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Tyrosine Kinase, p56^{lck}-induced Cell Motility, and Urokinase-type Plasminogen Activator Secretion Involve Activation of Epidermal Growth Factor Receptor/Extracellular Signal Regulated Kinase Pathways*

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Ganapati H. Mahabeleshwar, Riku Das, and Gopal C. Kundu‡

From the National Centre for Cell Science (NCCS), NCCS Complex, Pune 411007, India

We have recently reported that tyrosine kinase, p56^{lck} regulates cell motility and nuclear factor κB-mediated secretion of urokinase-type plasminogen activator (uPA) through tyrosine phosphorylation of IκBα following hypoxia/reoxygenation (Mahabeleshwar, G. H., and Kundu, G. C. (2003) *J. Biol. Chem.* 278, 52598–52612). However, the role of hypoxia/reoxygenation (H/R) on ERK1/2-mediated uPA secretion and cell motility and the involvement of p56^{lck} and EGF receptor in these processes in breast cancer cells is not well defined. We provide here evidence that H/R induces Lck kinase activity and Lck-dependent tyrosine phosphorylation of EGF receptor in highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells. H/R also stimulates MEK-1 and ERK1/2 phosphorylations, and H/R-induced phosphorylations were suppressed by the dominant negative form of Lck (DN Lck, K273R) as well as pharmacological inhibitors of EGF receptor and Lck indicating that EGF receptors and Lck are involved in these processes. Transfection of these cells with wild type Lck or Lck F505 (Y505F) but not with Lck F394 (Y394F) induced phosphorylations of EGF receptor followed by MEK-1 and ERK1/2, suggesting that Lck is upstream of EGF receptor and Tyr-394 of Lck is crucial for these processes. H/R also induced uPA secretion and cell motility in these cells. DN Lck and inhibitors of Lck, EGF receptor, and MEK-1 suppressed H/R-induced uPA secretion and cell motility. To our knowledge, this is the first report that p56^{lck} in presence of H/R regulates MEK-1-dependent ERK1/2 phosphorylation and uPA secretion through tyrosine phosphorylation of EGF receptor, and it further demonstrates that all of these signaling molecules ultimately control the motility of breast cancer cells.

Signal transduction by reactive oxygen species (ROS)[1] has recently become an important target in cellular biology studies (1, 2). The signaling properties of ROS are largely due to the reversible oxidation of redox-sensitive target proteins. The generated ROS act as intracellular second messengers to modulate signal transduction pathways. ROS play significant roles in regulating disease- and stress-induced cellular injuries such as ischemia/reperfusion, UV irradiation, and inflammation (3). The data also showed that the exposure of cultured cells to H_2O_2 resulted in tyrosine phosphorylation of several growth factor receptors (GFR) in the absence of growth factor (GF) stimulation (4). Tyrosine phosphorylation of GFR plays important role to induce the activation of downstream events similar to that observed upon GF stimulation.

Lck, a member of the Src family protein tyrosine kinase, is mostly expressed in T cells and some B cells. We and others have recently reported that Lck is expressed in breast cancer tissues and cell lines (5–7). Lck is a typical Src-like tyrosine kinase, and its activity is regulated by phosphorylation of a highly conserved tyrosine residue, Tyr-505, located near the carboxyl terminus (8). *In vitro*, Lck undergoes autophosphorylation at Tyr-394 (9), and the extent of phosphorylation at Tyr-394 correlates with Lck activity and appears to be required for maximum catalytic activity (10). T-cell antigen receptor ligation of an Lck-deficient Jurkat mutant, J.CaM1, failed to induce tyrosine phosphorylation and activation of p42^{MAPK} upon anti-CD3 or monoclonal anti-T-cell antigen receptor β antibody treatment. The same stimuli activated p42^{MAPK} in J.CaM1 cells transfected with p56^{lck}, demonstrating that Lck plays an important role in MAP kinase activation (11).

Epidermal growth factor receptor (EGFR) belongs to the erbB growth factor receptor family. EGF and its receptor are frequently up-regulated in many human cancers. It has also been described that c-Src catalytic activity is required for integrin-EGF receptor macromolecular complex formation and EGF receptor phosphorylation. Similarly, when cells were exposed to the Src kinase inhibitor pp1, tyrosine phosphorylation of EGF receptor induced by integrin-mediated adhesion was strongly reduced. Simultaneously, overexpression of a kinase negative form of c-Src strongly reduced integrin-dependent EGF receptor phosphorylation, suggesting that c-Src is upstream of EGF receptor (12). The EGF receptor has been shown to be instrumental in the activation of MAPK pathways and cell motility in different cell types (13, 14). Previous results indicated that extracellular-regulated kinase (ERK1/2), a member of the MAPK family, plays an important role in EGF receptor-mediated cell signaling. Inhibition of ERK1/2 by the MEK-1 inhibitor, PD98059, resulted in the significant inhibition of the basal cell migration (15). ERK1/2 also plays a key role in the regulation of AP-1 as its activation leads to the induction of c-Fos, which may associate with c-Jun to form a second AP-1 heterodimeric complex for the regulation of uPA

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‡ To whom correspondence should be addressed. Tel.: 91-20-25690931 (ext. 203); Fax: 91-20-25692259; E-mail: gopalkundu@hotmail.com.

[1] The abbreviations used are: ROS, reactive oxygen species; Lck, leukocyte-specific tyrosine kinase; ERK1/2, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK/ERK kinase; GF, growth factor; GFR, GF receptor; EGF, epidermal GF; EGFR, EGF receptor; uPA, urokinase-type plasminogen activator; DN, dominant negative.

expression (16). However, the molecular mechanism by which p56^{lck} in the presence of H/R regulates EGF receptor phosphorylation and ERK1/2 activation in human breast cancer cells is not well defined.

uPA is involved in cell adhesion, cell motility, tumor growth, and metastasis. It is also regulated at transcriptional level by multiple transcription factors. AP-1 transcription factor duplex plays a major role in the control of inducible uPA expression through binding of enhancer regions in the promoter of the *uPA* gene (17). The signaling pathways by which p56^{lck} in the presence of H/R controls the uPA secretion and cell motility through the activation of MEK-1/ERK1/2 pathways in breast cancer cells are not clearly understood.

In this study, we investigate the involvement of p56^{lck} in the redox-regulated activations of MEK-1 and ERK1/2 and secretion of uPA through the tyrosine phosphorylation of EGF receptor following H/R in MCF-7 and MDA-MB-231 cells. We demonstrate that H/R induced p56^{lck} kinase activity, which ultimately phosphorylates EGF receptor and activates ERK1/2 through the MEK-1-dependent pathway. These lead to the induction of uPA secretion and cell motility, which is prerequisite for proteolytic degradation, extracellular matrix invasion, and distant metastasis.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-p56^{lck}, anti-actin, anti-EGF receptor, anti-ERK1/2, anti-MEK-1, anti-phospho-MEK-1 and mouse monoclonal anti-phosphotyrosine, and anti-phospho-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology. The mouse monoclonal anti-uPA antibody was obtained from Oncogene. pp2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d] pyrimidine), emodin (6-methyl-1,3,8-trihydroxyanthraquinone), aminogenistein (4′-amino-6-hydroxyflavone), damnacanthal (3-hydroxy-1-methoxy anthraquinone-2-aldehyde), AG-99 (α-cyano-(3,4-dihydroxy) cinnamide), PD153035 [4-((3-bromophenyl)amino)-6,7-dimethoxyquinazoline], U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene), and PD98059 (2′-amino-3′-methoxyflavone) were purchased from Calbiochem. Rabbit muscle enolase was from ICN. [γ-^{32}P] ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). pTRACER-A vector and LipofectAMINE Plus reagent were purchased from Invitrogen. Boyden type cell migration chambers were from Corning. All other chemicals were analytical grade.

Cell Culture—The MDA-MB-231 and MCF-7 cells were purchased from ATCC (Manassas, VA). Both MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's modified Eagle's medium. The media were supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Hypoxic Cultures—The MCF-7 and MDA-MB-231 cells grown to 50–70% confluency were made hypoxic in evacuation chambers by intermittent application of vacuum and sparging with 95% N_2, 5% CO_2. Cells were analyzed at this point or maintained under hypoxic conditions in the presence of 100 mM dithionate (an oxygen scavenger) at 37 °C for 0–3 h. These cells were reoxygenated for the indicated periods by replacing the medium with fresh medium and incubating the cultures in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Plasmids and DNA Transfection—The wild type Lck, mutant Lck F394 (Y394F), and Lck F505 (Y505F) cDNAs in an expression vector (pCEP4) were generous gifts from Dr. B. M. Sefton (The Salk Institute, La Jolla, CA). The dominant negative form of Lck (DN Lck, K273R) in pcDNA3 was a kind gift from Dr. D. R. Branch (Canadian Blood Services, Toronto, Ontario). Both MCF-7 and MDA-MB-231 cells were transiently transfected with Lck cDNA using LipofectAMINE Plus according to the methods as described earlier (6). These transfected cells were used for Lck kinase activity, detection of phosphorylations of Lck, EGF receptor, MEK-1, and ERK1/2, and uPA secretion by Western blot analysis and cell migration.

The transfection efficiency was assessed as described previously (18). Briefly, both of these cells were cotransfected with wild type or mutant Lck cDNA and pTRACER-A vector (Invitrogen) containing green fluorescent protein in the presence of LipofectAMINE Plus. The cells were allowed to grow for 24 h and then analyzed under fluorescence microscopy. Transfection efficiency was assessed by dividing the number of green fluorescent cells by the total cell number in the same randomly chosen fields in each transfection.

Immunoprecipitation and Western Blot Analysis—To examine whether EGF receptor is involved in H/R-induced tyrosine phosphorylation of p56^{lck}, both MCF-7 and MDA-MB-231 cells were pretreated with different concentrations of EGF receptor inhibitors (0–35 pM PD153035 or 0–15 μM AG-99) and then induced by H/R for 90 min. The cells were lysed in lysis buffer (1% Triton X-100 solution containing 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 2 mM EDTA). Equal amounts of total proteins in lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody. The immunoprecipitated samples were analyzed by Western blot using mouse monoclonal anti-phosphotyrosine antibody. The same blots were reprobed with rabbit polyclonal anti-Lck antibody.

To delineate whether H/R plays any role in the regulation of EGF receptor phosphorylation, both of these cells were induced by H/R for 0–120 and 0–135 min, respectively. An equal amount of total proteins from the cell lysates were immunoprecipitated with rabbit polyclonal anti-EGFR antibody. The immunoprecipitated samples were immunoblotted with anti-phosphotyrosine antibody. The same blots were reprobed with anti-EGFR antibody. To check whether Lck plays any role in regulation of EGF receptor phosphorylation, both of these cells were individually treated with various concentrations of Lck inhibitors (0–16 μM emodin, 0–4 nM pp2, 0–2 μM aminogenistein, and 0–0.8 μM damnacanthal) for 24 h and then induced by H/R for 75 min in MCF-7 cells and for 90 min in MDA-MB-231 cells.

In separate experiments, cells were individually transfected with DN Lck and then induced by H/R. The dominant negative activity of Lck was also verified by Yousefi *et al.* (19). In other experiments, cells were transfected with wild type Lck, Lck F394, and Lck F505, and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors (16 μM emodin, 4 nM pp2, 2 μM aminogenistein, and 0.8 μM damnacanthal) and then induced by H/R as described above. Cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine antibody as described above. The same blots were reprobed with anti-EGFR antibody.

To examine whether H/R induces the phosphorylation of MEK-1 and whether H/R-induced MEK-1 phosphorylation is regulated by Lck or EGF receptor, MDA-MB-231 cells were transfected with DN Lck, wild type Lck, mutant Lck F394, or Lck F505 and wild type Lck transfected cells were treated with Lck inhibitors (16 μM emodin, 4 nM pp2, 2 μM aminogenistein, and 0.8 μM damnacanthal) for 24 h and then induced by H/R for 90 min. In other experiments, cells were treated with various concentrations of EGF receptor inhibitors (0–35 pM PD153035 and 0–15 μM AG-99) and then induced by H/R as described above. Equal amount of total proteins from treated or transfected cell lysates were analyzed by Western blot using rabbit anti-phospho-MEK-1 antibody. The same blots were reprobed with rabbit anti-MEK-1 antibody.

To check whether H/R regulates the ERK1/2 phosphorylation and H/R-induced ERK1/2 phosphorylation is governed by Lck, EGF receptor, and MEK-1, MDA-MB-231 cells were transfected with DN Lck, wild type Lck, mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with Lck inhibitors or EGF receptor inhibitors or MEK-1 inhibitors as described above. These cells were then induced with H/R, and cell lysates were immunoblotted with mouse monoclonal anti-phospho-ERK1/2 antibody. The same blots were reprobed with rabbit anti-ERK1/2 antibody.

To investigate the roles of Lck, EGF receptor, and MEK-1 on H/R-induced uPA secretion, MDA-MB-231 cells were transfected with DN Lck, wild type Lck, mutant Lck F394 or Lck F505, and wild type Lck transfected cells were treated with maximum concentrations of inhibitors of Lck, EGF receptor, or MEK-1 as described above and induced by H/R for 24 h. Cell lysates were analyzed by Western blot using mouse monoclonal anti-uPA antibody. As loading controls, the same blots were reprobed with rabbit polyclonal anti-actin antibody.

Immunoprecipitation and in Vitro Kinase Assay—To delineate the effect of H/R on Lck kinase activity, both of these cells were either transiently transfected with wild type Lck, mutant Lck F394, or Lck F505 or treated with different concentrations of EGF receptor inhibitors (0–35 pM PD153035 or 0–15 μM AG-99) and then induced by H/R for 90 min. Cell lysates containing an equal amount of total proteins were immunoprecipitated with rabbit polyclonal anti-Lck antibody. The immunoprecipitated samples were used for Lck kinase assay using acid denatured enolase as substrate as described earlier (10).

To examine whether H/R regulates the EGF receptor kinase activity and to check the specificity of Lck inhibitors in H/R-induced EGF receptor activation, the EGF receptor kinase assays were performed as described (20). Briefly, cells were induced by H/R, and the lysates were

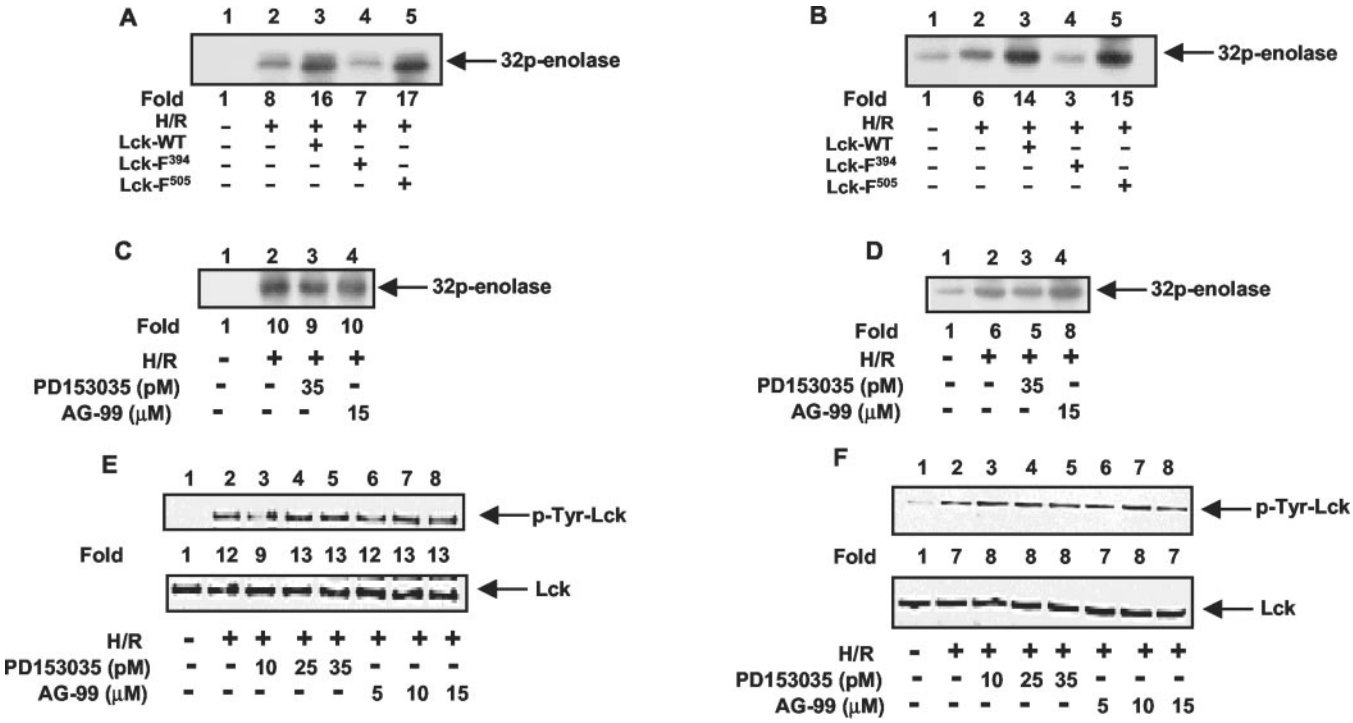

FIG. 1. As shown in *A* and *B*, H/R induces Lck kinase activity in low invasive (MCF-7) and highly invasive (MDA-MB-231) breast cancer cells. Both MCF-7 (*A*) and MDA-MB-231 (*B*) cells were individually transfected with wild type (*WT*) or mutant Lck (F394 or F505) and then induced by H/R for 90 min. Cell lysates were immunoprecipitated with rabbit polyclonal anti-Lck antibody. The immunoprecipitated samples were used for Lck kinase assay using rabbit muscle enolase as substrate. *Lane 1*, without H/R; *lane 2*, induced by H/R; *lane 3*, transfection with wild type Lck and induced by H/R; *lane 4*, transfection with Lck F394 and induced by H/R; and *lane 5*, transfection with Lck F505 and induced by H/R. As shown in *C* and *D*, H/R-induced Lck kinase activity is independent of EGF receptor activation. MCF-7 (*C*) and MDA-MB-231 (*D*) cells were pretreated with EGF receptor inhibitors and then induced by H/R as described. The cell lysates were immunoprecipitated with anti-Lck antibody and used for Lck kinase assay. *Lane 1*, without H/R; *lane 2*, induced by H/R; *lane 3*, treated with PD153035 and induced by H/R; and *lane 4*, treated with AG-99 and induced by H/R. As shown in *E* and *F*, H/R-induced tyrosine phosphorylation of Lck is independent of EGF receptor activation. MCF-7 (*E*) and MDA-MB-231 (*F*) cells were pretreated with various concentrations of EGF receptor inhibitors and then induced by H/R. The cell lysates were immunoprecipitated with anti-Lck antibody, and the immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody (*E* and *F*, *upper panels*, *lanes 1–8*). The same blots were reprobed with anti-Lck antibody (*E* and *F*, *lower panels*, *lanes 1–8*).

immunoprecipitated with anti-EGFR antibody. The immunocomplex was incubated with Lck inhibitors and used for EGF receptor kinase assay.

Cell Migration Assay—The migration assay was conducted using a Transwell cell culture chamber according to the standard procedure as described previously (21, 22). Briefly, both MCF-7 and MDA-MB-231 cells were exposed with H/R, and a cell suspension (5×10^5 cells/well) was added to the upper chamber of the polycarbonate membrane filter. The lower chamber was filled with fibroblast-conditioned medium, which acted as chemoattractant. In separate experiments, MDA-MB-231 cells were pretreated with Lck inhibitors (0–16 μM emodin, 0–4 nM pp2, 0–2 μM aminogenistein, and 0–0.8 μM damnacanthal) and induced by H/R for 24 h. In other experiments, both MCF-7 and MDA-MB-231 cells were individually transfected with DN Lck, wild type Lck, mutant Lck F394 or Lck F505, and wild type Lck transfected cells were treated with inhibitors of Lck, EGF receptor or MEK-1, or anti-uPA antibody as described earlier and then induced by H/R for 24 h. After treatment or transfection, cells were added to the upper chamber of the polycarbonate membrane filter and incubated at 37 °C for 16 h. The non-migrated cells on the upper side of the filter were scraped, and the filter was washed. The migrated cells in the reverse side of the filter were fixed with methanol and stained with Giemsa. The migrated cells on the filter were counted under an inverted microscope (Olympus). The experiments were repeated in triplicate. Preimmune IgG served as a non-specific control.

RESULTS

H/R Induces Lck Phosphorylation and Its Kinase Activity—We have investigated the effect of H/R on Lck-dependent MEK-1/ERK1/2-mediated uPA secretion through tyrosine phosphorylation of EGF receptor in breast cancer cells. Accordingly, we first examined whether H/R is able to induce tyrosine phosphorylation of Lck and its kinase activity in low invasive (MCF-7) and highly invasive (MDA-MB-231) breast cancer cells. Both of these cells were transiently transfected with wild type Lck, mutant Lck F394, and Lck F505 and induced with H/R for 90 min. These transfected cells were immunoprecipitated with anti-Lck antibody, and immunoprecipitated samples were used for kinase assay using enolase as substrate. The data revealed that H/R induced Lck kinase activity in MCF-7 (Fig. 1*A*, *lane 2*) and MDA-MB-231 (Fig. 1*B*, *lane 2*) cells as compared with cells grown under normoxic conditions (*lane 1*). Both wild type Lck and mutant Lck F505 enhanced the phosphorylation of enolase in MCF-7 (Fig. 1*A*, *lanes 3* and *5*) and MDA-MB-231 (Fig. 1*B*, *lanes 3* and *5*) cells as compared with cells transfected with Lck F394 (Fig. 1, *A* and *B*, *lane 4*). These results indicated that H/R induced the Lck kinase activity in both of these cells and also suggested that Tyr-394 of Lck is important for this process. To delineate whether p56^{lck} is located upstream of EGF receptor in ROS-mediated signaling pathway, both of these cells were pretreated with EGF receptor inhibitors, induced by H/R, and immunoprecipitated with anti-Lck antibody, and immunoprecipitated samples were used for Lck kinase assay. The data indicated that EGF receptor inhibitors have no effect on Lck kinase activity in both MCF-7 and MDA-MB-231 cells (Fig. 1, *C* and *D*, *lanes 1–4*), suggesting that Lck is located upstream of EGF receptor in these pathways.

To delineate whether EGF receptor plays any significant role in H/R-induced tyrosine phosphorylation of Lck, both of these cells were treated with EGF receptor inhibitors and then induced by H/R. Cell lysates were immunoprecipitated with anti-Lck antibody and immunoblotted with anti-phosphotyrosine antibody (Fig. 1, *E* and *F*, *upper panels*, *lanes 1–8*), and the same blots were reprobed with anti-Lck antibody as loading

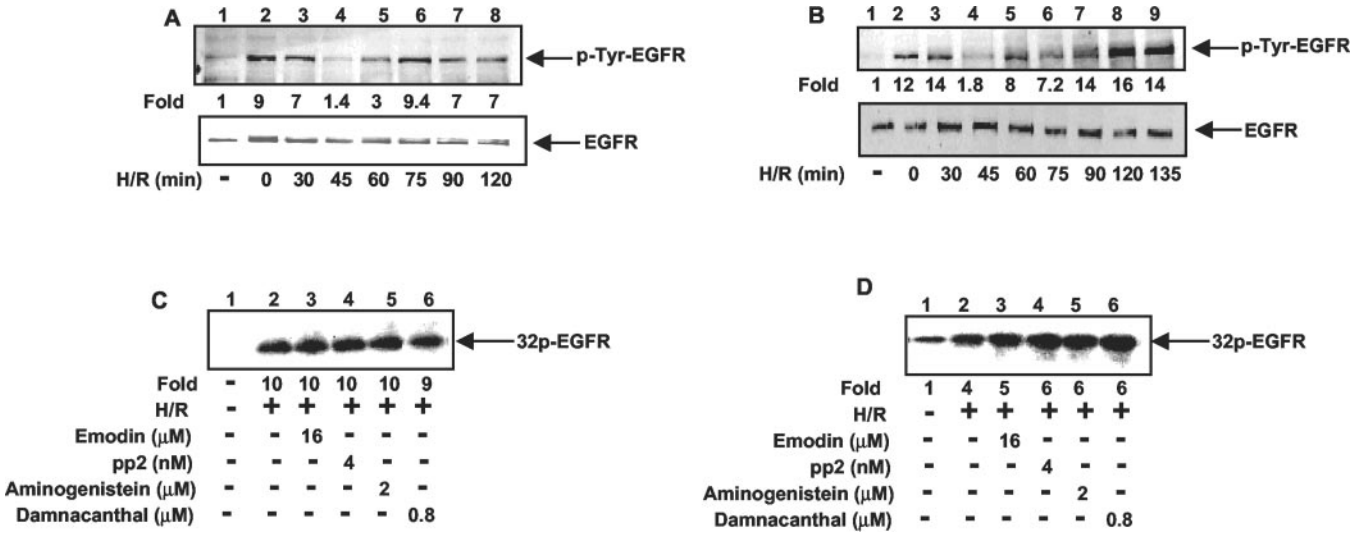

FIG. 2. As shown in *A* and *B*, H/R enhances EGF receptor phosphorylation. MCF-7 (*A*) and MDA-MB-231 (*B*) cells were induced by H/R, cell lysates were immunoprecipitated with anti-EGF receptor antibody, and the immunocomplexes were immunoblotted with anti-phosphotyrosine antibody. Note that H/R induced tyrosine phosphorylation of EGF receptor in MCF-7 (*A*, *upper panel*, *lanes 1–8*) and MDA-MB-231 (*B*, *upper panel*, *lanes 1–9*) cells. The same blots were reprobed with anti-EGF receptor antibody as loading control (*A*, *lower panel*, *lanes 1–8*; *B*, *lower panel*, *lanes 1–9*). *C* and *D*, effects of Lck inhibitors on H/R-induced EGF receptor kinase activity. MCF-7 (*C*) and MDA-MB-231 (*D*) cells were induced by H/R, and cell lysates were immunoprecipitated with anti-EGF receptor antibody. The immunocomplex was incubated in the absence or presence of Lck inhibitors, and a kinase assay was performed. Lck inhibitors have no effect on H/R-induced EGF receptor kinase activity in MCF-7 (*C*, *lanes 1–6*) and MDA-MB-231 (*D*, *lanes 1–6*) cells. All of these bands were analyzed densitometrically, and -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.

control (Fig. 1, *E* and *F*, *lower panels*, *lanes 1–8*). The inhibitors of EGF receptor have no effect on H/R-induced tyrosine phosphorylation of Lck. These data further confirmed that Lck is upstream of EGF receptor in these pathways. All of these bands were quantified by densitometric analysis, and the values of -fold changes are calculated.

To examine the transfection efficiency, both MCF-7 and MDA-MB-231 cells were cotransfected with Lck and pTRACER-A vector (Invitrogen) in the presence of LipofectAMINE Plus. The cells were analyzed under fluorescence microscopy. The transfection efficiency was quantified by dividing the number of green fluorescent cells by the total cell number in the same randomly chosen fields in each transfection, and a high level of transfection efficiency was observed in MCF-7 (82%) and MDA-MB-231 (78%) cells (data not shown).

H/R Enhances EGF Receptor Phosphorylation and Its Kinase Activity—We have examined the effect of H/R on phosphorylation of EGF receptor in breast cancer cells. Accordingly, MCF-7 cells were stimulated with H/R for 0–120 min, and MDA-MB-231 cells were induced by H/R for 0–135 min. The cell lysates were immunoprecipitated with rabbit polyclonal anti-EGF receptor antibody, and the immunocomplex was analyzed by Western blot using anti-phosphotyrosine antibody. The data showed that H/R induced EGF receptor phosphorylation in MCF-7 (Fig. 2*A*, *upper panel*, *lanes 1–8*) and in MDA-MB-231 (Fig. 2*B*, *upper panel*, *lanes 1–9*) cells. The same blots were reprobed with anti-EGF receptor antibody as loading control (Fig. 2, *A* and *B*, *lower panels*). The bands were quantified densitometrically, and the values of -fold changes are calculated.

To examine the effect of H/R on EGF receptor kinase activity and to check the specificity of Lck inhibitors on this activity, both of these cells were induced by H/R and immunoprecipitated with anti-EGF receptor antibody. The immunoprecipitated samples were treated in the absence or presence of Lck inhibitors and used for kinase assay. The results showed that H/R induced EGF receptor kinase activity and Lck inhibitors have no inhibitory effect on H/R-induced kinase activity of EGF receptor in both of these cells (Fig. 2, *C* and *D*, *lanes 1–6*).

p56^{lck} Is Involved in H/R-induced EGF Receptor Phosphorylation—To delineate the role of Lck in H/R-induced EGF receptor phosphorylation, both of these cells were pretreated with different concentrations of Lck-specific inhibitors and then induced by H/R. Cell lysates were immunoprecipitated with anti-EGF receptor antibody and immunoblotted with anti-phosphotyrosine antibody. The data showed that H/R-induced tyrosine phosphorylation of EGF receptor was dramatically suppressed by Lck inhibitors in a dose-dependent manner in MCF-7 (Fig. 3*A*, *upper panel*, *lanes 1–14*) and MDA-MB-231 (Fig. 3*B*, *upper panel*, *lanes 1–14*) cells. The same blots were reprobed with anti-EGF receptor antibody as loading control (Fig. 3, *A* and *B*, *lower panels*, *lanes 1–14*). These results clearly suggested that Lck plays a crucial role in H/R-induced EGF receptor phosphorylation.

To further confirm whether Lck plays any role in H/R-induced EGF receptor tyrosine phosphorylation, both of these cells were transfected with DN Lck and induced by H/R. Cell lysates were immunoprecipitated with anti-EGF receptor antibody and immunoblotted with anti-phosphotyrosine antibody. The data indicated that DN Lck suppressed the H/R-induced tyrosine phosphorylation of EGF receptor in MCF-7 (Fig. 3*C*, *upper panel*, *lanes 1–3*) and MDA-MB-231 (Fig. 3*D*, *upper panel*, *lanes 1–3*) cells. The same blots were reprobed with anti-EGF receptor antibody as loading control (Fig. 3, *C* and *D*, *lower panels*, *lanes 1–3*). In separate experiments, both of these cells were transiently transfected with wild type Lck, mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors and induced by H/R. The data showed that both wild type Lck and Lck F505 enhanced the H/R-induced tyrosine phosphorylation of EGF receptor in MCF-7 (Fig. 3*E*, *upper panel*, *lanes 3* and *5*) and MDA-MB-231 (Fig. 3*F*, *upper panel*, *lanes 3* and *5*) cells as compared with cells transfected with Lck F394 (Fig. 3, *E* and *F*, *upper panels, lane 4*) or induced by H/R alone (Fig. 3, *E* and *F*, *upper panels*, *lane 2*). As expected, no phospho-specific band was detected in cells grown under normoxic conditions (Fig. 3, *E* and *F*, *upper panels*, *lane 1*). Lck inhibitors drastically suppressed the H/R-induced EGF receptor phosphorylation in wild

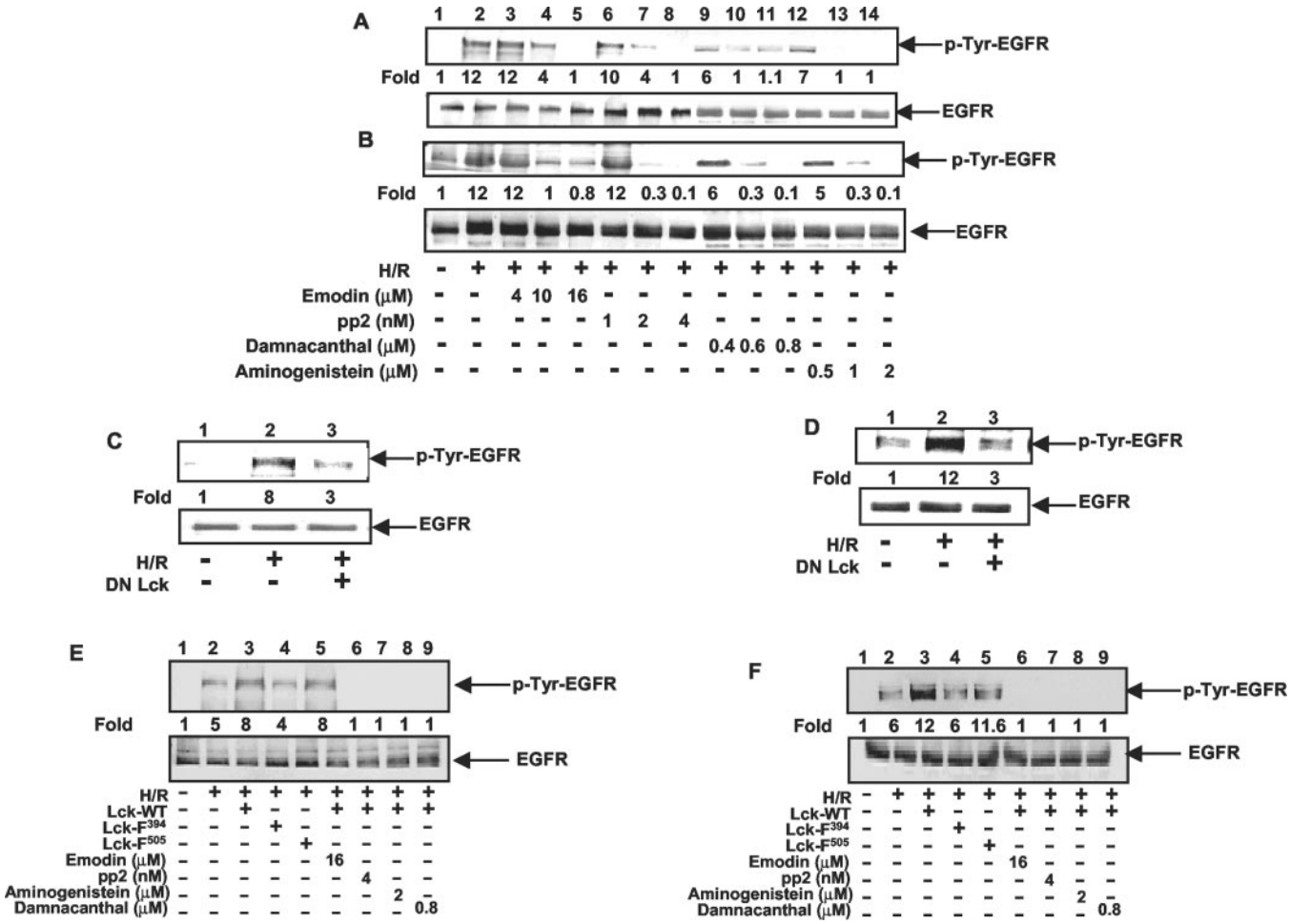

FIG. 3. **p56^{lck} is involved in H/R-induced EGF receptor phosphorylation.** As shown in A and B, MCF-7 (*A*) and MDA-MB-231 (*B*) cells were individually pretreated with various concentrations of Lck-specific inhibitors and then induced by H/R. Cell lysates were immunoprecipitated with rabbit anti-EGF receptor antibody, and immunoprecipitated samples were analyzed by Western blot using anti-phosphotyrosine antibody. The same blots were reprobed with anti-EGF receptor antibody. Note that H/R-induced tyrosine phosphorylation of EGF receptor was inhibited by Lck inhibitors in a dose-dependent manner (*A* and *B*, *upper panels*, *lanes 1–14*). The expression of non-phospho-EGF receptor was shown as loading control (*A* and *B*, *lower panels*, *lanes 1–14*). As shown in *C–F*, MCF-7 (*C* and *E*) and MDA-MB-231 (*D* and *F*) cells were transiently transfected with wild type (*WT*) Lck, DN Lck, mutant Lck F394, and Lck F505, and wild type Lck transfected cells were treated with Lck inhibitors. All of these transfected cells were induced by H/R, and cell lysates were immunoprecipitated with anti-EGF receptor antibody and immunoblotted with anti-phosphotyrosine antibody. Note that cells transfected with DN Lck suppressed the H/R-induced EGF receptor phosphorylation in MCF-7 (*C*, *upper panel*, *lanes 1–3*) and in MDA-MB-231 (*D*, *upper panel*, *lanes 1–3*) cells. MCF-7 (*E*) or MDA-MB-231 (*F*) cells transfected with wild type Lck or Lck F505 but not with Lck F394 induced the EGF receptor phosphorylation in the presence of H/R (*E* and *F*, *upper panels*, *lanes 2–5*). Lck-induced EGF receptor phosphorylation was completely inhibited by Lck inhibitors in the presence of H/R in these cells (*E* and *F*, *upper panels*, *lanes 3* and *6–9*). The same blots were reprobed with anti-EGF receptor antibody as loading control (*C–F, lower panels*). All of these bands were quantified by densitometric analysis, and the values of -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects.

type Lck transfected cells (Fig. 3, *E* and *F*, *upper panels*, *lanes 6–9*). The expression of non-phospho-EGF receptor was shown as loading control (Fig. 3, *E* and *F*, *lower panels*, *lanes 1–9*). This data clearly suggested that Lck is directly involved in H/R-induced EGF receptor phosphorylation and Tyr-394 of Lck plays a critical role in this phosphorylation process. All of these bands were quantified by densitometric analysis and the values of -fold changes are calculated.

H/R-induced Breast Cancer Cell Migration Is p56^{lck}-dependent—In this study, we have demonstrated that p56^{lck} is involved in EGF receptor phosphorylation upon H/R induction. Therefore, we sought to determine whether p56^{lck} has any role in breast cancer cell migration in the presence of H/R. MDA-MB-231 cells were pretreated with different concentrations of Lck inhibitors and then induced by H/R. These treated cells were used for a cell migration assay. The data indicated that MDA-MB-231 cells exposed with H/R showed higher migration (695%) than cells grown under normoxic conditions (100%) (Fig. 4, *A* and *B*). Pretreatment of MDA-MB-231 cells with emodin (398–132%), pp2 (320–109%), aminogenistein (415–134%), or damnacanthal (308–119%) showed drastic suppression of H/R-induced cell migration (Fig. 4, *A* and *B*). Similar results were obtained when MCF-7 cells were used for these studies (data not shown). In other experiments, both MCF-7 and MDA-MB-231 cells were individually transfected with wild type Lck, DN Lck (K273R), Lck F394, or Lck F505, and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors and induced by H/R. The high level of transfection efficiency was observed in both MCF-7 (82%) and MDA-MB-231 (78%) cells. These cells were used for migration assay, and both wild type Lck (880% in MCF-7 and 1076% in MDA-MB-231) and Lck F505 (872% in MCF-7 and 1072% in MDA-MB-231) transfected cells showed maximum migration as compared with Lck F394 transfected cells (560% in MCF-7 and 700% in MDA-MB-231) (Fig. 4, *C* and *D*). H/R-induced migration was suppressed when cells were transfected with DN Lck (K273R) in MCF-7 (Fig. 4*C*) and MDA-MB-231 (Fig. 4*D*) cells. Lck inhibitors dramatically blocked the H/R-induced cell mi-

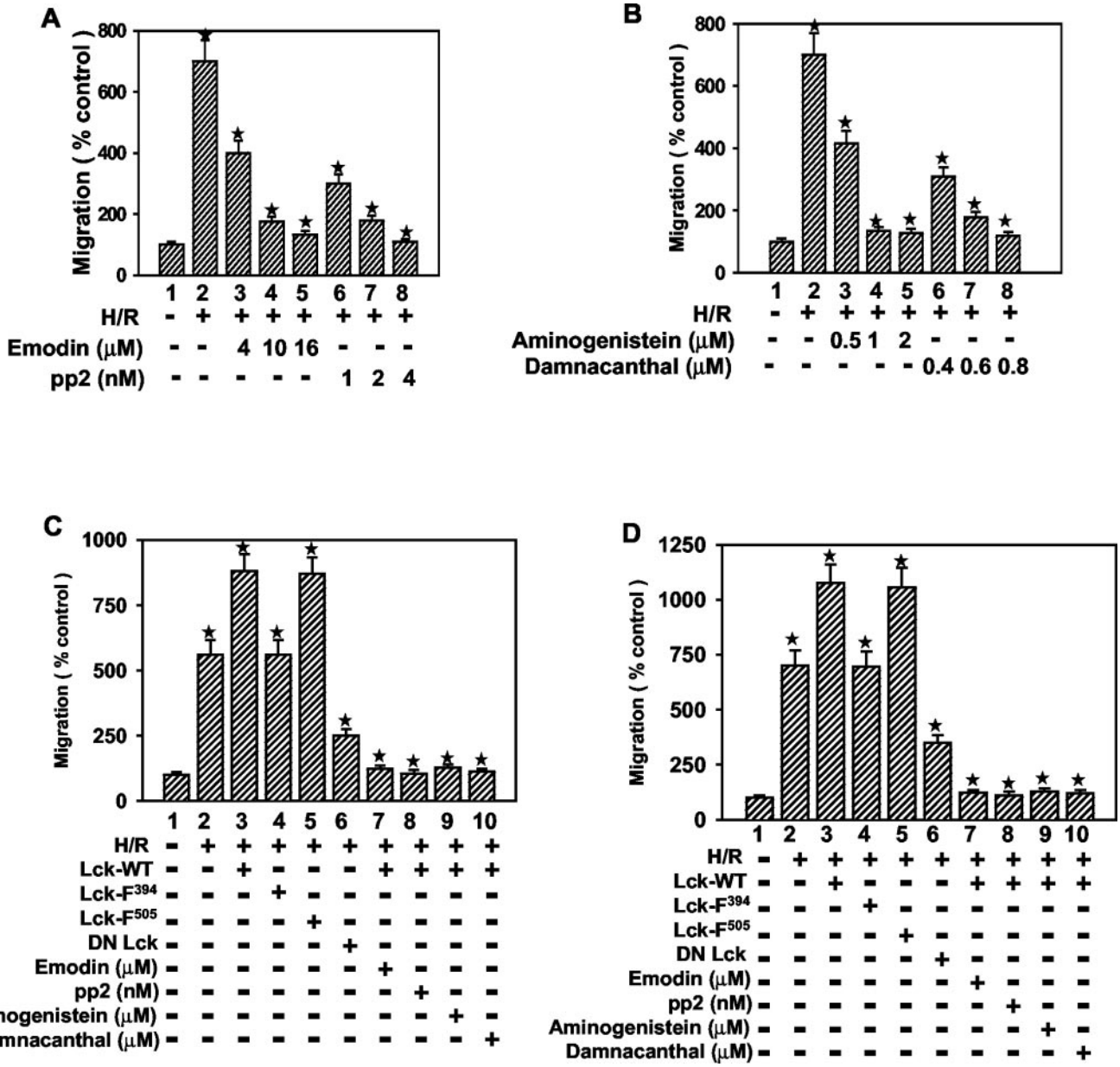

FIG. 4. **Role of p56^{lck} in H/R-induced cell migration.** As shown in *A* and *B*, MDA-MB-231 cells were pretreated with various concentrations of Lck-specific inhibitors and then induced by H/R. These treated cells were used for cell migration assay as described under "Experimental Procedures." The number of cells migrated under normoxic conditions was considered as 100%. H/R induced the cell migration, and Lck inhibitors suppressed the H/R-induced cell migration in a dose-dependent manner (*A* and *B*). As shown in *C* and *D*, MCF-7 (*C*) and MDA-MB-231 (*D*) cells were individually transfected with wild type (*WT*) Lck, DN Lck, mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors, induced by H/R, and used for migration assay. Both wild type Lck and Lck F505 transfected cells showed maximum cell migration as compared with Lck F394 transfected cells or cells induced by H/R alone. DN Lck transfected cells reduced the H/R-induced migration. Lck inhibitors suppressed H/R-induced cell migration in wild type Lck transfected cells. The results are expressed as the means ± S.E. of three determinations. The values were also analyzed by Student's *t* test ($p < 0.002$).

gration in wild type Lck-transfected MCF-7 and MDA-MB-231 cells (Fig. 4, *C* and *D*). These results suggested that H/R induced Lck-mediated breast cancer cell migration and Tyr-394 of Lck plays a crucial role in this process. The data are expressed as the means ± S.E. of three determinations. The values were also analyzed by Student's *t* test ($p < 0.002$).

H/R-induced p56^{lck} and EGF Receptor-dependent MEK-1 and ERK1/2 Phosphorylations—To examine the effect of H/R on phosphorylations of MEK-1 and ERK1/2 and whether Lck and EGF receptors are involved in these processes, MDA-MB-231 cells were transfected with wild type Lck, DN Lck (K273R), mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with various Lck inhibitors and then induced by H/R. In other experiments, cells were either treated with EGF receptor inhibitors or transfected with wild type Lck, DN Lck (K273R), mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with EGF receptor inhibitors or MEK-1 inhibitors and then induced by H/R. The transfection efficiency was monitored, and a high level of transfection efficiency was detected in both MCF-7 (82%) and MDA-MB-231 (78%) cells. Cell lysates were analyzed by Western blot using anti-phospho-MEK-1 or anti-phospho-ERK1/2 antibody. The data indicated that both wild type Lck and Lck F505 enhanced the H/R-induced MEK-1 phosphorylation as compared with Lck F394 transfected cells (Fig. 5*A*, *upper panel*, *lanes 2–5*). H/R-induced MEK-1 phosphorylation is reduced when cells were transfected with DN Lck (*lane 6*). A low level of phosphorylated MEK-1 was detected in cells grown under normoxic

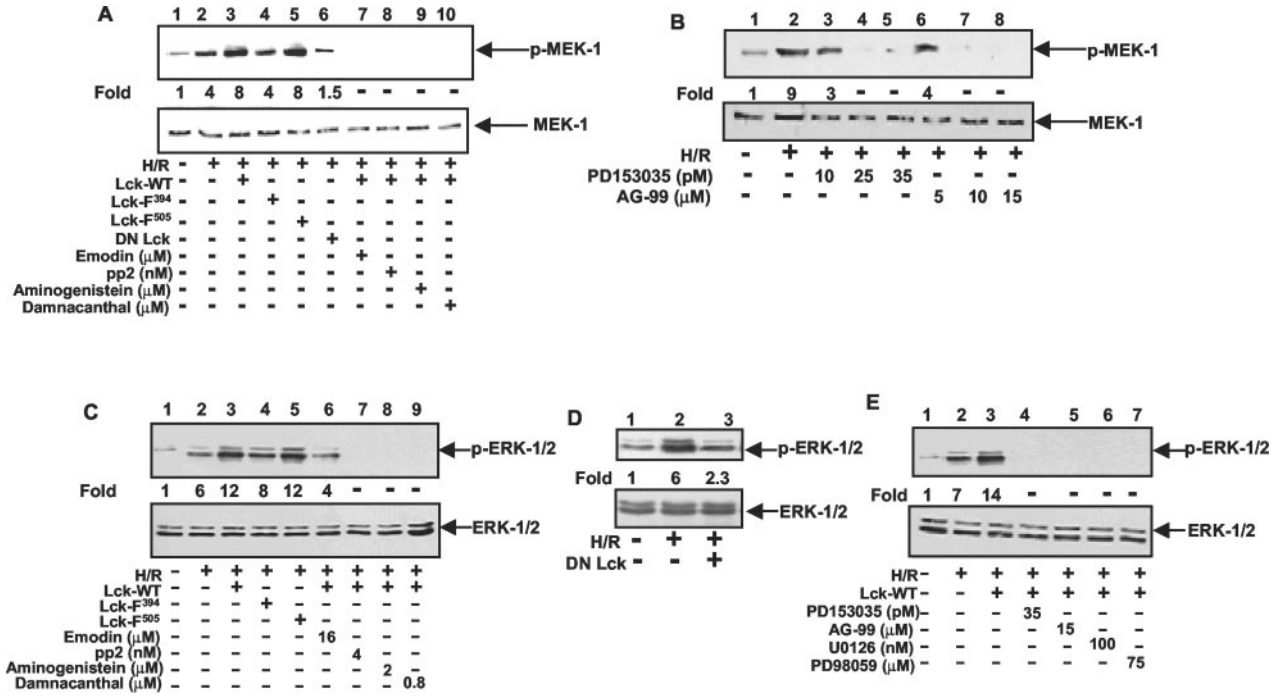

FIG. 5. **Roles of p56^{lck} and EGF receptor on H/R-induced MEK-1 phosphorylation (Ser-222 and -226).** As shown in *A*, MDA-MB-231 cells were transiently transfected with wild type (*WT*) Lck, DN Lck, mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with maximum doses of Lck inhibitors and induced by H/R. The levels of phospho-MEK-1 (*p-MEK-1*) and MEK-1 were detected by Western blot analysis. Cells transfected with wild type Lck or Lck F505 showed maximum phosphorylation of MEK-1 as compared with cells transfected with Lck F394 or induced by H/R alone (*A*, *upper panel*, *lanes 1–5*). DN Lck inhibited the H/R-induced MEK-1 phosphorylation (*lane 6*). Lck inhibitors drastically inhibited H/R-induced MEK-1 phosphorylation in wild type Lck-transfected cells (*lanes 7–10*). As shown in *B*, MDA-MB-231 cells were treated with various concentrations of EGF receptor inhibitors and induced by H/R, and cell lysates were immunoblotted with anti-phospho-MEK-1 antibody. The results indicated that EGF receptor inhibitors suppressed the H/R-induced MEK-1 phosphorylation in these cells (*B*, *upper panel*, *lanes 1–8*). The same blots were reprobed with anti-MEK antibody as loading control (*A* and *B, lower panel*). As shown in *C–E*, H/R-induced p56^{lck}-dependent ERK1/2 phosphorylation occurred through EGF receptor and MEK-1 mediated pathways. MDA-MB-231 cells were individually transfected with wild type Lck, DN Lck, mutant Lck F394, or Lck F505 and then induced by H/R. In separate experiments, wild type Lck transfected cells were treated with maximum doses of inhibitors of Lck, EGF receptor, or MEK-1 and induced by H/R. The level of phospho-ERK1/2 (*p-ERK-1/2*) was detected by Western blot using anti-phospho-ERK1/2 antibody. The phosphorylation of ERK1/2 was higher in cells transfected with wild type Lck or Lck F505 as compared with Lck F394 transfected cells or cells induced by H/R alone (*C*, *upper panel*, *lanes 1–5*). Lck inhibitors dramatically blocked the H/R-induced ERK phosphorylation of wild type Lck transfected cells (*lanes 6–9*). DN Lck suppressed the H/R-induced ERK1/2 phosphorylation (D, *upper panel*, *lanes 1–3*). Inhibitors of EGF receptor and MEK-1 suppressed the H/R-induced ERK1/2 phosphorylation in wild type Lck transfected cells (*E*, *upper panel*, *lanes 1–7*). As loading control, all of these blots were reprobed with anti-ERK1/2 antibody (*C–E*, *lower panels*). All of these bands were quantified by densitometric analysis, and the values of -fold changes are calculated. The data shown here represent three experiments exhibiting similar effects.

conditions (*lane 1*). Lck inhibitors drastically inhibited the H/R-induced MEK-1 phosphorylation in wild type Lck transfected cells (*lanes 7–10*). The data also showed that EGF receptor inhibitors dose-dependently blocked the H/R-induced MEK-1 phosphorylation (Fig. 5*B*, *upper panel*, *lanes 1–8*). Similarly, wild type Lck or Lck F505 enhanced the H/R-induced ERK1/2 phosphorylation as compared with Lck F394 transfected cells or cells induced by H/R alone (Fig. 5*C*, *upper panel*, *lanes 2–5*). Lck inhibitors dramatically suppressed the H/R-induced ERK1/2 phosphorylation in wild type Lck transfected cells (*lanes 6–9*). DN Lck blocked the H/R-induced ERK1/2 phosphorylation (Fig. 5*D*, *upper panel*, *lanes 1–3*). The results also indicated that inhibitors of EGF receptor or MEK-1 suppressed the H/R-induced ERK1/2 phosphorylation in wild type Lck transfected cells (*lanes 1–7*). The same blots were reprobed with either anti-MEK-1 or anti-ERK1/2 antibody as loading control (Fig. 5, *A–E*, *lower panels*). These results suggested that H/R induces MEK-1 and ERK1/2 phosphorylations through Lck and EGF receptor-mediated pathways. The same results were obtained in MCF-7 cells (data not shown).

H/R Induces uPA Secretion through p56^{lck}-dependent EGF Receptor and MEK-1-mediated Pathways—To delineate the roles of Lck, EGF receptor, and ERK in H/R-induced uPA secretion, MDA-MB-231 cells were transfected with wild type Lck, DN Lck, mutant Lck F394, or Lck F505, and wild type Lck transfected cells were treated with inhibitors of Lck, MEK-1, and EGF receptor and then induced by H/R for 24 h. The high level of transfection efficiency was observed in both MCF-7 and MDA-MB-231 cells. Cell lysates were analyzed by Western blot using mouse monoclonal anti-uPA antibody. The data indicated that both wild type Lck or Lck F505 transfected cells showed a higher level of uPA secretion as compared with H/R-induced or Lck F394 transfected cells (Fig. 6*A*, *upper panel*, *lanes 2–5*). Lck-specific inhibitors blocked the H/R-induced uPA secretion in wild type Lck transfected cells (*lanes 6–9*). DN Lck suppressed the H/R-induced uPA secretion (Fig. 6*B*, *upper panel*, *lanes 2* and *3*). The data also revealed that inhibitors of EGF receptor or MEK-1 suppressed the H/R-induced uPA secretion in wild type Lck transfected cells (Fig. 6*C*, *upper panel*, *lanes 2–7*). A low level of uPA was detected in cells grown under normoxic conditions (Fig. 6, *A–C*, *upper panels, lane 1*). All of these blots were reprobed with anti-actin antibody as loading controls (Fig. 6, *A–C*, *lower panels*) and quantified by densitometric analysis. The values were normalized with respect to actin, and -fold changes were calculated. These results demonstrated that H/R induced uPA secretion through Lck-dependent EGF receptor and ERK-mediated pathways. Similar results were obtained in MCF-7 cells (data not shown).

H/R Stimulates Lck-dependent EGF Receptor, MEK-1, and uPA-mediated Cell Migration—We and others have recently

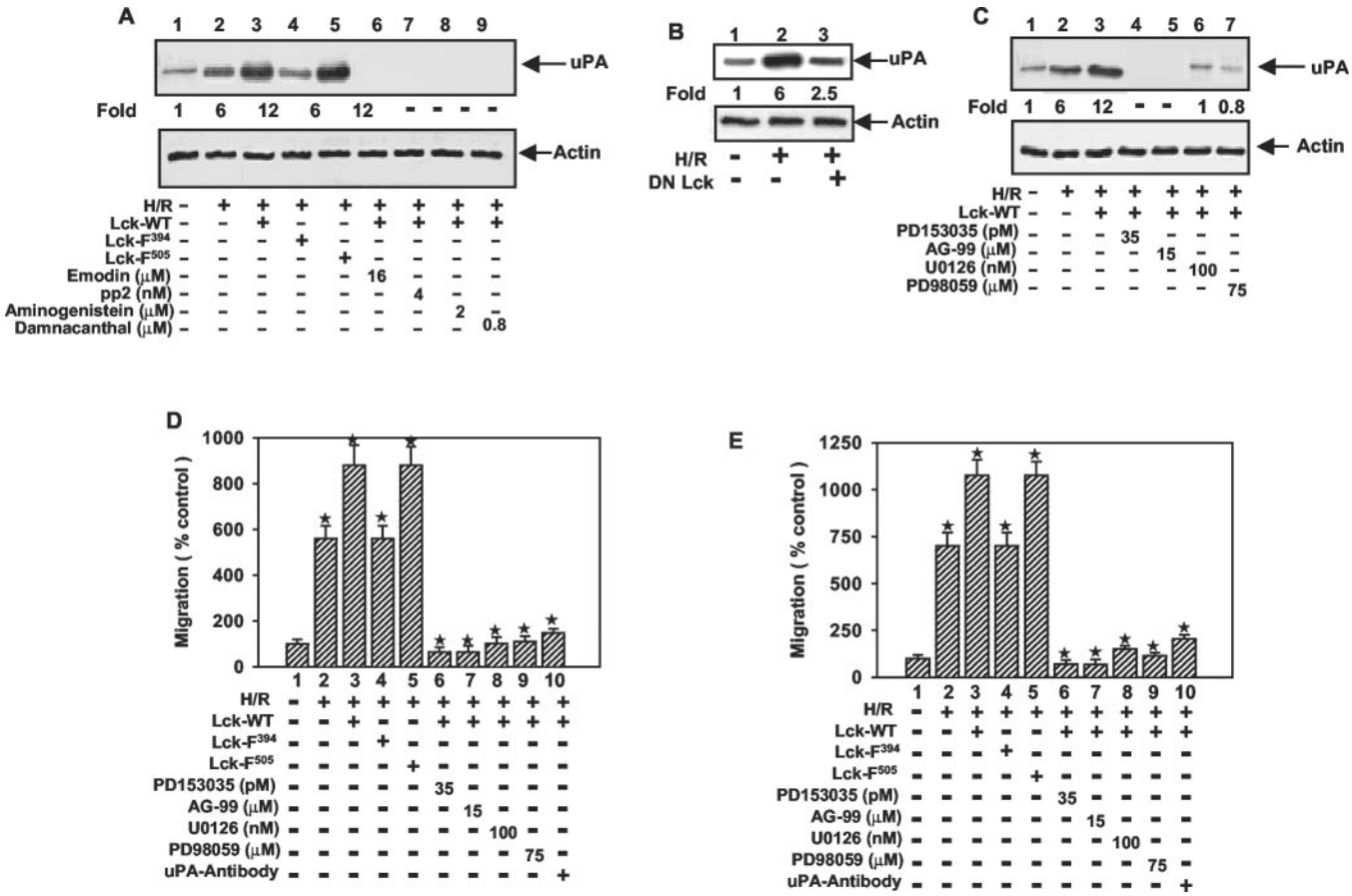

FIG. 6. As shown in *A–C*, H/R enhanced p56^{lck}-dependent uPA secretion occurred through EGF receptor and MEK-1-mediated pathways. MDA-MB-231 cells were transiently transfected with wild type (*WT*) Lck, mutant Lck F394, Lck F505. or DN Lck. and wild type Lck transfected cells were treated with inhibitors of Lck, EGF receptor, or MEK-1 and then induced by H/R. The expression of uPA was detected by Western blot using anti-uPA antibody. Wild type Lck or Lck F505 enhanced the uPA secretion as compared with Lck F394 transfected cells or cells induced by H/R (*A*, *upper panel*, *lanes 2–5*). A low level of uPA was detected in MDA-MB-231 cells grown under normoxic conditions (*lane 1*). Lck inhibitors drastically suppressed the H/R-induced uPA secretion in wild type Lck transfected cells (*lanes 6–9*). Cells transfected with DN Lck suppressed the H/R-induced uPA secretion (*B*, *upper panel*, *lanes 1–3*). Inhibitors of EGF receptor or MEK-1 suppressed the H/R-induced uPA secretion in wild type Lck transfected cells (*C*, *upper panel*, *lanes 1–7*). All of these blots were reprobed with anti-actin antibody (*A–C*, *lower panels*). The bands were quantified by densitometric analysis and normalized with respect to actin, and -fold changes are calculated. The results shown here represent three experiments exhibiting similar effects. As shown in *D* and *E*, EGF receptor, MEK-1, and uPA play crucial roles on H/R-induced Lck-mediated cell migration. MCF-7 (*D*) and MDA-MB-231 (*E*) cells were transiently transfected with wild type Lck, Lck F394, or Lck F505, and wild type Lck transfected cells were treated with inhibitors of EGF receptor or MEK-1 or with anti-uPA antibody and then induced by H/R. These cells were used for a migration assay. Cells transfected with either wild type Lck or Lck F505 followed by induction with H/R showed maximum cell migration as compared with Lck F394 transfected cells or cells induced by H/R alone (*D* and *E*), indicating that Tyr-394 of Lck is crucial for H/R-induced cell migration. Inhibitors of EGF receptor or MEK-1 or anti-uPA antibody suppressed the H/R-induced cell migration in wild type Lck transfected MCF-7 (*D*) and MDA-MB-231 (*E*) cells. The results are expressed as means ± S.E. of three determinations. The values were analyzed by Student's *t* test ($p < 0.001$).

demonstrated that uPA plays significant role in regulation of breast cancer cell migration (6, 23, 24). However, in this study, we have shown that Lck regulates the EGF receptor-mediated ERK-dependent uPA secretion in breast cancer cells in the presence of H/R. Therefore, we sought to determine whether EGF receptor and MEK-1 play any roles in Lck-mediated breast cancer cell migration in the presence of H/R. Accordingly, both MCF-7 (Fig. 6*D*) and MDA-MB-231 (Fig. 6*E*) cells were transfected with wild type Lck, Lck F394, or Lck F505, and wild type Lck transfected cells were treated with inhibitors of EGF receptor or MEK-1 and induced by H/R. The high level of transfection efficiency was observed in both of these cells. These cells were used for cell migration assay. The data indicated that both wild type Lck (880% in MCF-7 and 1076% in MDA-MB-231) and Lck F505 (872% in MCF-7 and 1072% in MDA-MB-231) transfected cells showed maximum migration as compared with Lck F394 transfected cells (560% in MCF-7 and 700% in MDA-MB-231) or cells induced by H/R alone (Fig. 6, *D* and *E*). The inhibitors of EGF receptor, PD153035 (65% in MCF-7 and 68% in MDA-MB-231 cells), or AG-99 (65% in MCF-7 and 70% in MDA-MB-231 cells) and MEK-1, U0126 (102% in MCF-7 and 150% in MDA-MB-231 cells), or PD98059 (110% in MCF-7 and 115% in MDA-MB-231 cells) suppressed the H/R-induced cell migration in both MCF-7 and MDA-MB-231 cells. The results showed that the H/R-induced cell migration is also blocked by anti-uPA antibody in wild type Lck-transfected MCF-7 (184%) and MDA-MB-231 (205%) cells. The data are expressed as the mean of three determinations ± S.E. The values were also analyzed by Student's *t* test ($p < 0.001$). These results suggested that EGF receptor and MEK-1 play crucial roles in H/R-induced Lck-dependent breast cancer cell migration. These data further demonstrated that p56^{lck} induces EGF receptor phosphorylation and subsequently stimulates MEK-1-ERK1/2-dependent uPA secretion, and all of these ultimately control the motility of breast cancer cells in the presence of hypoxia/reoxygenation (Fig. 7).

DISCUSSION

In a recent study (6), we have demonstrated that tyrosine kinase, p56^{lck}, regulates cell motility and NFκB-mediated se-

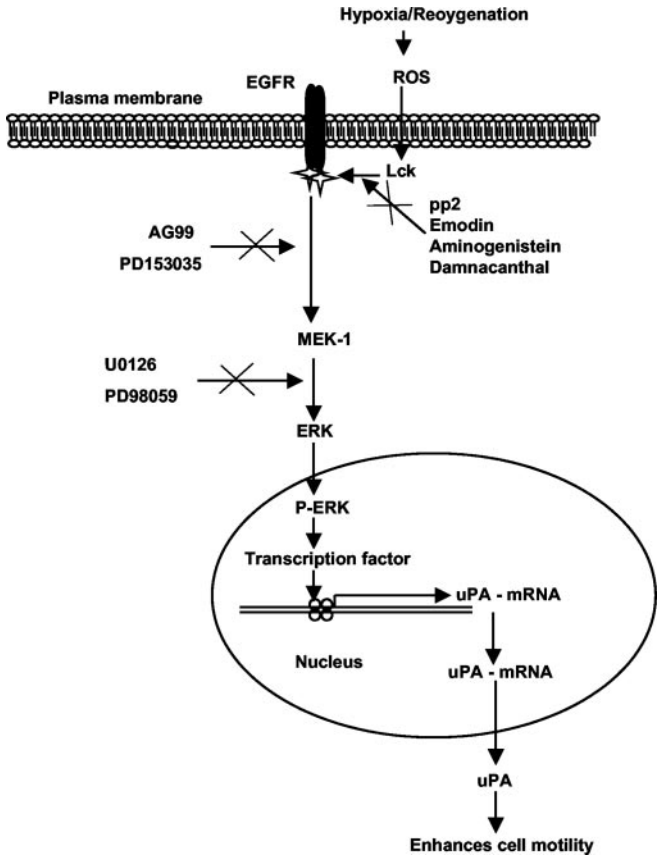

FIG. 7. **Molecular mechanism of H/R-induced Lck-dependent MEK-1-regulated ERK1/2 phosphorylation, uPA secretion, and cell motility through tyrosine phosphorylation of EGF receptor in breast cancer cells.** H/R induces Lck phosphorylation and its kinase activity through tyrosine phosphorylation of EGF receptor. Lck stimulates MEK-1-dependent ERK1/2 phosphorylation (*p-ERK*), uPA secretion, and cell motility in the presence of H/R in these cells. Emodin, pp2, aminogenistein, damnacanthal, AG-99, PD153035, U0126, PD98059, and DN Lck (K273R) specifically disrupt these signaling pathways.

cretion of uPA through tyrosine phosphorylation of IκBα following hypoxia/reoxygenation (H/R). However, it was not clear how p56^{lck} in the presence of H/R regulates EGF receptor phosphorylation and ERK-dependent uPA secretion in breast cancer cells. Accordingly, we have investigated the role of H/R on p56^{lck}-dependent EGF receptor phosphorylation in MCF-7 and MDA-MB-231 cells. In this report, we have shown that H/R induced p56^{lck} phosphorylation and Lck kinase activity in these cells. H/R also enhanced Lck-dependent EGF receptor phosphorylation. Lck induced the phosphorylation of MEK-1 and ERK1/2 in the presence of H/R, and both inhibitors of EGF receptors and Lck suppressed these effects, indicating that H/R-induced MEK-1/ERK1/2 phosphorylation occurred through Lck and EGF receptor-mediated pathways in breast cancer cells. Transfection of these cells with wild type Lck or Lck F505 but not with Lck F394 induced phosphorylations and activations of EGF receptor followed by MEK-1 and ERK1/2, suggesting that Tyr-394 of Lck plays important roles in these processes and Lck is upstream of EGF receptor. H/R also stimulated uPA secretion and cell motility in these cells. Pharmacological inhibitors of Lck, EGF receptor, and MEK-1 suppressed the H/R-induced uPA secretion and cell motility. Similarly, genetic inhibitor of Lck, DN Lck, also inhibited H/R-induced EGF receptor and ERK1/2 phosphorylations, uPA secretion, and cell motility. These data revealed that p56^{lck} in the presence of H/R regulates EGF receptor-mediated MEK-1-dependent ERK1/2 phosphorylation, uPA secretion, and cell motility in breast cancer cells.

Previous results indicated that phosphorylation of Lck at Tyr-394 by H_2O_2 is dominant over phosphorylation of Tyr-505 (25). It has been also documented that treatment of cells with pervanadate or diamide induces Lck phosphorylation at Tyr-394 and its kinase activity (26). Therefore, it is possible that these agents may act through a similar mechanism. Our data reveal that ROS generated by hypoxia/reoxygenation induced p56^{lck} phosphorylation at Tyr-394 and its kinase activity. In this study, we have demonstrated that p56^{lck} in the presence of H/R induced MEK-1 followed by ERK1/2 phosphorylation in MCF-7 and MDA-MB-231 cells. Both pharmacological (emodin, pp2, aminogenistein, and damnacanthal) and genetic (DN Lck) inhibitors of Lck suppressed the H/R-induced MEK-1 phosphorylation, indicating that Lck is present upstream of MAPK kinase.

Epidermal growth factor and its receptor are frequently upregulated in different types of human cancers. Earlier reports have indicated that expression of EGF receptor in colon carcinoma cells directly correlates with their metastatic potential (27). In this study, we have shown that H/R enhanced the Lck-dependent tyrosine phosphorylation of EGF receptor in breast cancer cells and further demonstrated that EGF receptor inhibitors have no effect on H/R-induced Lck phosphorylation and its kinase activity, suggesting that Lck is upstream of EGF receptor. EGF promotes the migration and invasion of a number of cell types, which has been linked to alternation in cell-extracellular matrix interactions (28). In this study, we have also demonstrated that EGF receptor is directly involved in H/R-induced Lck-mediated breast cancer cell migration. Previous data also indicated that EGF receptor plays a crucial role in the activation of MAP kinase pathways, induction of gene expression (29), and cell motility in different cell types (13, 14). In our study, we have shown that H/R induced EGF receptor phosphorylation, which leads to MEK-1-dependent ERK1/2 phosphorylation and cell motility in breast cancer cells. EGF receptor inhibitors blocked the H/R-induced ERK1/2 phosphorylation and cell motility in these cells.

uPA is a serine protease that plays a major role in tumor cell invasion, malignant progression, and distant metastasis. uPA is also responsible for regulation of matrix metalloproteinase activation and cell migration (30, 31). uPA is regulated at transcriptional level by multiple transcription factor. AP-1 transcription factor duplex plays a major role in the control of inducible uPA expression through the binding of enhancer regions in the promoter of the *uPA* gene (17). Therefore, it is possible that ERK1/2 might regulate the AP-1 transcription that ultimately controls the uPA secretion and cell migration in breast cancer cells. In this study, we have detected the level of uPA in both highly invasive (MDA-MB-231) and low invasive (MCF-7) breast cancer cells following H/R. H/R-induced uPA secretion is blocked by both genetic and pharmacological inhibitors of Lck and pharmacological inhibitors of MEK-1, suggesting that Lck in the presence of H/R regulates uPA secretion through MEK-1/ERK1/2-dependent pathways. H/R-induced Lck-mediated cell migration is also suppressed by uPA antibody, indicating that uPA plays an important role in breast cancer cell motility.

In summary, we have demonstrated for the first time that H/R induces Lck kinase activity and further indicated that Lck in the presence of H/R induces the cell migration through tyrosine phosphorylation of EGF receptor in low and highly invasive breast cancer cells. H/R also enhanced the phosphorylation of MAP kinase kinase and ERK1/2 in these cells. Both pharmacological and genetic inhibitors of Lck suppressed the

H/R-induced MEK-1/ERK1/2 phosphorylation, suggesting that Lck plays a significant role in these processes. Lck also induced uPA secretion and cell motility in the presence of H/R, and these are suppressed by inhibitors of Lck, EGF receptor, and MEK-1, indicating that these molecules are involved in uPA secretion and cell motility. Finally, these data showed that p56^{lck} in the presence of H/R regulates MEK-1-dependent ERK1/2 phosphorylation and uPA secretion through tyrosine phosphorylation of EGF receptor and further demonstrates that all of these signaling molecules ultimately control the motility of breast cancer cells. These findings may be useful in designing novel therapeutic interventions that may disrupt the redox-regulated p56^{lck}-dependent EGF receptor and MEK-1/ERK1/2-mediated signaling pathways, resulting in reduction of uPA secretion and consequent blocking of cell motility and metastatic spread of breast cancer.

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