The Ste20 Kinase MST4 Plays a Role in Prostate Cancer Progression

Victoria Sung, Wen Luo, Dapeng Qian, Isabelle Lee, Bahija Jallal, and Mikhail Gishizky


ABSTRACT

MST4, a member of the Sterile 20 serine/threonine kinase family, was found to be expressed in prostate carcinoma tumor samples and cell lines. In addition, expression levels appeared to correlate with tumorigenicity and androgen receptor status of the cells. Ectopic expression of wild-type and kinase-inactive MST4 was used to alter cellular MST4 activity levels in three widely studied human prostate tumor cell lines: LNCaP, DU 145, and PC-3. Overexpression of wild-type MST4 induced anchorage-independent growth of the LNCaP cell line, and increased both in vitro proliferation and in vivo tumorigenesis of the DU 145 cell line. On the other hand, expression of a kinase-inactive form reverted the anchorage-independent growth phenotype and highly tumorigenic behavior of the PC-3 cell line. MST4 kinase activity was stimulated significantly by epidermal growth factor receptor ligands, which are known to promote growth of prostate cancer cells. Together, our studies suggest a potential role for MST4 in the signal transduction pathways involved in prostate cancer progression.

INTRODUCTION

Prostate cancer is the second most common malignancy among males in the United States, with ~40,000 men dying annually from the disease (1). The importance of steroid hormone in prostate cancer progression was established many years ago, and AR blockade is usually used to treat patients in early stages of the disease (2). However, most prostate tumors eventually become androgen-insensitive, and proliferate and disseminate despite hormone ablation (3). One of the mechanisms responsible for hormone-independent growth of prostate cancer is thought to be altered growth factor signaling, which would allow for tumor progression even in the absence of androgen stimulation (4). Growth factors, which are essential for maintaining normal prostate tissue, such as members of the EGF family, platelet-derived growth factor, bFGF, and IGF, often become deregulated in malignant cells, and contribute to a transformed phenotype (5). Both increased expression and secretion of growth factors, and their corresponding receptors, as well as aberrant modulation of intracellular signaling cascades may lead to loss of growth factor regulation (6, 7). For example, although MAPK expression levels are similar in benign and malignant tissue, activation is increased ~15-fold in advanced stages of prostate cancer and in tumor samples from patients with recurrent, androgen-insensitive tumors (6).

Ste20 was originally identified as a component of the yeast pheromone response pathway and has been found to activate the MAPK cascade (8–10). Numerous mammalian Ste20 homologues have been cloned and implicated in regulation of cytoskeletal organization, disassembly of actin stress fibers, and modulation of apoptosis and the stress response (11–15). The Ste20 proteins are members of the STE kinase group (10) and are additionally divided into two subgroups: the p21-activated kinase subfamily (9), with COOH-terminal kinase domains and NH2-terminal p21-binding domains, and the GCK-like proteins (16), which possess an NH2-terminal kinase domain and a long COOH-terminal regulatory region lacking the p21-binding domain. Whereas members of both kinase subfamilies can signal through the MAPK pathway, only p21-activated kinases are known to be directly regulated by the small GTP-binding proteins Rac1 and Cdc42 (17).

In this paper we present evidence supporting a role for the GCK-like mammalian STE20 kinase 4 (MST4, also known as MASK; Ref. 18) in prostate cancer progression. MST4 RNA is expressed ubiquitously at low levels, but appears to be most strongly expressed in placenta, thymus, and PBLs (18–20). It is currently unclear whether MST4 can activate MAPK pathways. Although two independent studies found MST4 not to signal upstream of extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, or p38 (18, 19), another group demonstrated significant activation of extracellular signal-regulated kinase by MST4 via a Ras/Raf1-independent pathway (20). Phenotypic changes associated with MST4 expression and activity include increased proliferation and anchorage-independent growth, as well as induction of apoptosis (20, 18). We now demonstrate that MST4 protein is also expressed in normal prostate, prostate-derived tumor, and prostate cancer cell lines, where expression correlates inversely with AR status. MST4 overexpression can alter the morphology of prostate cancer cells, confer anchorage-independent growth in vitro, and promote tumor xenograft growth in vivo. In addition, MST4 kinase activity is stimulated by EGF treatment. These data suggest that MST4 may couple growth factor stimuli to downstream responses, and thus play a potential role in prostate cancer signal transduction and tumorigenesis.

MATERIALS AND METHODS

Cell Culture, Immunoblotting, and Proliferation Assay. The LNCaP, DU 145, and PC-3 human prostate cancer cell lines, and other tumor cell lines were originally obtained from the American Type Culture Collection (Manassas, VA), whereas the PC-3 AR cell line was a gift from Dr. Marco Marcelli (Department of Medicine, Baylor College of Medicine, and VA Medical Center, Houston, TX). PrEC cells (normal prostate epithelial cell line isolated from the prostate of a 42-year-old Asian male who did not have prostate cancer) were purchased from Clonetics (Walkersville, MD). All of the cell lines were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and 2 mM L-glutamine (Invitrogen) at 37°C with 5% CO2. Cell lines overexpressing WT, KE, or V were cultured in an identical manner as the parental cells from which they were derived. For growth factor treatment, cells were starved overnight in serum-free, phenol red-free RPMI 1640 (Invitrogen). The next day, growth factor was added for up to 1 h, and cells were lysed in NP40 buffer [20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP40, and 2 mM EDTA plus protease/phosphatase inhibitors]. After protein estimation using the BCA kit (Pierce Endogen, Rockford, IL), equal amounts of lysate were run on an 8% acrylamide gel, transferred to nitrocellulose, and probed using an MST4 antibody. Blots were also probed with a tubulin antibody to ensure equivalent loading.

LNCaP proliferation assay was performed in complete medium containing 10% FBS. Cells were plated either with or without 1 μM casodex and grown over the course of 5 days. Cells were trypsinized and counted using a hemo...
cytometer on days 1, 3, and 5 after plating, DU 145 proliferation assays were carried out as follows: cells were plated in duplicate in each well of a six-well tissue culture plate. After 24 h, growth medium was removed and replaced with serum-free and phenol red-free RPMI (Invitrogen). Cells were trypsinized and counted using a hemocytometer on days 1, 3, and 5 after serum starvation.

Reagents and Antibodies. Human EGF was purchased from Roche Molecular Biochemicals (Indianapolis, IN) and was used at a concentration of 10 ng/ml. Bicalutamide (Casodex) was a gift from AstraZeneca (Södertälje, Sweden), and unless otherwise described, all of the other growth factors and chemicals used was purchased from Sigma.

The MST4 polyclonal rabbit antibody was raised against a COOH-terminal peptide of the MST4 protein (Cys-EKFQKCSADESP) by JK antibodies (Mountain Ranch, CA) and column purified against the same peptide. The tubulin antibody was purchased from ICN (Aurora, OH). For immunofluorescence, Texas Red-conjugated phalloidin was purchased from Sigma Immunochemicals (St. Louis, MO).

Plasmid Constructs and Transfection. Full-length WT and KE (lysine to glutamic acid mutation at position 53; shown to be inactivating, Ref. 18) MST4 constructs were cloned into the pcDNA3 vector at the BamHI and XhoI restriction sites, and stably transfected into DU 145 and PC-3 cell lines using Lipofectamine reagent (Invitrogen). Stable lines and pools were generated by culturing the cells in neomycin sulfate (500 μg/ml; Invitrogen). As controls, pcDNA3 vectors without MST4 constructs were also transfected into the cell lines. The MST4 constructs were also cloned into the pBabeuro3 retroviral vector at the BamHI and BgIII sites to infect the LNCaP cells, which revealed a substantially higher rate of gene transfer via retroviral infection as compared with standard transfection. Stable clones were isolated by selection in puromycin (5 μg/ml; Sigma). Note that whereas only representative clones were included in this paper, several isolated clones were tested in each of the phenotypic assays below.

Northern Blot Analysis. A human multiple tissue Northern Blot (BD Biosciences Clontech, Palo Alto, CA) containing immobilized polyadenylated mRNA was used. A full-length MST4 DNA probe was labeled with [α-32P]dATP (Perkin-Elmer, Boston, MA) by random primed labeling. Hybridization and washing were carried out according to the manufacturer’s instructions. The membrane was also stripped and rehybridized with a β-actin probe.

Immunoprecipitation/Kinase Assay. Whole cell lysates were precleared with protein Sepharose A beads (Amersham Pharmacia Biotech, Uppsala, Sweden) and then incubated with 2 μg of MST4 antibody for 1 h, followed by addition of protein Sepharose A beads and incubation overnight at 4°C. The supernatant was removed by aspiration, and beads before assaying for kinase activity.

Kinase assays were carried out in a total volume of 20 μl kinase buffer (as above, with the addition of 5 mM MnCl₂, 1 μg MBP and 5 μCi [32P]γ-dATP). After incubating at room temperature for 15 min, reducing sample buffer was added to each sample, and the reaction boiled for 5 min and analyzed by SDS-PAGE (14% gel). The gel was then dried and exposed to X-ray film (Fuji Film, Stamford, CT).

Soft Agar Assay. This assay was performed to determine the ability of cells to grow in an anchorage-independent manner. Stock soft agar solution was prepared by autoclaving 1.6% bacto-agar (Difco; Becton Dickinson, Franklin Lakes, NJ) in H₂O. Stock agar was diluted in IMEM (Invitrogen) to 0.8% agar/FBS was layered on top of the cells. Assays were carried out for 4 weeks, with weekly “feeding” of 0.8% agar/FBS/IMEM. Colonies were observed and photographed using a Zeiss Axiosvert 100 inverted microscope.

Immunofluorescence. Cells were plated on glass coverslips, grown to ~70% confluence, fixed with 4% paraformaldehyde (EMS, Ft. Washington, PA), permeabilized with 0.2% Triton X-100/PBS and nonspecific sites blocked by incubation with 3% FBS. Coverslips were incubated with Texas red-conjugated phalloidin (dilution of 1:100) for 1 h, rinsed well, and mounted onto microscope slides and examined using the Nikon Microphot FXA microscope.

In Vivo Cell Inoculation. Cells were routinely prepared for inoculation by pelleting and washing twice by centrifugation followed by resuspension at 5 million cells/100 μl 1× PBS (cells were determined to be at least 90% viable). Cells were implanted s.c. without Matrigel into the right dorsal flank of 6–8-week old intact male NCr nude mice (Charles River Laboratories, Wilmington, MA). Beginning at ~2 weeks after injection, xenografted tumors were measured weekly, and monitored for growth and/or necrosis. Animals were sacrificed by CO₂ asphyxiation as tumors reached 2 cm³ or before, if they showed any sign of discomfort, paralysis, or emaciation. The DU 145 cell line was used to test whether WT MST4 could promote growth, whereas the PC-3 cell line was used to study whether kinase inactive MST4 could inhibit tumorigenesis.

RESULTS

MST4 Is Expressed in Normal Prostate and in Prostate Cancer Cells. Examination of a panel of RNA from normal human tissues indicated significant MST4 expression in the placenta, spleen, thymus, and PBLs, as well as low levels of expression in several of the examined tissues (Fig. 1; as published previously, Refs. 8–20). In addition, significant expression was detected in the prostate and testis, with prostate expression levels most comparable with those seen in thymus (Fig. 1). Using the same probe, we then examined MST4 expression in normal prostate and prostate tumor biopsies using the Clontech human tumor (matched tumor/normal) mRNA blot. Whereas MST4 was not differentially expressed in tumor versus normal samples, expression was found in all three pairs of prostate tissue (data not shown). MST4 protein was additionally detected in total cell lysates prepared from a small sample set of prostate cell lines: one normal prostate epithelial cell line (PrEC) and three well-established prostatic carcinoma cell lines, LNCaP, DU 145, and PC-3 (Fig. 2). The DU 145 and PC-3 cell lines were derived from brain and bone metastases, respectively, are AR-negative and poorly differentiated, whereas the LNCaP cell line, originally derived from a lymph node metastasis, has functional AR, grows less rapidly, and is less tumorigenic than the DU 145 and PC-3 lines (reviewed in Ref. 21). As shown in Fig. 2, we detected noticeably higher MST4 levels in DU 145 and PC-3 cells than in both the normal cell line and the well-differentiated AR-positive LNCaP line. Relative amounts of protein were normalized before loading, and the samples were also probed for tubulin to ensure equivalent protein loading. MST4 expression was not limited to prostate tumor cell lines, as abundant protein was found in a number of colon, breast, and lung cancer-derived cell lines (Fig. 3).

To test for a possible causal relationship between AR signaling and
MST4 protein expression, we used two different cell systems in which the AR status could be manipulated. Comparing MST4 expression in parental PC-3 cells to PC-3 cells in which functional AR has been stably reintroduced (PC-3AR) yielded a striking result, shown in Fig. 4. The PC-3AR cell line expressed significantly less MST4 than the parental line (see tubulin blot for protein normalization), suggesting that MST4 expression was reduced by re-expression of the hormone receptor. We also treated the LNCaP cell line, which has a functional AR, with bicalutamide (commercial name: Casodex), a nonsteroidal antiandrogen that has been shown to inhibit AR function and in some cases, growth in vitro (reviewed in Ref. 22). Inhibiting AR in the LNCaP cells resulted in increased expression of MST4 (Fig. 4A) without affecting cell proliferation (Fig. 4B), suggesting that a disruption in AR signaling rather than a decrease in cell number/viability is likely to account for our observation. Together, these findings suggest that MST4 protein expression is reduced by AR signaling in prostate cancer cell lines.

The MST4 antibody used in these experiments consistently detected two protein bands, prompting closer examination to determine the identity of both bands. Treatment of protein samples with phosphatase did not eliminate either band, ruling out the likelihood that one might represent a phosphorylated form of the protein (data not shown). Preincubation of the antibody with MST4 peptide (to which the antibody was raised) eliminated both bands, suggesting that the antibody is directed against two proteins that are MST4-related. In addition, overexpression of MST4 in the prostate cancer cell lines resulted in an increase in intensity of both bands (Figs. 5 and 7). Finally, because neither band was detected in the HT-29 cells (Fig. 3), and the intensity of both bands appeared to change proportionally to one another (Figs. 5 and 7), it is possible that our antibody detected an alternate form of MST4 or closely related protein.

Overexpression of MST4 in Prostate Cancer Cell Lines Results in Altered Morphology, Increased Proliferation, and Anchorage-independent Growth. To investigate whether MST4 expression may contribute to morphological changes in prostate tumor cells, we stably overexpressed WT MST4 and the corresponding kinase inactive form (Lys53Glu substitution shown previously to be inactivating: Ref. 18) in the three prostate cancer cell lines. Phenotypic changes were seen in both DU 145 and LNCaP cells. The Western blot in Fig. 5 shows MST4 expression levels in two independent DU 145 clones transfected with MST4 WT and KE. As demonstrated in the micrographs (Fig. 5, bottom panels), DU 145 cells overexpressing MST4 WT were

![Fig. 2. MST4 protein is found in prostate epithelial and tumor cell lines.](Image)

![Fig. 3. MST4 protein is expressed in a variety of tumor cell lines.](Image)

![Fig. 4. MST4 protein expression correlates inversely with AR status in prostate cancer cell lines.](Image)
more rounded than vector control cells and showed peripheral actin staining suggestive of lamellipodia formation. On the other hand, overexpression of MST4 KE resulted in cell elongation and flattening, with the appearance of longitudinal actin fibers. Infection of LNCaP cells with WT MST4 similarly altered cell morphology. LNCaP clones overexpressing WT MST4 also became rounded and eventually detached from the substrate, growing as viable clumps of suspension cells. Overexpression of MST4 KE in LNCaP cells had no noticeable effects on cell shape (data not shown).

In addition to morphological changes, we also examined growth of prostate cancer cells overexpressing WT or KE MST4. Whereas there was no change in proliferation of cells grown in 10% serum, stable overexpression of MST4 KE in DU 145 cells inhibited proliferation in serum-free medium compared with vector control cells (Fig. 6). We observed a small growth advantage in DU 145 WT clones compared with vector and KE clones, and despite slight clonal variation, the overall trend suggests that MST4 expression may contribute to cellular proliferation under stringent, serum-free conditions in this cell line. There was no apparent change in proliferation of the LNCaP or PC-3 cell lines overexpressing MST4 WT or KE.

An important hallmark of cellular transformation is anchorage-independent growth, and whereas normal cells are often polarized and require a solid substratum on which to proliferate, malignant cells gain the ability to grow regardless of their attachment status. To test whether MST4 can contribute to anchorage-independent growth of prostate cancer cells, we compared soft agar colony formation of vector control cells with those expressing WT and KE MST4. The upper panel in Fig. 7 demonstrates overexpression of MST4 WT and KE relative to vector controls in two clones each of LNCaP and PC-3 cells. The AR-positive LNCaP cells, which are minimally invasive and express low levels of endogenous MST4, do not usually form proliferating colonies in soft agar. However, overexpression of MST4 WT in these cells induced anchorage-independent growth in two of two clones (representative clone, WT5, shown in Fig. 7). We observed only a few colonies per plate, but they were large in size and clearly consisted of proliferating cells. In contrast, vector control and KE clones never formed colonies in soft agar. In contrast with the LNCaP cell line, PC-3 cells are very tumorigenic and express high endogenous levels of MST4. They usually grow well in soft agar, forming numerous colonies. Although transfecting these cells with MST4 WT resulted in slightly smaller colonies, the overall number of anchorage-independent colonies per dish was unaltered when compared with vector control cells. However, expression of inactive MST4 completely abrogated colony formation in two of two clones tested (rep-
resentative clone KE30 shown in Fig. 7, bottom right). We also tested the DU 145 cells in this assay, but expression of neither MST4 WT nor KE promoted nor inhibited soft agar colony formation. These results suggest that MST4 may contribute to prostate cancer tumorigenesis by minimizing the restrictions necessary for cell growth.

**MST4 Contributes to the in Vivo Growth of Prostate Cancer Cells.** Having observed a possible role for MST4 in prostate tumor cell growth *in vitro*, we asked whether it might also affect tumorigenesis *in vivo*. To test this hypothesis, we s.c. implanted representative DU 145, PC-3, and LNCaP stable clones overexpressing MST4 WT and KE in nude mice, and measured xenograft growth weekly in the following months. DU 145 cells stably overexpressing WT MST4 (WT12) demonstrated an increase in tumor incidence compared with those cells expressing vector and KE MST4 (KE14; Fig. 8A). Although we might not have expected the KE cells to grow well, we

**Fig. 6.** Overexpression of MST4 modifies proliferation of DU 145 prostate cancer cells. In two separate experiments (A and B), stable transfection of KE MST4 inhibited proliferation compared with V and WT clones over a period of 5 days (KE 9/14 compared with V and WT12/15). Cells were plated overnight in serum-supplemented RPMI 1640, then switched to serum-free medium and counted on days 1, 3, and 5 thereafter. Results are represented on a semi-log plot.

**Fig. 7.** Expression of WT and KE MST4 influences anchorage-independent growth of LNCaP and PC-3 prostate cancer cell lines. A. Stable LNCaP clones expressing WT and KE MST4 were generated by retroviral infection, whereas PC-3 stable clones were made through standard transfection of MST4 constructs. Clones were screened for expression using the MST4 antibody, and the overexpression of clones relative to vector controls is shown in the Western blots (LNCaP WT 4 and 5, KE1 and 2; PC-3 WT 8 and 11, KE30 and 31). B. Micrographs illustrate the growth of representative LNCaP and PC-3 WT and KE clones in soft agar. Overexpression of WT MST4 by infection conferred a growth advantage to LNCaP cells (WT 5), whereas transfection of inactive MST4 into PC-3 cells inhibited growth compared with vector control cells (KE30). 1 × 10^5 cells were plated in 0.4% agar in serum-supplemented IMEM. Assays were carried out for 4 weeks, with weekly feedings of 0.8% agar/10% FBS. Colonies were observed and photographed using a Zeiss Axiovert 100 inverted microscope. Magnification = ×10.

**Fig. 8.** MST4 expression alters the growth of DU 145 and PC-3 cell lines in nude mice. Stable overexpression of WT MST4 potentiated s.c. growth of DU 145 cells (A), whereas expression of KE MST4 abrogated PC-3 xenograft growth (B). DU 145 WT12/KE14 and PC-3 WT8/KE30 clones were injected s.c. into the right dorsal flank of 6–8-week old intact male NCr nu/nu mice. Five million cells were resuspended in PBS and implanted without Matrigel into groups of five mice per group, and tumors were measured weekly after initial detection of tumors. Plots show the average tumor volume over time for each set of cell lines; bars, ±SD.
were somewhat surprised that our isolate of DU 145 parental and vector cells did not grow in nude mice. One explanation for the lack of growth could be that our implantation conditions were slightly stringent (i.e., cells were resuspended in PBS and implanted without Matrigel). PC-3 stable clones were similarly implanted into nude mice, resulting in significant tumor formation by cells expressing both vector control and WT MST4 (clone 8; Fig. 8B). In contrast, cells overexpressing kinase-inactive MST4 (KE30) were dramatically growth-inhibited. We continued this study for up to 3 months and observed no growth of the PC-3 KE cells. Taken together, these in vivo data advocate a role for MST4 in tumorigenesis of prostate cancer cells.

EGF Activates MST4 Kinase Activity in Prostate Cancer Cells. Prostate cancer tumorigenesis and progression to androgen independence is largely dependent on growth factor-stimulated signaling pathways. We wished to determine whether stimulation of the prostate cancer cell lines with specific polypeptide growth factors could activate MST4 and result in the initiation of a signaling process. The EGFR-stimulated MAPK pathway is one of the most well-studied signaling pathways to be associated with prostate tumorigenesis, and signaling through this receptor has been correlated with increased prostate tumor cell motility, androgen independence, and invasion. Indeed, treatment of all three of the cell lines with EGF resulted in significant MST4 activity as demonstrated by phosphorylation of MBP (Fig. 9). However, the peak and duration of activity was cell line-dependent, with DU 145 and PC-3 cells undergoing maximum activation at 5–15 min, whereas the LNCaP cell line manifested a more gradual activation, which peaked at approximately 15–30 min. To ensure that these data were specific and MBP phosphorylation attributable only to MST4 activity, we ran parallel experiments using peptide-blocked MST4 antibody for immunoprecipitation. Addition of the peptide to block MST4 resulted in the complete removal of EGF-induced kinase activity (data not shown).

In addition to EGFR, prostate cancer cells also secrete and express a number of other growth factors/receptors (21, 23). To determine whether EGF is unique in its ability to activate MST4, we treated the DU 145 cell line with TGF-α, bFGF, IL-6, and IGF, and measured subsequent MBP phosphorylation. Although TGF-α (also an agonist of EGFR) induced robust MST4 activation, bFGF and IL-6 treatment resulted in substantially lower levels of MST4 activity, whereas IGF did not appear to activate MST4 at all (data not shown). These findings suggest that MST4 may function downstream of the EGFR family.

**DISCUSSION**

The results presented here suggest that the Ste20 family member MST4 is involved in progression of prostate cancer. MST4 is expressed in both normal and malignant prostate samples, and expression levels appear to be higher in tumorigenic cell lines, which have lost AR function. Whereas normal prostate epithelial cells and well-differentiated, AR-positive LNCaP cells express very little MST4, the invasive DU 145 and PC-3 cell lines express higher protein levels. We also associate increased MST4 expression with loss of AR function in casodex-treated LNCaP cells, and conversely, show that PC-3 cells engineered to express functional AR have less MST4 than parental PC-3 cells. Finally, overexpression of MST4 can alter prostate tumor cell morphology, proliferation, anchorage-independent growth, and in vivo tumorigenesis.

There are a growing number of signaling molecules including intracellular calcium, inositol triphosphates, diacylglycerol, MAPK, and Ste20 kinases that are AR-regulated and thought to play a role in prostate cancer progression (24–26). PSK, a novel Ste20 family member isolated from surgically removed prostate tumor, activates the c-Jun NH₂-terminal kinase pathway and stimulates actin organization (27). Another Ste20 kinase, SPAK, was isolated from LNCaP cells and is induced by androgen (28). SPAK is most homologous to MST3 in the GCK/Ste20 family and was originally cloned from a transformed rat pancreatic β cell line and shown to activate p38 in cotransfection studies (29). Similar to MST4, SPAK is expressed in spleen, thymus, and testis, and in both hormonally regulated and nonhormonally regulated cancer cell lines. However, whereas casodex treatment results in elevated MST4 expression in LNCaP cells, it blocks androgen-induced SPAK protein expression in the same cell line, suggesting that SPAK is regulated differently than MST4. We have yet not examined whether the AR agonist dihydrotestosterone can stimulate MST4 expression and/or signaling.

Our study was focused on examining the role of MST4 in cellular transformation of prostate to locally invasive cancer and finally to metastatic disease. Abnormal proliferation is one characteristic of neoplastic transformation and is attributable in part to altered expression of growth factors, their receptors, and subsequent signaling pathways (reviewed in Refs. 4, 30). Overexpressing MST4 in DU 145 cells grown in serum-free conditions potentiates in vitro growth, whereas expression of kinase inactive MST4 decreases proliferation to levels below vector control cells. In addition, overexpression of WT MST4 in DU 145 cells induces s.c. tumor formation in nude mice, whereas KE MST4 inhibits PC-3 xenograft growth. Experiments by Lin et al. (20) also demonstrate that overexpression of MST4 causes increased growth of Phoenix cells in both serum and serum-free conditions. In contrast, Dan et al. (18) observed an apoptotic phenotype in MCF-7 breast tumor cells overexpressing MST4. We also overexpressed MST4 in a number of tumor cell lines, but did not detect cell death, only rounding of the cells. This difference may be because of expression levels and subsequent localization of the kinase; whereas we and Lin et al. (20) made stable clones overexpressing the protein, Dan et al. (18) reported apoptosis in transiently transfected cells expressing substantially higher levels of MST4.

The interaction between tumor cells and the surrounding ECM is another characteristic associated with neoplastic transformation. Survival of normal epithelial cells is closely related to attachment and interaction with the ECM, and cross-talk between stroma and epithelia is essential in regulation of cell growth (reviewed in Ref. 31). As tumor cells become transformed, they often lose these interactions and proliferate in the absence of adhesion to ECM (anchorage-independence growth; Ref. 31). In a previous study by Lin et al. (20), overexpression of MST4 in Phoenix and HeLa cells resulted in increased survival of normal epithelial cells is closely related to attachment and interaction with the ECM, and cross-talk between stroma and epithelia is essential in regulation of cell growth (reviewed in Ref. 31). As tumor cells become transformed, they often lose these interactions and proliferate in the absence of adhesion to ECM (anchorage-independence growth; Ref. 31). In a previous study by Lin et al. (20), overexpression of MST4 in Phoenix and HeLa cells resulted in increased
anchorage-independent growth. We extend these observations to prostatic cell lines and demonstrate that MST4 can confer anchorage-independent growth on LNCaP cells, which normally do not grow well in soft agar. On the other hand, the ability of PC-3 cells to grow anchorage-independently and to form numerous colonies is abrogated by expression of kinase-inactive MST4.

In addition to increased proliferation and loss of anchorage-dependent growth, tumor cells often acquire morphological changes potentiating motility and cellular invasion (reviewed in Ref. 30). Overexpression of MST4 in LNCaP and DU 145 causes cell rounding and detachment (LNCaP) from the substrate. The rounded and detached cells are viable and continue to proliferate in suspension, undergoing dramatic changes in actin organization. Our finding that MST4 is involved in morphological changes contributes to the growing number of studies that link expression and activation of Ste20 family members to cytoskeletal reorganization (12, 13, 32). For example, Tsutsumi et al. (32) have described the translocation of proline- and alanine-rich Ste20-related kinase to the cytoskeleton in response to cellular stress, and Traf2- and Nck-interacting kinase, another member of the GCK/Ste20 family, inhibits cell spreading through disruption of the actin cytoskeleton (13). Also, a recently characterized Dictyostelium discoideum Ste20 kinase (which is highly homologous to MST4) phosphorylates the actin-binding protein severin, associated previously with cell motility and pseudopod extension (12, 33).

Whereas the effects of MST4 on cell growth and transformation have been explored (20), there are no studies focused on the signaling pathways that lie upstream of MST4. To address this, we examined the ability of various growth factors to stimulate MST4 phosphorylation in prostate cancer cells. Whereas some stimulation is seen with bFGF and IL-6, treatment of serum-starved cells with EGF and TGF-α results in significant MST4 activation. Alongside studies published previously (8), our results suggest that MST4 may function downstream of EGFR and act as a MAP4K, providing another EGF growth factor signaling pathway.

The importance of the EGFR family in prostate cancer progression is well documented. Expression of the receptor closely mirrors disease stage, and inhibitors of EGFR as well as EGFR/Her-2 antibodies suppress proliferation of DU 145 and PC-3 cells in vitro and in vivo (34, 35). EGFR family signaling in prostate cancer progression has also been associated with increased regulation of prostate tumor cell growth through autocrine activation of EGFR by EGF and TGF-α (36). Furthermore, stimulation and activation of the EGFR promotes migration and invasion of DU 145 cells in vitro, possibly mediated by the interaction of EGF with the actin cytoskeleton (37–42).

EGFR signaling is also important in cross-talk between growth factor-mediated signaling and AR activity in prostate cancer. Whereas early stages of the disease are treatable with androgen ablation, tumors eventually become androgen-insensitive and proliferate/disseminate despite hormone withdrawal (3). This change is thought to be attributable in part to a shift in response to growth factors such as EGF, which gain the ability to activate AR. Overexpression of the EGFR family receptor Her-2/neu activates AR and increases growth through MAPK signaling (43, 44). Moreover, androgen-independent cells appear to express higher levels of Her-2/neu, and forced overexpression of this growth factor receptor in the LAPC-4 prostate cancer model confers androgen-independent growth (45). If MST4 signaling is common to both hormone and growth-factor signaling pathways, it may prove to be important in understanding the progression of prostate and breast cancer to hormone independence.

In summary, our present studies suggest a role for the serine threonine kinase MST4 in prostate cancer progression. MST4 is expressed by prostate tumor cell lines and patient-derived prostate specimens, and is involved in prostate cancer cell proliferation, anchorage-independent growth, and in vivo tumorigenesis. Although its mechanism of action is not yet understood, MST4 may signal in an EGFR pathway because activity is stimulated by both EGF and TGF-α. Collectively, these observations point to a role for MST4 in prostate carcinoma progression and suggest that a specific inhibitor against MST4 may be therapeutically effective in treating patients with prostate disease.

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