 ng/ml EGF, 10 μM of Pep2-YAC caused phosphorylation of EGFR, of ERK1/2 at Thr 202/Tyr 204, and of Akt1 at Ser 473 (Fig. 2B). However, treatment with EGFR siRNA inhibited Pep2-YAC from inducing phosphorylation of EGFR, ERK1/2, and Akt1 in a dose-dependent manner. Next, we compared the effect of EGF or Pep2-YAC on the degradation of EGFR. EGF or Pep2-Yac treatment induced EGFR degradation in a time-dependent manner (Fig. 2C). In addition, we performed a proliferation assay to assess whether Pep2-YAC has a mitogenic effect on HaCaT cells through EGFR. EGF and Pep2-Yac increased the proliferation of HaCaT cells, which was prevented by EGFR siRNA transfection in a concentration-dependent manner (Fig. 2D). Taken together, these data showed that Pep2-YAC agonistic activity is dependent on EGFR.

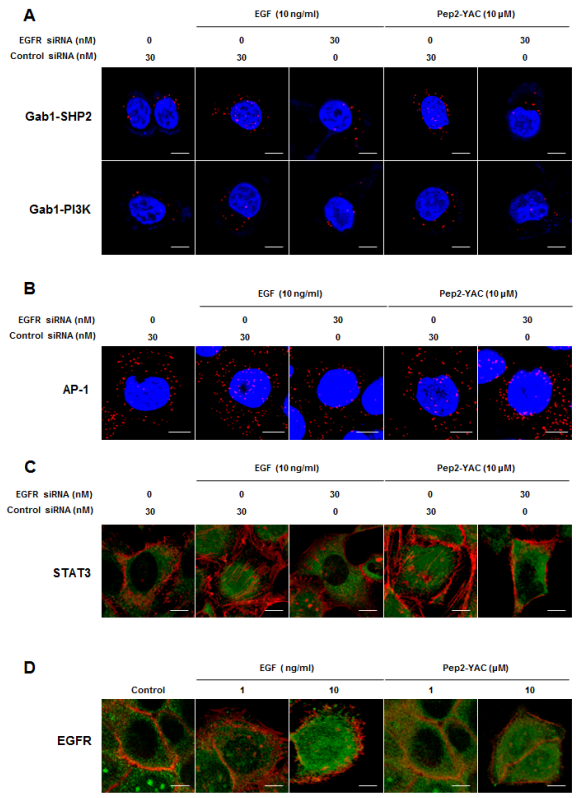

Fig. 3. Pep2-YAC induces not only interaction of EGFR signaling molecules but nuclear translocation of transcription factors via EGFR. (A) and (B) HaCaT cells were transfected with control siRNA (30 nM), or EGFR siRNA (30 nM) for 48 h and then treated with EGF (10 ng/mL) or Pep2YAC (10 μM) for 15 min. After fixation, *in situ* PLA for Gab1-SHP2, Gab1-PI3K, and c-Jun-c-Fos were performed with specific antibodies. Nuclei were stained with DAPI (blue). Red spots represent physical interaction between the two indicated molecules. (C) HaCaT cells treated as described above were subjected to immunofluorescent staining for analyzing nuclear-translocation of STAT3. Cells were incubated with phalloidin-rhodamine and then with an anti-STAT3 mAb and FITC-conjugated anti-mouse IgG antibody. (D) HaCaT cells were treated with EGF (1 or 10 ng/ml) or Pep2-YAC (1 or 10 μM) for 15 min. Untreated cells were used as controls. Thereafter, immunofluorescence was performed to analyze EGFR nuclear-translocation, as in (C). Anti-EGFR mAb and FITC conjugated anti-mouse IgG antibody were used to detect EGFR. In all figures, the scale bar = 5 μm. Original magnification, ×600.

Pep2-YAC induced not only association of Gab1 with SHP2 or PI3K but also nuclear translocation of transcription factors via EGFR

To further confirm the dependence of Pep2-YAC activity on EGFR, we examined the effect of EGFR siRNA transfection on the physical interactions of Gab1-SHP2 and Gab1-PI3K in HaCaT cells treated with EGF or Pep2-YAC peptide. Increased association of Gab1 with SHP2 or PI3K was observed in HaCaT cells treated with Pep2-YAC, as with EGF, which was abolished, to control levels, in EGFR knockdown cells (Fig. 3A). Previous studies showed that EGFR activation results in the nuclear translocation of c-Jun, c-Fos, and STAT3 and transcription of target genes in cells with a high proliferative index (13). Notably, the nuclear interaction of c-Jun and c-Fos and nuclear translocation of STAT3 was observed in the HaCaT cells stimulated with Pep2-YAC, but not in EGFR knockdown cells (Fig. 3B, C).

Finally, we investigated whether Pep2-YAC induced the nuclear translocation of EGFR. Previous studies have shown that EGFR activation leads to its internalization and subsequent nuclear translocation, which regulates gene expression required for cell proliferation by interacting with STAT3 (14). Like EGF, Pep2-YAC induced the nuclear translocation of EGFR, suggesting that Pep2-YAC may promote cell proliferation through EGFR nuclear translocation (Fig. 3D). Collectively, our results demonstrated that Pep2-YAC has the ability to promote cell proliferation by activating the EGFR signaling pathway.

DISCUSSION

Here, we demonstrated that Pep2-YAC, an EGF-derived tripeptide, has a similar ability to EGF to activate EGFR. The treatment of HaCaT cells with Pep2-YAC induced phosphorylation of EGFR and organization of signaling complexes, which consist of Grb2, Gab1, SHP2, and PI3K. Furthermore, it enhanced phosphorylation of ERK1/2 and Akt1 and subsequent nuclear translocation of EGFR, STAT3, c-Jun, and c-Fos, which would promote keratinocyte proliferation.

Pep2-YAC was identified based on a sequence homology search. To search for EGFR agonist peptides, we focused on amino acid sequences in the EGF B loop region, which have

been shown to play a key role in the activation of EGFR (6). Previous structural analyses showed that Met21, Ile23, and Leu26 in the B loop of EGF interact with EGFR (8). However, peptide mimics containing these residues inhibited EGF from interacting with its receptor (15). Additionally, these residues are not evolutionarily conserved, suggesting that these amino acid sequences may not be directly involved in the activation of EGFR. These observations caused us to focus on the β-sheet sequence (residues 19-23 and 28-32) in the B loop of EGF that contains relatively highly evolutionarily conserved regions (16). The evolutionary information in a multiple sequence alignment showed the conserved amino acid sequences covering the 19-21 and 29-32 region of EGF. Thus, we synthesized them as three EGFR agonist peptide candidates. Our data revealed that one of them, Pep2-YAC, was functionally significant in the activation of EGFR.

In the present report, we demonstrated that Pep2-YAC promoted keratinocyte proliferation by EGFR activation. Our *in situ* PLA analysis showed that Pep2-YAC increased the physical interaction between EGFR and Grb2, Gab1 and SHP2 or PI3K through EGFR. Binding of EGF to its receptor leads to receptor dimerization and the activation of an intrinsic tyrosine kinase (17). Autophosphorylated EGFR then recruits Gab1 through Grb2. Subsequently, Gab1 activates SHP2 (18) and the p85/p110 subtype of phosphoinositide 3-kinase (PI3K) on juxtamembrane EGFR (19, 20), which are important for EGFR-mediated biological responses. In an immunoblotting assay, Pep2-YAC phosphorylated EGFR, ERK1/2, and Akt1 through EGFR, suggesting that it induces the activation of major EGF/EGFR signaling pathways, including the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway and phosphoinositide 3 kinase (PI3K)-Akt pathways (21, 22). Furthermore, Pep2-YAC, like its parent EGF, induced EGFR degradation, which plays an important role in the regulation of the EGFR-mediated ERK and Akt signaling pathway in pathological and physiological conditions (23).

Here, we showed that Pep2-YAC induced the nuclear translocation of EGFR and STAT3. EGF signaling can be transmitted, via EGFR nuclear translocation, directly from the cytoplasmic membrane to the transcriptional targets in the nucleus. EGFR has transcriptional activity and is able to translocate into the nucleus, but requires a DNA-binding transcription cofactor for its transcriptional function, such as STAT3, due to its lack of a DNA-binding domain (24). STAT3 represents an essential effector pathway of Rho GTPases in regulating multiple cellular functions, including actin cytoskeleton reorganization, cell migration, gene activation, and proliferation (25), and transit from the cell membrane to the nuclear region in response to growth factor stimulation (26). Additionally, nuclear translocation of c-Jun/c-Fos and their interaction were increased by Pep2-YAC or EGF treatment. EGF activates different mitogen-activated protein kinases including ERK, JNK, and p38, which, in turn, activate c-Jun and c-Fos, leading to cell growth and differentiation (13). Consistent with these results, our proliferation assay showed Pep2-YAC significantly enhanced keratinocyte proliferation. Overall, our results demonstrated that Pep2-YAC can function as an EGFR agonist, and, therefore, may be useful as a therapeutic agent in skin regeneration and wound-healing.

MATERIALS AND METHODS

Cell culture

HaCaT keratinocytes were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium containing 10% FBS, 5% penicillin/streptomycin and maintained at 37°C, 5% CO_2.

RNA interference

Small-interfering RNAs (siRNAs) against human EGFR and a non-specific siRNA control were purchased from Santa Cruz Biotechnology. HaCaT cells were cultured to 50-60% confluence, and then transfected with the siRNA duplex using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. Non-specific siRNA was also transfected as a control.

In situ PLA

To determine changes in protein-protein interactions by EGFR signaling, we performed an *in situ* PLA assay. The *in situ* PLA studies on fixed HaCaT keratinocytes were performed as follows (O-LINK Bioscience, Upsalla, Sweden). EGFR or control siRNA transfected cells were grown on slide glasses for at least 16 h, and treated with EGF or EGF-pep for 15 min in 5% CO_2 at 37°C and washed with PBS twice. *In situ* PLA detection was carried out using the appropriate DUOLINK II *in situ* kit components according to the manufacturer's protocol. Briefly, cells were washed once with PBS, and then subjected to blocking using the DUOLINK blocking solution (1 drop) at 37°C in a wet chamber for 30 min. After tapping off the blocking solution from the slides, antibodies were added at a dilution of 1 : 100 in 40 μl DUOLINK antibody diluent and incubated in a wet chamber at 37°C for 30 min. The slides were washed twice with wash buffer A for 5 min each, and then secondary antibodies (DUOLINK anti-rabbit PLA-plus probe, DUOLINK anti-goat PLA-minus probe) were added and incubated at 37°C for 1 h. Two washes with wash buffer A were then followed by addition of the ligation mix and incubation at 37°C for 30 min, followed by another two washes. Then, the amplification reaction was carried out at 37°C for 100 min. Subsequently, the slides were washed twice with wash buffer B, and once with 0.1×wash buffer B. Mounting was done with mounting solution containing DAPI. Antibodies used for PLA were as follow: rabbit anti-EGFR Ab (Santa Cruz Biotechnology), mouse anti-Grb2 Ab (Santa Cruz Biotechnology), rabbit anti-Gab1 Ab (Cell signaling technology), mouse anti-SHP2 Ab (Santa Cruz Biotechnology), rabbit anti-Gab1 Ab (Cell Signaling Technology), mouse anti-PI3K p85 Ab (Santa Cruz Biotechnology), rab-

bit-c-jun (Santa Cruz Biotechnology), and mouse-c-fos (Santa Cruz Biotechnology).

Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM sodium orthovanadate, 1% NP-40, and a protease inhibitor cocktail) for 30 min on ice, and centrifuged (13,000 rpm, 20 min, 4°C). Supernatants were collected and total protein concentration was determined using a Bradford assay kit (Bio-Rad, USA). Equal amounts of protein were loaded and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane and probed with appropriate antibodies. Primary antibodies against EGFR-pY845, EGFR (Santa Cruz Biotechnology), ERK1/2-pT202/Y204, ERK1/2 (Millipore), AktpS473 and Akt1 (Santa Cruz Biotechnology; diluted 1 : 1,000 in 0.5% bovine serum albumin), were used and detected by incubation with HRP-conjugated secondary antibodies (1 : 10,000 in 0.5% bovine serum albumin) using an ECL system.

Immunofluorescence and confocal microscopy

Cells transfected with EGFR or control siRNA were treated with EGF (10 ng/ml) or Pep2-YAC (10 μM) for 15 min and washed by cold PBS twice. Cells were fixed in 4% formaldehyde in PBS for 15 min at room temperature. Subsequently, the cells were washed with PBS twice, permeabilized for 5 min in 0.1% Triton X-100 in PBS, and washed with PBS. Cells were incubated with phalloidin-rhodamine for 40 min, and washed with PBS for 5 min twice. Further cells were incubated with a primary antibody (1 : 100) for 40 min, washed with PBS twice, and incubated with FITC-conjugated anti-IgG antibody (1 : 100) for 40 min, washed with PBS for 5 min three times and mounted in mounting medium. Primary antibodies against EGFR and STAT3 (Santa Cruz Biotechnology) were used. Confocal images were acquired by using a confocal microscope (Olympus fluoview FV1000; Olympus, Tokyo, Japan).

Proliferation assay

Cell proliferation was assessed using a CCK8-based assay. Briefly, EGFR or control siRNA transfected cells were seeded at 5×10^3 per well in 96-well culture plates and incubated in medium containing 10% FBS. After 24 h, cells were treated with EGF (10 ng/ml) or EGF-derived peptides (10 μM) in 90 μl of SFM for 24, 48, and 72 h. At the indicated time points, cells were washed once and incubated with 10 μl of CCK-8 solution at 37°C for 2 h. Then, the absorbance was measured at 450 nm using a microplate reader (Versa Max, USA). Independent experiments were repeated in triplicate.

Statistical analysis

Data are expressed as the average of mean values obtained ± SD. Statistical significance was determined using Student's *t*-test with the statistical software GraphPad Prism (ver. 4.0). All experiments were conducted three times or more to ensure reproducible results. Representative data are shown in the figures.

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