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# A Novel Signaling Pathway of Tissue Kallikrein in Promoting Keratinocyte Migration: Activation of Proteinase-Activated Receptor 1 and Epidermal Growth Factor Receptor

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## Abstract

Biological functions of tissue kallikrein (TK, KLK1) are mainly mediated by kinin generation and subsequent kinin B2 receptor activation. In this study, we investigated the potential role of TK and its signaling pathways in cultured human keratinocyte migration and in a rat skin wound healing model. Herein, we show that TK promoted cell migration and proliferation in a **concentration**- and time-dependent manner. Inactive TK or kinin had no significant effect on cell migration. Interestingly, cell migration induced by active TK was not blocked by icatibant or L-NAME, indicating an event independent of kinin B2 receptor and nitric oxide formation. TK's stimulatory effect on cell migration was inhibited by small interfering RNA for proteinase-activated receptor 1 ($PAR_1$), and by $PAR_1$ inhibitor. TK-induced migration was associated with increased phosphorylation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK), which was blocked by inhibition of protein kinase C (PKC), Src, EGFR and ERK. TK-induced cell migration and EGFR phosphorylation were blocked by metalloproteinase (MMP) inhibitor, heparin, and antibodies against EGFR external domain, heparin-binding EGF-like growth factor (HB-EGF) and amphiregulin (AR). Local application of TK promoted skin wound healing in rats, whereas icatibant and EGFR inhibitor blocked TK's effect. Skin wound healing was further delayed by aprotinin and neutralizing TK-antibody. This study demonstrates a novel role of TK in skin wound healing and uncovers new signaling pathways mediated by TK in promoting keratinocyte migration through activation of the $PAR_1$-PKC-Src-MMP pathway and HB-EGF/AR shedding-dependent EGFR transactivation.

### Keywords

tissue kallikrein; keratinocyte; migration; proteinase-activated receptor 1; epidermal growth factor receptor



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## Introduction

It is well known that true tissue kallikrein (TK, KLK1), a member of tissue kallikrein-related peptidase family, exerts its biological effects through kinin formation from low molecular weight (LMW) kininogen substrate with subsequent kinin B2 receptor stimulation [1]. Though kinin formation plays an important role in physiological actions of the kallikrein-kinin system, recent studies showed that TK could directly activate kinin B2 receptor independent of kininogen and kinin release in cultured CHO cells [2,3]. This novel finding is consistent with our reports that TK directly induced rat uterine contraction and elicits cardio-protection by direct kinin B2 receptor activation [4,5]. Moreover, TK has also been shown to induce inflammation and paw edema through activation of proteinase-activated receptor 4 ($PAR_4$), independent of nitric oxide (NO), prostaglandin release and kinin B1 receptor activation [6]. These combined findings suggest that TK may exert biological functions by acting on proteinase-activated receptors or other substrates in addition to LMW kininogen.

Proteinase-activated receptor 1 ($PAR_1$) was recognized as one of the thrombin receptors [7, 8,9]. Recent studies showed that members of the TK gene family (KLK5, KLK6 and KLK14) can activate $PAR_1$, $PAR_2$ and $PAR_4$ [10,11]. A previous study showed that $PAR_1$ activation resulted in cardiac fibroblast proliferation through intracellular activation of protein kinase C (PKC) and Src family tyrosine kinases, epidermal growth factor-like growth factor receptor (EGFR) transactivation and subsequent activation of extracellular signal-regulated kinase (ERK)1/2 [12]. Moreover, EGFR was transactivated by metalloproteinase (MMP)-mediated release of heparin-binding epidermal growth factor-like growth factor (HB-EGF) in the migration of human keratinocytes [13]. Ligands other than HB-EGF that bind to EGFR include EGF, amphiregulin (AR) and transforming growth factor-α (TGF-α) [14,15]. Moreover, TK was found to promote hypertrophy and hyperplasia in cultured human submucosal gland cells by cleaving pro-EGF and leading to EGFR and ERK phosphorylation [16]. Whether TK can trigger $PAR_1$ signaling pathway and EGFR activation in skin cells has not been investigated.

Wound healing in mammals is a complex cellular process involving epithelial cells, fibroblasts, blood vessels and recruited inflammatory cells [17]. The wound healing process consists of fibrin deposition and clot formation, early inflammatory response, granulation and reepithelialization. At the late stage, keratinocytes may be activated, break down their cellular junctions, migrate across the wound and facilitate the restoration of epithelial integrity [18]. TK was reported to be widely expressed and specifically localized in normal human skin, as well as in salivary gland, pancreas and kidney [2,19,20]. As a serine proteinase, TK may take part in balance and maintenance of barrier function of skin and promote skin wound healing. In this study, we determined the novel signaling pathways mediated by TK via $PAR_1$ and EGFR activation in the migration of cultured HaCaT keratinocytes, and its role in skin wound healing *in vivo*.

## Materials and methods

### Culture of HaCaT keratinocyte cells

Immortalized human HaCaT keratinocyte cell line was developed by Drs. Petra Boukamp and Norbert Fusenig at the German Cancer Research Center (DKFZ) and was purchased from CLS Cell Lines Service (69214 Eppelheim, Germany). The cells were maintained in high glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine (Invitrogen) and 10% FBS (Atlanta Biologicals, Lawrenceville, GA).

### Inactivation of TK by aprotinin

TK was purified from rat salivary gland using DEAE-cellulose and aprotinin-affinity column chromatography as previously described [21,22]. An extinction coefficient of $E_{280nm}$=1.5 was used for 1.0 mg/ml of TK (21). TK was dissolved in $Ca^{2+}$, $Mg^{2+}$-free phosphate-buffered saline (PBS). TK was inactivated by pre-incubation with 5-fold molar excess of aprotinin (Sigma, St. Louis, MO) for 1 hour at 37°C [23]. Kallikrein activity was analyzed using assay buffer (50 mM Tris·HCl, 100 mM NaCl, pH 7.4), which contained 1 mM of chromogenic substrate S-2266 (Chromogenix Instrumentation Laboratory SpA, Milano, Italy). Absorbance at 405 nm was measured periodically at 37°C and enzymatic slope was recorded. TK pre-incubated with aprotinin exhibited less than 5% of enzymatic activity.

### Cell proliferation assay

Cell proliferation assay was performed using CellTiter AQueous One Solution (Promega, Madison, WI) following the supplied instructions. Briefly, HaCaT keratinocytes were seeded on 48-well plates ($1 \times 10^4$ cells/well) and were incubated with graded concentrations of TK. At 24, 48 and 72 hours after treatment with TK, CellTiter 96 AQueous One solution was added into each well and absorbance at 490 nm was recorded 2 hours later.

### Cell migration by scratch method

Cells were seeded on 12-well plates coated with collagen I (BD Bioscieces, San Jose, CA) and blocked with BSA (Sigma). The cells were grown to confluence for 2 days. The cultures were then scratch-wounded with a 0.2 ml pipette tip. Two perpendicular wounds were created. Loose cells were removed by washing with PBS. Treatments were carried out in $Ca^{2+}$-free DMEM (Invitrogen) supplemented with 1% FBS as previously described [24]. After 48 hours of treatment, the cells were fixed with 4% formaldehyde (Fisher scientific, Pittsburgh, PA) and stained with crystal violet (Sigma). Cell migration was conducted at 1% FBS because at this concentration cells are viable and attached, but do not migrate spontaneously. Antagonist for $PAR_1$ (SCH79797) was obtained from TOCRIS (Bristol, UK). **EGF (receptor grade)** and icatibant were from Sigma. Antagonists against EGFR (AG1478, AG), PKC (chelerythrine, CHE), Src kinase (PP2), **MMP (GM6001)** and ERK (PD58059, PD) were purchased from Calbiochem (La Jolla, CA).

### Cell migration by modified Boyden chambers

Cells ($1\times 10^6$ cells per ml in DMEM containing 0.1% BSA in a total volume of 150 ml) were added to the upper chamber of modified Boyden chambers (Corning Incorporated, Corning, NY) [25]. After addition of appropriate treatments to the bottom chamber in 0.6 ml of the same medium, cells were incubated at 37°C for 18 hours. The upper surface of the membranes was wiped with a cotton tip to mechanically remove non-migratory cells. The migrant cells attached to the lower surface were fixed in 4% formalin at room temperature for 30 minutes, and then stained with crystal violet for 20 minutes. On the lower surface of the filter, cells from 4 random fields were chosen and quantified.

### Immunoprecipitation and western blot

Cells were starved overnight and were washed with FBS-free DMEM. Protein (600 μg) from cells lysate was immunoprecipitated with 10 μg antibody against total EGFR (Catalog number: sc-03, Santa Cruz Biotechnology, Santa Cruz, CA) as previously described [26]. Antibodies against phosphotyrosine (PY99, Catalog number: sc-7020) and total EGFR (Santa Cruz Biotechnology, 1:500) were used to determine the levels of phosphorylated and total EGFR, respectively. Western blot analysis was performed to detect phosphorylated and total forms of ERK (phospho- and total p44/42 Map Kinase antibody, Cell Signaling Technology, Danvers, MA, 1:1000). Membranes were incubated with secondary antibody conjugated to LumiGLO

chemiluminescent reagent. Chemiluminescence was detected using an ECL-Plus kit (Perkin Elmer Life Science, Boston, MA) and visualized by Kodak X-ray film. Protein concentration was measured by Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA).

### Transfection of siRNAs

Cells were transfected with non-targeting siRNA (Catalog number: D-001810-01, Dharmacon RNAi Technologies, Lafayette, CO) or $PAR_1$-siRNA for human G protein-coupled receptor 172A (Catalog number: L-010743-00, Dharmacon RNAi Technologies) according to the manufacturer's protocol. After 48 hour incubation, expression of $PAR_1$ mRNA in keratinocytes was reduced by 82.9% using real-time PCR analysis.

### Measurement of HB-EGF and amphiregulin (AR) gene expression

Cells were starved overnight and were incubated with or without TK (1.0 mM) for 18 hours. Cells were harvested and total mRNA was prepared by Trizol reagent (Invitrogen). cDNA was transcribed using a High cDNA Archive Kit according to the manufacturer's protocol (Applied Biosystems, Foster, CA). Primers of HB-EGF were: 5'-TCC TCC AAG CCA CAA GCA CT-3' and 5'-GCC CAT GAC ACC TCT CTC CA-3'. Primer of Gene Expression Assay (Hs00155832_ml) for AR was purchased from Applied Biosystems. Real-time PCR analysis was then performed using the SYBR Green Expression Assay (Bio-Rad) for HB and TaqMan Gene Expression Assay (Applied Biosystems) for AR. Gene expression was normalized by the housekeeping gene GAPDH (Mm99999915_gl).

### Skin wound healing model in rats

Surgical procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Male Sprague-Dawley rats (220–250 g, Harlan, Indianapolis, IN) were anesthetized and eight mm diameter excisions were made with a sterile biopsy punch (Robbins Instruments, Chatham, NJ) on the dorsum. Three wounds on each side were patterned 1.5 cm from the dorsal midline according to the previously described protocol [27]. Active TK (3 μg) with or without AG1478 (2 μg) (Calbiochem) and icatibant (12 μg) (Sigma), as well as aprotinin (3 μg) (Sigma), neutralizing antibody to rat TK, control IgG (50 μg), or saline in 100 μl final volume was injected intradermally at 6 different sites around the wound edge once a day for 8 days. Wounds were left undressed and rats were housed separately after surgery.

## Results

### TK promotes keratinocyte migration and proliferation

Effects of TK on the migration of cultured HaCaT keratinocytes were determined by both scratch wound method and modified Boyden chambers. Representative images show that TK-induced cell migration in monolayer in a **concentration**-dependent manner by the scratch method (Fig. 1A). As a positive control, EGF also stimulated cell migration in **concentration**-dependent manner under the same conditions (Fig. 1A). Moreover, TK stimulated proliferation of HaCaT keratinocytes in a **concentration**- and time-dependent manner (Fig. 1B). Maximum migration and proliferation of TK were obtained at 48 and 72 hours, respectively.

### Active TK stimulates keratinocyte migration independent of kinin, kinin B2 receptor and NO formation

As shown in Fig. 2A & B, TK but not kinin induced keratinocyte migration. Icatibant (kinin B2 receptor antagonist) and L-NAME ($N^{\omega}$-nitro-L-arginine methyl ester, nitric oxide synthase (NOS) inhibitor) had no effect on TK-induced cell migration in scratch wound healing (Fig.

2A). This observation was confirmed by quantitative analysis using modified Boyden chamber method (Fig. 2B). Although active TK promoted cell migration, inactive TK pretreated with aprotinin only had a slight effect on cell migration (Fig. 2A & B). Consistent with the cell migration data, Western blot analysis also showed that active but not inactive TK induced EGFR and ERK phosphorylation in HaCaT keratinocytes (Fig. 2C & D).

### TK-induced keratinocyte migration is dependent on $PAR_1$ activation and EGFR phosphorylation

HaCaT cell migration induced by TK was completely blocked by $PAR_1$-siRNA using both scratch method and modified Boyden chamber method (Fig. 3A & B). In addition, TK-induced cell migration was inhibited by SCH79797 (a $PAR_1$ antagonist) and AG1478 (an inhibitor of EGFR) (Fig. 3C). The inhibitory effects of AG1478 and SCH79797 on TK-induced cell migration were confirmed by quantitative analysis in modified Boyden chambers (Fig. 3D). These results indicate that $PAR_1$ and EGFR activation account for HaCaT cell migration induced by TK. To confirm the effects of TK on $PAR_1$ activation and EGFR transactivation, we further demonstrated that AG1478 and SCH79797 blocked TK-induced EGFR phosphorylation, as determined by immunoprecipitation followed by Western blot (Fig. 3E & F). EGF-induced EGFR phosphorylation was used as a positive control. Similarly, inhibitors of $PAR_1$ and EGFR abolished TK-induced ERK phosphorylation (Fig. 3G & H).

### PKC and Src act upstream of EGFR transactivation and ERK phosphorylation

CHE (Chelerythrine, PKC antagonist) and PP2 (Src antagonist) inhibited TK-induced HaCaT keratinocyte migration in both scratch wound and modified Boyden chamber methods (Fig. 4A & B). Furthermore, PD (PD98059, specific antagonist to ERK pathway) completely terminated TK-induced cell migration (Fig. 4A & B), suggesting that the ERK pathway is directly involved in HaCaT cell migration. EGFR phosphorylation was also abolished by CHE and PP2, as confirmed by immunoprecipitation followed by Western blot (Fig. 4C & D). Moreover, ERK phosphorylation is also blocked by inhibitors of PKC and Src (Fig. 4E & F). These results indicate that PKC and Src act upstream of EGFR transactivation and ERK phosphorylation.

### Src and ERK act downstream of EGFR transactivation

Aprotinin, SCH79797 (SCH, a $PAR_1$ antagonist) and Chelerythrine (CHE, a PKC antagonist) had no effect on EGF-induced keratinocyte migration in both scratch wound and modified Boyden chamber methods (Fig. 5 A & B). However, EGF-induced keratinocyte migration was blocked by AG1478 (AG, EGFR antagonist), PP2 (Src antagonist) and PD98059 (PD, ERK antagonist). These results indicated that Src and ERK are responsible for EGF-induced keratinocyte migration. Activation of Src has an important role in EGF-mediated keratinocyte migration, as well as in TK-induced EGFR transactivation. Moreover, EGF-induced keratinocyte migration is independent of protease activity.

### TK-induced keratinocyte migration involves MMP-mediated shedding of EGFR ligands

GM6001 (GM, non-specific MMP antagonist) and LA1 (antibody to EGFR external domain) completely blocked TK-induced HaCaT keratinocyte migration in both scratch wound and modified Boyden chamber methods, demonstrating that TK-stimulated cell migration was related to MMP-mediated release of EGFR ligands (Fig. 6A & B). Heparin, HB-EGF and amphiregulin (AR) antibodies also inhibited TK-induced cell migration (Fig. 6A & B). GM6001 inhibited TK-induced EGFR and ERK phosphorylation as confirmed by Western blot and quantitative analysis (Fig. 6C & D). Although LA1 significantly reduced TK-induced ERK phosphorylation, LA1 partially inhibited TK-induced EGFR phosphorylation (Fig. 6C & D). Heparin or specific antibodies for HB-EGF and AR were used to examine the type of ligand

binding with EGFR in keratinocytes. Heparin, HB-EGF and AR antibodies greatly decreased TK-induced EGFR phosphorylation in HaCaT cells (Fig. 6C). However, HB-EGF and AR antibodies had no significant effect on TK-induced ERK phosphorylation (Fig. 6D). Additionally, TK significantly increased HB-EGF and AR gene expression (Fig. 6E & F). Taken together, these results indicate involvement of MMP-mediated shedding of EGFR ligands in TK-induced keratinocyte migration.

### TK promotes skin wound healing in vivo

Local application of TK treatment significantly reduced wound diameter in a time-dependent manner in a rat excisional skin model. At 7 days after surgery, injection of TK improved wound closure by 65%, compared to the saline control group by 40% (Fig. 7). Inhibition of EGFR by AG1478 effectively blocked TK-induced wound closure process and icatibant (kinin B2 receptor antagonist) partially inhibited TK-induced wound healing. Moreover, aprotinin or neutralizing antibody for TK significantly delayed wound closure compared with control rats injected with saline or normal IgG. These results indicate that exogenous TK application promotes skin wound healing, whereas inhibition of endogenous TK retards wound closure, implicating a role of endogenous TK in promoting skin wound healing. In addition, skin wound healing promoted by TK in rats is dependent on EGFR activation, and is partially involved with kinin B2 receptor-dependent processes.

## Discussion

The present study elucidated a novel signaling mechanism and function of true TK in promoting skin cell migration and wound healing. We showed that TK promoted, whereas aprotinin alone or TK antibody retarded, wound closure in a skin-wounded rat model, demonstrating that endogenous TK might play an important role in the wound healing process. A previous study has shown that PAR1 activation resulted in the proliferation of cardiac fibroblasts via EGFR transactivation through PKC and Src signaling [12]. Consistent with this finding, we further uncovers new signaling pathways mediated by TK in promoting keratinocyte migration through activation of membrane-bound PAR1, TK stimulated intracellular PKC and Src pathway to further activate EGFR and phosphorylate ERK or Src via MMP-mediated release of EGF ligands (as shown in Fig. 8). Our present study reveals a novel mechanism by which TK promotes skin cell migration through direct $PAR_1$ activation and subsequent EGFR transactivation. In addition, keratinocyte migration and proliferation, as well as angiogenesis and extracellular matrix deposition, are vital in the wound healing process. Our study also showed that TK promoted HaCaT keratinocyte migration by more than 30-fold (Fig. 2*B*), whereas TK only stimulated keratinocyte proliferation by 1.5-fold (Fig. 1*B*), suggesting that migration but not proliferation plays a vital role in TK's effects.

### EGFR transactivation signaling pathway plays a vital role in TK-induced keratinocyte migration

Human TK is known to release bioactive Lys-bradykinin (kallidin) from LMW kininogen substrate [1,28]. In rats, however, TK generates bradykinin from LMW kininogen [29]. Our previous study showed that TKs purified from different species exhibited similar substrate specificity by cleaving the Arg-Ser bond at the C-terminal domain of LMW kininogen to release kinin peptide [30]. In this study, we used purified rat TK, as our preliminary results showed that rat and human TK exerted a similar effect on promoting keratinocyte migration. In our initial study, we found that TK at 0.01 to 1.0 μM stimulated HaCaT keratinocyte cell migration in a **concentration**-dependent manner, and that TK at 1.0 μM maximally stimulated cell migration. Therefore, we chose to use this concentration for the subsequent experiments. Since TK stimulation is **concentration**-dependent, we assume that TK at lower concentrations can induce cell migration at a significant but less than maximal rate *in vivo*. In addition, TK may

be produced by specialized cells during wound healing and effectively create a localized supply of high TK levels to promote cell migration *in vivo*.

Our results showed that TK promoted HaCaT keratinocyte migration through both EGFR and ERK activation, which coincided with a previous report that β2-adrenergic receptor agonist activated pro-migratory and pro-proliferative pathways in dermal fibroblasts through transactivation of EGFR followed by ERK phosphorylation [31,32]. Together, these findings indicate that EGFR activation may play a vital role in keratinocyte biology including growth, migration and suppression of terminal differentiation. The auto-phosphorylation sites of EGFR are important for the initiation of downstream signaling (e.g., ERK phosphorylation) in cell proliferation and migration [33,34,35]. Consequently, ERK phosphorylation stimulates keratinocyte proliferation and migration [36,37]. In this study, we demonstrated that TK promotes keratinocyte migration via ERK phosphorylation which occur downstream of EGFR transactivation.

### $PAR_1$ acts as a membrane receptor transmitting TK-induced intracellular signaling

We showed that TK promoted HaCaT keratinocyte migration by transactivation of EGFR through $PAR_1$ activation. EGFR and G-protein-coupled receptors (GPCRs) are connected and involved in the regulation of epithelial cell proliferation and migration. Through activation of GPCR, thrombin promotes cell migration, proliferation and EGFR transactivation in cultured cells [14]. Transactivation of EGFR is related to activation of $PAR_1$ in proliferation of human colon cancer cells, as well as of cardiac fibroblasts and cardiomyocytes [12,38]. Furthermore, thrombin activates pro-inflammatory reaction through $PAR_1$, which could be inhibited by aprotinin [39]. Knockdown of $PAR_1$ by siRNA and inhibition of $PAR_1$ by pharmacological antagonist indicated that TK can directly activate the $PAR_1$-mediated signaling pathway, independent of kinin B2 receptor activation.

### MMP-mediated release of EGFR ligands induced by intracellular signaling

Our results demonstrated that MMP inhibitor (GM) blocked TK-induced HaCaT keratinocyte migration, indicating that MMPs were involved in the TK-dependent signaling events. As previously reported, numerous MMPs, especially membrane-type MMPs (MT-MMPs) such as MMP3, MMP7, disintegrin and metalloproteinase 9 (ADAM9), ADAM10 and ADAM12, are functional in the process of EGFR ligand shedding [14,40]. Heparin binding factors of EGF family are also reported to directly stimulate keratinocyte growth [41,42]. Angiotensin II-induced keratinocyte migration is known to be initiated by activation of GPCR angiotensin II receptor, heparin-related EGF ligand shedding, such as HB-EGF, and subsequent activation of ERK [17,43]. HB-EGF is thought to be required for breast carcinoma cell migration and GPCR-mediated EGFR transactivation, which is tightly coupled with MMP-mediated ligand release [44,45,46]. EGFR contains a single trans-membrane domain whose intracellular portion harbors the tyrosine kinase domain and the extracellular portion contains a ligand binding domain [14]. Using antibodies for EGFR external segment (LA1), and EGFR ligands including HB-EGF and amphiregulin (AR), our results confirmed that MMP-mediated shedding of EGFR ligand was responsible for TK-induced EGFR transactivation [47,48]. Though the exact identity of the MMP responsible for the release HB-EGF and AR has not been identified, MMP3 and MMP7 have recently been recognized as MMPs capable of cleaving membrane-anchored HB-EGF at a specific juxta-membrane sequence [49]. LA1 completely inhibited TK-induced keratinocyte migration and ERK phosphorylation and significantly decreased TK-induced EGFR phosphorylation, while antibodies for HB-EGF and AR partly decreased TK-induced keratinocyte migration and EGFR phosphorylation, suggesting that HB-EGF and AR are both involved in this process. Moreover, TK also significantly increased HB-EGF and AR synthesis, demonstrating that constant EGFR activation also attributes to increased EGFR

ligand synthesis. Taken together, these findings indicate that TK promotes skin cell migration through EGFR transactivation by MMP-mediated-HB-EGF and AR shedding.

### HB-EGF and amphiregulin (AR) are both required ligands that bind with EGFR

Our results indicated that heparin-binding site was required for EGFR-binding ligands to bind with EGFR. HB-EGF and AR are both involved in TK-induced transactivation of EGFR in keratinocytes. We showed that HB-EGF and AR antibodies had no apparent affect on TK-induced ERK phosphorylation, whereas heparin and ERFR antibody (LA1) did. We speculate that this is attributed to activation of EGFR by multiple independent heparin-related ligands, such as HB-EGF and AR. Therefore, inhibition of AR alone by its antibody can not prevent HB-EGF from binding to EGFR and thus ERK activation. Similarly, inhibition of HB-EGF binding to EGFR alone has no effect on AR-induced ERK activation. However, LA1's ability to completely block EGFR and ERK phosphorylation may possibly be due to masking of HB-EGF and AR binding sites on EGFR. Although a previous report showed that AR, but not HB-EGF, regulates EGFR transactivation in keratinocytes [47], our current findings are consistent with the report that AR and HB-EGF were both rapidly and markedly elevated in regenerative wound healing model [50]. The potential role of TGF-α as a ligand of EGFR in keratinocytes awaits further study.

### Intracellular activation of PKC-Src links $PAR_1$ activation with MMP-mediated EGFR transactivation

As previously reported, activity of PKC and Src kinase was involved in thrombin or EGF-induced cell migration through EGFR transactivation [12,51,52]. Our data showed that Chelerythrine and PP2 effectively inhibit EGFR activation, ERK phosphorylation and keratinocyte migration, demonstrating that PKC and Src acted upstream of EGFR transactivation induced by TK. Due to the fact has been verified that MMP activation is regulated by PKC and Src phosphorylation in GT1–7 neurons [53], PKC and Src activity appear to be linked with the activation of MMPs in our study. Furthermore, Src is also involved in downstream of EGF-mediated keratinocyte migration.

In conclusion, we demonstrate that TK promotes keratinocyte migration and skin wound closure through a novel trans-membrane mechanism: $PAR_1$-mediated PKC-Src signaling pathway, leading to EGFR transactivation resulting from MMP-induced EGFR ligand shedding. Keratinocyte migration, which is independent of kinin B2 receptor activation, is one of the multiple processes involved in skin wound healing. Our study indicates the potential of therapeutic application of TK for patients with peripheral arterial occlusive disease as well as those who possess difficulty in dermal wound healing such as deep vein thrombosis, diabetes, pressure sores and burns.

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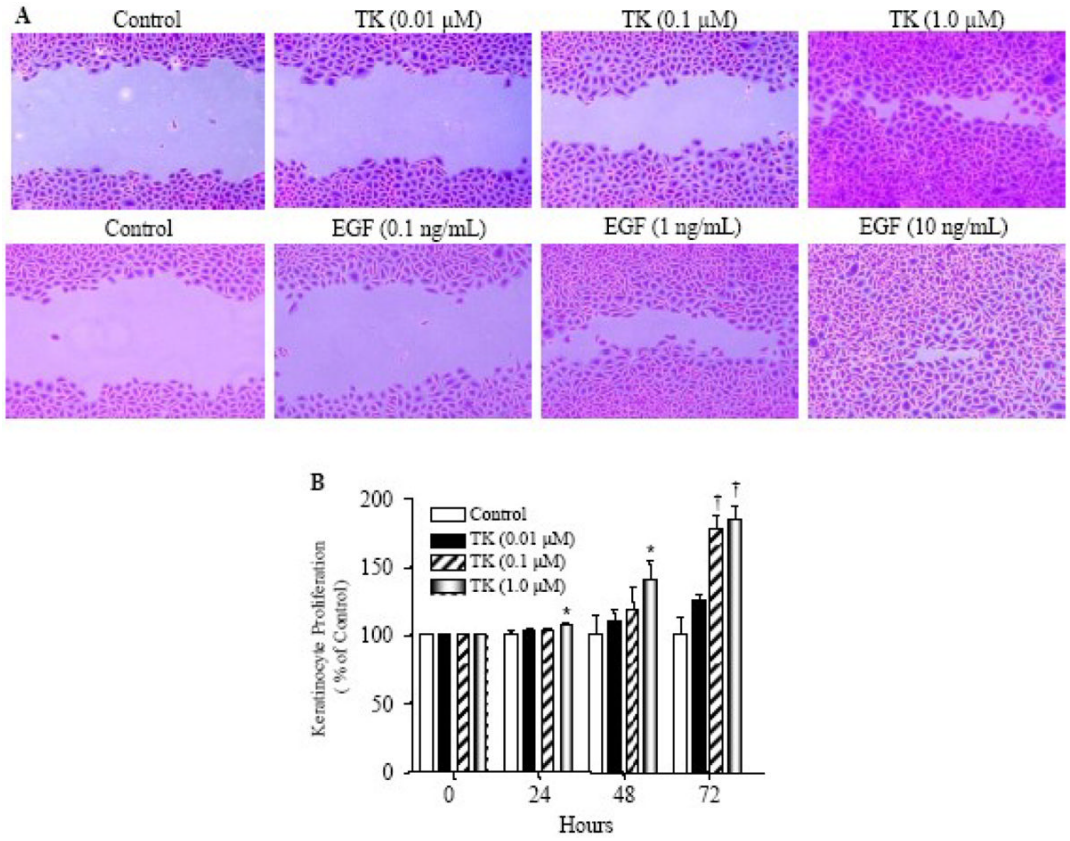


**Fig. 1.**

TK promotes skin wound healing by stimulating HaCaT cell migration and proliferation in a time- and **concentration**-dependent manner. (A) Treatment with TK for 48 hours stimulated cell migration in a **concentration**-dependent manner by scratch method (upper lane). Treatment with EGF for 36 hours stimulated cell migration in a **concentration**-dependent manner by the scratch method (lower lane). (B) TK enhanced cell proliferation in a time- and **concentration**-dependent manner. Proliferation was quantified by Promega CellTiter Aqueous One Solution Proliferation Assay. Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. **P*<0.05 vs. Control at 24 and 48 hours, †*P*<0.01 vs. Control & TK (0.01 μM) at 72 hours.

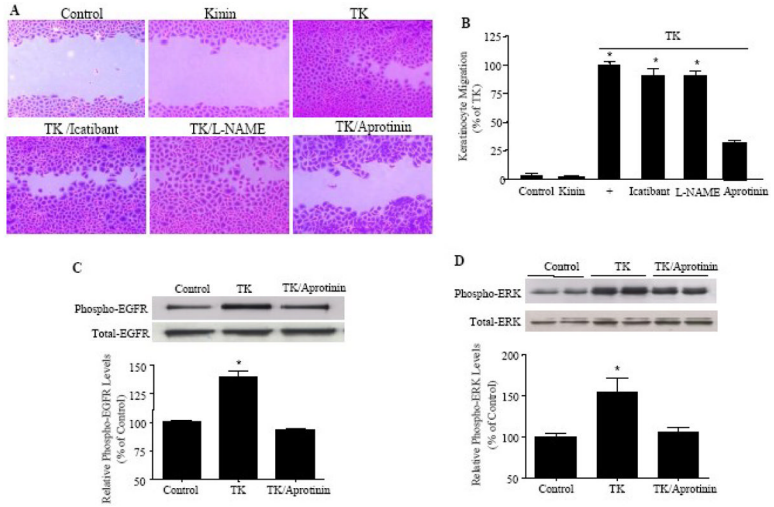


**Fig. 2.**

Active TK, but not inactive form, induces HaCaT cell migration independent of kinin, kinin B2 receptor and NO formation, and stimulates EGFR and ERK phosphorylation. (A) Treatment with kinin (1 μM) or inactive TK (1 μM) for 48 hours had no effect on cell migration by scratch method. Cells were pretreated with kinin B2 receptor antagonist (icatibant, 200 μM) or NOS inhibitor (L-NAME, 200 μM) for 45 minutes, and then stimulated with TK (1 μM) for 48 hours. (B) TK (1 μM), but not kinin (1 μM), stimulated cell migration after 18 hour incubation in modified Boyden chambers. Aprotinin (5 μM), but not icatibant (200 μM) or L-NAME (200 μM) blocked TK-induced cell migration. (C) Stimulation with active TK (1 μM), but not inactive TK (1 μM), for 15 minutes induced EGFR phosphorylation as determined by immunoprecipitation and Western blot. (D) Stimulation with active TK (1 μM), but not inactive TK (1 μM) for 15 minutes, induced ERK phosphorylation. Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. **P*<0.01 vs. Control, Kinin, and TK/Aprotinin.

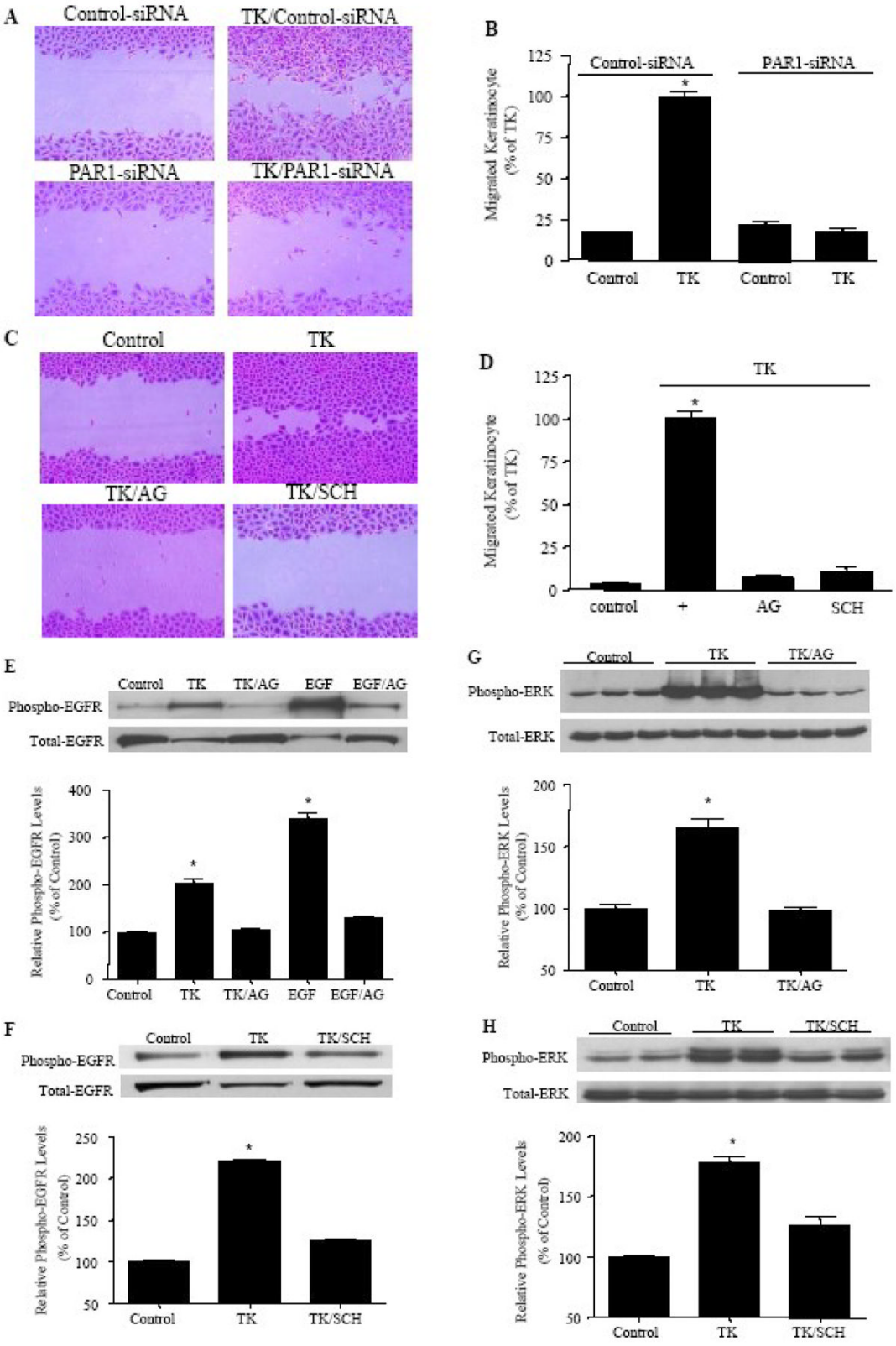


**Fig. 3.**

$PAR_1$-siRNA, EGFR antagonist (AG) and $PAR_1$ antagonist (SCH) block TK-induced HaCaT cell migration through EGFR and ERK phosphorylation. (A) Knockdown of $PAR_1$ with siRNA abolished TK-induced cell migration by scratch method. Cells were transfected with $PAR_1$-siRNA for 48 hours, followed by TK (1 μM) incubation for another 48 hours. (B) TK-induced cell migration was blocked by $PAR_1$-siRNA transfection using modified Boyden chambers. Cells were transfected with $PAR_1$-siRNA for 48 hours, then transferred to modified Boyden chamber and incubated with TK (1 μM) for another 18 hours. (C) AG and SCH diminished TK-induced cell migration by scratch method. Cells were pretreated with AG (300 nM) and SCH (1 μM) for 45 minutes, then stimulated with TK (1 μM) for 48 hours. (D) AG and SCH blocked kallikrein-induced cell migration in modified Boyden chambers. Cells were incubated with TK (1 μM) in the presence or absence of AG (300 nM) and SCH (1 μM) for 18 hours. (E) AG and (F) SCH blocked TK-induced EGFR phosphorylation as determined by immunoprecipitation and Western blot. (G) AG and (H) SCH blocked TK-induced ERK phosphorylation as determined by Western blot. (E – H) cells were pretreated with AG (300 nM) or SCH (1 mM) for 45 minutes, then stimulated with TK (1 μM) for 15 minutes. Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. *$P$<0.01 vs. other groups.

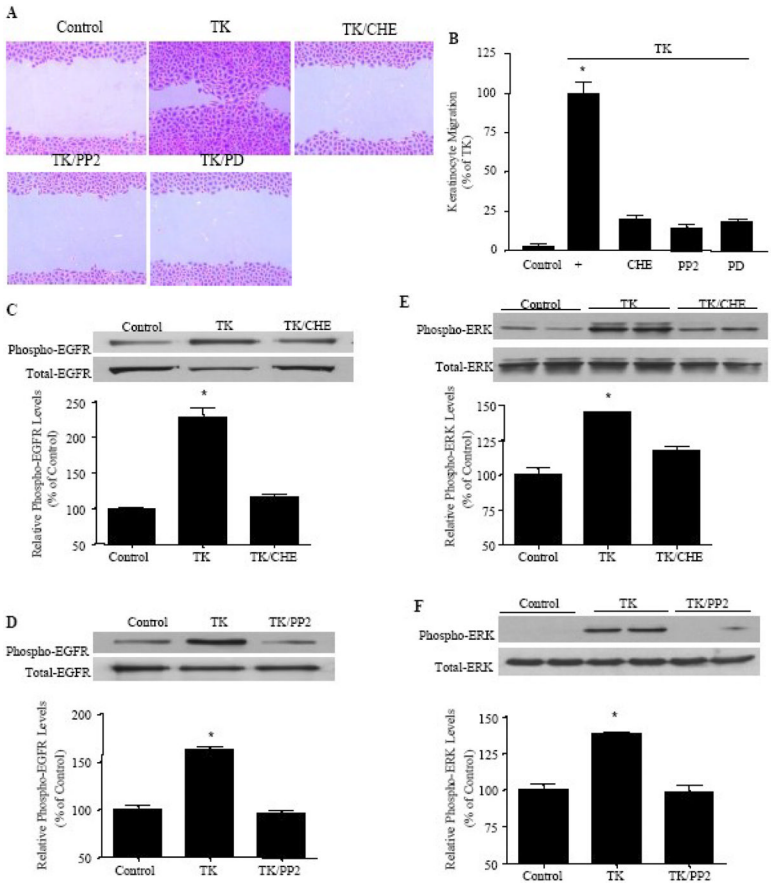


**Fig. 4.**

PKC and Src activities are required for EGFR and ERK phosphorylation-mediated HaCaT cell migration induced by TK. (A) PKC antagonist (CHE), Src antagonist (PP2) and ERK antagonist (PD) blocked TK-induced cell migration by scratch method. Cells were pretreated with CHE (5 μM), PP2 (5 μM) and PD (5 μM) for 45 minutes, then stimulated with TK (1 μM) for 48 hours. (B) CHE, PP2 and PD blocked TK-induced cell migration in modified Boyden chambers. Cells were treated with TK (1 μM) in the presence or absence of CHE (5 μM), PP2 (5 μM) and PD (5 μM) for 18 hours. (C) CHE and (D) PP2 blocked TK-induced EGFR phosphorylation as determined by immunoprecipitation and Western blot. (E) CHE and (F) PP2 blocked TK-induced ERK phosphorylation as determined by Western blot. (C – F) cells were pretreated with CHE (5 μM) or PP2 (5 μM) for 45 minutes, then stimulated with TK (1 μM) for 15 minutes. Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. **P*<0.01 vs. other groups.

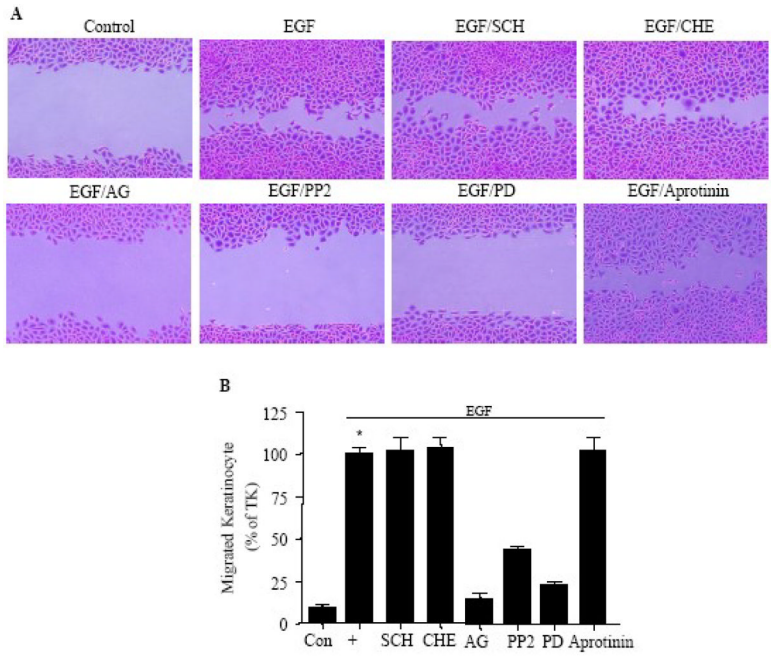


**Fig. 5.**

EGFR, Src or ERK activition are required for EGF-induced HaCaT cell migration. (A) EGFR antagonist (AG), Src antagonist (PP2) and ERK antagonist (PD), but not $PAR_1$ antagonist (SCH), PKC antagonist (CHE) or aprotinin blocked TK-induced cell migration by scratch method. Cells were pretreated with AG (300nM), SCH (1 μM), CHE (5 μM), PP2 (5 μM) or PD (5 μM) for 45 minutes, then stimulated with EGF (5 ng/mL) for 48 hours. (B) AG, PP2 and PD, but not SCH, CHE or aprotinin blocked EGF-induced cell migration in modified Boyden chambers. Cells were treated with EGF (5 ng/mL) in the presence or absence of AG (300nM), SCH (1 μM), CHE (5 μM), PP2 (5 μM) or PD (5 μM) for 18 hours. Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. **P*<0.01 vs. Control, AG, PP2 and PD.

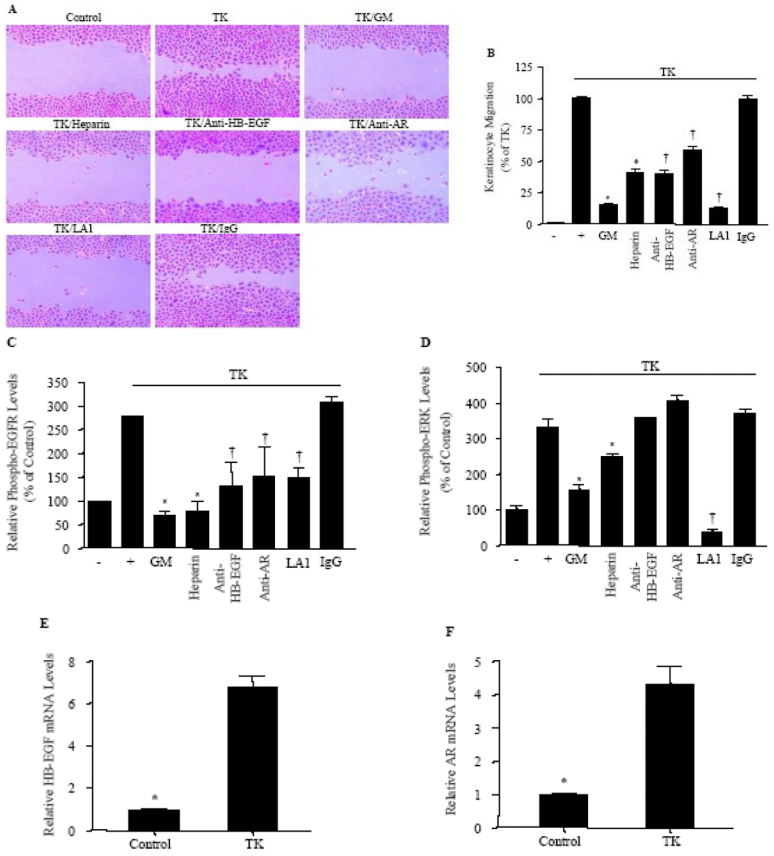


**Fig. 6.**

TK promotes HaCaT cell migration, EGFR and ERK phosphorylation through MMP-mediated shedding of EGFR ligands. TK-induced cell migration was blocked by MMP antagonist (GM) and EGFR extracellular domain antibody (LA1), and partially inhibited by heparin, HB-EGF antibody and AR antibody by (A) scratch method and (B) modified Boyden chambers. (A) Cells were pretreated with GM6001 (GM, 5 μM), heparin (100 ng/ml), HB-EGF antibody (20 μg/ml), AR antibody (20 μg/ml), LA1 (20 μg/ml) and normal mouse IgG (20 μg/ml) for 45 minutes, then stimulated with TK (1 μM) for 48 hours. (B) Cells were incubated with TK (1 μM) in the presence or absence of GM (5 μM), heparin (100 ng/ml), HB-EGF antibody (20 μg/ml), AR antibody (20 μg/ml), LA1 (20 μg/ml) and normal mouse IgG (20 μg/ml) for 18 hours. (C) TK-induced EGFR phosphorylation was inhibited by GM, heparin, HB-EGF antibody, AR antibody and LA1 as determined by immunoprecipitation and Western blot. (D) TK-induced ERK phosphorylation was inhibited by GM, heparin and LA1, but not by HB-EGF antibody or AR antibody as determined by Western blot. (C & D) cells were pretreated with GM (5 μM), heparin (100 ng/ml), HB-EGF antibody (20 μg/ml), AR antibody (20 μg/ml), LA1 (20 μg/ml) and normal mouse IgG (20 μg/ml) for 45 minutes, then stimulated with TK (1 μM) for 15 minutes. (E & F) HB-EGF and AR gene expression in HaCaT cells treated with TK (1 μM). Three independent measurements were performed in triplicate. Values are represented as mean ± SEM. **P*<0.01 vs. other groups, †*P*<0.01 vs. TK/IgG.

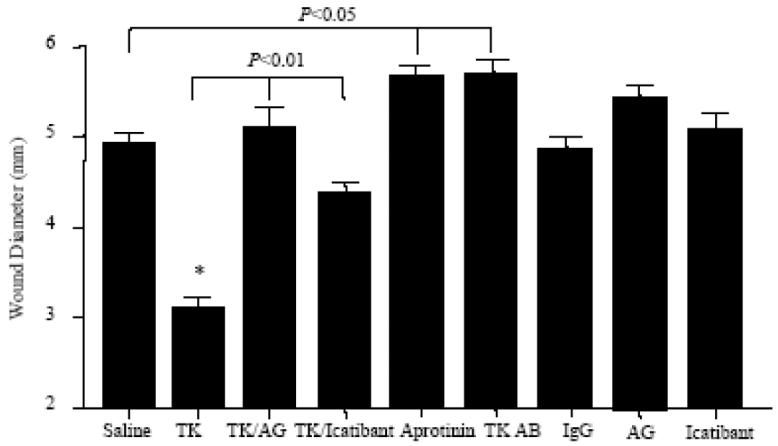


**Fig. 7.**

TK promoted skin wound healing in an excisional skin wound rat model. Values were obtained from 18 wounds in 3 rats per group (n=18). **P*<0.001 vs. other groups.

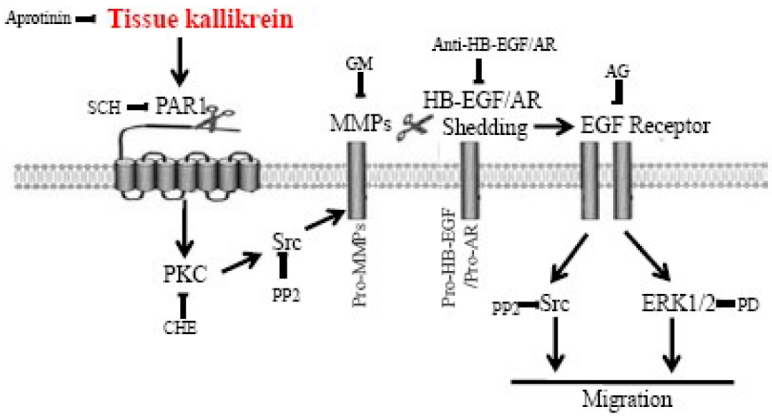


**Fig. 8.**
Schematic diagram of TK-mediated signaling pathway promoting HaCaT keratinocyte migration.