

Hypoxia Regulates Cross-talk between Syk and Lck Leading to Breast Cancer Progression and Angiogenesis*

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Hypoxia is a key parameter that controls tumor angiogenesis and malignant progression by regulating the expression of several oncogenic molecules. The nonreceptor protein-tyrosine kinases Syk and Lck play crucial roles in the signaling mechanism of various cellular processes. The enhanced expression of Syk in normal breast tissue but not in malignant breast carcinoma has prompted us to investigate its potential role in mammary carcinogenesis. Accordingly, we hypothesized that hypoxia/reoxygenation (H/R) may play an important role in regulating Syk activation, and Lck may be involved in this process. In this study, we have demonstrated that H/R differentially regulates Syk phosphorylation and its subsequent interaction and cross-talk with Lck in MCF-7 cells. Moreover, Syk and Lck play differential roles in regulating Sp1 activation and expressions of melanoma cell adhesion molecule (MelCAM), urokinase-type plasminogen activator (uPA), matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor (VEGF) in response to H/R. Overexpression of wild type Syk inhibited the H/R-induced uPA, MMP-9, and VEGF expression but up-regulated MelCAM expression. Our data also indicated that MelCAM acts as a tumor suppressor by negatively regulating H/R-induced uPA secretion and MMP-9 activation. The mice xenograft study showed the cross-talk between Syk and Lck regulated H/R-induced breast tumor progression and further correlated with the expressions of MelCAM, uPA, MMP-9, and VEGF. Human clinical specimen analysis supported the *in vitro* and *in vivo* findings. To our knowledge, this is first report that the cross-talk between Syk and Lck regulates H/R-induced breast cancer progression and further suggests that Syk may act as potential therapeutic target for the treatment of breast cancer.

Hypoxia plays a crucial role in regulating breast tumor progression through a multistep process that includes oncogene activation or inhibition of tumor suppressor genes (1). Most tumors develop regions of chronically or transiently hypoxic cells during growth (2). Hypoxic tumor regions may show increased expression of many genes because of hypoxia-induced activation of transcription factors (3–5). Low extracellular pH, glucose depletion, high lactate levels, and regions with low oxygen tension (6, 7) characterize most tumors. Low oxygen tension in tumors has been associated with poor outcome, enhanced local or locoregional spread, and enhanced metastatic potential (8). Hypoxia is a key parameter, which modulates the expression of a variety of genes that are involved in tumor angiogenesis, malignant progression, and distant

metastasis (9). The signaling properties of reactive oxygen species are because of the reversible oxidation of redox-sensitive target proteins (10). The generated reactive oxygen species act as intracellular second messengers in various signal transduction pathways and hence play a crucial role in regulating disease and stress-induced cellular injuries such as ischemia/reperfusion, UV irradiation, and inflammation (10).

Previous reports have indicated that areas of hypoxia/reoxygenation (H/R)³ are a typical feature of rapidly growing and metastasizing tumors (11, 12). It was also demonstrated that both hypoxia and consecutive hypoxia/reoxygenation exert a variety of influence in tumor cell biology that ultimately regulates tumor progression (13). Earlier reports also showed that hypoxia and H/R regulate the activation of various mitogen-activated protein kinase signaling pathways (13) and induce the activation of several transcriptional factors such as HIF-1 α , NF κ B, AP-1, and Sp1 (13–15). However, very recently it was demonstrated that H/R rather than hypoxia alone appears to induce the expression and activation of several oncogenic molecules and plays an important role in tumor progression (13, 16).

The nonreceptor protein-tyrosine kinase Syk is widely expressed in hematopoietic cells (17, 18). It has tandem amino-terminal Src homology 2 domains and a carboxyl-terminal kinase domain (19). The Src homology 2 domains bind phosphorylated immunoreceptor tyrosine-based activation motifs and hence play a significant role in immunoreceptor and cytokine signaling (20). The expression of Syk has also been reported in cell lines of epithelial origin (21), but its function in these cells is not well understood. It has been documented that Syk is commonly expressed in normal human breast tissue, benign breast lesions, and low tumorigenic breast cancer cell lines (22). Previous data indicated that Syk suppresses cell motility and NF κ B-mediated urokinase-type plasminogen activator (uPA) secretion by inhibiting phosphatidylinositol 3-kinase activity in breast cancer cells (23). Lck, a member of the Src family nonreceptor protein-tyrosine kinase, is mostly expressed in T cells, breast cancer tissues, and cell lines and also in some B cells (24). Lck binds to the cytoplasmic domain of CD4 and CD8 and plays an essential role in T cell activation and development (25). Earlier reports have indicated that p72^{Syk} plays a crucial role in activation of p56^{Lck} through physical association and amino-terminal tyrosine phosphorylation at residues Tyr-518 and Tyr-519. Mutation of these residues to phenylalanines abolished its activity *in vitro* and toward cellular substrates *in vivo* and reduced its tyrosine phosphorylation by ~90% (26). However, the molecular mechanism by which H/R regulates Syk phosphorylation and its subsequent interaction with Lck leading to downstream signaling events in breast cancer cells are not well defined.

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³ The abbreviations used are: H/R, hypoxia/reoxygenation; Syk, splenic tyrosine kinase; Lck, leukocyte-specific kinase; HIF-1 α , hypoxia-inducible factor 1 α ; EMSA, electrophoretic mobility shift assay; MelCAM, melanoma cell adhesion molecule; uPA, urokinase-type plasminogen activator; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; WT, wild type; Mut, mutant; DN, dominant negative; NMRI, Naval Medical Research Institute.

MelCAM, previously known as MUC18 or MCAM, a newly recognized cell adhesion molecule with an apparent molecular mass of 113 kDa, belongs to the immunoglobulin superfamily (27–29). The presence of putative binding sites for the transcription factors Sp1, AP-1, AP-2, and cAMP-response element-binding protein in the promoter region suggests that MelCAM expression can be modulated by exogenous factors (30). At the cellular level, phorbol ester and cyclic AMP have been shown to modulate MelCAM expression (31). Earlier reports have indicated that MelCAM acts differently in the progression of breast carcinomas. It is expressed in normal and benign proliferative breast epithelium, and its expression is frequently lost in *in situ* and infiltrating breast carcinomas (32, 33). The MelCAM core promoter contains four binding sites for the Sp1 transcription factor, and deletion analyses have indicated that removal of all putative Sp1 sites reduced the promoter activity by 80%, suggesting that Sp1 is an important regulator of MelCAM expression (34). However, the molecular mechanism by which H/R regulates Syk activation and Syk-dependent Lck-mediated Sp1 activation leading to the regulation of MelCAM expression in MCF-7 cells is not well defined.

Degradation of extracellular matrix plays an important role in tumor metastasis. uPA is a member of the serine protease family that interacts with uPA receptor and facilitates the conversion of inert zymogen plasminogen into widely acting serine protease plasmin (35, 36). MMP-9, also referred as gelatinase-B, is not only associated with invasion and metastasis but also has been implicated in angiogenesis, rheumatoid arthritis, retinopathy, and vascular stenosis and hence is considered to be a prioritized therapeutic target (37, 38). Several reports have indicated the positive correlation between uPA/MMP-9 activation and the metastatic potential of tumors (39). However, the molecular mechanism by which H/R regulates Syk/Lck-dependent MelCAM expression and uPA secretion and the uPA-dependent pro-MMP-9 activation in breast carcinoma cells is not well understood. Moreover, the roles of these molecules in regulating H/R-induced tumorigenesis and its clinical implications are not well defined.

Hypoxia-induced VEGF production provides one of the main driving forces that stimulate the angiogenesis, which accompanies tumor progression (40, 41). To date, VEGF is considered as the key factor that guided and regulated tumor angiogenesis (42). Tumor cell-derived VEGF binds to its specific receptors and regulates tumor progression through neovascularization via autocrine and paracrine pathways (43, 44). Recent evidence suggested that the VEGF promoter contains an Sp1-response element (45). However, the role of Lck and Syk in H/R-induced VEGF expression in breast cancer is not defined clearly.

In this study we have demonstrated the differential role of Syk and Lck in the H/R-induced uPA, MMP-9, and VEGF expression. Our findings suggested that H/R down-regulates Syk activation leading to enhanced uPA, MMP-9, and VEGF expression. Furthermore, over-expression of Syk restored H/R-induced down-regulation of MelCAM expression. In hypoxic cells, Lck also physically associates with Syk, and this association plays a crucial role in regulating the downstream signaling. The *in vivo* relevance of our study was further validated in a xenografted nude mice model, which also supports our *in vitro* findings. Clinical data also indicated that the higher grades of tumors showed significant HIF-1 α expression compared with that of lower grades or normal breast tissue and also demonstrated an inverse correlation between Syk/MelCAM and uPA/MMP-9/VEGF expression, which further correlates with enhanced tumorigenic potential and neovascularization.

EXPERIMENTAL PROCEDURES

Materials—The rabbit polyclonal anti-Syk, anti-Lck, anti-Sp1, anti-MelCAM, anti-uPA, anti-MMP-9, anti-actin, mouse monoclonal anti-Lck, anti-Syk, anti-phosphotyrosine antibodies, and MelCAM blocking peptide were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-VEGF was from Oncogene. Rabbit polyclonal anti-vWF antibody was from Sigma. Rabbit polyclonal anti-HIF-1 α was from Upstate Biotechnology, Inc. Lipofectamine Plus was obtained from Invitrogen. pp2, aminoguanidine, and damnacanthal were from Calbiochem. The Sp1 consensus oligonucleotide was purchased from Bangalore Genei. Matrigel was purchased from BD Biosciences. The [γ -³²P]ATP was purchased from the Board of Radiation and Isotope Technology (Hyderabad, India). The female nude mice (NMRI, nu/nu) were from National Institute of Virology (Pune, India). All other chemicals were of analytical grade.

Cell Culture—The MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Hypoxic/Reoxygenation Cultures—The MCF-7 cells grown to 50–70% confluence were made hypoxic in evacuation chambers by intermittent application of vacuum and sparging with 95% N₂, 5% CO₂. Cells were analyzed at this point or maintained under hypoxic conditions in the presence of 100 mM dithionite (an O₂ scavenger) at 37 °C for the indicated time point. These cells were reoxygenated for the indicated periods by replacing the medium with fresh medium and incubating the cultures in humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Immunofluorescence and Immunohistochemistry—To detect the effect of H/R on cellular localization of Syk, cells grown in monolayer on glass slides were induced by hypoxia for 2 h and reoxygenated for 45 min. The cells were fixed and incubated with rabbit polyclonal anti-Syk antibody (1:50 dilution) followed by FITC-conjugated anti-rabbit IgG at room temperature. The role of H/R in regulating Syk-Lck colocalization was determined by immunofluorescence studies using a mixture of mouse monoclonal anti-Syk and rabbit polyclonal anti-Lck antibody followed by a mixture of TRITC- and FITC-conjugated IgG. The cells were washed and mounted with coverslips. All these samples were analyzed under confocal microscopy (Zeiss).

The clinical specimens were analyzed by immunohistopathological studies. Formalin-fixed paraffin-embedded sections (4 μ m) were subjected to antigen retrieval, and the Syk-Lck colocalization was determined by immunofluorescence studies using a mixture of rabbit polyclonal anti-Syk and mouse monoclonal anti-Lck antibodies followed by a mixture of FITC- and TRITC-conjugated IgGs. The levels of HIF-1 α expression in these clinical samples were determined by Western blot analysis. The levels of MelCAM, uPA, MMP-9, and VEGF expressions in these samples were detected by immunofluorescence studies by using their specific antibodies. The tumor microvessel densities were detected by immunostaining with anti-vWF antibody. All these samples were analyzed under confocal microscopy (Zeiss).

Plasmids and DNA Transfection—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). The wild type and kinase-negative Syk cDNA in pcDNA 3.1 were the generous gifts from Dr. Susette C. Mueller (Department of Oncology, Georgetown University Medical School, Washington, D. C.). MCF-7 cells were transfected with specific cDNA using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions as described previously (46). These transfected cells were used for Syk-Lck colocalization and interaction studies, detection of Sp1, Syk, Lck, MelCAM, uPA,

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MMP-9, and VEGF by Western blot analysis, and *in vivo* tumorigenicity experiments.

Zymography Experiments—The gelatinolytic activity was measured as described (46, 47). To examine whether H/R regulates MMP-9 activation and to investigate the effect of inhibition of MelCAM expression on H/R-induced MMP-9 activation, cells were either induced with H/R for 24 h or pretreated with MelCAM blocking peptide (50 μ g) and then exposed with H/R. The conditioned media were collected, and the samples were analyzed by zymography as described (47). Negative staining showed the zones of gelatinolytic activity.

Immunoprecipitation—To delineate the role of H/R in the regulation of tyrosine phosphorylation of Syk, cells were exposed to hypoxia for 2 h followed by reoxygenation for 0–60 min. In separate experiments, cells were pretreated with Lck inhibitors, pp2 (4 nM), aminogestine (2 μ M), and damnacanthal (0.8 μ M), and then exposed to H/R. Cell lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody and analyzed by Western blot using anti-phosphotyrosine antibody. The same blots were reprobed with anti-Syk antibody. To analyze whether Syk interacts with Lck and to determine whether H/R regulates this process, cells were exposed to H/R for 45 min. In other experiments, cells were transfected with wild type and kinase-negative Syk and then subjected to H/R. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin). The lysates containing equal amount of total proteins were immunoprecipitated with rabbit anti-Lck antibody and analyzed by Western blot using anti-Syk antibody. The same blots were reprobed with anti-Lck antibody.

Western Blot Analysis—To analyze the roles of Syk and Lck in regulating MelCAM, VEGF expression, and MMP-9 activation, cells were transfected with wild type and kinase-negative Syk or dominant-negative Lck and then exposed to H/R for 24 h as described earlier. The cell lysates were analyzed by Western blot using anti-MelCAM or anti-VEGF antibody. The levels of Lck and Syk in nontransfected or transfected cell lysates were also detected by Western blot using anti-Lck or anti-Syk antibody. The level of active MMP-9 in conditioned media was detected by Western blot.

To analyze the effect of inhibition of MelCAM on H/R-induced uPA secretion, cells were pretreated with MelCAM-specific blocking peptide (50 μ g/ml) for 24 h and subjected to H/R treatment. Cell lysates were analyzed by Western blot using anti-uPA antibody. The level of HIF-1 α expression in the normal and breast tumor specimens of different grades was also detected by Western blot using anti-HIF-1 α antibody. The same blots were reprobed with anti-actin antibody as loading control.

Nuclear Extracts and Western Blot—To check whether H/R regulates Sp1 expression, cells were subjected to H/R for 0–180 min at 37 °C. The nuclear extracts were prepared as described earlier (24). Briefly, cells were incubated in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and allowed to swell on ice for 10 min. Cells were homogenized in a Dounce homogenizer. The nuclei were separated by spinning at 3300 \times g for 5 min at 4 °C. The supernatant was used as cytoplasmic extract. The nuclear pellet was extracted in nuclear extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and centrifuged at 12,000 \times g for 30 min. The supernatant was used as nuclear extract. The nuclear extracts were resolved by SDS-PAGE, and the level of Sp1 was detected by Western blot using rabbit anti-Sp1 antibody. In separate experiments, to examine the

effects of Syk and Lck on H/R-induced Sp1 expression, cells were transfected with wild type and kinase-negative Syk or DN Lck and then induced with H/R. The nuclear extracts were prepared and analyzed by Western blot using anti-Sp1 antibody. The levels of Lck and Syk in nuclear extracts were also detected by Western blot using anti-Lck or anti-Syk antibody. The level of expression of HIF-1 α in hypoxia or H/R-induced nuclear extracts was detected by Western blot using anti-HIF-1 α antibody. Actin was used as loading control.

EMSA—To examine whether H/R induces the Sp1-DNA binding, cells were exposed to H/R for 0–180 min as described earlier. To check whether Syk and Lck play any role in regulating H/R-induced Sp1-DNA binding, cells were transfected with wild type and kinase-negative Syk or pretreated with Lck inhibitors, pp2 (4 nM), aminogestine (2 μ M), and damnacanthal (0.8 μ M) followed by exposed to H/R for 1 h. The nuclear extracts were prepared as described above and incubated with 16 fmol of ³²P-labeled double-stranded Sp1 oligonucleotide (5'-ATTCGATCGGGCGGGGCG-3') in binding buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) containing 1 μ g of poly(dI-dC). The DNA-protein complex was resolved on a native polyacrylamide gel and analyzed by autoradiography.

In Vivo Xenograft Tumor Model—The tumorigenicity experiments were performed as described (47, 48). Briefly, female athymic nude mice, NMRI, nu/nu (BALB/c) obtained from National Institute of Virology (Pune, India) were housed under specific pathogen-free conditions and used for *in vivo* tumorigenicity studies. Cells were either exposed to H/R or transfected with wild type or mutant Syk and then exposed to H/R for 24 h. Cell viability was determined by the trypan blue exclusion test, and only a single cell suspensions (5 \times 10⁶/0.2 ml) of >90% viability were mixed with Matrigel and injected subcutaneously into the flanks of female athymic NMRI (nu/nu) mice (6–8 weeks old). Five mice were used in each set of experiments. Growth of tumors was monitored weekly by measuring the tumors with calipers. The mice were killed after 4 weeks of injection, and the levels of uPA, VEGF, and MelCAM in the tumors were analyzed by Western blot. The level of Sp1-DNA binding in the nuclear extracts of the tumor samples was determined by EMSA.

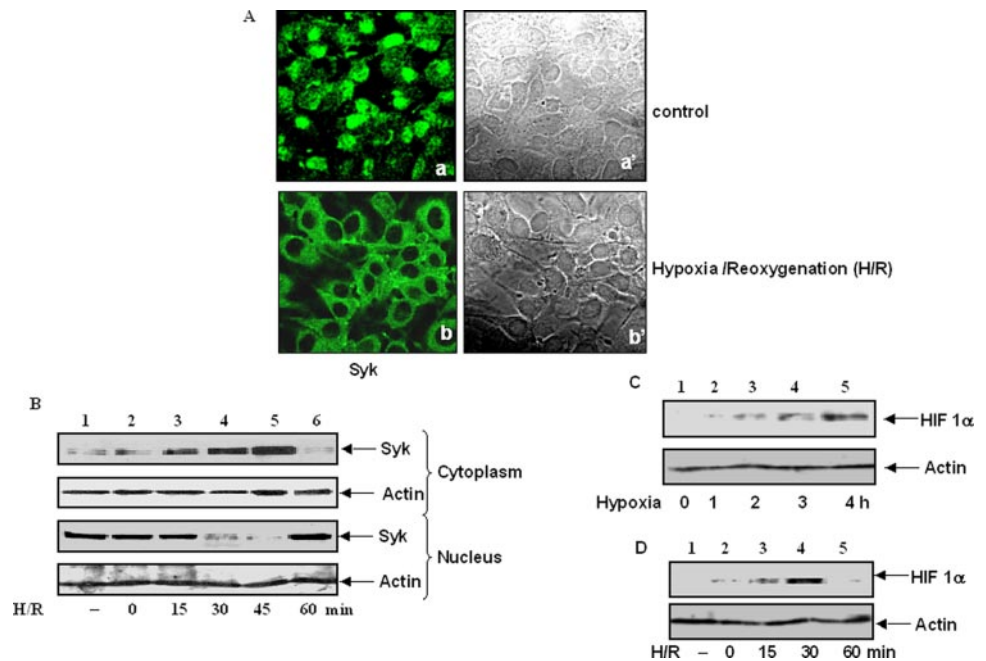
The tumor samples were also processed for histopathological studies. Formalin-fixed paraffin-embedded sections (4 μ m) were subjected to antigen retrieval and then stained with rabbit polyclonal anti-MMP-9, anti-uPA antibody, or mouse monoclonal anti-MelCAM antibody followed by FITC-conjugated anti-rabbit or anti-mouse IgG. The tumor microvessel densities were detected by immunostaining with anti-vWF antibody. The level of colocalization of Syk and Lck in these tumor specimens was determined by immunofluorescence using a mixture of mouse monoclonal anti-Syk and rabbit polyclonal anti-Lck antibody followed by a mixture of FITC- and TRITC-conjugated IgG. All these samples were analyzed under confocal microscopy (Zeiss).

Human Breast Tumor Specimen Analysis—Human breast tumor specimens with different grades and normal breast tissues were collected from a local hospital with informed consent and were flash-frozen. The immunohistochemical and Western blot analyses of those specimens were performed as described above.

RESULTS

H/R Regulates the Cellular Localization of Syk—To determine whether H/R regulates the cellular localization of Syk, MCF-7 cells (2 \times 10⁵ cells/ml) seeded on 35-mm tissue culture plate were exposed to hypoxia for 2 h and then reoxygenated for 45 min. After that, cells were fixed and incubated with rabbit polyclonal anti-Syk antibody followed by FITC-conjugated anti-rabbit IgG. The data indicated that H/R induces the cytoplasmic translocation of Syk at 45 min in these cells

FIGURE 1. H/R regulates cellular localization of Syk. *A*, MCF-7 cells were exposed to hypoxia for 2 h and reoxygenated for 45 min. The cellular localization of Syk was determined by immunofluorescence staining using anti-Syk antibody and analyzed by confocal microscopy. *a* and *b*, immunofluorescence photographs; *a'* and *b'*, phase contrast photographs. *B*, cellular localization of Syk was analyzed by Western blot. Cells were exposed to hypoxia for 2 h and then reoxygenated for 0–60 min. Cytoplasmic and nuclear extracts were prepared, and the cellular localization of Syk was detected by Western blot using anti-Syk antibody. Actin was used as loading control. Note that maximum level of cytoplasmic translocation of Syk was observed at 45 min of exposure with H/R, and its least expression was noticed in the nucleus at 45 min. *C* and *D*, effects of hypoxia and H/R on nuclear localization of HIF-1 α . Cells were exposed to hypoxia for 0–4 h or exposed to hypoxia for 2 h followed by reoxygenation for 0–60 min. The level of HIF-1 α in the nuclear extracts was detected by Western blot. Note that the higher level of HIF-1 α was observed at 4 h of hypoxic treatment or at 30 min of H/R exposure. Actin was used as loading control. The results shown here represent three experiments exhibiting similar effects.



(Fig. 1A). To further confirm the cellular localization of Syk in response to H/R, cells were exposed to hypoxia for 2 h followed by reoxygenation for 0–60 min. The cytoplasmic and nuclear fractions were isolated, and the level of Syk was detected by Western blot. The results indicated that cytoplasmic translocation of Syk was found at 45 min in response to H/R exposure (Fig. 1B). The same blots were reprobed with anti-actin antibody as loading control. Previous studies demonstrated that HIF-1 α is considered as key transcription factor and acts as marker under hypoxic conditions (15). Accordingly cells were exposed to hypoxia for 0–4 h or exposed to hypoxia for 2 h and then reoxygenated for 0–60 min. The nuclear extracts were prepared and subjected to Western blot analysis using anti-HIF-1 α antibody. The results indicated that nuclear expression of HIF-1 α was found after 4 h of exposure with hypoxia (Fig. 1C, lanes 1–5), whereas maximum nuclear expression of HIF-1 α was detected when cells were exposed to hypoxia for 2 h and reoxygenation for 30 min and rapidly returned to basal level at 60 min (Fig. 1D, lanes 1–5). The level of expression of HIF-1 α in response to hypoxia or reoxygenation in nuclear extracts was detected as positive control. Actin was used as loading control.

H/R Suppresses Syk Phosphorylation—To ascertain the role of H/R on tyrosine phosphorylation of Syk, cells were exposed to hypoxia for 2 h and then reoxygenated for 0–60 min. The cell lysates were immunoprecipitated with rabbit polyclonal anti-Syk antibody and analyzed by Western blot using anti-phosphotyrosine antibody (Fig. 2A, upper panel, lanes 1–5). The data revealed that cells exposed to H/R suppressed tyrosine phosphorylation of Syk at 45 min (Fig. 2A, upper panel, lane 4) and were restored at 60 min (lane 5). These data suggested that dephosphorylation of Syk at 45 min leads to cytoplasmic translocation, whereas rephosphorylation causes its nuclear translocation at 60 min (Figs. 1B and 2A). The same blots were reprobed with anti-Syk antibody (Fig. 2A, lower panel).

To delineate whether p56^{Lck} plays any role in Syk phosphorylation in response to H/R, cells were pretreated with Lck inhibitors (pp2, aminoguanidine, and damnacanthal) and then treated with H/R. Cell lysates were immunoprecipitated with anti-Syk antibody and immunoblotted with anti-Tyr(P) antibody (Fig. 2B, upper panel, lanes 1–5). The same blots were reprobed with anti-Syk antibody (Fig. 2B, lower panel). The

results indicated that cells treated with Lck inhibitors restored the Syk phosphorylation, which was down-regulated upon exposure to H/R.

H/R Regulates the Interaction between Syk and Lck—To examine the role of H/R in regulating the interaction between Syk and Lck, cells were either exposed to H/R or transfected with wild type and mutant Syk and then exposed to H/R. Cell lysates were immunoprecipitated with anti-Lck antibody and analyzed by Western blot with anti-Syk antibody (Fig. 2C, upper panel). Cells transfected with WT Syk but not with Mut Syk resulted in restoration of Syk and Lck interaction in response to H/R. The same blots were reprobed with anti-Lck antibody (Fig. 2C, lower panel). The cellular colocalization between Syk and Lck in response to H/R was further confirmed by immunofluorescence study (Fig. 2D). These results suggested that H/R down-regulates the physical association between Syk and Lck, and this is due to cytoplasmic translocation of Syk in response to H/R at 45 min.

Syk Negatively Regulates H/R-induced Sp1 Activation—To determine the role of H/R on Sp1 nuclear translocation and DNA binding, MCF-7 cells were exposed to H/R for 0–180 min as described earlier, and the cytoplasmic and nuclear extracts were prepared. Nuclear extracts were subjected to Western blot using anti-Sp1 antibody (Fig. 3A, upper panel, lanes 1–5). Similarly, cytoplasmic extracts were also used for Western blot using anti-Sp1 antibody (Fig. 3A, 2nd middle panel). Actin was used as loading control (Fig. 3A, 1st middle and lower panels). To examine whether H/R regulates Sp1-DNA binding, nuclear extracts were analyzed by EMSA using Sp1-specific oligonucleotide (Fig. 3C, lanes 1–5). The results showed that H/R induces maximum Sp1 nuclear translocation and DNA binding at 60 min (Fig. 3, A and C). To examine the roles of Syk and Lck in H/R-induced Sp1 nuclear translocation and Sp1-DNA binding, cells were individually transfected with WT and Mut Syk or DN Lck or treated with Lck-specific inhibitors (pp2, aminoguanidine, and damnacanthal) and were then exposed to H/R. Nuclear extracts were analyzed by Western blot (Fig. 3B, upper panel, lanes 1–5) and EMSA (Fig. 3, D and E). The blots were reprobed with anti-Syk or anti-Lck antibody in order to detect the expression of Syk and Lck (Fig. 3B, 1st middle and 2nd middle panels). Actin was used as loading control (Fig. 3B, lower panel). The results demonstrated that Syk negatively regulates H/R-induced Sp1 nuclear translocation and DNA binding,

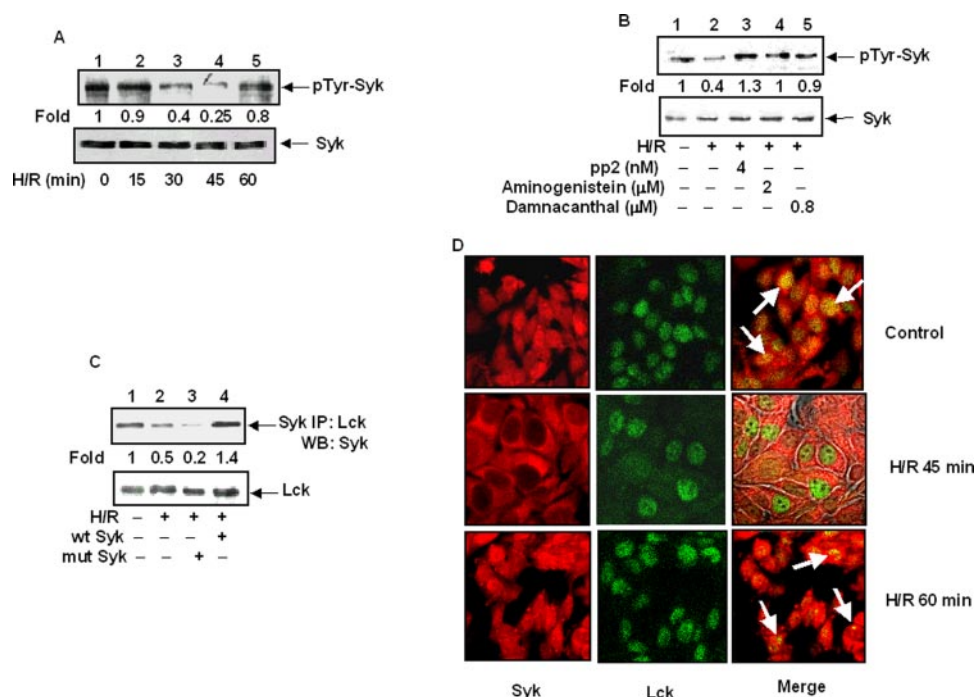
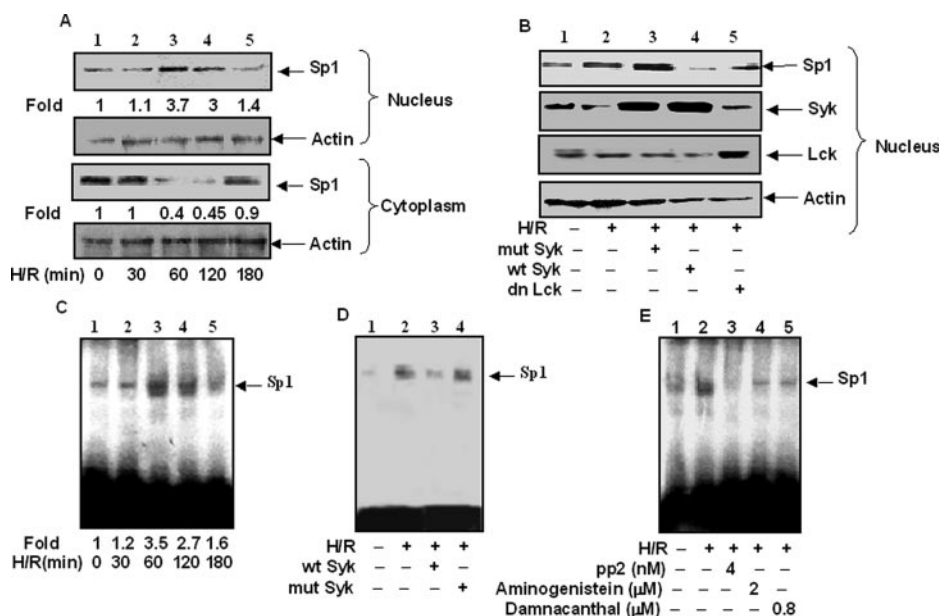


FIGURE 2. A and B, role of H/R in regulation of Lck-dependent Syk phosphorylation. A, cells were exposed to hypoxia for 2 h followed by reoxygenation for 0–60 min as described. Cell lysates were immunoprecipitated with anti-Syk antibody followed by Western blot with anti-phosphotyrosine antibody. The same blot was reprobed with anti-Syk antibody. Note that significant down-regulation of Syk phosphorylation was observed at 45 min of exposure to H/R. B, cells were pretreated with Lck inhibitors (pp2 (4 nM), aminoginestien (2 μ M), and damnacanthal (0.8 μ M)) and then exposed to H/R. The level of phosphorylated Syk in the cell lysates was detected by Western blot as described earlier. Note that H/R down-regulates Syk phosphorylation, and inhibition of Lck restored the Syk phosphorylation in response to H/R. C, H/R down-regulates Syk-Lck interaction. Cells were either exposed to H/R or individually transfected with WT or Mut Syk and then exposed to H/R. Cell lysates were immunoprecipitated with anti-Lck antibody and analyzed by Western blot (WB) using anti-Syk antibody. The same blot was reprobed with anti-Lck antibody. All these bands were quantified by densitometric analysis, and the fold changes were calculated. D, role of H/R in regulation of colocalization between Syk and Lck by immunofluorescence. Note that Syk was stained with TRITC-conjugated IgG, and Lck was stained with FITC-conjugated IgG. The enhanced colocalization was observed at initial stage (control) and restored at 60 min of H/R treatment as indicated by arrows. However, no colocalization was observed at 45 min due to cytoplasmic translocation of Syk. The results shown here represent three experiments exhibiting similar effects.

FIGURE 3. H/R regulates Syk-Lck-mediated Sp1 nuclear translocation and DNA binding.

A, cells were exposed to hypoxia for 2 h followed by reoxygenation for 0–180 min. The level of Sp1 in nuclear and cytoplasmic extracts was detected by Western blot using anti-Sp1 antibody. Actin was used as loading control. B, cells were individually transfected with WT or Mut Syk or DN Lck and then exposed to H/R for 60 min as described earlier. The Sp1 level in the nuclear extract was determined by Western blot. The levels of Syk and Lck in the nuclear extracts were also analyzed by Western blot using anti-Syk or anti-Lck antibody. The same blots were reprobed with anti-actin antibody as loading control. C, experiments paralleling that of A but measuring Sp1-DNA binding by EMSA. D and E, cells were individually transfected with WT and Mut Syk or pretreated with pp2 (4 nM) or aminoginestien (2 μ M) or damnacanthal (0.8 μ M) and then exposed to H/R. The Sp1-DNA binding was performed by EMSA. The results shown here represent three experiments exhibiting similar effects.



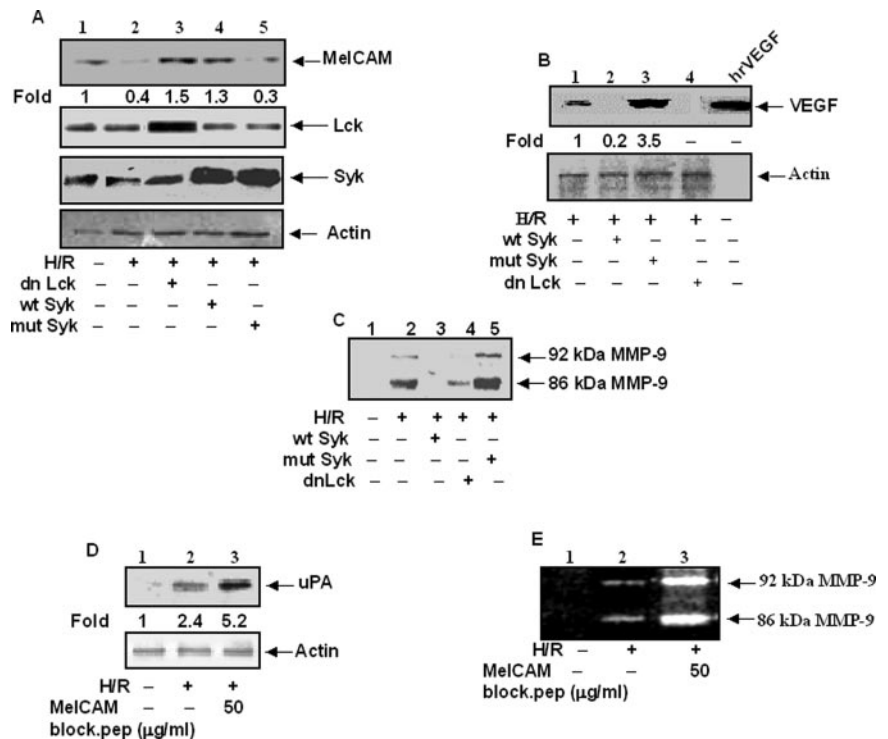
whereas pharmacological or genetic inhibitors of Lck significantly suppressed H/R-induced Sp1 activation, suggesting that Lck and Syk differentially regulate the Sp1 activation in response to H/R.

Syk and Lck Differentially Regulate MelCAM, VEGF, and MMP-9 Expressions in Response to H/R—To delineate the roles of Syk and Lck in differentially controlling the expressions of MelCAM and VEGF and the activation of MMP-9 in the presence of H/R, cells were transfected with DN Lck or WT and Mut Syk and then exposed to H/R. The levels of

MelCAM, VEGF, and MMP-9 were detected by Western blots using their specific antibodies. The results showed that DN Lck and WT Syk but not Mut Syk restored the H/R-suppressed MelCAM expression (Fig. 4A, lanes 1–5). The levels of Lck and Syk expression were also detected by Western blot analysis using their specific antibodies (Fig. 4A, 1st and 2nd middle panels). Actin was used as loading control (Fig. 4A, lower panel). In contrast, WT Syk and DN Lck suppressed the H/R-induced VEGF expression and MMP-9 activation (Fig. 4, B, lanes 1–4,

FIGURE 4. Syk-Lck interplay differentially regulates H/R-induced expression of downstream effector molecules.

A, cells were individually transfected with WT and Mut Syk or DN Lck and exposed to H/R. The level of MelCAM expression in the cell lysates was analyzed by Western blot. The levels of Syk and Lck were analyzed by Western blot. The same blots were reprobed with anti-actin antibody as loading control. **B** and **C**, the expression of VEGF in the above transfected cell lysates and the activation of MMP-9 in the above conditioned media were detected by Western blot using their specific antibodies. Human recombinant VEGF was used as positive control. **D** and **E**, cells were pretreated with MelCAM blocking peptide and then exposed to H/R. The cell lysates and conditioned media were used to detect the levels of uPA by Western blot (**D**) and MMP-9 by zymography (**E**), respectively. Note that H/R down-regulates MelCAM expression but enhances VEGF expression and MMP-9 activation. Blocking of MelCAM by its blocking peptide further stimulates H/R-induced uPA expression and MMP-9 activation.



and **C**, lanes 1–5). However, cells transfected with Mut Syk further enhanced the H/R-induced VEGF expression and MMP-9 activation (Fig. 4, **B**, lane 3, and **C**, lane 5). These data suggested that Syk, which is considered a negative regulator of tumor progression, can also regulate the expression of MelCAM, a breast tumor suppressor molecule.

MelCAM Negatively Regulates H/R-induced uPA Secretion and MMP-9 Activation—Our previous study demonstrated that uPA plays a crucial role in MMP-9 activation (47). To examine the role of MelCAM in regulation of uPA secretion and MMP-9 activation, cells were either exposed to H/R or pretreated with MelCAM blocking peptide and then exposed to H/R. Cell lysates were analyzed by Western blot using anti-uPA antibody. Similarly, the conditioned media were used to detect the MMP-9 activation by zymography. The results clearly indicated that H/R enhances uPA secretion and MMP-9 activation, which is further up-regulated in the presence of MelCAM blocking peptide (Fig. 4, **D**, lanes 1–3, and **E**, lanes 1–3). Our findings clearly demonstrated that a molecular link exists between Syk/Lck and MelCAM and between VEGF and MMP-9, and all of these ultimately control the tumor progression and angiogenesis.

Effect of Syk on Xenograft Tumor Growth—Our *in vitro* results prompted us to extend our studies to the *in vivo* system. Accordingly, MCF-7 cells were transfected with WT or Mut Syk, exposed to H/R, and then implanted into the nude mice. After 4 weeks, mice were sacrificed, and tumor specimens were excised. A portion of tumor samples was used for histopathological studies. The histopathological analysis data indicated that Mut Syk-transfected tumors showed higher infiltration toward the extravasations, nuclear polymorphism, and mitotic features compared with the control or WT Syk-transfected tumors (Fig. 5A, panels a–c). The immunohistochemical studies were also performed with a mixture of anti-Syk and anti-Lck antibodies or with anti-MelCAM, anti-uPA, anti-MMP-9, and anti-vWF antibodies. The data indicated that tumors generated by injecting WT Syk-transfected cells revealed higher nuclear localization and enhanced interaction between Syk and Lck, whereas Mut Syk-transfected tumors showed enhanced cytoplasmic localization of Syk (Fig. 5A, panels d–f). Most inter-

estingly, the expressions of MMP-9, uPA, and neovascularization (vWF expression) were also higher in the Mut Syk-transfected tumor (Fig. 5E, panels a–i) that further correlated with our *in vitro* study. As expected, the MelCAM expression was higher in WT Syk-transfected tumor (Fig. 5A, panels g–i). This was also confirmed by Western blot analysis (Fig. 5C, lanes 1–3). Western blot data also suggested that the levels of uPA and VEGF are higher in Mut Syk-transfected tumors (Fig. 5B, lanes 1–3). Similar results were obtained in Sp1-DNA binding as detected by EMSA (Fig. 5D, lanes 1–3). Thus our studies clearly indicated that Syk acts as a tumor suppressor and anti-angiogenic candidate gene that differentially regulates MelCAM expression that ultimately controls uPA-dependent MMP-9 activation. Syk also negatively regulates VEGF expression. These signaling molecules ultimately control the tumor progression and angiogenesis.

Human Breast Tumor Specimen Analysis—The *in vitro* and *in vivo* mouse model data further prompted us to extend these studies with human clinical breast tumor samples. Human solid breast tumor specimens were collected with the informed consent from a local hospital. The tumor grading was determined by histopathology analysis by hematoxylin and eosin staining using a modified Scarff-Bloom-Richardson system, and photographs were taken with a Nikon microscope (Fig. 6A, panels a–d). The cellular localizations of Syk and Lck in human clinical breast tumor sections were detected by incubating with a mixture of anti-Syk and anti-Lck antibodies. Our results indicated that in normal breast tissue there was colocalization of Syk and Lck, but with the increasing grade of tumor specimens, no colocalization was observed. Furthermore, in higher grades of tumors, the expression of Syk was totally diminished (Fig. 6A, panels e–h). As expected, MelCAM expression was significantly lower in higher grades of tumors compared with lower grades or normal tissue (Fig. 6A, panels i–l). The expression of HIF-1 α was detected by Western blot using the same specimens, and the data showed that the level of HIF-1 α was significantly higher in grade II and III tumors (Fig. 6B, lanes 1–4), which indicated the hypoxic status in the tumor microenvironment. Higher expressions of MMP-9, uPA, VEGF, and vWF in these tumor specimens further correlated with

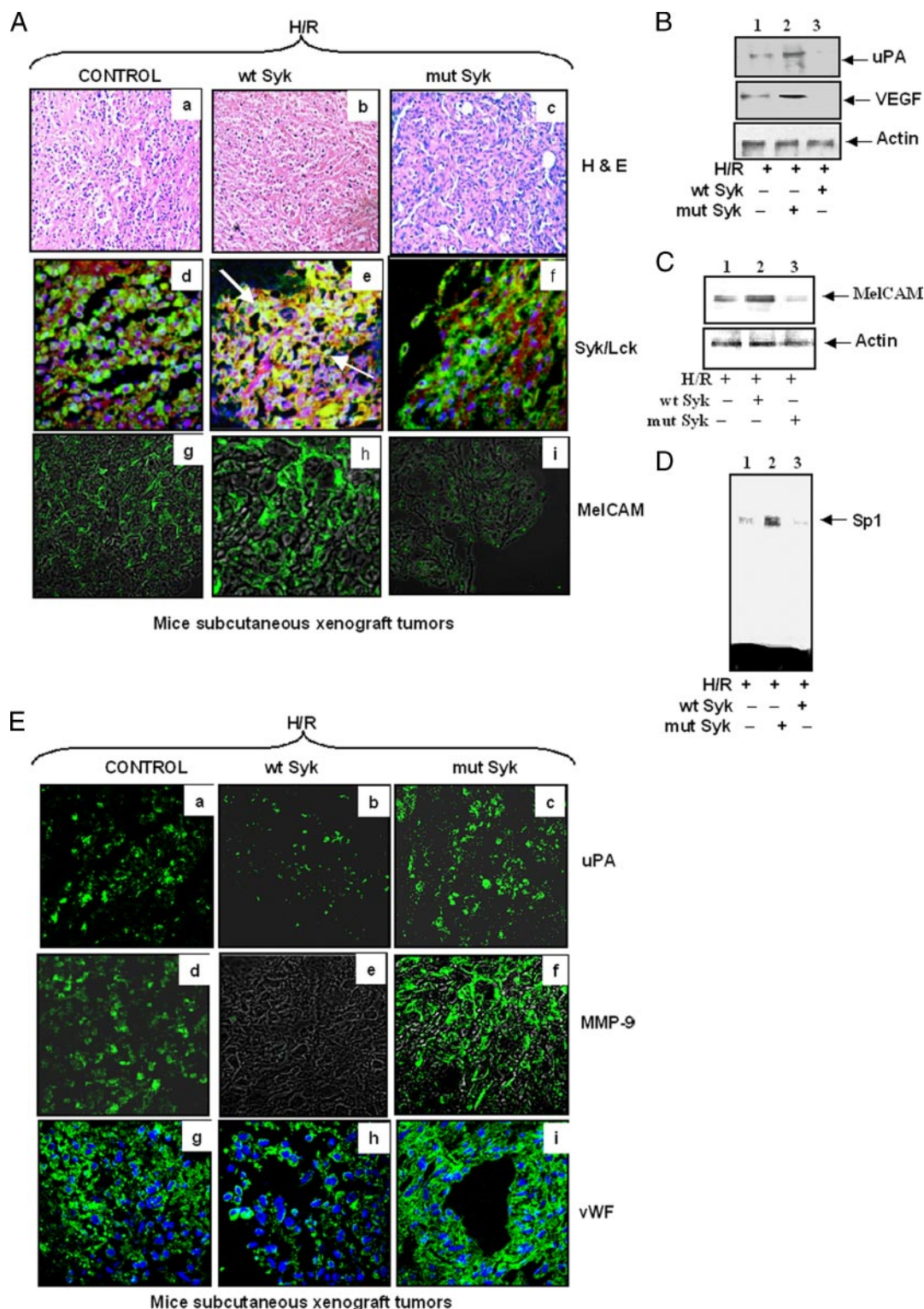


FIGURE 5. H/R regulates tumor growth and angiogenesis through differential interplay between Syk and Lck. *A*, MCF-7 cells ($5 \times 10^6/0.2$ ml) were either exposed to H/R for 24 h or transfected with WT or Mut Syk and then exposed to H/R. The cell suspension was mixed with cold Matrigel and injected subcutaneously in the dorsal region of mice. After 4 weeks, tumors were excised and subjected to histopathological analysis by hematoxylin and eosin staining (panels *a–c*). Note that increased infiltration and nuclear polymorphism were observed in a tumor generated by injecting Mut Syk but not WT Syk-transfected cells. The physical association between Syk and Lck in these tumors was detected by immunohistochemistry (panels *d–f*). Note that tumors generated by injecting WT Syk-transfected cells showed higher nuclear localization and enhanced interaction between Syk and Lck, whereas Mut Syk-transfected tumors showed enhanced cytoplasmic localization of Syk (panels *d–f*). Syk was stained with FITC-conjugated IgG (green), and Lck was stained with TRITC-conjugated IgG (red). The nuclei of the cells was visualized by DAPI (blue) staining. The MelCAM expression in these tumors was analyzed by immunofluorescence using anti-MelCAM antibody (panels *g–i*). *B* and *C*, the expressions of uPA, VEGF, and MelCAM in the tumor extracts were also analyzed by Western blot using their specific antibodies. *D*, the Sp1-DNA binding in the nuclear extracts was performed by EMSA. *E*, the levels of uPA, MMP-9, and vWF in the above tumor specimens were analyzed by immunofluorescence using their specific antibodies (panels *a–i*). Note that there was drastic reduction in uPA and MMP-9 levels in a tumor generated by injecting WT Syk but not Mut Syk-transfected cells. The microvessel density was detected by staining with anti-vWF antibody (endothelial cell-specific marker) in these tumors (panels *g–i*). The nuclei were stained with DAPI (blue). The enhanced vWF expression observed in Mut Syk-transfected tumors indicated higher vascularization compared with control or WT Syk-transfected tumors.

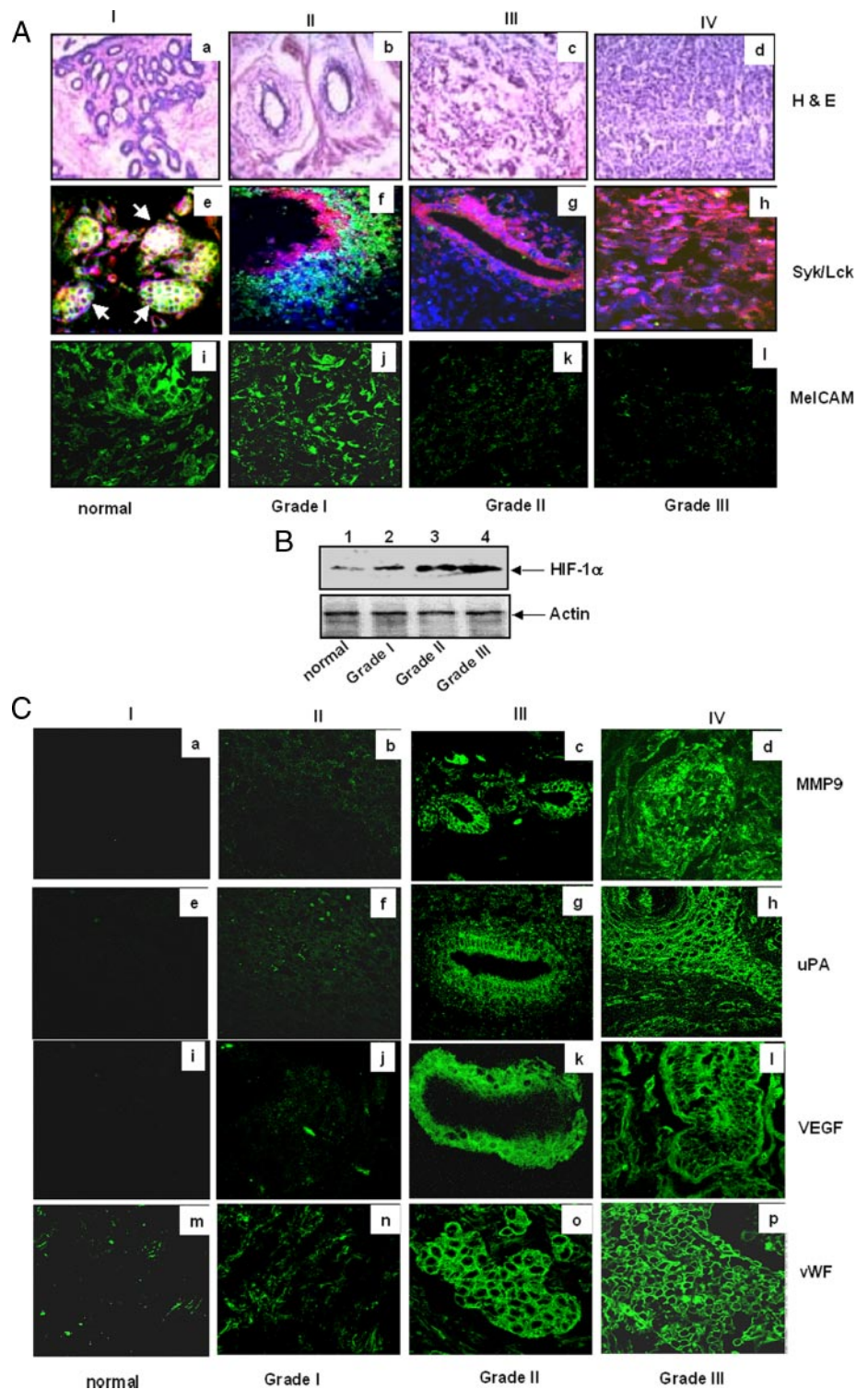


FIGURE 6. Immunohistochemical analysis of human breast tumor specimens of different pathological grades. *A*, normal and tumorigenic human breast specimens were collected from a local hospital with informed consent, and the gradations of these samples were performed using a modified Scarff-Bloom-Richardson system (panels *a–d*). Expression and cellular colocalization of Syk and Lck were analyzed by immunohistochemistry (panels *e–h*). Syk was stained with FITC, and Lck was stained with TRITC, and colocalization was visualized as yellow. The nuclei were stained with DAPI (blue). Note that in normal breast tissue the cellular colocalization of Syk-Lck was observed. The expression of both Syk and Lck were observed in grade I tumor. However, Lck but not Syk expression was observed in tumors of higher grades (grades II and III). There was significant expression of MelCAM in normal and lower grade tumors but was significantly diminished in tumors of higher grades (panels *i–l*). *B*, hypoxic level of these specimens was determined from the tumor lysates by Western blot analysis using anti-HIF-1α antibody. Actin was used as loading control. *C*, expression levels of MMP-9 (panels *a–d*), uPA (panels *e–h*), VEGF (panels *i–l*), and vWF (panels *m–p*) were analyzed by immunohistochemistry using their specific antibodies. Note that the expression status of all of these molecules was significantly enhanced in higher grades of tumors.

higher grades (Fig. 6C, panels *a–p*). Taken together, our data indicated that the Syk and MelCAM act as a negative regulator of tumor progression and angiogenesis and can act as a potential therapeutic target in breast cancer.

DISCUSSION

Our study indicated that Syk and Lck play a crucial regulatory factor in hypoxia-induced tumor progression and angiogenesis. The data also demonstrated that Syk acts as a negative regulator of H/R-induced

tumor progression, whereas Lck as a positive regulator, and H/R plays a pivotal role in regulating the cross-talk between Syk and Lck. Our previous study indicated that inhibition of Syk activity by Syk-specific antisense S-oligonucleotide resulted in enhanced uPA expression and cell motility in MCF-7 cells (23). In this study, we showed that H/R plays a crucial role in inactivation of Syk by inhibiting its phosphorylation, thereby resulting in its cytoplasmic translocation. Furthermore, the data indicated that the physical association between phosphorylated Syk and Lck in the nucleus resulted in inactivation of Lck. Upon H/R treatment

The diagram illustrates the signaling pathway of the Lck-Syk complex in hypoxia/reoxygenation-induced angiogenesis and tumor progression. The process is divided into the Cytoplasm and the Nucleus.

Cytoplasm:

- HYPOXIA/REOXYGENATION** (indicated by a pink box) triggers the process.
- MeICAM** (a blue oval) is involved in the initial signaling.
- Syk** (a blue circle) is activated, leading to **Cytoplasmic translocation and inactivation of Syk** (indicated by a red arrow).
- Lck** (a blue rectangle) is activated, leading to **Activation of Lck** (indicated by a red arrow).
- Sp1** (a green hexagon) is involved in the signaling pathway.

Nucleus:

- DNA** (a blue line) is the target of the signaling pathway.
- Pro MMP9** (a pink rectangle) is produced, leading to **Active MMP9** (a green rectangle).
- uPA** (a light blue rectangle) is produced.
- VEGF** (a pink rectangle) is produced.

Outcomes:

- Tumor growth and metastasis** (indicated by a blue arrow) is promoted by **Active MMP9**.
- Angiogenesis and tumor progression** (indicated by a blue arrow) is promoted by **VEGF**.

VEGF, and MelCAM that promotes tumor growth and angiogenesis (Fig. 7).

Our *in vivo* and clinical specimen analysis clearly supports our *in vitro* findings. In a xenograft study, tumors generated by injecting wild type Syk-transfected cells significantly suppressed the tumor growth, whereas mutant Syk-transfected cells showed enhanced tumor growth. The levels of uPA, MMP-9, VEGF, as well as microvessel density were significantly reduced, whereas the physical interaction between Syk and Lck and expression of MelCAM were significantly enhanced in wild type but not in Mut Syk-transfected tumors. In clinical specimen analysis, we observed that in higher grades of tumors (grade II and III), the level of HIF-1 α is significantly higher, which indicated the enhanced hypoxic condition in tumor microenvironment. The low level of HIF-1 α was observed in normal breast tissue and in lower grades of tumor. There was significant expression of Syk in normal breast tissues and lower grades of tumor. However, the physical association between Syk and Lck was observed in normal breast tissues. Moreover, the increased expression of Lck but not Syk was visualized in higher grades of tumors. Furthermore, the higher grades of tumors showed enhanced expression of uPA, MMP-9, and VEGF, and the increased microvessel density was also characterized in these tumors. In addition, the MelCAM expression was significantly diminished in tumors of higher grades that corroborated with both *in vitro* and *in vivo* findings.

In summary, to our knowledge, this is the first report that H/R differentially regulates the cross-talk between Lck and Syk, which ultimately control tumor progression. Previous reports have indicated that an increased hypoxic or consecutive hypoxia/reoxygenation condition in the tumor microenvironment plays a crucial role in determining the oncogenic potential of various cancers. Therefore, an in-depth understanding of the H/R-regulated signaling mechanism may be beneficial in designing a novel therapeutic approach for the treatment of cancer. We have shown that Syk, a tumor suppressor, positively regulates the expression of the breast cancer-specific tumor suppressor MelCAM. Interestingly, overexpression of Syk inhibits H/R-induced expression of several tumorigenic molecules, including uPA, MMP-9, and VEGF. Our results indicate that the mechanism demonstrated in the mouse model underlies human pathology, and a clear understanding of such mecha-

nisms may facilitate the development of novel therapeutic approaches to suppress hypoxia/reoxygenation-regulated Syk/Lck-mediated uPA, MMP-9, and VEGF expression, thereby controlling tumor growth and angiogenesis.

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