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Src-Family Tyrosine Kinases in Wound- and Ligand-Induced Epidermal Growth Factor Receptor Activation in Human Corneal Epithelial Cells

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Abstract

Purpose—The authors have previously demonstrated that wounding of human corneal epithelial cells (HCECs) transactivates epidermal growth factor (EGF) receptor (EGFR) and its downstream signaling pathways and that this EGFR signaling is required for epithelial wound healing. In this study, the authors sought to identify the underlying mechanisms for EGFR transactivation in response to wounding in HCECs.

Methods—SV40-immortalized HCEC (THCE) monolayer was wounded and allowed to heal in the presence or absence of a selective inhibitor of the Src family kinases PP2 and EGFR ligand heparin-binding EGF-like growth factor (HB-EGF). Wound closure was monitored by photographing of the injury immediately or 24 hours after wounding. Activation of EGFR in THCE cells and in primary HCECs was analyzed by immunoprecipitation of EGFR, followed by Western blotting with phosphotyrosine antibody. Phosphorylation of extracellular signal–regulated kinase (ERK), AKT (a major substrate of phosphatidylinositol 3′-kinase [PI3K]), Src at tyrosine Y416, and EGFR at Y845 was analyzed by Western blotting with antibodies specific to phosphorylated proteins. Effects of PP2 on THCE cell migration were determined by Boyden chamber migration assay.

Results—Among several inhibitors tested, PP2 blocked wound-induced EGFR phosphorylation in THCE cells. PP2 at 12.5 μM effectively inhibited EGFR transactivation in response to wounding and to the phosphorylation of ERK and AKT in THCE cells and primary HCECs. Consistent with the inhibition of EGFR transactivation, PP2 also attenuated epithelial migration and wound closure with or without exogenously added HB-EGF. PP2 at a concentration as high as 50 μM exhibited no effects on HB-EGF induced ERK phosphorylation. On the other hand, AKT phosphorylation was much more sensitive to PP2 than ERK or EGFR phosphorylation because 3.13 μM PP2 effectively inhibited wound- or HB-EGF-induced AKT phosphorylation.

Conclusions—These results suggest that Src kinase mediates wound-induced EGFR transactivation and participates in a pathway to activate the PI3K-AKT pathway downstream of EGFR in HCECs.

Corneal epithelial cells respond rapidly to injury, resulting in a healing process of cell migration as a sheet to cover the defect and to reestablish epithelial barrier function.[1] Critical for wound healing are cell migration and proliferation, which are driven by growth factors and cytokines released coordinately into the injured bed. Prominent among these epithelium-derived factors are ligands for the epidermal growth factor receptor (EGFR), the EGF family.[1] Numerous

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studies have shown that epithelial wound healing is, at least in part, mediated by EGFR-ligand (EGF) interactions in an autocrine fashion.[1–3] We recently showed that wounding induces EGFR transactivation through ectodomain shedding of heparin-binding EGF-like growth factor (HB-EGF) and that this wound-induced activation of EGFR and its coreceptor, erbB2, is required for wound closure in cultured HCECs and in cultured porcine corneas.[4,5]

HB-EGF is synthesized as a type 1 transmembrane protein that can be shed enzymatically to release a soluble 14- to 20-kDa growth factor; the process has been termed *ectodomain shedding*.[6–8] The transmembrane form of HB-EGF acts in a juxtacrine manner to signal neighboring cells.[9] The soluble form of HB-EGF is a potent mitogen and chemoattractant for many types of cells, including keratinocytes and other epithelial cells.[10,11] The released HB-EGF acts through the stimulation of specific cell-surface receptors.[12,13] Four related receptor tyrosine kinases have been identified.[14–16] These are EGFR/erbB1/HER1, erbB2/HER2/neu, erbB3/HER3, and erbB4/HER4.[12] Shed EGFR ligands such as HB-EGF act as autocrine/paracrine EGFR ligands and stimulate EGFR activation. Phosphorylation of EGFR creates docking sites for adaptor proteins such as Grb2, Shc, and Gab1 and leads to activation (tyrosine phosphorylation) of effectors such as extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K). These two pathways have been shown to be involved in corneal epithelial wound healing.[17–22] Ectodomain shedding is a highly regulated process.[23] The mechanism by which intracellular signaling pathways lead to an increase in sheddase activity has not been fully established. PKC or intracellular Ca^{2+} pathways are shown to up-regulate the activity of sheddase.[24–27] Src nonreceptor tyrosine kinases have also been suggested as upstream mediators of EGFR transactivation,[28] particularly in response to the stimulation of G-protein–coupled receptor ligands.[29,30]

Recently, Src was found to be activated in cells along the wounding edge of cultured mouse corneal epithelial cells; blocking this activation with the Src kinase inhibitor, PP_1, inhibited wound closure.[31] *c-Src* is the prototype of a closely related family of nine genes encoding nonreceptor membrane–associated protein tyrosine kinases (PTKs). In a variety of cell types, Src family members participate in the regulation of diverse functions, including proliferation, cell cycle, migration, adhesion, and differentiation.[32] Only three of the nine members of the Src family are found in epithelial cells, and these include Src, Fyn, and Yes, which are ubiquitously expressed.[32] The members of the Src family have a similar structure and share common pathways of regulation and function.[33,34] They are also integral components of the signal transduction apparatus used by growth factor receptor tyrosine kinases.[35]

To determine signaling pathways regulating wound-induced EGFR transactivation, we used several inhibitors and found that PP2, a selective inhibitor of the Src family kinases, attenuated wound-induced EGFR transactivation and wound closure in cultured human corneal epithelial cells with or without exogenously added EGFR ligands. We also investigated the effects of Src inhibition on EGFR downstream signaling pathways. The study suggests that Src mediates wound-induced EGFR transactivation and plays a role in growth factor–mediated corneal epithelial cell migration and wound closure.

Materials and Methods

Materials

Keratinocyte basic medium (KBM) and keratinocyte growth medium (KGM; KBM supplemented with bovine pituitary extract, epinephrine, hydrocortisone, transferrin, insulin, and EGF) were from BioWhittaker (Walkersville, MD). Human recombinant HB-EGF was obtained from R&D Systems (Minneapolis, MN). Antibodies against human EGFR, ERK2 (p42 MAPK), phospho-ERK1/2 (p42/p44), c-Src (Src 2), pY20, and pY99 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against a major substrate of PI3K—AKT,

phospho-AKT, and phospho-Src (Y416)—were obtained from Cell Signaling (Beverly, MA). Rabbit anti-EGFR (Y845) was from Biosource (Camarillo, CA). The EGFR inhibitor tyrphostin AG 1478 was from Sigma-Aldrich (St. Louis, MO). The Src inhibitor PP2 (4-amino-5-(4-chloro-phenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine), the phospholipase C (PLC) inhibitor U73122, and the PI3K inhibitor LY294002 were from Calbiochem (La Jolla, CA). Boyden chamber and polycarbonate membranes (14-μm pores) were purchased from Neuroprobe (Cabin John, MD) and Osmonics, Inc. (Livermore, CA), respectively. Fibronectin collagen coating mix (FNC) was from Biological Research Faculty & Facility (Ljamsville, MD). All other chemicals were purchased from Sigma-Aldrich.

Cell Culture and Migration Studies

THCE cells, an accepted SV40-immortalized corneal epithelial cell line, were generously provided by Kaoru Araki-Sasaki.[36] THCE cells were grown in KGM in a humidified 5% CO_2 incubator at 37°C. For wounding experiments, cells were seeded onto 12-well plates or 100-mm culture dishes coated with FNC.

For in vitro wound closure assay, THCE cells were grown to 80% confluence in 12-well tissue culture plates. Cells were then starved in KBM overnight and wounded with a sterile 0.1- to 10-μL pipette tip (TipOne; USA Scientific, Ocala, FL) to remove cells by two perpendicular linear scrapes. After washing away suspended cells, the cells were refed with KBM in the presence or absence of HB-EGF (50 ng/mL) with or without PP2 (12.5 μM). The progress of migration was photographed immediately or 24 hours after wounding near the crossing point with an inverted microscope equipped with a digital camera (Spot Digital Camera; Diagnostic Instruments, Inc., Sterling Heights, MI).

To confirm the results obtained from THCE cells, primary HCECs were isolated from human donor corneas obtained from the Michigan Eye Bank. The epithelial sheet was separated from underlying stroma after overnight dispase (2.5 U/mL; Sigma) treatment at 4°C. The dissected epithelial sheet was trypsinized, and cells were collected by centrifugation (500*g*, 5 minutes). Primary HCECs were cultured in T25 flasks coated with FNC and used at passage 3 or 4.

Determination of EGFR Phosphorylation

To determine EGFR tyrosine phosphorylation, growth factor–starved THCE cell monolayers on 100-mm dishes were pretreated with PP2, U73122 (5 μM), LY294002 (40 μM), or AG1478 (1 μM) for 1 hour and then wounded by multiple linear scratches using a cut of 48-well sharks tooth comb for DNA sequencing (Bio-Rad, Hercules, CA) going from one side to the other of the dish. The dish was then rotated, and scrapes were made the same way at 45°, 90°, and 135° to the original scrapes. Cells with no scrape wound were used as control. Damaged cells were washed away before the cells were fed with fresh KBM. Cells were cultured for the indicated time. At the end of culture, cells were lysed with RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktails, and 0.1 mM phenylmethylsulfonyl fluoride). For each sample, 600 μg proteins were immunoprecipitated with 4 μg antibody against EGFR (agarose conjugated) and immunoblotted with mouse anti-pY99 antibody (1:4000). ERK2 and ERK1/2 phosphorylation were determined using monoclonal antibodies against ERK2 (1:4000) or phospho-ERK1/2 (1:500 dilution).

Boyden Chamber Analysis of Cell Migration

A 48-well Boyden chamber was used to measure the migratory response of THCE cells to HB-EGF. Cultured cells were starved in KBM overnight and pretreated with or without PP2 (12.5 μM) in KBM for 1 hour. Before treatment, cells were detached by 0.05% trypsin and 0.53 mM

EDTA and washed with 10% fetal bovine serum to neutralize the trypsin, and the number of cells was adjusted to 3.6×10^5/mL. A polycarbonate membrane (14-μm pore size; Osmonics, Inc.) was coated with FNC on the surface facing the lower chambers and air dried. The bottom chambers were then filled with KBM with and without 50 ng/mL HB-EGF as a chemoattractant. PP2-treated and untreated cells were placed into each of the top chambers, separated from the bottom chambers by the polycarbonate membrane. Cells were then incubated at 37°C in 5% CO_2 and allowed to migrate for 3 hours. After incubation, cells on the top surface of the polycarbonate membrane were scraped off. The membrane was then stained with a modified stain kit (Diff-Quik; Dade Behring Inc., Dudingen, Switzerland). Cell migration was quantified as the number of migrated cells on the lower surface of the polycarbonate membrane in three random fields of ×400 magnification.

Results

We previously demonstrated that wounding of HCECs induces EGFR transactivation and activation of its downstream signaling pathways in an HB-EGF shedding-dependent manner.[5] To determine the mechanisms underlying EGFR transactivation in response to wounding, we assessed the role of Src, PLC, and PI3K in wound-induced EGFR transactivation. These pathways are known to be the downstream of EGFR signaling or are implicated in mediating EGFR transactivation in other cell types.[28–30,37,38] As shown in Figure 1, wound-induced phosphorylation of EGFR was attenuated by PP2, a potent inhibitor of the Src family tyrosine kinases,[39] in THCE cells. However, U73122, an inhibitor of PLC, and LY294002, an inhibitor of PI3K, did not affect wound-induced EGFR phosphorylation. As expected, AG1478, an inhibitor of EGFR kinase, slowed wound-induced EGFR phosphorylation in THCE cells.

To understand the roles of Src in corneal epithelial wound healing, we analyzed Src phosphorylation, which reflects its activation in THCE cells and in the primary culture of HCECs (Fig. 2). Src has two major phosphorylation sites at its C terminus, Y416 and Y527. Phosphorylation of Y416, but not Y527, activates Src.[40] Thus, phosphospecific antibody toward Src Y416 was used for the assessment of Src activity. As shown in Figure 2, a detectable increase in the phosphorylation of Src Y416 was observed 5 minutes after wounding, and the increase became more apparent 10 minutes after wounding. The levels of total c-Src remained largely unchanged during the time course of the study (Fig. 2A). Phosphorylation of EGFR Y845, a Src-mediated and EGFR ligand–dependent phosphorylation site, was also induced by wounding in THCE cells in parallel (Fig. 2A). Wounding also induced the phosphorylation of EGFR at Y845 and Src at Y416 in primary HCECs (Fig. 2B). Although EGFR phosphorylation was sensitive to EGFR and Src inhibitors, only PP2, but not AG1478, attenuated wound-induced Src Y416 phosphorylation to a level similar to the basal level in unwounded primary HCECs, suggesting that Src kinases may act as upstream mediators of EGFR signaling in HCECs in response to wounding.

To determine the effect of Src activation on wound-induced EGFR signaling, a dose-dependent inhibition of PP2 was performed. The results shown in Figure 3 indicated that 12.5 μM PP2 was sufficient to inhibit wound-induced EGFR phosphorylation (total phosphorylation), whereas phosphorylation of EGFR at the Src-related site Y845 was more sensitive to PP2 inhibition, with apparent inhibition of wound-induced EGFR Y845 phosphorylation observed at 3.13 μM PP2. As was the total phosphorylation of EGFR, wound-induced ERK phosphorylation was inhibited by 12.5 μM PP2, suggesting that wound-induced ERK phosphorylation is EGFR dependent. Interestingly, wound-induced phosphorylation of AKT was more sensitive to PP2 than that of total EGFR or ERK in THCE cells, suggesting that Src may function as a bridge between EGFR activation and PI3K-AKT pathway in HCECs in response to wounding.

We previously showed that EGFR transactivation was required for wound closure in cultured bovine corneas and in THCE cells and that exogenously added EGFR ligands, such as HB-EGF, enhanced epithelial wound healing.[5] To determine the effects of Src inhibition on spontaneous wound healing, confluent THCE cells were scratch wounded and allowed to heal in the presence or absence of PP2 for 24 hours (Fig. 4). PP2 in the culture medium inhibited spontaneous wound healing. This result is consistent with results of a previous study reporting that the inhibition of Src with PP1, a close relative of PP2, inhibited the closure of a scratch wound of cultured mouse corneal epithelial cells by 85%.[31] To determine the effects of PP2 on EGFR ligand-enhanced wound closure, wounded cells were cultured in the presence of HB-EGF with or without PP2 (Fig. 4). HB-EGF accelerated HCEC wound closure, as reported previously,[5,41] and PP2 slowed epithelial wound closure in the presence of HB-EGF.

The effects of PP2 on HCEC migration were determined by Boyden chamber migration assay. We first tested spontaneous haptotactic migration of HCECs toward FNC (a 1:3 mix of fibronectin and collagen 1) with no EGFR ligands added. As shown in Figure 5, the presence of PP2 delayed migration to some extent; however, the effects of the Src inhibitor on haptotactic migration of HCECs in basal medium was not significant. We then tested chemotaxis induced by HB-EGF. The presence of HB-EGF in the bottom chamber increased the migration of THCE cells, and pretreatment of PP2 significantly reduced chemotactic response of the cells to HB-EGF to a level similar to that of the control.

Inhibition of wound-induced EGFR transactivation by PP2 suggests that Src acts upstream of EGFR activation. On the other hand, PP2 inhibition of HB-EGF–induced HCEC migration and wound closure implicates that Src functions as a downstream mediator of EGFR signaling. To assess the effects of PP2 on EGFR signaling, ligand-induced EGFR activation and downstream ERK and AKT phosphorylation were determined (Fig. 6). HB-EGF induced massive EGFR phosphorylation and degradation, as evidenced by the reduced amount of EGFR precipitated from HB-EGF–treated THCE cells and primary HCECs. In the presence of PP2, a significant amount of EGFR phosphorylation was still observed, whereas EGFR degradation was not affected by PP2 in THCE cells or primary HCECs (Fig. 6A). HB-EGF induced site-specific tyrosine phosphorylation of EGFR was also examined (Fig. 6B). Similar to total EGFR phosphorylation, HB-EGF–induced phosphorylation at EGFR Y1068 was not affected by PP2. PP2, however, inhibited HB-EGF–induced Y845 phosphorylation at 25 μM, a concentration higher than that needed for blocking wound-induced Y845 phosphorylation. Similar to total and Y1068 phosphorylation of EGFR, PP2 exhibited little effect on HB-EGF–induced ERK1/2 phosphorylation at 25 μM; however, HB-EGF–induced AKT phosphorylation was completely inhibited by PP2 at a concentration as low as 3.13 μM.

Discussion

Stimulation of the EGFR by its ligands initiates the activation of ERK and AKT, two important signaling effectors. Wounding also activates EGFR, ERK1/2, and PI3K-AKT through the release of HB-EGF as an endogenous ligand; however, the mechanisms by which wounding triggers the activation of these signaling pathways are incompletely understood. In this study, we used a commonly used inhibitor of the Src family kinases, PP2, and demonstrated that Src regulated epithelial wound closure by mediating wound-induced EGFR transactivation and its downstream signaling pathways, including ERK and PI3K-AKT pathways. In addition to attenuating EGFR transactivation–mediated wound closure, Src kinase inhibition also blocked HB-EGF–enhanced cell migration and wound healing, but not HB-EGF–induced EGFR activation, indicating that one or more EGFR downstream signaling pathways required for wound healing were affected by Src inhibition. Further analysis of the effects of Src kinase inhibition on EGFR downstream signaling revealed that Src had minimal effect on HB-EGF–induced ERK1/2 activation, in contrast to that induced by wounding, whereas HB-EGF–

induced AKT phosphorylation appeared to require Src activity. These findings provide new insight into the molecular mechanisms by which Src activates separate pathways influencing upstream or downstream EGFR. The potential involvement of Src kinases in participating HCEC wound healing and EGFR signaling is summarized in Figure 7.

How might wounding trigger EGFR transactivation in HCECs? Several intracellular signaling pathways, such as PKC, Ca^{2+}, and Src, have been suggested in EGFR transactivation.[24–30] In this study, we demonstrated that the pathophysiological condition, wounding, induced Src phosphorylation at Y416, which is the indicator for Src activation. Inhibition of Src activation by PP2 prevented this wound-induced Src tyrosine phosphorylation and EGFR activation, suggesting that Src participates in mediating EGFR transactivation in response to wounding in HCECs. Recent studies revealed that EGFR transactivation induced by G-protein–coupled receptor ligands and by pathophysiological stimuli occur primarily through autocrine/paracrine release of soluble EGFR ligands from transmembrane pro-form, a process termed *ectodomain shedding* and known to be catalyzed by ADAM (a disintegrin and metalloprotease) proteins.[42–45] Pro-HB-EGF has been shown to be a common EGFR ligand subjected to ectodomain shedding, leading to the transactivation of EGFR in a variety of cells.[37,46,47] Using the matrix metalloproteinase inhibitor GM6001 and the HB-EGF inhibitor CRM 197, we previously showed that wound-induced EGFR activation occurred through shedding of pro-HB-EGF by a metalloprotease-sensitive process in corneal epithelial cells.[5] Because PP2 does not inhibit EGFR activation induced by exogenously added HB-EGF, we suggest that Src is an upstream signaling molecule for HB-EGF ectodomain shedding in wounded HCECs.

Consistent with the essential role of EGFR transactivation in mediating corneal epithelial wound healing, we observed that PP2 blocked scratch wound closure in cultured HCECs. A previous study[31] revealed that Src was activated in corneal epithelial cells along the wound edge and that blocking this activation with PP1 inhibited wound closure. Furthermore, it formed a complex with Cdk5, a member of the cyclin-dependent kinase family, to regulate epithelial cytoskeletal reorganization and cell migration. Our study suggested another essential role of Src for wound closure of HCECs in vitro: to regulate the release of soluble HB-EGF in response to wounding. Given that the inhibition of Src activation slowed wound-induced EGFR transactivation, the presence of PP2 in the culture medium, as expected, attenuated wound closure in cultured THCE cells. Interestingly, the inhibition of Src kinase activity by PP2 also blocked HB-EGF–induced HCEC wound closure, but not EGFR activation, suggesting that Src also acts as a downstream regulator for EGFR signaling.

ERK1/2 and PI3K are two effectors of EGFR signaling pathways. Epithelial wounding resulted in rapid activation of these two pathways in an EGFR-dependent manner.[5] Inhibition of either pathway resulted in the slowing of epithelial wound closure in corneal epithelial cells.[5,19,48] Consistent with the concept that EGFR transactivation in wounded HCECs involves Src activation, PP2 at a concentration that inhibits the EGFR phosphorylation also blocked ERK1/2 phosphorylation in wounded cells. Interestingly, PP2 at a concentration that had minimal effect on wound-induced EGFR phosphorylation was able to block wound-induced AKT phosphorylation, suggesting a role for the Src family in activating PI3K and AKT. The sensitivity of PI3K-AKT pathway to PP2, independent of EGFR phosphorylation, was further observed in HB-EGF–induced EGFR signaling in HCECs. PP2 at 12.5 μM had minimal effect on HB-EGF–induced overall EGFR phosphorylation. If ERK phosphorylation in response to HB-EGF was assessed as an indicator of EGFR activation, PP2, up to 50 μM (tested; data not shown), exhibited no noticeable effects on the phosphorylation of ERK in HB-EGF–treated cells. Although HB-EGF induced strong phosphorylation of AKT, as little as 3.13 μM PP2 completely inhibited HB-EGF–induced AKT phosphorylation. The effects of PP2 were not on ATK levels because similar immunoreactivity was detected in cells treated with or without PP2. We could find studies showing that Src inhibition prevents AKT phosphorylation, but

not ERK phosphorylation, when EGFR is transactivated by G-protein–coupled receptor ligands.[49,50] However, we are not aware of Src inhibition leading to the inhibition of AKT activation induced by EGFR ligands, which usually induce much stronger EGFR activation than that induced by EGFR transactivation. AKT is the major substrate and downstream effector of PI3K, and its phosphorylation, a commonly used parameter for PI3K activation, has been shown to be PI3K dependent in HCECs.[4,5] Thus, to our knowledge, this is the first example of different requirements for EGFR ligand–induced activation of the ERK1/2 and PI3K-AKT pathways in the same cell type. Moreover, our findings support the conclusion that the signal generations by EGFR transactivation induced by wounding or direct HB-EGF binding are indistinguishable.

It is well established that PI3K-AKT and ERK signaling pathways are two key downstream intracellular signaling molecules of EGFR on ligand binding.[51,52] How might Src be involved in wound- or ligand-induced ATK phosphorylation? First, EGFR-mediated PI3K-AKT activation requires the phosphorylation of EGFR Y845 in HCECs. Phosphorylation, but not autophosphorylation, of this highly conserved tyrosine is mediated by c-Src or related kinases and is dependent on EGFR ligand stimulation.[53–55] PI3K, but not ERK, activation may require phosphorylation of EGFR at Y845; thus, the inhibition of Src leads to the blocking of PI3K, but not ERK, downstream signaling pathways of EGFR (trans)activation. Consistent with this assumption, mutation at this site was found to retain EGFR full kinase activity and its ability to activate the adapter protein SHC and ERK2 in response to EGF.[54] The effects of the mutation on PI3K-AKT activation have not been reported. Our study, however, showed that AKT phosphorylation is more sensitive to PP2 inhibition in response to HB-EGF stimulation than that of EGFR Y845 phosphorylation, suggesting that the activation PI3K-AKT pathway is unlikely to depend on phosphorylation on this site. In addition, Src might be directly activated by EGFR. The activated Src can directly phosphorylate PI3K, as suggested in COS7 cells stimulated with G-protein–coupled receptor ligand gonadotropin[50] and in isolated rabbit hearts stimulated with acetylcholine.[56] Src can also indirectly activate PI3K by phosphorylating IRS-1. Phosphor-IRS-1 in turn recruits and activates PI3K, as shown in Chinese hamster ovary cells in response to gastrin challenge.[57] Further studies are required to clarify the role of Src tyrosine kinases as downstream signaling mediators in response to wounding and EGFR ligand stimulation in HCECs.

Three members of the Src family, Src, Fyn, and Yes, are known to be ubiquitously expressed in epithelial cells.[32] Although the members of the Src family are generally believed to share common pathways of regulation and function,[33,34] these members may theoretically exert different effects on epithelial wound healing and EGFR activation. PP2 has been shown to be a potent and selective inhibitor of the Src family kinases; however, PP2 cannot differentiate among the various members of the Src family.[39] As such, it remains to be determined which member(s) of the Src family is involved in corneal epithelial wound healing. It is possible that a particular member is involved in EGFR transactivation and another one in the activation of EGFR-mediated PI3K-AKT pathway. Further studies, such as those making use of siRNA gene silencing or dominant-negative mutant expression, are required to clarify the issue.

In summary, the data presented here reveal that the nonreceptor Src tyrosine kinases play a role in corneal epithelial wound healing by regulating EGFR transactivation. Furthermore, wounding or HB-EGF induces the activation of PI3K-AKT and ERK pathways through EGFR activation, but Src family tyrosine kinases only participate in the pathway to activate the PI3K-AKT pathway.

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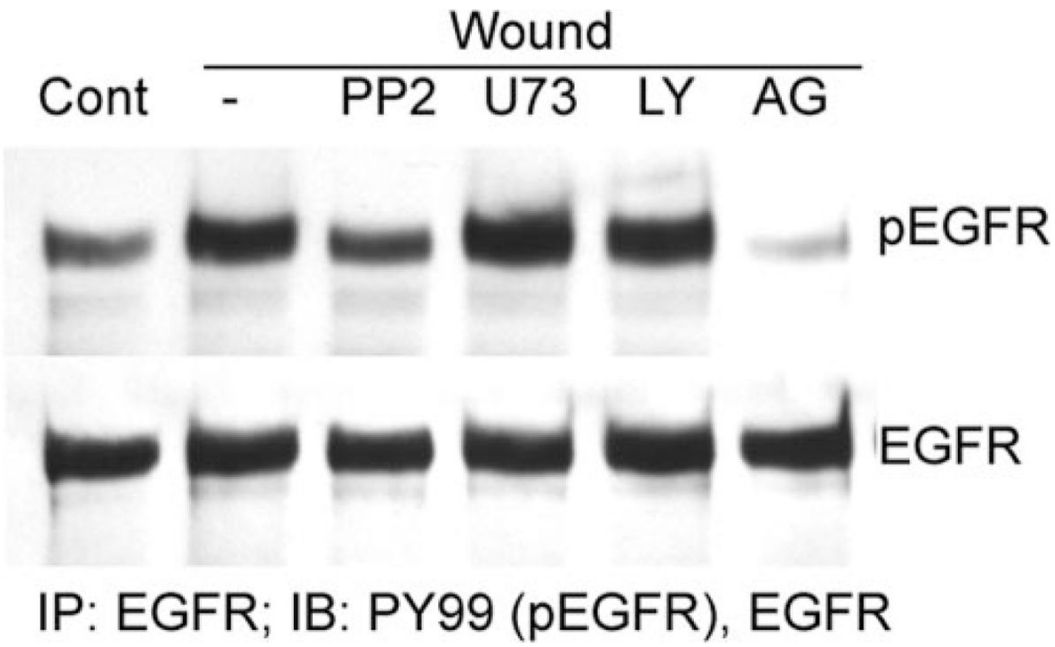

Figure 1.
Inhibition of wound-induced EGFR transactivation in HCECs by the Src inhibitor PP2. THCE cells were cultured in 100 mm dishes and starved in KBM overnight (growth factor starvation). Cells were pretreated with PP2 (12.5 μM), U73122 (5 μM), LY294002 (40 μM), and tyrphostin AG1478 (1 μM) for 1 hour, extensively injured by sequencing comb scratching, and incubated for 1 hour in KBM. Wounded THCE cells were then lysed, and 600 μg protein of each condition was immunoprecipitated (IP) with 10 μg agarose-conjugated rabbit anti-EGFR, subjected to SDS-PAGE, and immunoblotted with mouse anti–pY99 antibody (pEGFR). The stripped membrane was reprobed with EGFR antibody (EGFR) to assess the amount of protein precipitated.

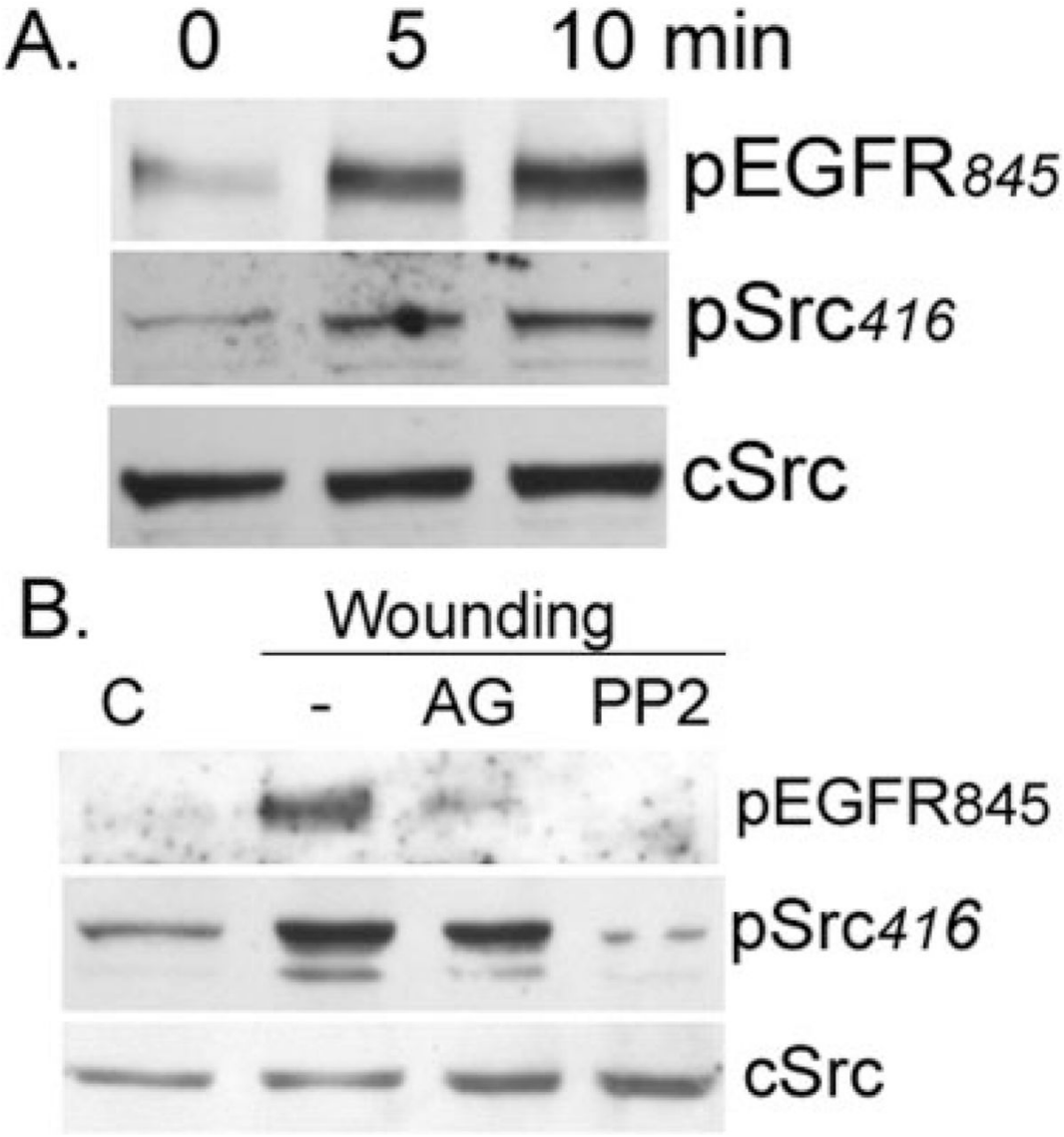

Figure 2.

Wound-induced, PP2-sensitive Src and EGFR phosphorylation in HCECs. THCE cells (**A**) or primary HCECs (p3) (**B**) were cultured in 100-mm dishes and starved in KBM overnight. Primary HCECs were pretreated with PP2 (12.5 μM), AG1478 (1 μM), or no pretreatment as control for 1 hour before wounding. Cells were then extensively injured by sequence comb scratching and incubated for 5 (**A**) or 10 (**A**, **B**) minutes in KBM. Wounded cells were lysed, and phospho-Y845 of EGFR and phospho-Y416 of Src in the cells were analyzed with phosphotyrosine-specific antibodies. Stripped membrane of Src Y416 phosphorylation was reprobed with c-Src antibody to assess the equal protein loading.

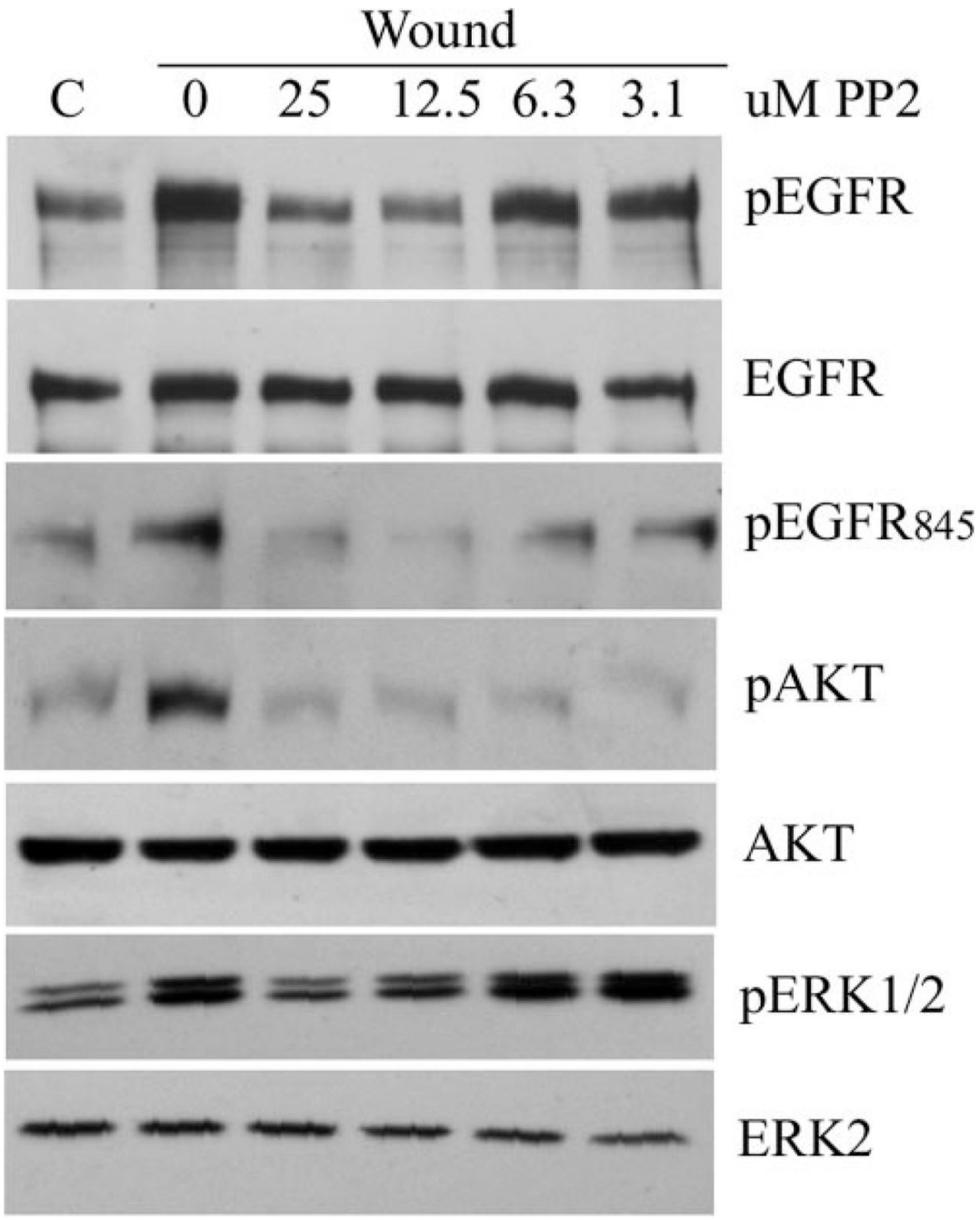

Figure 3.
Effects of PP2 on wound-induced EGFR signaling in a dose-dependent manner. Growth factor–starved THCE cells were pretreated with different concentrations of PP2 (μM) for 1 hour and then extensively injured by sequence comb scratching. The wounded cells were incubated for another 15 minutes in KBM in the presence of PP2, with unwounded cells as control (C). EGFR phosphorylation (pEGFR) was determined by EGFR immunoprecipitation, followed by Western blotting with pY99. Stripped membrane was reprobed with EGFR for an equal amount of EGFR precipitated. To assess ERK and AKT phosphorylation, cell lysates of the same samples were subjected to Western blotting with anti–phospho-AKT (pAKT) and AKT (AKT) or anti–phospho-ERK1 and 2 (pERK1/2) and ERK2 (ERK2).

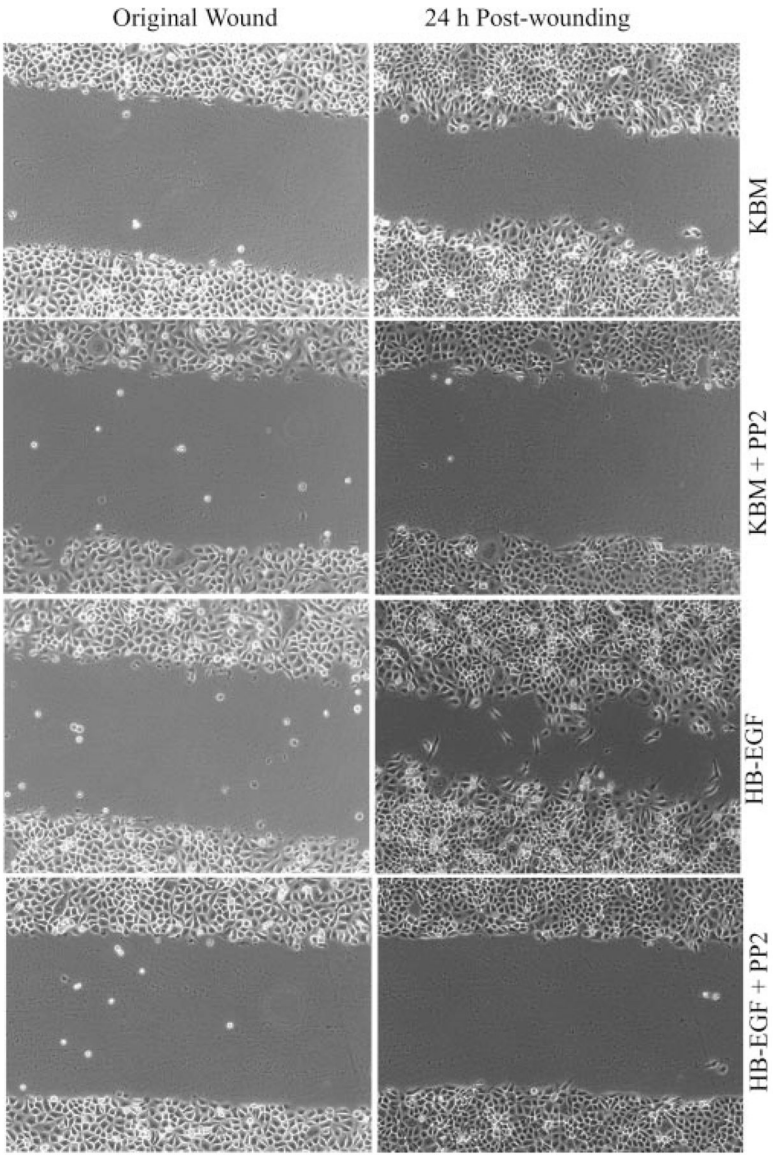

Figure 4.

Effects of PP2 on basal and HB-EGF–enhanced HCEC wound closure. THCE cells cultured in 12-well plates were growth factor starved in KBM overnight. Cells were pretreated with PP2 (12.5 μM) for 1 hour and then wounded with 10-μL pipette tip by the creation of two perpendicular linear scrapes. Cells were allowed to heal in KBM in the presence or absence of HB-EGF (50 ng/mL) with or without PP2. The progress of migration was monitored by photographing the wound near the crossing point immediately (original wound) or 24 hours later (24 h post-wounding) with an inverted microscope equipped with a digital camera. Micrographs represent one of three samples performed each time.

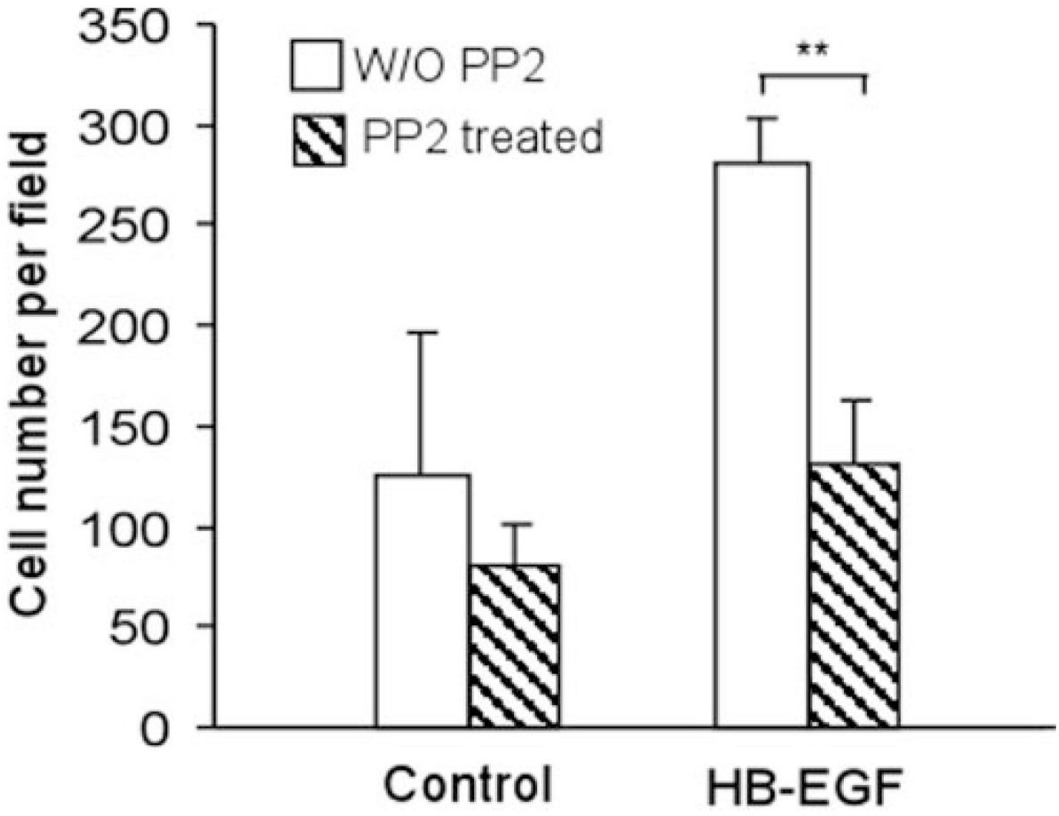

Figure 5.
Effects of PP2 on migratory response of HCECs toward HB-EGF. Growth factor–starved HCECs underwent trypsin digestion and adjustment of cell numbers. Cells were pretreated with PP2 (12.5 μM) for 1 hour. Subsequently, cells were plated onto top chambers that were separated by polycarbonate membrane with 14-μm pore size from the lower chambers. Cells were cultured with or without HB-EGF (50 ng/mL) in the bottom chambers for 3 hours. Numbers of migrated cells per field were counted. Number of cells per field is the average of wells counted. Data represent the mean ± SD of counts in three wells.** $P < 0.01$ (unpaired t test; $N = 6$).

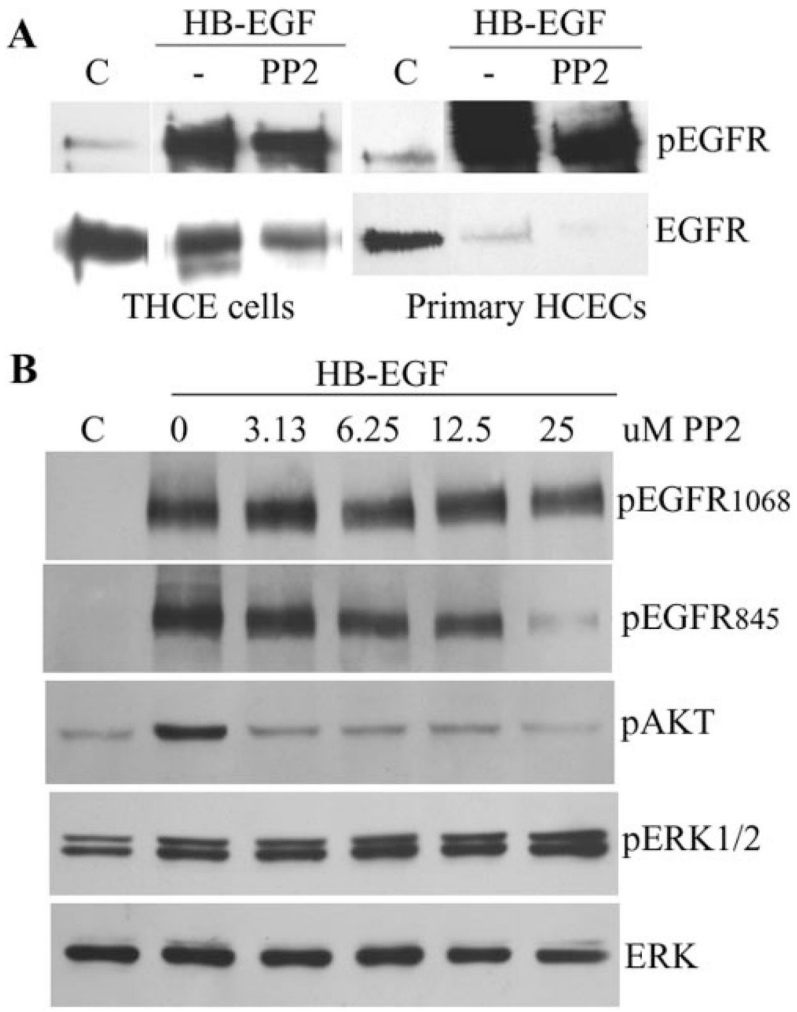

Figure 6.

PP2 inhibition of HB-EGF-induced PI3K/AKT, but not ERK, activation in HCECs. (**A**) Growth factor–starved THCE cells or primary HCECs were pretreated with PP2 (12.5 μM) for 1 hour. Cells were stimulated with HB-EGF (50 ng/mL) in the presence of PP2 and were lysed with RIPA buffer. Cell lysates were immunoprecipitated with anti–EGFR antibody, followed by probing with pY99 antibody (pEGFR). Stripped membrane was reprobed with EGFR antibody (EGFR). (**B**) Growth factor–starved HCECs were pretreated with different concentrations of PP2 for 1 hour followed by stimulation of HB-EGF (50 ng/mL) for 15 minutes. Cell lysates were subjected to Western blotting with phosphor-EGFR Y1086, phosphor-EGFR Y845, phosphor-ERK1/2(pERK), and phosphor-AKT (pAKT). ERK levels were also assessed for equal protein loading.

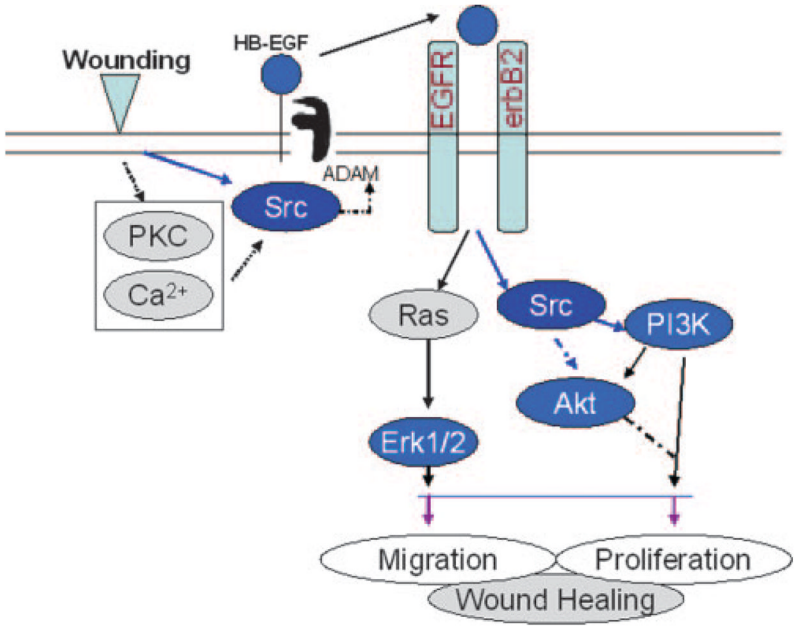

Figure 7.

Proposed Src involvement in HCEC wound healing. This scheme summarizes the pathway from wounding to HCEC wound closure and includes EGFR transactivation and its downstream signaling. Wounding activates Src, which leads to the release of HB-EGF. The soluble form of HB-EGF then binds EGFR, which transduces the signals into the intracellular signaling pathways, including the activation of Src. After EGFR activation, HCEC migration, proliferation, and wound healing are induced through PI3K, ERK, and other intracellular signaling pathways. Activation of PI3K, but not ERK, pathway in HCECs after EGFR (trans) activation requires Src activation. *Light solid arrows*: novel findings reported in the present paper. *Dash lines*: remain to be determined.