MST3 is a member of the sterile-20 protein kinase family with a unique preference for manganese ion as a cofactor in vitro; however, its biological function is largely unknown. Suppression of endogenous MST3 by small interference RNA enhanced cellular migration in MCF-7 cells with reduced expression of E-cadherin at the edge of migrating cells. The alteration of cellular migration and protruding can be rescued by RNA interference-resistant MST3. The expression of surface integrin and Golgi apparatus was not altered, but phosphorylation on tyrosine 118 and tyrosine 31 of paxillin was attenuated by MST3 small interfering RNA (siRNA). Threonine 178 was determined to be one of the two main autophosphorylation sites of MST3 in vitro. Mutant T178A MST3, containing alanine instead of threonine at codon 178, lost autophosphorylation and kinase activities. Overexpression of wild type MST3, but not the T178A mutant MST3, inhibited migration and spreading in Madin-Darby canine kidney cells. MST3 could phosphorylate the protein-tyrosine phosphatase (PTP)-PEST and inhibit the tyrosine phosphatase activity of PTP-PEST. We conclude that MST3 inhibits cell migration in a fashion dependent on autophosphorylation and may regulate paxillin phosphorylation through tyrosine phosphatase PTP-PEST.

The group of Ste20 kinases is related to yeast sterile 20 proteins, which are a component of the pheromone-response pathway in budding yeast (1). Ste20 belongs to the STE group, which is among the newest groups added in the new classification of protein kinases (2, 3). Ste20 is placed upstream of the mitogen-activated protein kinase (MAPK)2 in the mating pathway of Saccharomyces cerevisiae. The mammalian Ste20 family consists of two structurally distinct subfamilies, including P21-activated kinases (PAK) and germinal center kinases (GCK). PAK regulates several critical signal pathways through the interaction with many proteins, including Rho GTPase (4, 5). Autophosphorylation plays an important role in the activation of PAK (6, 7).

GCK includes GCK, YSK1, and mammalian Ste20-like (MST)1–4 kinases (8). MST1 and MST2 undergo autophosphorylation and are activated by a variety of stress stimuli (9–11). MST2 plays a role in suppressing RAF-1-mediated apoptosis (12) and activates the human large tumor suppressor kinase LATS1 (13). YSK1 is activated by the Golgi matrix protein GM130 and plays a role in migration (14,15). MST4 enhances cell transformation and plays a role in prostate cancer progression (16–18). MST3 does not alter any known MAPK pathway by overexpression of either wild type MST3 or kinase-dead MST3 (19). Nuclear translocation and activation of MST3 occur during apoptosis (20), and the nuclear translocation signal of MST3 has been determined (21). One unique feature of MST3 is the preference for Mn2+ as the cofactor in the kinase reaction, an unusual feature for a serine/threonine kinase (19). Recently, we have further demonstrated that Zn2+ is also a cofactor for MST3 (22).

Cell migration is an important process that is essential for development and life long physiological processes (23, 24). Migration can be viewed as a cyclical process. First, cells begin to polarize and make protrusions in response to out-

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2. The abbreviations used are: MAPK, mitogen activated protein kinase; siRNA, small interfering RNA; DMEM, defined minimal essential medium; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; FN, fibronectin; GCK, germinal center kinase; MDCK, Madin-Darby canine kidney; MBP, myelin basic protein; NDR, nuclear Dbf2-related kinases; PAK, P21-activated kinase; PBS, phosphate-buffered saline; PIPES, piperazine-N,N’-bis-(2-ethanesulfonic acid); PTP-PEST, protein-tyrosine phosphatase-PEST; PTP-1B, protein-tyrosine phosphatase 1B; RNAi, RNA interference; shRNA, short hairpin RNA; Ab, antibody; shRNA, short hairpin RNA; HA, hemagglutinin.
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side stimuli. Second, cells form adhesions that attach the protrusion to the substratum. Third, these adhesions partially act as tracking points for migration. Finally, contraction moves the cell body forward and releases the attachment through disassembly of the adhesion at the rear end, allowing the cycle to begin again.

Several types of adhesion occur in different cell types on various substrata. One type of adhesion involves integrin, a heterodimeric receptor consisting of α and β chains. Binding of ligand changes the interaction between the α and β chain cytoplasmic domains and integrin clustering (25). Activated integrins preferentially localize to the site of new adhesion and establish interactions with several proteins, including focal adhesion kinase, paxillin, p130 Crk-associated protein (p130 CAS), and others (26–28). Paxillin is usually recruited to the leading edges of migrating cells, where it acts as a scaffold for protein interactions (29), and paxillin is essential for protein-tyrosine phosphatase (PTP)-PEST inhibition of cell spreading and membrane protrusion (30). Regulation of tyrosine phosphorylation on Tyr-31/118 is observed during cell migration and adhesion (31, 32). Replacement of Tyr-31/118 with phenylalanine reduces cell motility with reduced Crk-II association (33), whereas coexpression of the Crk-II adaptor protein and protein-tyrosine phosphatase 1B promotes cell migration accompanying decreased tyrosine phosphorylation on paxillin (34). Although paxillin is postulated as being a positive regular of cell migration (35), small interference RNA (siRNA) directed against paxillin can enhance cell migration at certain circumstances (36, 37).

In contrast to other members of the MST family, the biological functions of MST3 are less understood. RNA interference (RNAi) technology has become a powerful tool for studying protein function (38–40); therefore, we investigated the altered phenotype generated by silencing the expression of MST3 with RNAi. Our results indicate that autophosphorylated MST3 inhibits cellular migration associated with alteration of paxillin phosphorylation. In addition, MST3 can phosphorylate PTP-PEST and inhibit its phosphatase activity, which may account for the change of paxillin phosphorylation.

MATERIALS AND METHODS

Cell Culture and Antibodies—MCF-7 and MDCK cells were maintained at 37 °C and 5% CO₂ in defined minimal essential medium (DMEM) supplemented with 10% fetal calf serum (DMEM/FCS). MDA-MB-231 cells were grown in Leibowitz's L15 medium, supplemented with 14 mM NaHCO₃ and 10% fetal calf serum. MCF-10A cells were kindly provided by Dr. Mien-Chie Hung and cultured in DMEM/F-12 medium containing 0.5% equine serum, 0.5 mM L-alanine reduces cell motility with reduced Crk-II association (33), whereas coexpression of the Crk-II adaptor protein and protein-tyrosine phosphatase 1B promotes cell migration accompanying decreased tyrosine phosphorylation on paxillin (34). Although paxillin is postulated as being a positive regulator of cell migration (35), small interference RNA (siRNA) directed against paxillin can enhance cell migration at certain circumstances (36, 37).

In contrast to other members of the MST family, the biological functions of MST3 are less understood. RNA interference (RNAi) technology has become a powerful tool for studying protein function (38–40); therefore, we investigated the altered phenotype generated by silencing the expression of MST3 with RNAi. Our results indicate that autophosphorylated MST3 inhibits cellular migration associated with alteration of paxillin phosphorylation. In addition, MST3 can phosphorylate PTP-PEST and inhibit its phosphatase activity, which may account for the change of paxillin phosphorylation.

RNA Interference—siRNA targeting MST3 was constructed within pHsU6 vector as described previously (40, 41). The target sequence of MST3 was 5’-GGAGAAAGGCAGCGGTGC-3’ (nucleotides 1127–1145 of MST3 from the start codon). Cells were cotransfected with the pHsU6 vectors containing shRNA of targeting sequences and pCMV-neo by using Lipofectamine 2000 transfection reagent (Invitrogen). The stable transfectants were selected with G418. To monitor the efficiency of MST3-RNAi, the expression of MST3 in MCF7 stable transfectants was analyzed by Western blotting and probed with anti-MST3 antiserum.

MST3 Mutant Plasmids—Wild type HA-MST3 and the indicated mutants were constructed between NotI and EcoRI sites in pcDNA3.0 with HA epitope tags added to the N terminus. PCR-directed mutagenesis was used to achieve the desired mutations. The primers for MST3 T178A were as follows: forward primer, 5’-AAAAAGGCCTTCTTCTGTTGGGC-3’; reverse primer, 5’-GCCCGAGAAGCAGCCCTT-3’. The primers for MST3 T264A were as follows: forward primer; 5’-CTTTAGACCCGCTGCTAAGGAGT-3’; reverse primer, 5’-AATCTCCTAGCCAGGGGTCTAAAG-3’. The underlined base pairs represent the mutated amino acids. The sequence altered in RNAi-resistant MST3 is GGA-GAAAGCCAAGCTTGC (nucleotides 1127–1145) without altering the amino acid sequence. All mutant MST3 plasmids were confirmed by DNA sequencing.

Wound-healing Assays—Cells were plated onto glass-based dishes coated with 10 μg/ml fibronectin (FN) at a saturation density (1.2 × 10⁶ cells/35-mm diameter). Cells were then scratched manually with a blade 24 h after plating. The wounded regions were allowed to heal for a defined time in DMEM/FCS. Images for these cells were observed by phase-contrast microscopy using a CKII microscope (Olympus, Japan).

Migration Assays—Cells were cultured to confluence in DMEM/FCS. The lower compartment of a Boyden chamber was filled with 1 μg/ml FN (26 μl/well), and 5 × 10⁵/ml cells in serum-free medium were added to the upper compartment of the chamber (50 μl/well). The chambers were separated by a polycarbonate filter (25 × 80 mm, 8 μm pore size, polyvinylidene difluoride; GE Osmonics, Tarevo, PA). The cells were allowed to migrate for a defined time at 37 °C in a humidified atmosphere containing 5% CO₂. Nonmigrated cells remaining on the upper surface of the filter were removed mechanically by scraping with a cotton swab. Cells that had migrated through the pores were fixed in methanol for 8 min and then stained with 10% Giemsa stain-modified solution (Fluka, Buchs, Switzerland). Cells were examined and counted with U-5RE-2 microscope (Olympus, Japan).

Immunoprecipitation and in Vitro Kinase Assay—Cells were washed three times with cold phosphate-buffered saline (PBS) and then lysed using 300 μl of lysis buffer containing 150 mM NaCl, 50 mM NaH₂PO₄, 2 mM H₂O, 6 mM deoxycholate, 1% Igepal-Ca-630 (Sigma), 1 mM sodium orthovanadate, 2 mM EGTA, 0.5% aprotinin (Sigma), and 1 mM phenylmethlysulfonyl fluoride. After a 15-min centrifugation at 8000 rpm, supernatant containing 300 μg of total proteins was incubated for 2 h at 4 °C with 1 μg of anti-
Ab (Roche Applied Science). The immunocomplex was harvested with 35 μl of protein A-Sepharose (Amersham Biosciences) for 1.5 h at 4 °C. Immunoprecipitates were washed with lysis buffer three times and 1/100 Tris-buffered saline three times and then suspended in 30 μl of Tris-buffered saline buffer. The HA-MST3-immunoprecipitate bound to protein A-Sepharose was incubated with 30 μl of kinase buffer containing 40 mM PIPES, 20 μM cold ATP, 20 μCi of [γ-33P]ATP (2500 Ci per mmol), 4 μg of histone H3, or myelin basic protein (MBP) and 2 mM Mn2+ or Mg2+ at room temperature for 20 min. The reaction was stopped by the addition of 6 μl of 10× SDS loading buffer. After boiling for 5 min, proteins in the kinase reactions were separated by SDS-PAGE. The gel was dried, and substrate phosphorylation was visualized by autoradiography.

**In Vitro Kinase Assay with Protein-tyrosine Phosphatase**—0.5 μg of recombinant MST3 was incubated with 1 μg of recombinant PTP1B (amino acids 1–321) enzyme (Exalpha Biologicals), 0.5 μg of myelin basic protein, or 0.5 μg of recombinant PTP-PEST (amino acids 2–300) enzyme (Exalpha Biologicals) in kinase buffer described as above. Kinase reactions were performed at room temperature for 15 min and stopped by the addition of 6 μl of 10× SDS loading buffer. After boiling for 5 min, proteins in the kinase reactions were separated by SDS-PAGE. The gel was dried, and substrate phosphorylation was visualized by autoradiography.

**MST3 Substrate Peptide Assay**—The sequences of peptide 178, 264, G peptide, and 178A are (respectively) TQIKRN-TFVTPFWMAPFVIKQS (residues 172–194 of MST3), KEPSFRPTAKELLKHFI (residues 257–274 of MST3), KPGFSQPSSRSSESEEVY, and TQIKRNATGFTFWMAPEVIKQS. The sequence of peptide NDR is YKNKD-WFVFINITYKRFEGLTAR (residues 433–454 of NDR kinase). Peptide 264 was predicted to be the potential autophosphorylation residue of MST3. One mM of peptide 178, peptide 264, NDR peptide, G peptide, or whole MBP protein was reacted with 1 μM baculovirus-expressed MST3 or immunoprecipitated MST3 with 2 mM Mn2+ and [33P]ATP. The amount of 33P incorporated into a given amount of synthetic peptide was quantified by spotting the phosphorylated peptide on P81 paper and counting the radioactivity in a Cerenkov counter. Kinetic parameters were determined by varying the concentration of peptide from 0.4 to 2.4 mM while maintaining the ATP concentration at 10 μM.

**Phosphopeptide Analysis**—MST3 (178) peptide (25 μg), His-tagged recombinant MST3 (1 μg), or both were incubated in a 25-μl reaction mixture containing 0.2 mM [γ-32P]ATP (1 pmol of ATP = 1000 cpm), 20 mM Mg2+ at 30 °C for 30 min. The reaction was stopped by adding 4× Laemmli sample buffer, and the reaction products were resolved in 16.5% SDS-PAGE. The bands of 32P-labeled T178 peptide and of 32P-autophosphorylated MST3 were excised and digested by 10 μg of trypsin at 37 °C for 48 h. The resulting tryptic phosphopeptides from 32P-

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**FIGURE 1. Influence of MST3 expression on cell migration in MCF7 cells.** A, reduction of MST3 proteins by siRNA targeting MST3. Lysates of MCF7 parental cells, MCF7 cells expressing empty vector (Vector-1 and Vector-2), or the MST3 siRNA plasmid (RNAi-1 and RNAi-2) were subjected to immunoblotting with the anti-MST3 antisemur. Tubulin served as a loading control. B, effect of MST3-knockdown on collective migration in MCF7 cells. Cells were plated onto FN-coated glass-based dish and subjected to wound-healing assay for 24 and 72 h. C, decrease of E-cadherin expression in migrating edge of MST3 RNAi transfectants. Cells in the wound-healing assay were fixed and subjected to E-cadherin immunostaining at 72 h after scratch. Images of cadherin were acquired by confocal microscopy. D, migration of MST3 RNAi transfectants in Boyden chamber assay. Cells were plated on the membranes of the chambers in the serum-free medium in the wells, fibronectin as chemoattractant, and the migrated cells were calculated 24 h after plating. The values are represented as the means ± S.D. **, p < 0.01 when compared with control vector clones in Student’s t test. V-1, vector-1; V-2, vector-2.
labeled T178 peptide (~3,000 cpm), 32P-autophosphorylated MST3 (~3,000 cpm), or both 32P-labeled MST3 (178) peptide (~1,500 cpm) and 32P-autophosphorylated MST3 (~1,500 cpm) were subjected to two-dimensional phosphopeptide map analysis as described before (42). The samples were analyzed in the first dimension on cellulose-coated TLC plates (Merck) in a solution (formic acid/acetic acid/water, 50:156:1794 by volume) at 1 kV for 40 min followed by ascending chromatography in the second dimension in a solution (butanol-1/pyridine/acetic acid/water, 15:10:3:12 by volume) for 6–8 h. The air-dried plates were exposed to x-ray films for autoradiography.

**Immunofluorescence Microscopy**—Cells were plated on 10 µg/ml fibronectin-coated glass-based dish. Cells were then fixed with 3.7% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 in PBS. The permeabilized cells were washed three times with PBS and then incubated with anti-GM130, anti-paxillin, and anti-cadherin antibody. Alexa 488 goat anti-mouse IgG was used to visualize proteins. Images for these proteins were obtained by fluorescent microscopy (Nikon 80I fluorescence microscope, Japan) or confocal microscopy (LSM510 Pascal, Carl Zeiss, Germany).

**Cell Sorting with Flow Cytometry**—Cells were cotransfected with the indicated plasmids and pEGFP-N1 plasmid (Clontech) by Lipofectamine 2000 transfection reagent (Invitrogen). Twenty four hours after transfection, cells were mechanically dissociated to obtain a single cell suspension in a solution containing 2% bovine serum albumin in PBS. Cells were harvested by centrifugation (1500 rpm, 5 min, 4 °C), filtered through a 0.35-µm filter (Falcon), and kept on ice until FACS analysis. Cell sorting was performed on a FACSAria flow cytometer (BD Biosciences) equipped with 488-nm argon and 633-nm He-Ne lasers. The green fluorescent protein-expressing cells were seeded on a FN-coated dish to observe cell protrusion or sorted for analysis with Boyden chamber migration assay.

**Protein-tyrosine Phosphatase Activity**—PTP-PEST activity was measured by using 6,8-difluoro 4-methylumbelliferyl phosphate (Molecular Probes, Eugene, OR). Cells were lysed on ice with 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 50 mM Tris, pH 7.4. Cell lysate was centrifuged at 4 °C and 1000 × g for 10 min. The supernatant was subjected to immunoprecipitation with PTP-PEST antibody (AG10, Cell Signaling). The immunoprecipitated PEST was added to the substrate-coated assay wells. Samples were incubated for 1 h, and phosphatase activity was determined by using a fluorimeter at an excitation/emission
wavelength of 358/452 nm. The PTP-PEST plasmid was purchased from OriGene Technologies Inc.

**Statistical Analysis**—All the statistical analyses were performed with Student’s paired t test.

### RESULTS

**Establishment of MST3-RNAi Transfectants**—To investigate the biological function of MST3, we constructed U6 promoter plasmid-based shRNA targeting at nucleotides 875, 1127, and 1170 on the MST3 gene. The efficacy of the three shRNA-MST3 constructs was determined by cotransfection of the shRNA and HA-MST3 expression plasmids into COS-1 cells. The shRNA targeting at nucleotide 1170 had no effect, whereas the shRNA targeting nucleotide 1127 was the best among the three, inhibiting more than 90% of HA-MST3 expression (data not shown). The MCF-7 breast cancer cell line was cotransfected with the plasmid expressing the plasmid expressing shRNA against MST3 and a neomycin marker. Two stable clones (RNAi-1 and RNAi-2) were established by selection with G418. The other two stable clones (vector-1 and vector-2) had no shRNA integration. The expression of endogenous MST3 of RNAi-1 or RNAi-2 cells was less than 20% of control transfectants or MCF-7 parental cells (Fig. 1A). The growth rates of MST3-RNAi transfectants were similar to that of parental cells and control transfectants (data not shown).

**Down-regulation of MST3 Enhanced Cell Migration**—Because YSK1, a member of MST family, played a role in cellular migration (14), we further examined whether MST3 affected cellular migration. A wound-healing assay was performed to monitor cell migration. Cell migration toward the wound was recorded at 24 and 72 h after scratching. MST3 RNAi transfectants migrated faster than control transfectants (Fig. 1B). Decreased expression of E-cadherin and loss of cellular contacts is usually observed at the migrating cell edge in collec-
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tive cell migration (36). The expression of E-cadherin was much lower in the leading cell edges of MST3 RNAi transfectants (Fig. 1C, lower panel). Conversely, the expression of E-cadherin in the center of the cell mass was not significantly altered between MST3 RNAi and control transfectants (Fig. 1C, upper panel).

In addition to the wound-healing assay for measuring collective cell migration (36), Boyden chamber assay was used to assess the cell migration without cell-cell interaction (Fig. 1D). Both RNAi transfectants displayed a significant enhancement of cell migration than control transfectants.

**Effect of Down-regulation of MST3 on Paxillin Phosphorylation and Cellular Protruding**—The expression of integrins α3 and β1 was not altered by the expression of MST3 siRNA (data not shown). Antibody to activated integrin (conformation-specific antibody for β44) did not produce a significant difference upon flow cytometric analysis (data not shown). In contrast, decreased phosphorylation on residue 118 on paxillin was evident on MST3 RNAi transfectants at 24 or 48 h after plating (Fig. 2, A and B). A minor decrease of phosphorylation of Tyr-31 on paxillin was observed in MST3 RNAi transfectants. To assess if the altered paxillin phosphorylation was associated with change of distribution for MST3 RNAi transfectants, cells were plated for 24 h, and the localization of paxillin was determined using an immunofluorescence analysis. Paxillin formed speckles on one edge of the MST3 RNAi transfectants (Fig. 2C). However, the distribution of paxillin in RNAi transfectants was not altered in the spread cells. The RNAi transfectants spread faster than the control transfectants, and the percentage of protruded cells was determined in triplicate experiments (Fig. 2D). Because YSK1 might affect Golgi distribution, we examined the GM130 distribution in RNAi transfectants. The GM130 distribution was not altered in the transfectants (data not shown).

The **Enhancement of Cell Migration and Protruding Can Be Rescued by RNAi-resistant MST3**—To further examine the specificity of MST3 siRNA, we performed the assay with RNAi-resistant HA-MST3 (Rnai-R-HA-MST3). Three nucleotides of wild type MST3 were mutated to create resistance to shRNA-MST3 siRNA, and the encoding protein was not altered. The shRNA-MST3 could only weakly degrade the RNAi-resistant HA-MST3 in transiently transfected COS-7 cells (Fig. 3A, right panel). The MCF-7-Rnai-2 cells were further transfected with pcDNA3.0, HA-MST3, or Rnai-R-HA-MST3 plasmid and examined for the expression of HA-tagged MST3. The expression of HA-MST3 was only detected in cells transfected with Rnai-R-HA-MST3 (Fig. 3A, left panel). The protruding rate of MCF-7-Rnai-2 cells expressing Rnai-R-HA-MST3 was significantly lower than that of the pcDNA3.0 plasmid transfectants (Fig. 3B). This indicated that the RNAi-resistant MST3 could rescue the enhancement of spreading by shRNA-MST3 in MCF-7-Rnai-2 cells. The transfected cells were further sorted by flow cytometry and analyzed for cell migration. Reintroduction of Rnai-R-HA-MST3 significantly attenuated the enhancement of cellular migration (Fig. 3C). Re-expression of RNAi-resistant HA-MST3 in the MCF-7-Rnai-2 cells recovered the migration speed to a similar level of the MCF-7-V-2 cells (Fig. 1D and Fig. 3C), which suggested the RNAi-resistant MST3 can rescue the migratory phenotype induced by shRNA-MST3.

**Autophosphorylation of Thr-178 in MST3**—To further understand the structure-function relationship of MST3, we identified the MST3 autophosphorylation site. When the amino acid sequence between kinase subdomain VII (DFG) and VIII (APE) of the Ste20 kinase family members is aligned, the number in the parentheses indicates the accession number. Amino acid residues identical in every sequence are boxed in black, and those amino acids with similar chemical properties are boxed in gray. The conserved threonine or serine residues that may be autophosphorylated are denoted by a black rectangle. B, MST3 phosphorylation of peptide containing Thr-178 was assayed in different metal ion as indicated. C, peptide T178 containing Thr-178, peptide T264 containing Thr-264, or whole MBP protein was reacted with 1 μM baculovirus expressing MST3, 2 mM Mn2+, and [33P]ATP. The amount of 33P incorporated into each synthetic peptide was determined in a Cerenkov counter and plotted against reaction time. G-peptide, cAMP-dependent protein kinase substrate, was used as negative control. C, effect of Mn2+, Mg2+, or Zn2+ as a cofactor on peptide T178 phosphorylation. Phosphorylation of peptide T178 by MST3 was assayed in different metal ion as indicated.
whereas MBP was used as the positive control. Recombinant MST3 could phosphorylate the peptide containing Thr-178 (peptide 178), but not Thr-264 (peptide 264) (Fig. 4B), indicating that peptide 178 was a specific substrate for MST3. MST3 could phosphorylate peptide 178 using Mn\(^2^+\)/H\(_{11001}\), or Mg\(^2^+\)/H\(_{11001}\), or Zn\(^2^+\)/H\(_{11001}\) as cofactor but had very little activity in the presence of Zn\(^2^+\)/H\(_{11001}\) (Fig. 4C). Based on a Lineweaver-Burk reciprocal plots, the calculated apparent \(K_m\) was 1 mM, and the \(V_{max}\) was \(-31.2\) pmol/min/mg when Mn\(^2^+\) was used as the cofactor for MST3.

As there are multiple serine and threonine residues in peptide 178, the phosphorylated peptide 178 was subjected to mass spectrometry analysis. Thr-178 was detected as the single phosphorylation site (Fig. 5A). Peptide T178A containing alanine instead of threonine at position 178 was examined with kinase reaction by MST3. Peptide T178A could not be phosphorylated by MST3 (Fig. 5B). To further demonstrate that Thr-178 was the main autophosphorylation of MST3, tryptic digests of autophosphorylated baculovirus-expressed MST3 (22) were subjected to two-dimensional electrophoresis, and the presence of two radioactively labeled phosphopeptides was detected. One of these two peptides overlapped with peptide T178 (Fig. 5C), indicating that Thr-178 was one of the two main MST3 autophosphorylation sites.

Role of Thr-178 Autophosphorylation in Mammalian Cells—To determine the role of Thr-178 autophosphorylation of MST3 in vivo, Thr-178, Thr-264, or both in MST3 were mutated to alanine. COS-1 cells were transfected with the above HA-tagged MST3 expression constructs, immunoprecipitated with anti-HA antibody, and subjected to the in vitro kinase assays with Mn\(^2^+\) as cofactor. Mutant T178A MST3 exhibited a very low level of autophosphorylation activity and had very low kinase activity in the presence of Mn\(^2^+\). In contrast, T264A mutant retained autophosphorylation and kinase activities. Autophosphorylated MST3 showed an additional shifted band when the immunoprecipitates were subjected to Western blotting (Fig. 6A).

To further define the role of Thr-178 in MST3-regulated migration, fast-migrating cell line MDCK cells were selected for
FIGURE 6. Thr-178 roles in autophosphorylation, kinase activity and migratory function of MST3 in MDCK cells. A, effect of Thr-178 substitutions on MST3 activity. COS-1 cells were transiently transfected with each WT-MST3, T178A/T264A-MST3, T178A-MST3, or T264A-MST3, respectively, and immunoprecipitated (IP) with anti-HA antibody. The immunoprecipitates were assayed for kinase activity with myelin basic protein and Mn$^{2+}$ as the cofactor. Radiolabeled samples were resolved by 15% SDS-PAGE followed by autoradiography. The radiolabeled samples were also subjected to 8% SDS-PAGE and probed with anti-MST3 antibody. IB, immunoblot. B, influence of WT-MST3 or T178A-MST3 expression on cell migration in MDCK cells. MDCK cells stably expressing WT-MST3 or T178A MST3 were subjected to wound-healing assay, and cells were photographed 12 and 24 h later. C, the longest migration distance in wound-healing assay was quantitated in three independent experiments. D, migration of various MST3 MDCK transfectants in Boyden chamber assay. Cells were plated on the membranes of the chambers in the serum-free medium in the wells, fibronectin as chemoattractant, and the migrated cells were calculated 7 h after plating. ** represents $p < 0.01$, and *** represents $p < 0.001$ when compared with control MDCK cells.
target cells. MDCK cells were transfected with either wild type MST3 or T178A MST3 and selected for stable clones. The wound-healing assay revealed that the collective migration rate of MST3 stable transfectants was lower than parental MDCK cells, whereas the migration rates of MDCK-T178A-MST3 stable clones are higher than that of MDCK cells (Fig. 6B). Quantitation and statistical analysis of the longest migration distance in three independent experiments were shown in Fig. 6C. Expression of wild type MST3 also significantly inhibited the cell migration with Boyden chamber assay, whereas T178A mutant MST3 could significantly enhance cell migration (Fig. 6D). The growth rates of MDCK-MST3 and MDCK-T178A-MST3 transfectants were similar (data not shown).

Cell protrusion was also compared among these mutant MST3 transfectants at 1, 2, and 24 h after seeding the cells onto the plates coated with fibronectin (Fig. 7A). All cells completely protruded at 24 h. MST3 apparently inhibited protrusion, whereas the T178A MST3 enhanced protruding. The percent-age of protruded cells were calculated (Fig. 7B). We further examined paxillin phosphorylation among the MST3 mutants. Expression of wild type MST3 increased Tyr-118 and Tyr-31 phosphorylation of paxillin (Fig. 7C), whereas expression of...
T178A MST3 decreased Tyr-118 and Tyr-31 phosphorylation. The quantitations of paxillin phosphorylation in three independent experiments were shown in Fig. 7D.

MST3 Phosphorylates PTP-PEST—MST3 is a kinase involved in phosphorylation at serine or threonine residues, and so is unlikely to directly change the tyrosine phosphorylation of paxillin. Therefore, we hypothesized that MST3 may act through protein-tyrosine phosphatases to alter paxillin phosphorylation. PTP-1B and PTP-PEST were known to affect paxillin phosphorylation (30, 34) and were tested as MST3 substrates. Recombinant MST3 can directly phosphorylate recombinant PTP-PEST but was unable to phosphorylate PTP-1B in vitro (Fig. 8A). MDCK cells were cotransfected with PTP-PEST expression plasmid and MST3 expression plasmid, and the tyrosine phosphatase activity of immunoprecipitated exogenous PTP-PEST was measured. MST3 inhibited the protein-tyrosine phosphatase activity of PTP-PEST and raised paxillin phosphorylation (Fig. 8B). Furthermore, the protein-tyrosine phosphatase activity of PTP-PEST was enhanced in MCF7-Rnai-2 cells (Fig. 8C).

MST3 Kinase Activity in Breast Cancer Cell Lines—Finally, we wished to study the status of MST3 in immortalized MCF-10A cells, poorly metastatic MCF-7, and highly metastatic MDA-MB-231 cancer cell lines. The expression of MST3 was similar among the three breast cell lines (Fig. 9A). Recently, MST3 has been shown to regulate NDR protein kinase through phosphorylation (44). The immunoprecipitated MST3 kinase activity toward the NDR peptide was not significantly different among these three cell lines. On the other hand, the MST3 kinase activity toward peptide T178 was varied among these cell lines (Fig. 9B).

DISCUSSION

We demonstrate that MST3 regulates cellular migration with alteration of PTP-PEST activity and paxillin phosphorylation. Either overexpression or down-regulation of MST3 affects cellular protrusion and migration. The effects of MST3 on cellular migration are dependent on its autophosphorylation. Mutation of the autophosphorylation site of MST3 inactivates the observed effect on cellular migration. Altogether, we conclude that autophosphorylated active MST3 inhibits cellular migration.

Golgi apparatus is positioned at the edge of wounds and both migrating and polarized cells. MST4 and YSK1 have been reported to play a role in cellular migration through targeting Golgi (14). Cdc42, a master regulator of cellular polarity, binds to an MST3 homologue PAK4, and the binding is involved in the translocation of PAK4 to Golgi apparatus (45). On the other hand, MST3 may affect protrusion and migration through a different pathway, because we presently observed that MST3 does not localize to Golgi, and ablation of MST3 does not affect Golgi distribution during protrusion.

Tyrosine phosphorylation of paxillin is required for the temporal regulation of focal adhesion formation in motile cells. Phosphorylation of residues 31 and 118 of paxillin occurs in cell adhesion, polarization, and migration in lymphoid cells, hepatoma MM1 cells, and NMuMG cells (31, 46, 47). On the other hand, phosphorylation-null mutation, in which Tyr-31, -40, -118, or -181 of paxillin is mutated to phenylalanine, enhances migration activity in COS-7, NMuMG, or MM1 cells on collagen-coated dishes (48). In addition, aberrant large protrusion and increased migration are induced by suppression of paxillin with siRNA (36). We observed that down-regulation of MST3 enhances cellular migration with a decrease of tyrosine phosphorylation on paxillin. Similar discrepancy was also observed for another paxillin adaptor molecule PTP-PEST. Either overexpression or inactivation of PTP-PEST inhibits cell motility (49–51), suggesting that the balanced coordination of multiple gene products rather than the expression level or tyrosine phosphorylation of a single gene controls cellular motility.

Phosphorylation of paxillin 31 and 118 is highly inducible upon integrin activation (31). We observed that knockdown of MST3 did not apparently affect the expression level of integrin α3 and β1 and only slightly affected the active form of integrin β1. Another receptor for fibronectin, integrin αvβ3, was not determined, because integrin β3 expression is...
not detected in MCF7 (52). Integrin signaling may not be the main pathways that MST3 involved in migration. In contrast, we demonstrated that MST3 directly phosphorylated PTP-PEST but not PTP-1B. The PTP-PEST expression plasmid was cotransfected with control vector or MST3 into MDCK cells. The tyrosine phosphatase activity of the immunoprecipitated PTP-PEST was measured, and the amount of immunoprecipitated PTP-PEST was determined by Western blotting. The tyrosine phosphatase activity was normalized with the amount of immunoprecipitated PTP-PEST. The PTP-PEST was immunoprecipitated in MCF7-RNAi-2 cells, and the activity was normalized with the amount of immunoprecipitated PTP-PEST. **p < 0.001.

**FIGURE 8.** MST3 phosphorylates PTP-PEST and inhibits tyrosine phosphatase activity. A, recombinant MST3 was incubated with PTP-PEST or PTP-1B in a kinase reaction. The reaction products were separated by 15% SDS-PAGE and analyzed with autoradiography (right panel), and the amounts of substrates were revealed by Coomassie Blue stain (left panel). MBP was used as a positive control. B, MST3 inhibited PTP-PEST activity. The PTP-PEST expression plasmid was cotransfected with control vector or MST3 into MDCK cells. The tyrosine phosphatase activity of the immunoprecipitated PTP-PEST was measured, and the amount of immunoprecipitated PTP-PEST was determined by Western blotting. The tyrosine phosphatase activity was normalized with the amount of immunoprecipitated PTP-PEST. C, MST3NAi enhanced PTP-PEST activity. The PTP-PEST was immunoprecipitated in MCF7-RNAi-2 cells, and the activity was normalized with the amount of immunoprecipitated PTP-PEST. **p < 0.001.

**FIGURE 9.** The status of MST3 in breast cell lines. A, the MST3 protein expression in three breast cell lines was determined with Western blotting and probed by anti-MST3 antiserum. IB, immunoblot. B, the endogenous MST3 in MCF-7, MCF-10, and MDA-231 cells was immunoprecipitated (IP) with anti-MST3 antiserum and assayed for the kinase activity on the T178 peptide or NDR peptide. The amounts of immunoprecipitated MST3 were shown by Western blotting.

De novo, these measurements indicate that MST3 may regulate cell migration through other pathways in addition to PTP-PEST. In contrast, the enhancement of PTP-PEST activity in MST3 RNAi transfectants may account, at least in part, for the decrease of paxillin phosphorylation. However, MST3 may regulate cell migration through other pathways in addition to PTP-PEST. MST3 can regulate NDR protein kinase by phosphorylation (44), and the NDR protein can regulate cell cycle progression and morphology. MST3 may act through both NDR and PTP-PEST to regulate cellular morphology and migration. In addition, it was interesting to note that ligand/hormone or endogenous stimuli for activation of NDR have not been identified (44). Similarly, attempts to identify the stimulus for MST3 have failed (19, 20). PTP-PEST is a potential substrate for MST3; therefore, the stimuli associated with cellular migration and PTP-PEST may be the potential ligands for MST3, such as vascular injury, CD2 in T-cell activation, and insulin-like growth factor-1 (53–55). Investigation of these stimuli may further reveal the physiologic role of MST3. In an initial attempt to analyze the MST3 kinase activity in three different breast cancer cell lines with varied metastatic ability, we did not find the correlation between the status of MST3 and migration potential of individual cell lines. It is possible that post-translational modification of MST3 or its interacting partners may further refine the physiologic roles of MST3 in vivo.
MST3 is activated by autophosphorylation (19); however, the site and the function in vivo have not been demonstrated before. In this study, we have identified Thr-178 as one of the major autophosphorylation sites, and mutation of this residue inactivates the MST3 kinase activity in vitro and effects on migration in vivo. Previously, several autophosphorylation sites, including Thr-183 and Thr-187, have been identified in MST1 (10). Mutation of these two residues may cause autophosphorylation at other sites. In contrast, Thr-178 is critical for MST3 function. Mutation of the single residue almost completely inactivates its function and phosphorylation on other sites, at least in the MDCK cell line we tested. However, we do not exclude the possibility that MST3 may autophosphorylate itself at sites other than Thr-178 in other cell types or under different stimuli. Mutation on Thr-178 appears to influence the rate of migration and spreading, which may affect many physiological processes. For example, at the wound sites of gastric mucosa, cells initially spread rapidly and migrate into the lesion without proliferation (56). This repairing process may be impaired by the expression of T178A MST3. To further investigate the role of these mutations and the physiological role of MST3, transgenic mice for MST3 will be used to study the physiological function of various forms of MST3 in our laboratory.

In summary, MST3 regulates cell migration involving paxillin and PTP-PEST. Furthermore, Thr-178 is an important autophosphorylation site of MST3; mutation of this site inactivates the migration regulatory function of MST3.

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