Hyperphosphorylated Cortactin in Cancer Cells Plays an Inhibitory Role in Cell Motility

Lin Jia, Takamasa Uekita, and Ryuichi Sakai

Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan

Abstract
Cortactin is frequently overexpressed in cancer cells, and changes of the levels of its tyrosine phosphorylation have been observed in several cancer cells. However, how the expression level and phosphorylation state of cortactin would influence the ultimate cellular function of cancer cells is unknown. In this study, we analyzed the role of cortactin in gastric and breast cancer cell lines using RNA interference technique and found that knockdown of cortactin inhibited cell migration in a subset of gastric cancer cells with a lower level of its tyrosine phosphorylation, whereas it greatly enhanced cell migration and increased tyrosine phosphorylation of p130Cas in other subsets of cells with hyperphosphorylated cortactin. Consistent results were obtained when hyperphosphorylation of cortactin was induced in MCF7 breast cancer cells by expressing Fyn tyrosine kinase. Additionally, immunostaining analysis showed that knockdown of hyperphosphorylated cortactin resulted in the recruitment of p130Cas to focal adhesions. These results suggest that cortactin hyperphosphorylation suppresses cell migration possibly through the inhibition of membrane localization and tyrosine phosphorylation of p130Cas.


Introduction
Protein phosphorylation by tyrosine kinases functions as a major switch in cellular biological signaling events through modulating protein-protein interaction and protein conformation. Substrates of Src family kinases (SFK) play essential roles in various cellular events by mediating tyrosine phosphorylation dependent signals. Because cortactin was originally identified as a v-Src substrate, it has been shown to play a critical role in the organization of the cytoskeleton (1). The cortactin gene EMSI is located on chromosome 11q13, a region amplified in several cancers such as head and neck squamous carcinoma and breast cancer (2-6). Cortactin is a modular protein that contains several motifs and domains involved in protein-protein interactions. An NH2-terminal acidic domain mediates its binding to Arp2/3, which regulates actin assembly, followed by an adjacency of six-and-a-half tandem repeats of 37 amino acids called cortactin repeats domain. There is a proline-rich domain immediately upstream of the SH3 domain, which also contains tyrosine residues phosphorylated by Src family kinases (7, 8). At the carboxyl terminus, there is a Src homology 3 (SH3) domain binding to several proteins, including cortactin binding protein1 (CortBP1/Shank2; ref.9) and N-WASP (10). Cortactin is a substrate of tyrosine kinases, including SFKs, Fyn, and Syk (11-13), and of serine/threonine kinases, including Erk and PAK (14, 15). Among the Src family, Fyn kinase seems to play a specific role in the cortactin function in some tumors because highly phosphorylated cortactin was shown to associate with Fyn kinase in metastatic murine melanoma in our previous study (16). However, how cortactin phosphorylation affects intercellular signaling pathways for cell dynamics control and other functions is not understood.

It has been shown that cell motility involves coordination of multiple signaling pathways regulating cell-substrate adhesion or actin polymerization (17, 18). A docking protein, p130Cas (Crk-associated substrate), is one of the key components of integrin-mediated signaling pathways, which conducts cell migration and actin filament reorganization in a tyrosine phosphorylation-dependent manner (19, 20). The COOH-terminal domain of p130Cas has both consensus SH3 and SH2 domains, binding sites for SFKs, which are mainly responsible for the phosphorylation of p130Cas (21).

In this study, we investigated the role of cortactin in human gastric cancer cell lines using RNA interference technique and discovered that knockdown of cortactin led to suppression of cell migration of the cells in which phosphorylation of cortactin is at basal level, whereas it increased cell migration of the cells in which cortactin is highly phosphorylated. It was also observed that knockdown of cortactin resulted in enhancement of cell motility of breast cancer cell line MCF7 in which phosphorylation level of cortactin was elevated by exogenously introduced Fyn kinase. In both cases, marked elevation in tyrosine phosphorylation of p130Cas was specifically and consistently observed by knockdown of hyperphosphorylated cortactin. We propose that tyrosine phosphorylation of cortactin may function as a molecular switch buffering the change in cell motility.

Results
Effect on Cortactin Knockdown on Cell Migration of Gastric Cancer Cells
Levels of tyrosine phosphorylation of cortactin were examined in human gastric cancer cell lines HSC57,
HSC44As3, HSC44PE, and HSC58As9 established in National Cancer Center Research Institute, Japan (22), along with a breast carcinoma cell line MCF7 that possesses amplification of cortactin gene, EMS1 (2). Expression of total cortactin was at similar levels (Fig. 1A, bottom), whereas there was a significant difference in tyrosine phosphorylation among these cell lines (Fig. 1A, top). HSC57, HSC44As3, and MCF7 cells exhibited a low level of tyrosine phosphorylation of cortactin, whereas HSC44PE and HSC58As9 showed hyperphosphorylation of cortactin as also indicated by the ratio of tyrosine-phosphorylated cortactin to total cortactin (Fig. 1A).

We knocked down cortactin expression by small interfering RNA (siRNA) to investigate the function of cortactin in the regulation of cell motility in various cancer cells using the Transwell assay. It was confirmed by immunoblotting that >80% of cortactin expression was down-regulated at 72 hours after initiation of siRNA treatment (Fig. 1B). Interestingly, knockdown of cortactin increased cell migration in HSC44PE and HSC58As9 cells with hyperphosphorylated cortactin, whereas it impaired cell motility in HSC57, HSC44As3, and MCF7 cells with a low level of phosphorylated cortactin (Fig. 1C). Essentially similar effect on cell migration was seen by another siRNA, cort-siRNA2 (data not shown). To further confirm the migration-promoting effect of cortactin siRNA in HSC44PE cells, we expressed mouse cortactin (mcort-WT), which is well conserved to human cortactin by retrovirus vector (4), along with the mutant mouse cortactin (mcort-Mut), which lacks all three putative tyrosine phosphorylation sites by exchanging tyrosine residues 421, 466, and 482 to phenylalanine (F421F466F482). Wild-type cortactin but not the mutant

![Image](image_url)

**FIGURE 1.** Changes in cell motility by knockdown of cortactin in gastric and breast cancer cells. A. To evaluate expression and tyrosine phosphorylation of cortactin, cell lysates of gastric and breast cancer cell lines were immunoprecipitated (IP) with anti-cortactin antibody (2 μg/mL) and immunoblotted by anti-phosphotyrosine antibody (4G10) and anti-cortactin antibody. Arrows, positions of cortactin isoforms (p85/80). MCF7, HSC57, and HSC44As3 cells exhibited a low level of tyrosine phosphorylation of cortactin, whereas HSC44PE and HSC58As9 showed hyperphosphorylation of cortactin. Quantification of tyrosine-phosphorylated cortactin is noted under the panel. Bottom, the expression of total cortactin. B. Amounts of cortactin in these cell lines at 72 h after siRNA treatment. C. Cell motility in various cancer cell lines was evaluated by numbers of migrated cells on the membrane. Knockdown of cortactin by siRNA led to inhibition of cell migration in HSC57, HSC44As3, and MCF7 cells with hypophosphorylated cortactin, whereas it resulted in increased cell migration in HSC44PE and HSC58As9 cells with hyperphosphorylated cortactin. D. HSC44PE cells (Parent) and HSC44PE cells stably expressed mouse cortactin (mcort-WT) or F421F466F482 triple mutant of mouse cortactin (mcort-Mut) fused with GFP were treated with or without cortactin siRNA. Cells were lysed 72 h after treatment and immunoblotted for cortactin and GFP. The concentration of total proteins was confirmed by the same membrane rehybridized with anti–α-tubulin antibody. Cortactin siRNA down-regulates endogenous human cortactin (endo) but not exogenous mouse cortactins (exo). Tyrosine phosphorylation of mouse cortactins was analyzed by immunoprecipitation of total cortactin and immunoblotting with anti-phosphotyrosine antibody (4G10). The quantification of immunoprecipitated cortactin is shown in the bottom (IB: cortactin). E. Effect of the rescue of mouse cortactin expression on cell migration in cortactin knockdown HSC44PE cells was analyzed as described. Rescue of tyrosine-phosphorylated cortactin (mcort-WT) affected the inhibition of cell migration but nontyrosine-phosphorylated cortactin (mcort-Mut) did not.
cortactin could block the elevation of cell migration induced by siRNA of cortactin in HSC44PE cells, suggesting that cortactin is actually suppressing migration of HSC44PE cells in a tyrosine phosphorylation–dependent manner. These results indicate that cortactin might differentially exert negatively and positively regulating functions in cell migration depending on its tyrosine phosphorylation.

Cortactin was originally identified as a substrate of SFKs. Among SFKs expressing in solid tumor cells, Fyn kinase was shown to play central roles in the tyrosine phosphorylation of cortactin in murine melanoma cells in our previous study (16). Treatment by a Src family specific inhibitor PP2 significantly reduced tyrosine phosphorylation of cortactin in HSC44PE cells (Supplementary Fig. S1A), suggesting that Src family kinases are actually responsible for tyrosine phosphorylation of cortactin in HSC44PE cells. Relatively high expression of Fyn kinase along with stable association between Fyn and cortactin was observed in HSC44PE cells (Supplementary Fig. S1B and S1C), suggesting the possibility that Fyn kinase is involved in hyperphosphorylation of cortactin in HSC44PE cells.

Knockdown of Cortactin Enhanced Tyrosine Phosphorylation of p130Cas in the Cells with Hyperphosphorylation of Cortactin

By knockdown of cortactin, tyrosine phosphorylation of a 125 to 130 kDa protein was remarkably enhanced in HSC44PE cells but not in HSC57 cells (Fig. 2A). In HSC44PE cells treated with cortactin siRNA, dramatically increased tyrosine phosphorylation of p130Cas was observed using phosphospecific antibody of p130Cas (P-Cas460Y) at the exactly same position where the 125 to 130 kDa protein was detected by 4G10 (Fig. 2B), whereas anti–phospho-FAK (Tyr 397) and anti–phospho-paxillin (Tyr118) antibodies failed to detect a significant change of phosphorylation state (Fig. 2B). We also generated another phosphospecific antibody against p130Cas (P-Cas766Y) and found consistent elevation in tyrosine phosphorylation of p130Cas by P-Cas766Y antibody in HSC44PE cells treated with two independent cortactin siRNAs was confirmed by specific anti–phospho-Cas antibodies (P-Cas460Y and P-Cas766Y). The effect of cortactin siRNA on cell migration of MCF7-Fyn clones was analyzed as described.
in the phosphorylation level of FAK or paxillin by the cortactin siRNAs in either HSC44PE cells or HSC57 cells (Fig. 2B).

To confirm the role of hyperphosphorylated cortactin in these cells, we also generated MCF7 cell lines that have elevated tyrosine phosphorylation of cortactin. Original MCF7 cells showed high expression of cortactin with the minimum level of tyrosine phosphorylation (Fig. 1A) and low expression of Fyn kinase (Fig. 2C). A vector expressing the Flag-tagged Fyn kinase was introduced into MCF7 cells, and several clones with stable expression of Fyn kinase were isolated, and two of these clones were named as MCF-Fyn1 and MCF-Fyn2. The amount of Fyn protein in both cells was ~10-fold greater than that of endogenous Fyn kinase in original MCF7 cells (Fig. 2C). Marked hyperphosphorylation of cortactin was observed in these two clones where Fyn kinase was introduced whereas original MCF7 cells or mock-transfected cells (MCF-Vec) were not (Fig. 2C). Knockout of cortactin greatly enhanced tyrosine phosphorylation of p130Cas both in MCF-Fyn1 cells and MCF-Fyn2 cells but not in MCF7-Vec cells (Fig. 2D and data not shown). These consistent results suggest that hyperphosphorylated cortactin may inhibit tyrosine phosphorylation of p130Cas in both HSC44PE and MCF-Fyn cells. Knockdown of cortactin also increased cell motility in both MCF-Fyn1 and MCF-Fyn2 cells whereas it blocked cell migration in parental MCF7 cells and MCF-Vec cells (Figs. 1C and 2E; data not shown). Similar results were obtained in the study using gastric cancer cells and it was shown that hyperphosphorylation of cortactin switches the response of cell motility.

Because many reports have shown the significant role of phosphorylation of p130Cas in cell migration, we examined whether knockdown of p130Cas by siRNA impairs the cell motility of HSC44PE cells treated or not treated with cortactin siRNA. Double treatment of Cas-siRNA and cort-siRNA completely impaired cell motility as well as treatment by Cas-siRNA alone, showing that p130Cas plays a dominant role in the regulation of cell motility of HSC44PE cells (Fig. 3B). These results indicate the possibility that hyperphosphorylated cortactin may suppress cell migration through inhibiting tyrosine phosphorylation of p130Cas.

Recruitment of p130Cas to Focal Adhesion by Treatment of Cortactin siRNA

To further investigate the cause of tyrosine phosphorylation of p130Cas induced by knockdown of cortactin, we analyzed the subcellular localization of p130Cas, cortactin, and focal adhesion proteins in HSC44PE cells. Total cortactin was widely expressed near the cell membrane in HSC44PE cells (Fig. 4A and B) whereas phosphorylated cortactin detected by a phosphospecific antibody against cortactin Y421 appeared at a specific domain, which seems to be focal adhesion within the cell membrane (Fig. 4B). It was further shown that phosphotyrosine-containing cortactin was clearly colocalized with vinculin, which is expressed at focal adhesion (Fig. 4C). By the treatment with cortactin siRNA, both the signals detected by total cortactin and cortactin Y421 were significantly weakened, suggesting these signals are cortactin specific (Fig. 4A).

In the control cells, p130Cas is mainly distributed in the cytoplasm and only slightly expressed at focal adhesion (Fig. 5B, top). When cortactin is knocked down, a substantial amount of p130Cas comes to locate at focal adhesion (Fig. 5B, bottom), suggesting the loss of cortactin-induced membrane localization of p130Cas. On the other hand, tyrosine-phosphorylated p130Cas detected by phosphospecific antibody was specifically localized at the focal adhesion although the amount of tyrosine phosphorylation was greatly increased by the suppression of cortactin expression (Fig. 5A). This observation may support the model that tyrosine-phosphorylated cortactin expressed predominantly in focal adhesions interferes with the localization of p130Cas at the focal adhesion, which causes tyrosine-phosphorylated p130Cas. Loss of hyperphosphorylated cortactin could therefore recruit p130Cas to the focal adhesion, which results in tyrosine phosphorylation of p130Cas, followed by enhancement of cell motility.

Discussion

We explored the role of cortactin in cell motility by using RNA interference technique in several gastric cancer cell lines that show various phosphorylation states of cortactin. It was...
revealed that knockdown of cortactin results in enhanced cell motility along with increased tyrosine phosphorylation of p130Cas in the cells that have hyperphosphorylated cortactin, whereas it impairs cell migration in the cells with a low level of tyrosine-phosphorylated cortactin. In addition, knockdown of hyperphosphorylated cortactin caused recruitment of p130Cas to focal adhesion, which might result in enhanced cell migration. In this study, for the first time, we showed that cortactin has a dual function in the regulation of cell motility, which depends on its tyrosine phosphorylation state.

Amplification and overexpression of the cortactin gene, EMS1, have been identified in various cancers (2, 3, 6, 23). In our previous study, overexpression and elevated tyrosine phosphorylation of cortactin was selectively observed in the metastatic subgroup of murine melanoma cells with high migratory potential (16). Based on these results, we first hypothesized that the hyperphosphorylated cortactin may promote cell migration in cancer cells. In this study, gastric cancer cells that have hyperphosphorylated cortactin showed at least similar or higher migration potential compared with those cells with basal levels of tyrosine phosphorylation of cortactin (Fig. 1A and C). However, unexpectedly, knockdown of cortactin in these cells further enhanced cell migration. The effect of cortactin knockdown was opposite in the cells with a low level of cortactin phosphorylation. This paradoxical outcome by cortactin knockdown was also confirmed in breast cancer cells MCF7, which expressed a significant amount of cortactin although the level of tyrosine phosphorylation was quite low. Introduction of Fyn kinase to MCF7 cells significantly enhanced the tyrosine phosphorylation of cortactin. In this condition, loss of cortactin by siRNA enhanced cell migration whereas it had a negative effect on cell migration in the parental cells. Based on this finding, cortactin hyper-phosphorylation may be induced as a negative feedback mechanism when cells acquire highly migratory potential.

Some of the studies have shown that knockdown of cortactin results in impaired cell motility in hepatocellular carcinoma and head and neck squamous cell carcinoma cells (24, 25), whereas other studies have shown no significant effect on cell motility (8, 26), although all these studies lack information on the phosphorylation states of cortactin. Because it is suggested that cortactin differentially functions in cell migration depending on its phosphorylation state, the previous controversial results from the knockdown of cortactin should be reevaluated from the viewpoint of its tyrosine phosphorylation state. A previous study shows positive effect of the cortactin phosphorylation on the cell migration by overexpression of normal cortactin and phosphorylation-defective mutants in the endothelial cells (27). Although this study does not mention about the level of tyrosine phosphorylation of endogenous cortactin either, it might be weak in normal endothelial cells. Therefore, the positive effect of cortactin expression shown in the study might be consistent with our result using HSC57 or MCF7 cells, whereas the dominant-negative effect of phosphorylation-defective mutant might indicate some different function of cortactin in the endothelial cells.

In our observation, knockdown of cortactin induced outstanding enhancement in tyrosine phosphorylation of
p130Cas. Because there is no significant change in the phosphorylation of other substrates of SFKs such as FAK and paxillin, it is indicated that there exists some specific mechanism underlying the regulation of tyrosine phosphorylation state of p130Cas by cortactin. Unphosphorylated p130Cas is mainly expressed in the cytoplasm as shown in Fig. 5B, whereas localization of p130Cas in the focal adhesion is thought to be required for tyrosine phosphorylation of p130Cas (28, 29). The results of immunocytostaining indicate that tyrosine-phosphorylated cortactin predominantly exists in the focal adhesion, and thus purging p130Cas from focal adhesion to lose the chance of being phosphorylated. Therefore, knockdown of cortactin might give chance for p130Cas to localize at the focal adhesion to be phosphorylated (Fig. 5). p130Cas was originally found as a prominent substrate of SFKs, including c-Src and Fyn during the transformation of cells (21, 30). It has been shown that phosphorylated p130Cas in focal adhesion plays a regulatory role in cell spreading and cell migration (19, 31-33), and knockdown of p130Cas actually abrogated cell migration in our gastric cancer cells, HSC44PE, even in the absence of cortactin (Fig. 3A and B). These results indicate that activation of p130Cas-mediated signal might be responsible for the enhancement of cell motility in the cells where hyperphosphorylated cortactin is knocked down.

Our study sheds new light on the cross-talk between cortactin and p130Cas, both of which are Src substrates. Such cross-regulation between SFK substrates might give more optimized outcome out of overlapping or conflicting effects of SFK signals. However, there is still a possibility that phosphorylated cortactin may also transduce specific signals regulating cell migration, which is independent of a function of p130Cas. As far as we examined, there was no significant effect on the morphology, cell-matrix adhesion, or number of focal adhesions in HSC44PE cells by suppression of cortactin expression (Fig. 5A and data not shown). On the other hand, it has been suggested that cortactins originally support cell migration through several pathways and therefore its tyrosine phosphorylation may have an inhibitory effect on these pathways. Binding of cortactin with N-WASP via its SH3 domain may synergize in causing actin polymerization

**FIGURE 5.** Changes in localization of p130Cas by treatment with cortactin siRNA. A, HSC44PE cells were transfected with cortactin siRNA or control siRNA for 72 h before localization of phosphorylated p130Cas was analyzed by immunocytostaining with P-Cas766Y antibody. Anti-vinculin antibody was used to visualize focal adhesions. Merged images indicate that tyrosine-phosphorylated p130Cas, which was greatly increased by cortactin siRNA, specifically localizes at focal adhesion. B, In HSC44PE cells treated with control or cortactin siRNA, localization of p130Cas was analyzed by anti-Cas3 antibody. A portion of p130Cas shows distinct colocalization with vinculin at focal adhesion in cells treated with cortactin siRNA, whereas staining of vinculin and p130Cas did not significantly overlapped in cells treated with control siRNA.
preceding cell migration, which is promoted by a serine kinase Erk and conversely inhibited by Src kinase (34). We showed all these signal pathways of phosphorylated cortactin in a schema (Fig. 6).

Materials and Methods

Cell Culture

Human breast cancer cell line MCF7 was maintained in DMEM containing 10% (v/v) fetal bovine serum (Life Technologies) and 50 μg/mL penicillin-streptomycin antibiotics. Human gastric cancer cell lines HSC57, HSC44As3, HSC44PE, and HSC58As9 was supplied by Central Animal Laboratory, National Cancer Center Institute, Tokyo, Japan (24). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

Antibodies and Reagents

Anti–phosphotyrosine (4G10) and anti-cortactin (clone 4F11) antibodies were obtained from Upstate Biotechnology. Polyclonal antibodies Fyn3 and anti-FAK were purchased from Santa Cruz Biotechnology. Anti-paxillin and anti–green fluorescent protein (GFP) antibodies were purchased from Zymed and MBL, respectively. Anti–phospho-paxillin (Tyr118), anti–phospho-FAK (Tyr397), and anti–phospho-cortactin (Tyr421) antibodies were purchased from Cell Signaling Technology, Upstate Biotechnology, and Chemicon, respectively. Anti–Flag-M2 antibody was obtained from Sigma. Polyclonal antibodies against p130Cas (Cas2 and Cas3) and phoshosphospecific polyclonal antibody P-Cas460Y were used as described previously (30, 35). Another phosphospecific anti–phosphotyrosine antibody, P-Cas766Y, was generated by immunizing rabbits with a synthetic phosphopeptide, CMEDpYDpYVHL, which includes the phosphotyrosine-containing motifs in the Src-binding domain of p130Cas, after being conjugated with thymoglobulin. As secondary antibodies, horseradish peroxidase–conjugated anti-rabbit and anti-mouse IgG (Amersham) were used. Polyclonal antibodies Fyn3 and anti-FAK were purchased from Santa Cruz Biotechnology. Anti-paxillin and anti–green fluorescent protein (GFP) antibodies were purchased from Zymed and MBL, respectively. Anti–phospho-paxillin (Tyr118), anti–phospho-FAK (Tyr397), and anti–phospho-cortactin (Tyr421) antibodies were purchased from Cell Signaling Technology, Upstate Biotechnology, and Chemicon, respectively. Anti–Flag-M2 antibody was obtained from Sigma. Polyclonal antibodies against p130Cas (Cas2 and Cas3) and phoshosphospecific polyclonal antibody P-Cas460Y were used as described previously (30, 35). Another phosphospecific anti–phosphotyrosine antibody, P-Cas766Y, was generated by immunizing rabbits with a synthetic phosphopeptide, CMEDpYDpYVHL, which includes the phosphotyrosine-containing motifs in the Src-binding domain of p130Cas, after being conjugated with thymoglobulin. As secondary antibodies, horseradish peroxidase–conjugated anti-rabbit and anti-mouse IgG (Amersham) were used. Polyclonal, fibronectin, and cycloheximide were purchased from Sigma. Inhibitor of SFKs, 4-amino-5-(4-chlorophenyl)-7-(butyl) pyrazolo[3,4-d] pyrimidine (PP2), and the inactive structural analogue 4-amino-7-phenylpyrazol[3,4-d] pyrimidine (PP3) were obtained from Calbiochem-Novabiochem Ltd.

RNA Interference Experiments for Cortactin and p130Cas

Two independent siRNA of human cortactin, cort-siRNA1 and cort-siRNA2, for RNA interference experiment were generated by Invitrogen Life Technologies. Cort-siRNA1 targets cortactin mRNA at 5′-CCCAGAAAGACUAGUGGAAGGGGUU-3′ and Cort-siRNA2 targets at 5′-GGAGAAGCAGCA-GUCACAGAGAU-3′. The siRNA of human p130Cas was also generated by Invitrogen Life Technologies, using the target sequence 5′-CCAGAGACUUUGUGGCCGACAGCAA-3′. For transient transfection of siRNA, the cells were plated at 1.5 × 10⁴ per well on a six-well plate. Transfection of siRNAs was done with Lipofectamine 2000 (Invitrogen Co.). After transfection for 72 h, the cells were harvested for the biochemical analyses.

Infection of Retroviral Constructs

Retrovirus vectors were used to express mouse cortactin fused with GFP (pJL6) and F421F466F482 triple mutant of mouse cortactin fused with GFP (pJL12; ref. 4). Briefly, the retroviral vector and the packaging construct pCL-10A1 were cotransfected into 293T cells and culture fluid was harvested 72 h posttransfection. HSC44PE cells were infected with the viral fluid in the presence of 4 mg/mL polybrene, and the infected cells were selected in the presence of 800 μg/mL Geneticin G418 (Sigma) for a period of 2 to 3 wk.

Establishment of Stable MCF7 Clones Expressing Fyn Kinase

Breast cancer cells MCF7 were grown in DMEM containing 10% fetal bovine serum and antibiotics. On the night before transfection, the cells were seeded onto a 10-cm dish at a density of 9.0 × 10⁴. Transfection of a vector expressing Fyn kinase with a tag of Flag (Fyn-Flag) and an original vector (pcDNA3.1) was done according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were subjected to selection by Geneticin G418 (Sigma) at a concentration of 800 μg/mL for a period of 2 to 3 wk.

Immunoblotting and Immunoprecipitation

For immunochemical analysis, cells were cultured in the incubator at 37°C with 5% CO₂ for 48 to 72 h, before the cells were lysed in 1% Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 100 mmol/L NaF, 1 mmol/L Na₃VO₄, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride), and insoluble material was removed by centrifugation for 10 min. Protein concentration was analyzed by BCA Protein Assay (Pierce), and the protein...
aliquots were separated by SDS-PAGE. Gels were transferred to the polyvinylidene difluoride membrane (Millipore) and subjected to immunoblotting. After blocking with 5% skim milk in TBST [100 mM NaCl, 0.05% Tween 20] for 1 h, blots were incubated with primary antibodies. In the case of 4G10, blocking was done with Blocking One Solution (Nakarai Co.). Membranes were then washed thrice with TBST, incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature, washed thrice by TBST again and once by TBS [100 mM NaCl, 150 mM NaCl], and visualized by autoradiography using a chemiluminescence reagent (Western Lighting, Perkin-Elmer).

For immunoprecipitation, 500 µg of protein were mixed with 2 µg of antibodies and incubated for 1 h on ice, and then samples were rotated with protein G-Sepharose beads (Amersham Pharmacia) for 2 h at 4°C. The beads were washed thrice with 1% Triton X-100 buffer and boiled in sample buffer [2% SDS, 0.1 mol/L Tris-HCl (pH 7.0), 10% glycerol, 0.01% bromphenol blue, 0.1M DTT] for SDS-PAGE analysis.

### Immunocyto staining

Approximately 5 × 10⁴ cells were plated on 12-mm circle cover glasses on a 24-well plate, which were grown in DMEM with 10% fetal bovine serum at 37°C with 5% CO₂ for 24 h. The 12-mm circle cover glasses were coated by fibronectin 10 µg/mL in PBS overnight before seeding the cells. Then, cells were fixed in 4% paraformaldehyde in 0.1 mol/L sodium phosphate (pH 7.0) for 5 min, washed thrice with PBS, and permeabilized with 0.1% Triton X-PBS for 10 min before blocking with 5% bovine serum albumin with TBST [0.15 mol/L NaCl, 1% Tris (pH 7.0), 0.05% Tween 20] for 10 min. Then, the cells were incubated with the first antibody for 1 h at room temperature. Cells were washed with PBS thrice, and incubated with appropriate second Alexa antibodies (Molecular Probe; 1:500) in 5% goat serum with 3% bovine serum albumin/TBST. All cover glasses were mounted in 1.25% DABCO, 50% PBS, and 50% glycerol. The staining was visualized using a Radiance 2100 confocal microscopic system (Bio-Rad).

### Cell Migration Assay

Migration assay was done using modified Transwell chambers with polycarbonate Nucleopore membrane (Falcon, BD). Precoated filters (6.5 mm in diameter, 8-µm pore size, fibronectin 10 µg/mL) were rehydrated with 100 µL medium. Then, 4 × 10⁴ cells in 100 µL serum-free DMEM supplement were seeded onto the upper part of each chamber, whereas the lower compartments were filled with 600 µL of the same medium with 10% fetal bovine serum. Following incubation for 8 h at 37°C, nonmigrated cells on the upper surface of the filter were wiped out with a cotton swab, and the migrated cells on the lower surface of the filter were fixed and stained with Giemsa stain solution (Azur-Eosin-Methylene Blue Solution, Muto Pure Chemicals, Co., Japan). The total number of migrated cells was determined by counting cells in five microscopic fields per well at a magnification of ×100, and the extent of migration was expressed as the average number of the cells per microscopic field.

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### References

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