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# Tissue kallikrein induces SH-SY5Y cell proliferation via epidermal growth factor receptor and extracellular signal-regulated kinase1/2 pathway

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ABSTRACT

Tissue kallikrein (TK) is well known to take most of its biological functions through bradykinin receptors. In the present study, we found a novel signaling pathway mediated by TK through epidermal growth factor receptor (EGFR) in human SH-SY5Y cells. We discovered that TK facilitated the activation of EGFR, extracellular signal-regulated kinase (ERK) 1/2 and p38 cascade. Interestingly, not p38 but ERK1/2 phosphorylation was severely compromised in cells depleted of EGFR. Nevertheless, impairment of signaling of ERK1/2 seemed not to be restricted to EGFR phosphorylation. We also observed that TK stimulation could induce SH-SY5Y cell proliferation, which was reduced by EGFR down-regulation or ERK1/2 inhibitor. Overall, our findings provided convincing evidence that TK could mediate cell proliferation via EGFR and ERK1/2 pathway *in vitro*.



## 1. Introduction

Tissue kallikrein (TK), an important component of the kallikrein–kinin system (KKS), is a serine proteinase capable of processing low molecular weight kininogen to release vasoactive kinins [1,2]. Most of the cellular effects of TK in the regulation of inflammatory response, blood pressure, coagulation and pain are thought to be mediated by bradykinin though G protein-coupled bradykinin receptors. However, recent findings have revealed that TK could activate epidermal growth factor receptor (EGFR) signaling pathway independent of bradykinin receptors to trigger its biological functions [3,4]. Moreover, the potential role of TK on inducing cell proliferation has received relatively little attention.

EGFR is one of four members of the ErbB family receptor tyrosine kinases that modulate a series of signaling pathways to control cell survival, growth, differentiation and proliferation [5,6]. There are several endogenous EGFR ligands, such as EGF, heparin-binding EGF, transforming growth factor-α, amphiregulin and epiregulin, each of which is synthesized as precursors and cleaved to generate the mature one [5,6]. Ligand binding results in multiple phosphorylation events of EGFR, which in many cases mediates simultaneous activation of downstream cascades, especially the mitogen-activated protein (MAP) kinase pathway [7].

MAP kinase signaling pathways are widely distributed and can be activated by multiple extracellular stimuli [8,9], which may be classified into three main functional groups: the extracellular signal-regulated kinase (ERK) pathway, the Jun N-terminal kinase (JNK)/stress activated protein kinase (SAPK) pathway and the p38/high osmolarity glycerol (HOG) pathway [8,9]. MAP kinase function in protein kinase cascades plays a critical role in the regulation of cell growth, differentiation, proliferation, and control of cellular responses to cytokines and stress [8,9].

By agglomerating our preceding experiment data it can be concluded that TK played an important part in various physiological and pathological processes through the activation of B2 bradykinin receptor and ERK1/2 [10–14]. In this study, we determined the novel signaling pathway mediated by TK through EGFR and ERK1/2 in the proliferation of cultured human SH-SY5Y cells.

## 2. Materials and methods

### 2.1. Materials

TK was from Techpool Bio-Pharma Co. (Guangzhou, China). Phospho-EGFR (Tyr1068), EGFR, phospho-ERK1/2 (Thr202/

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Tyr204), ERK1/2, phospho-p38 (Thr180/Tyr182), p38, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, cyclin D1 and β-Tubulin monoclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies coupled to horseradish peroxidase (HRP) were from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor 488-conjugated secondary antibody was from Invitrogen (Grand Island, NY, USA). The lipofectamine 2000 transfection reagent was from Invitrogen (Grand Island, NY, USA). PD98059 and mitochondrial conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma–aldrich (St. Louis, MO, USA).

### 2.2. Cell cultures and transfections

Human SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C and 5% $CO_2$. The following small interfering RNA (siRNA) duplex sequences against EGFR mRNA were used: sense 5′-GGCUCUGGAGGAAAAGAAATT-3′ and antisense 5′-UUUCUUUUCCUCCAGAGCCTT-3′. The commercial siRNA with non-targeting sequence was used as a negative control (NC). Cells were transiently transfected with Lipofectamine 2000 according to the manufacturer's instructions, and experiments were carried out at 48 h after transfection.

### 2.3. Drug treatment

For drug treatment, SH-SY5Y cells were serum-starved overnight before various concentration of TK (0.25–1.0 μM) were given for 5–30 min or 24–48 h. In some experiments cells were treated with ERK kinase inhibitor PD98059 (10 μM) for 1 h previous to TK stimulation.

### 2.4. Western blot

The cells were lysed in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS containing phosphatase and protease inhibitor mixture) supplemented with 1 mM PMSF on ice for 30 min. Equal amounts of protein were separated by 10% or 8% SDS–PAGE and then transferred to PVDF membrane (Millipore). The membranes were subsequently incubated overnight at 4 °C with the following primary antibodies: anti-phospho-EGFR (diluted 1:1000), anti-EGFR (diluted 1:1000), anti-phospho-ERK1/2 (diluted 1:2000), anti-ERK1/2 (diluted 1:1000), anti-phospho-p38 (diluted 1:1000), anti-p38 (diluted 1:1000), anti-phospho-JNK (diluted 1:1000), anti-JNK (diluted 1:1000), anti-cyclin D1 (diluted 1:2000) or anti-β-Tubulin (diluted 1:5000). Secondary antibodies conjugated with HRP were used, and blotted proteins were visualized using an enhanced chemiluminescence assay. The phosphorylated bands of interest were quantified by scanning densitometry using Scion Image software and normalized against the total amount of the respective protein unless stated; otherwise β-Tubulin was used as an equal loading control.

### 2.5. Immunocytochemistry

Treated with TK, SH-SY5Y cells were fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin (Sigma–aldrich) in TBS-T at room temperature, and then were incubated with anti-phospho-EGFR primary antibody (diluted 1:800) overnight at 4 °C. Thereafter cells were exposed to Alexa Fluor 488-conjugated secondary antibody (diluted 1:1000) at room temperature in the dark for 1 h. Cell nuclei were stained by Hoechst 33258. The cells were observed under a fluorescence microscope (Olympus, Japan).

### 2.6. MTT assay

SH-SY5Y cells were plated on 96-well plates in 100 μl total volume at density of 4000 cells/well. The viability of cells was examined by MTT assay. After drug treatment, each well was supplemented with 10 μl MTT solution (5 mg/ml). The plates were incubated in 37 °C, 5% $CO_2$ for another 4 h. Cell proliferation was determined by the optical density, and values were read on a microplate reader at 570 nm wavelength after addition of 150 μl dimethyl sulfoxide.

### 2.7. Statistical analysis

In general, all experiments presented in this paper were performed at least three times. Data were shown as mean ± S.D. Differences between groups were considered statistically significant according to one-way ANOVA followed by the Bonferroni post hoc tests for parametric data or the Kruskal–Wallis for non-parametric data. Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. TK activates EGFR phosphorylation in a concentration-dependent manner

To explore whether EGFR activation was involved in the TK stimulation, we first performed the experiment with different dose of TK. In both immunocytochemistry and Western blot analyses, as shown in Fig. 1, serum-starved SH-SY5Y cells exposed to TK at 0.25–1.0 μM for 5 min exhibited a concentration-dependent increase in EGFR phosphorylation. Thus, SH-SY5Y cells were treated with 1.0 μM TK in the next experiments.

### 3.2. Knockdown of EGFR impairs TK-induced ERK1/2 phosphorylation

Phosphorylation events of EGFR resulted in activation of various signaling pathways, especially the MAP kinase cascades [7], which were also involved in TK effects [3,15]. To determine whether TK could activate MAP kinase pathway via EGFR, we assayed the phosphorylation of MAP kinase signaling proteins (ERK, p38, JNK) by Western blot experiments. As shown in Fig. 2, serum-starved SH-SY5Y cells exposed to TK within 30 min exhibited a significant increase in EGFR, ERK1/2 and p38 phosphorylation, but had no effect on JNK. Interestingly enough, the time course of ERK1/2 response paralleled with EGFR activation. Using transient transfection of siRNAs, we generally obtained a knockdown efficiency of about 78.8% down-regulation of EGFR protein level (data not shown). With the depletion of EGFR, we found there was a remarkable reduction of ERK1/2 phosphorylation in TK-stimulated cells, while no major difference in p38 or JNK phosphorylation was observed. Taken together, these findings suggested that TK was able to activate ERK1/2 pathway through EGFR.

### 3.3. Inhibition of TK-induced ERK1/2 activation does not affect EGFR phosphorylation

In order to verify the relationship between TK-induced ERK1/2 activation and EGFR phosphorylation, cells were treated with ERK kinase inhibitor (PD98059) for 1 h previous to TK stimulus in the present study. The level of phospho-ERK1/2 in SH-SY5Y cells was substantially inhibited by PD98059, but there was no

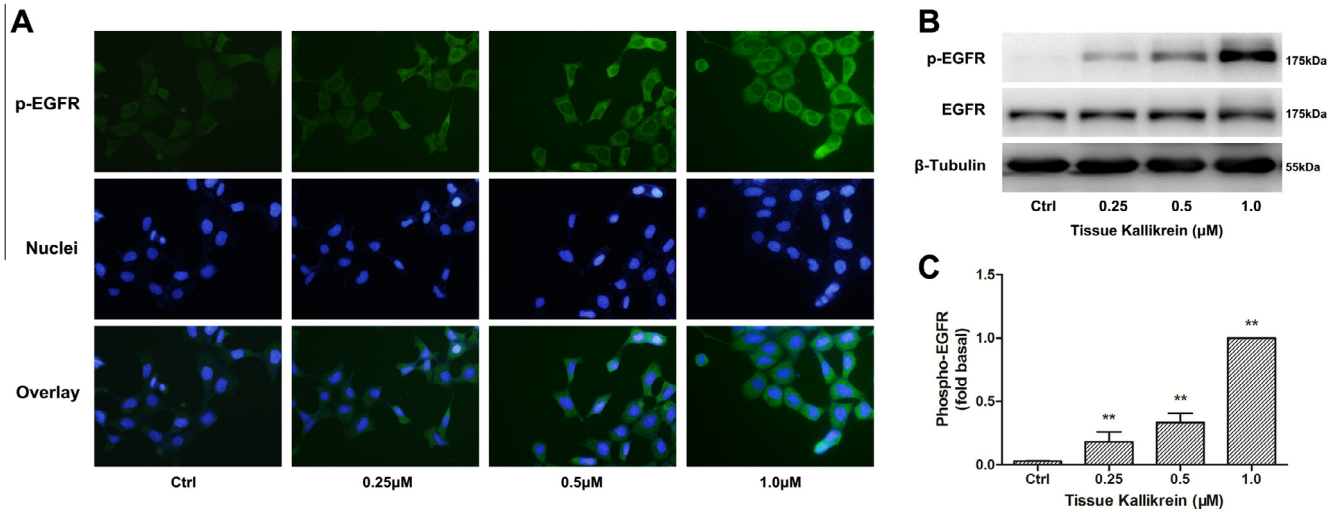


**Fig. 1.** TK results in the activation of EGFR in a concentration-dependent manner in SH-SY5Y cells. (A) SH-SY5Y cells were treated with 0.25–1.0 μM TK for 5 min, and EGFR phosphorylation was assayed by immunocytochemistry. Images capture at 20×. (B, C) SH-SY5Y cells were exposed to 0.25, 0.5, or 1.0 μM TK for 5 min, and phospho-EGFR was detected by Western blot. Cell total EGFR was used as an equal loading control. Bars represent the mean ± S.D. of at least three individual experiments. $^{**}P < 0.01$ compared to control group.

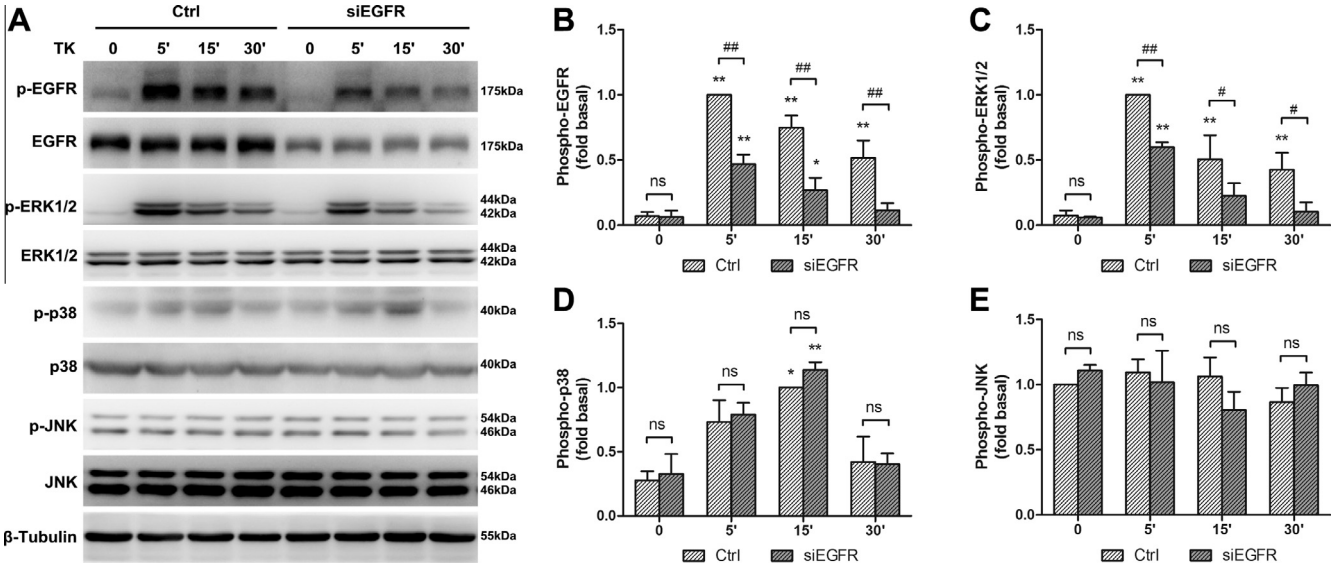


**Fig. 2.** Knockdown of EGFR impairs TK-induced ERK1/2 phosphorylation. SH-SY5Y cells were transiently transfected with EGFR siRNAs. After transfection 48 h cells were treated with or without 1.0 μM TK for 5, 15, or 30 min, the level of EGFR or MAP kinase was detected by Western blot. The p-EGFR was normalized to β-Tubulin, and phosphorylated MAP kinase proteins were normalized against the total amount of the respective proteins. At least three individual experiments were performed. Values are represented as mean ± S.D. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to non-stimulated control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to TK-stimulated control group; ns, not significant.

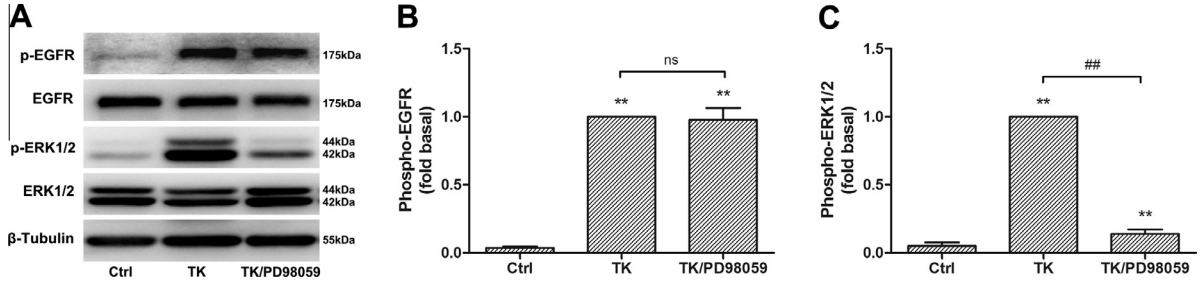


**Fig. 3.** TK-induced ERK1/2 phosphorylation was the downstream event of EGFR signaling. SH-SY5Y cells were pre-incubated for 1 h with or without the ERK1/2 antagonist, PD98059 (10 μM), previous to treatment with 1.0 μM TK for 5 min, and EGFR or ERK1/2 phosphorylation was detected as described. The p-EGFR and p-ERK1/2 were normalized to total EGFR and ERK respectively. At least three individual experiments were performed. Values are represented as mean ± S.D. $^{**}P < 0.01$ compared to control group; $^{\#\#}P < 0.01$ compared to TK stimulated group; ns, not significant.

significant change in TK-induced EGFR phosphorylation (Fig. 3). These data confirmed that TK gave rise to phosphorylation of ERK1/2, which was the downstream event of EGFR signaling.

### 3.4. TK mediates the proliferation of SH-SY5Y cells in a concentration- and time-dependent manner

To study whether TK could promote cell proliferation, serum-starved SH-SY5Y cells were exposed to TK at 0.25–1.0 μM for 24 h or 48 h. The effects of TK on proliferation were evaluated by MTT assay. As shown in Fig. 4A, TK could stimulate cell proliferation in a concentration- and time-dependent manner. Maximum proliferation of TK was obtained at 1.0 μM for 48 h. Therefore, SH-SY5Y cells were incubated with 1.0 μM TK for 48 h in the subsequent experiments.

### 3.5. Down regulation of EGFR inhibits TK-induced cell proliferation

We have already proved that TK could activate EGFR phosphorylation, which was upstream event of ERK1/2 pathway. Then we aimed to determine whether EGFR activation was involved in the TK-stimulated cell proliferation. As shown in Fig. 4B, the TK effect on cell proliferation was down-regulated by EGFR silencing. Interestingly, we found that cyclin D1, a key regulator of cell proliferation, increased in the TK stimulation, while decreased by EGFR knockdown (Fig. 4D and F).

### 3.6. Blockage of ERK1/2 activation impairs TK-induced cell proliferation

To further evaluate the role of TK-induced ERK1/2 phosphorylation in cell proliferation, serum-starved SH-SY5Y cells were incubated with PD98059 for 1 h prior to TK treatment. With the reduction of cyclin D1 protein level, the TK-mediated cell proliferation was also blocked by ERK kinase inhibitor (Fig. 4C, E and G).

## 4. Discussion

The KKS is highly expressed and widely localized in all components of the neurovascular unit [16]. The complex function of the KKS in health and disease underscores the importance of finding out its molecular mechanisms. Accumulating information has demonstrated that TK gene transfer could protect against cerebral ischemia/reperfusion injury by promoting neurogenesis [17,18]. However, the exact signaling chain of TK in neurogenesis was still unknown. In this study, we provided convincing evidence that TK could promote cell proliferation through EGFR and ERK1/2 pathway in neuronal cell line.

It was recently reported that activation of EGFR could transduce signal to several downstream molecules like MAP kinase and cyclin D1 [2,7]. In the present study, we found that TK facilitated the activation of EGFR, ERK1/2 and p38 cascade. Interestingly enough, not p38 but ERK1/2 phosphorylation was severely compromised in cells depleted of EGFR. Nevertheless, impairment of signaling of ERK1/2 seemed not to be restricted to EGFR phosphorylation. Correlative research showed that TK might activate the proteinase-activated receptor 1 and stimulate intracellular protein kinase C and Src kinase pathway, and further lead to EGFR transactivation resulting from metalloproteinase-induced EGFR ligand shedding [3]. These combined results revealed a novel signaling pathway mediated by TK in the phosphorylation process of EGFR and ERK1/2.

The above experimental data raised the question what was the biological function of TK-induced both EGFR and ERK1/2 activation. Published findings indicated that the auto-phosphorylation sites of EGFR were critical for the initiation of the downstream

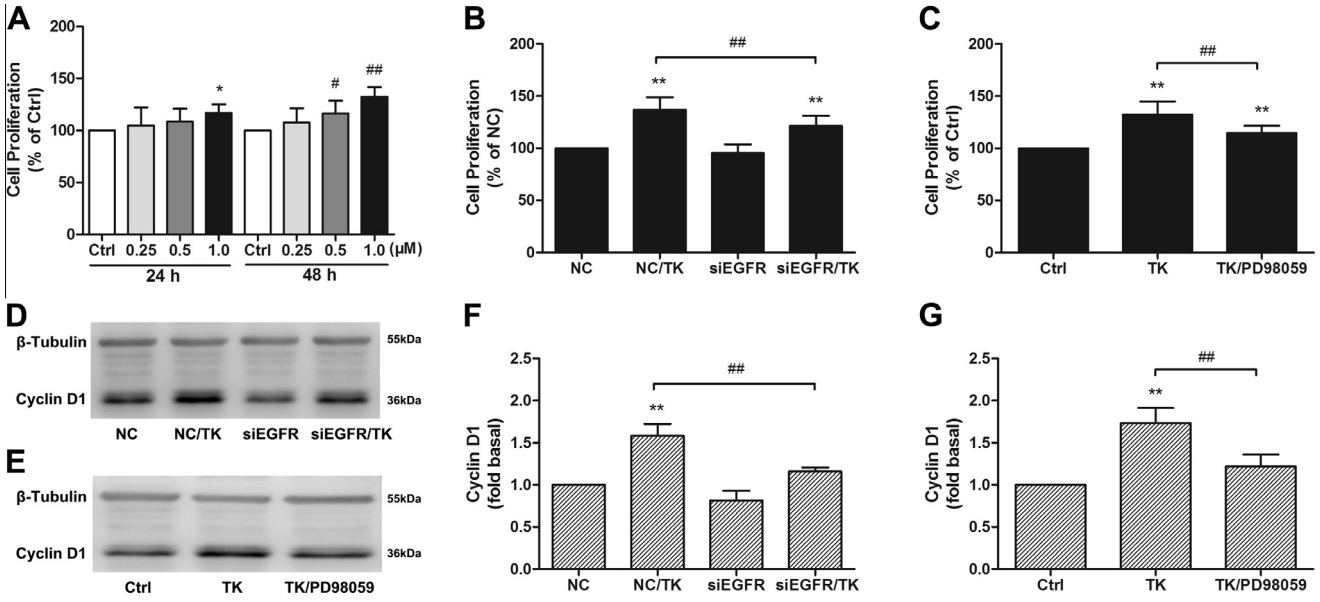


**Fig. 4.** TK induced SH-SY5Y cell proliferation via EGFR and ERK1/2. (A) Cultured SH-SY5Y cells were exposed to TK at increasing concentration for 24 or 48 h, and then cell proliferation was examined by MTT assay. $^{*}P < 0.05$ compared to cells in 24 h control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to cells in 48 h control group. (B) SH-SY5Y cells were transiently transfected with EGFR siRNAs. After transfection 48 h cells were treated with or without 1.0 μM TK for 48 h, cell proliferation was examined by MTT assay. $^{**}P < 0.01$ compared to NC group; $^{\#\#}P < 0.01$ compared to NC/TK group. (C) SH-SY5Y cells were pre-incubated for 1 h with or without the ERK1/2 antagonist, PD98059 (10 μM), prior to treatment with 1.0 μM TK for 48 h, and then cell proliferation was examined by MTT assay. $^{**}P < 0.01$ compared to control group; $^{\#\#}P < 0.01$ compared to TK stimulated group. (D, F) SH-SY5Y cells were transiently transfected with EGFR siRNAs. After transfection 48 h cells were treated with or without 1.0 μM TK for 48 h, the level of cyclin D1 was detected by Western blot. Tubulin was used as an equal loading control. Bars represent the mean ± S.D. of at least three individual experiments. $^{**}P < 0.01$ compared to NC group; $^{\#\#}P < 0.01$ compared to NC/TK group. (E, G) SH-SY5Y cells were pre-incubated for 1 h with or without PD98059 (10 μM) prior to treatment with 1.0 μM TK for 48 h, the protein level of cyclin D1 was detected as described. Tubulin was used as an equal loading control. At least three individual experiments were performed. Values are represented as mean ± S.D. $^{**}P < 0.01$ compared to control group; $^{\#\#}P < 0.01$ compared to TK group.

ERK signaling [19,20], which could stimulate keratinocyte cell proliferation [21,22]. Moreover, TK could promote proliferation in HaCaT keratinocyte cell and DU145 cell through the ERK1/2 pathway [3,15]. Consistent with these studies, we observed that TK stimulation could induce human SH-SY5Y cell proliferation, which was reduced by EGFR down-regulation or ERK1/2 inhibitor.

In conclusion, our results here demonstrated that TK induced SH-SY5Y cell proliferation through EGFR and ERK1/2 pathway. Combining our previous work with updated findings, we revealed the potential of TK as a therapeutic application for neural injury. However, future studies on neurogenesis of TK effects will be useful. Also, experiments in the neurovascular unit are needed.

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