

## Novel Role of MDA-9/Syntenin in Regulating Urothelial Cell Proliferation by Modulating EGFR Signaling

Santanu Dasgupta<sup>1,2,3</sup>, Mitchell E. Menezes<sup>1</sup>, Swadesh K. Das<sup>1,2</sup>, Luni Emdad<sup>1,2,3</sup>, Aleksandar Janjic<sup>1</sup>, Shilpa Bhatia<sup>1</sup>, Nitai D. Mukhopadhyay<sup>4</sup>, Chunbo Shao<sup>5</sup>, Devanand Sarkar<sup>1,2,3</sup>, and Paul B. Fisher<sup>1,2,3</sup>

### Abstract

**Purpose:** Urothelial cell carcinoma (UCC) rapidly progresses from superficial to muscle-invasive tumors. The key molecules involved in metastatic progression and its early detection require clarification. The present study defines a seminal role of the metastasis-associated gene MDA-9/Syntenin in UCC progression.

**Experimental Design:** Expression pattern of MDA-9/Syntenin was examined in 44 primary UCC and the impact of its overexpression and knockdown was examined in multiple cells lines and key findings were validated in primary tumors.

**Results:** Significantly higher ( $P = 0.002-0.003$ ) expression of MDA-9/Syntenin was observed in 64% (28 of 44) of primary tumors and an association was evident with stage ( $P = 0.01$ ), grade ( $P = 0.03$ ), and invasion status ( $P = 0.02$ ). MDA-9/Syntenin overexpression in nontumorigenic HUC-1 cells increased proliferation ( $P = 0.0012$ ), invasion ( $P = 0.0001$ ), and EGF receptor (EGFR), AKT, phosphoinositide 3-kinase (PI3K), and c-Src expression. Alteration of  $\beta$ -catenin, E-cadherin, vimentin, claudin-1, ZO-1, and T-cell factor-4 (TCF4) expression was also observed. MDA-9/Syntenin knockdown in three UCC cell lines reversed phenotypic and molecular changes observed in the HUC-1 cells and reduced *in vivo* metastasis. Key molecular changes observed in the cell lines were confirmed in primary tumors. A physical interaction and colocalization of MDA-9/Syntenin and EGFR was evident in UCC cell lines and primary tumors. A logistic regression model analysis revealed a significant correlation between MDA-9/Syntenin:EGFR and MDA-9/Syntenin:AKT expressions with stage ( $P = 0.04$ , EGFR;  $P = 0.01$ , AKT). A correlation between MDA-9/Syntenin: $\beta$ -catenin coexpression with stage ( $P = 0.03$ ) and invasion ( $P = 0.04$ ) was also evident.

**Conclusions:** Our findings indicate that MDA-9/Syntenin might provide an attractive target for developing detection, monitoring, and therapeutic strategies for managing UCC. *Clin Cancer Res*; 19(17); 4621–33. ©2013 AACR.

### Introduction

Urothelial cell carcinoma (UCC) ranks ninth in worldwide cancer with cigarette smoking being the most important risk factor (1). It is more frequent in men than in women (1). In the United States, UCC is the fourth most commonly diagnosed cancer in men and eighth most

common cancer in women (2). It is estimated that 72,570 cases of UCC will be diagnosed and 15,210 deaths will occur in 2013 (3). Of all newly diagnosed cases of UCC, about 70% are superficial tumors (noninvasive; ref. 4). Approximately, 50% to 70% of those superficial tumors will recur and roughly 10% to 20% will progress to muscularis propria invasive (muscle-invasive) disease (4). Predicting progression from superficial to muscle-invasive disease and delineating approaches for early detection remain major challenges (4). Several genetic alterations including losses on chromosomal regions 3p, 5q, 10q, 11p, 13q, 14q, 17p, and 18q and activation of several proto-oncogenes, such as EGF receptor (EGFR), H-Ras, Bcl-2, mdm-2, FGFR3, and c-myc, have been implicated in UCC progression (5, 6). Epithelial-to-mesenchymal transition (EMT) has also been implicated in the development of muscle-invasive disease (6). However, the key molecules and genetic pathways controlling the development of invasive disease remain largely unknown.

MDA-9/Syntenin is a member of the family of PDZ domain-containing scaffold proteins displaying a diverse

**Authors' Affiliations:** Departments of <sup>1</sup>Human and Molecular Genetics, <sup>2</sup>VCU Institute of Molecular Medicine, <sup>3</sup>VCU Massey Cancer Center, <sup>4</sup>Department of Biostatistics, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298 USA, <sup>5</sup>Department of Otolaryngology, Johns Hopkins University, Baltimore, MD 21231

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**Corresponding Authors:** Paul B. Fisher, Virginia Commonwealth University, 1101 East Marshall Street Sanger Hall Building, Room 11-015, Richmond, VA 23298. Phone: 804-628-3506; Fax: 804-827-1124; E-mail: pbfisher@vcu.edu; and Santanu Dasgupta, Virginia Commonwealth University, 1220 E. Broad Street, MMRB Room # 7042, Richmond VA 23219. Phone: 804-628-3346, Fax: 804-827-1124; Email: sdasgupta@vcu.edu.

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### Translational Relevance

Urothelial cell carcinoma (UCC) is an unpredictable disease that rapidly progresses from superficial to muscle-invasive cancer, which poses significant obstacles to current treatment approaches. Identifying the key genes and relevant regulatory pathways leading to rapid spread of UCC is mandatory if one hopes to develop novel disease management strategies. The present study identifies MDA-9/Syntenin (human chromosome 8q12) as an abundantly overexpressed molecule from early stages of UCC development and associates with disease progression. MDA-9/Syntenin regulates UCC growth and metastatic progression in concert with various growth and epithelial-to-mesenchymal transition (EMT) regulatory molecules. This protein directly interacts with EGF receptor (EGFR), which is a therapeutic target for many solid tumors, in UCC cell lines and patient samples. MDA-9/Syntenin alone or in combination with EGFR may provide a viable target to develop novel therapeutics as well as monitoring strategies for UCC.

array of biologic functions, including binding to syndecans, clustering of membrane receptors, inducing pseudopodia formation, and intracellular trafficking (7–13). Overexpression of MDA-9/Syntenin occurs in multiple human cancer cell lines including melanoma, breast, and gastric cancer cells and is associated with increased cell migration and invasion (7–15). Cross-talk between MDA-9/Syntenin, c-Src, and NF- $\kappa$ B in mediating invasion and metastasis has also been shown (12). However, a comprehensive analysis of the expression pattern of MDA-9/Syntenin and its precise role in UCC progression remains to be determined.

In the present study, we examined the expression pattern of MDA-9/Syntenin in primary UCC tumors and examined the impact of its gain-of-function (GOF) and loss-of-function (LOF) in several UCC cell lines. Overexpression of MDA-9/Syntenin was evident in primary tumors and we show a direct relationship between MDA-9/Syntenin overexpression and UCC progression and elucidate the molecular mechanism underlying these changes. MDA-9/Syntenin could represent a useful target for diagnosis and potential therapy for UCC.

### Materials and Methods

#### Tissue specimens

Preconstructed tissue microarray slides containing primary tumors and corresponding adjacent normal tissues in duplicate from 44 patients with UCC were collected from commercial sources (US Biomax Inc.). All tumors were transitional cell carcinoma (TCC) and graded according to the current World Health Organization (WHO) system. The demographic data along with MDA-9/Syntenin alteration in expression patterns are represented in Supplementary Table S1.

#### Cell lines and reagents

Urothelial cell lines SV-HUC-1 (indicated as HUC-1), T24, SW780, and SCaBER were purchased from American Type Culture Collection (ATCC) and cultured in ATCC-recommended medium. HEK293 cells were also procured from ATCC and cultured in ATCC-recommended medium. The cell lines were periodically checked for *Mycoplasma* contamination (Sigma; # MP-0025). All tissue culture media and reagents were purchased either from ATCC or Invitrogen.

#### Immunohistochemistry

Immunohistochemistry (IHC) was conducted using specific antibody in paired primary UCC specimens of different stages as described earlier (16). Each experiment was repeated at least two times for counting positively stained cells and intensity measurements using MetaMorph software (Universal Imaging) were conducted. At least 10 fields were chosen at random for counting and data were expressed as mean  $\pm$  SE. For intensity measurements, the lowest value ( $\leq 50.00$ ) was represented by a single "+" sign and each fold increase was represented by an additional "+" sign (16). MDA-9/Syntenin antibody was a mouse monoclonal antibody obtained from Abnova Corporation (H00006386-MOI; dilution 1:200) and detects a single band at 33 kDa. The EGFR (#4267, clone D38B1, rabbit monoclonal), AKT (#4691, clone C67E7, rabbit monoclonal), AKT-S473 (#4060, clone D9E, rabbit monoclonal), E-cadherin (#3195, clone 24E10, rabbit monoclonal), and CTNNB1 (#9582, clone 6B3, rabbit monoclonal) antibodies were obtained from Cell Signaling Technology, Inc. (dilution, 1:200) and C-Src-Y418 (#44660G, rabbit polyclonal; dilution, 1:200) from Invitrogen. Anti-mouse (#115-065-003) and anti-rabbit (#111-065-003) secondary antibodies were obtained from Jackson ImmunoResearch (dilution, 1:1,000).

#### Plasmid constructions and transfections

The wild-type (wt) MDA-9/Syntenin cDNA (Gene ID: 6386) was subcloned into *Sall* and *NotI* sites of the phosphorylated cytomegalovirus pCMV/myc/cyto plasmid, which has an N-terminal myc tag (Invitrogen). The resultant plasmid was resequenced using the ABI BigDye Cycle Sequencing Kit (Applied Biosystems) for verification of the insert sequence. SV-HUC-1 cells were transfected with MDA-9/Syntenin expression plasmid using FuGene 6 transfection reagent (Roche Diagnostics). An empty vector was used as control. Transfected cells were selected in 300  $\mu$ g/ml of G418. The expression of c-myc-tagged MDA-9/Syntenin fusion protein was confirmed by Western blot or immunofluorescence analysis using the anti-c-myc antibody (Invitrogen) in the stable clones. For knockdown experiments, MDA-9/Syntenin-specific short hairpin RNA (shRNA) was constructed and cloned into the *BamI*–*HindIII* sites of pRNA3.1 vector (GeneScript Corporation). The sequence of the shRNA is GGATCCGCCCTAATGGACCACACCATTCAAGAGAATGGTGTGGTCCATTAGGCC GAA-GCTT. Stable clones of T24, SCaBER, and SW780 cells with MDA-9/Syntenin knockdown were generated using

the above plasmid. Transfected cells were selected in 200 µg/ml of G418. Western blot analysis determined the level of knockdown. We have also constructed full-length MDA9/Syntenin with a C-terminal hemagglutinin (HA) tag into pcDNA3.1/Hygro (+; Invitrogen) between *Bam*HI and *Xho*I, after PCR using the following primers: sense 5'-CGCGGATCCGCCACCATGTCTCTCTATCCATCTCTC-3' and antisense 5'-CCGCTCGAGCGGTTAAGCGTAATCTGGAACATCGTATGGGTAACCTCAGGAATGGTGTG-3' (Supplementary Fig. S4). Another deletion construct containing only PDZ1-PDZ2 domains (Supplementary Fig. S4) was also constructed in the same vector as above after PCR using the sense 5'-CGCGGATCCGCCACCATGTCTCTCTATCCATCTCTC-3' and antisense 5'-CCGCTCGAGCGGTTAAGCGTAATCTGGAACATCGTATGGGTAACCTGTCACGAATGGTCATG-3' primers. All the constructs generated were verified by sequencing. In transfection, HEK293 cells were transfected with the HA-tagged plasmids. Pooled clones selected in the presence of 50 µg/ml of hygromycin after 1 week were further analyzed by Western blot analysis for the detection of HA (Sigma; dilution, 1:1,000).

#### Cell proliferation assay

Proliferation of the transfected cells plated in triplicate wells was determined by a bromodeoxyuridine (BrdUrd) incorporation assay kit (Roche Diagnostics) as per the manufacturer's protocol. Data are presented as mean ± SE of duplicate experiments.

#### Dual-color FISH

Dual-color FISH was conducted on formalin-fixed paraffin-embedded (FFPE) sections obtained from 44 patients with UCC. All tumors had corresponding normal tissue. Following deparaffinization, pretreatment consisted of 10 minutes steam cooking in 10 mmol/L citrate acid solution followed by pepsin (4 mg/mL) digestion at 45°C for 30 minutes. Bacterial artificial chromosome (BAC)-derived test probe targeting *mda-9/syntenin* (8q12, RP11-23K11; BACPAC Resources Center, Oakland CA) was labeled with Spectrum Orange; this was paired for dual-target hybridization diluted at 1:50 in DenHyb hybridization buffer (Insitus Laboratories) with control probe CEP8 (probe targeting centromeric region of chromosome 8; Abbott Laboratories). The centromeric probe (CEP) probe provided enumeration of chromosome copy number for chromosome 8. Ten milliliter of the hybridization mix was applied to the sections, with simultaneous denaturing of probe and target at 80°C for 2 minutes, and 50°C for 45 minutes. Overnight hybridization at 37°C occurred in a humidified chamber and post-hybridization washes included 50% formamide/1 × saline-sodium citrate (SSC) (5 minutes) and 2 × SSC (5 minutes). 4',6-Diamidino-2-phenylindole (DAPI; 0.125 ng/mL; Abbott Laboratories) served as a nuclear counterstain. Sections showing sufficient hybridization efficiency (majority of nuclei with signals) were considered informative and were scored. Non-neoplastic brain specimens served as the controls. Specimens were considered ampli-

fied for *mda-9/syntenin* when they showed nuclei containing numerous red test probe signals with a test probe: control (CEP) probe ratio more than 2. Cases showing an increased number of test to control probe signals, but in a ratio of more than 1.2 and 2 or less were scored as a low-level gain for that respective test probe. Cases, in which both the test and control probes were equally increased in number, were considered to show polysomy for chromosome 8.

#### Cell invasion assay

Cell invasion capacity was assessed using the Cell Invasion Assay Kit (BD Biosciences; ref. 17). The assay was conducted in triplicate. Briefly,  $1 \times 10^4$  cells in 500 µL of serum-free medium were plated in triplicate in the invasion chambers (24-well format). The bottom wells of the 24-well plate containing the individual chamber were supplemented with 750 µL of serum-containing medium. Plates were incubated for 24 hours in a tissue culture incubator followed by fixation in methanol for 5 minutes and staining with 0.5% crystal violet. At least 10 fields were randomly selected for counting the cells that invaded through the membrane from each group. Data were presented as mean ± SE of duplicate experiments.

#### Immunofluorescence

Cells were cultured in tissue culture-treated chamber slides and fixed in 4% paraformaldehyde as described previously (18) and stained with the EGFR (Cell Signaling Technology; # 4267, 1:200 dilution) overnight at 4°C followed by staining with fluorescein isothiocyanate (FITC) or Texas red-tagged secondary antibody (Jackson ImmunoResearch; 1:1,000) for 1 hour at room temperature. Cells were then washed thoroughly with PBS and mounted with Prolong Gold antifade reagent (Invitrogen) and observed immediately under a fluorescent microscope. At least 10 fields were randomly selected for examining the staining intensity and distribution pattern of the proteins.

#### Western blot and coimmunoprecipitation analysis

Preparation of whole-cell lysates and Western blot analysis was conducted following standard protocols as described earlier (19). The coimmunoprecipitation (Co-IP) analysis was conducted using Dynabead Protein G kit (#100.07D) and DynaMag-2 system (# 123.21D) and protocols from Invitrogen. Antibodies used for Co-IP analysis are described earlier. We obtained whole-cell lysates of primary UCC tumors and corresponding normal tissues from Protein Biotechnologies, Inc. (<http://www.protein-biotechnologies.com/>).

#### Colony focus formation assay

Stably transfected cells were plated in duplicate at low density for determining formation of single cell colonies in 10-cm plates. Cells were cultured in the presence of appropriate complete medium containing 200 µg/ml of G418 for 3 weeks. Plates were then stained with 5% crystal

violet, photographed, and colonies were counted from at least 10 randomly selected fields. Data represent the mean  $\pm$  SE of duplicate experiments.

### ***In vivo* metastasis analysis**

For tumor growth,  $1 \times 10^5$  cells in 100  $\mu$ L PBS were injected through the intracardiac route into athymic, 4- to 6-week-old female nude mice (Charles River Laboratories). All experiments were carried out in accordance with the Animal Care and Use Committee guidelines of the Virginia Commonwealth University (VCU; Richmond, VA). Each group consisted of 6 mice and each experiment was repeated two times. Mice were examined every day and mice showing signs of morbidity were immediately sacrificed according to the University guidelines. After 12 weeks, mice were sacrificed and lungs were removed. Focal tumor lesions in the lungs were counted in each mouse and FFPE sections were prepared and stained with standard hematoxylin and eosin (H&E) method for visualization of focal metastases and immunohistochemical analysis. Data were presented as mean  $\pm$  SE of duplicate experiments.

### **MDA-9/Syntenin promoter methylation analysis**

Twelve paired (matched normal and tumor) UCC samples showing overexpression of MDA-9/Syntenin were analyzed. For bisulfite treatment and genomic sequencing, EpiTect Bisulfite kit (Qiagen) was used according to the manufacturer's instructions. To span CpG islands in the promoter region of MDA-9/Syntenin, specific sets of primers were designed by MethPrimer (20). The primer sequences did not contain any CG dinucleotides. Methprimer's default criteria used for CpG islands were: (i) an observed/expected CpG ratio more than 0.6, (ii) the percentage of G plus C more than 50%, and (iii) a window size of at least 100 bp. Using these criteria, clustered CpG islands within  $-315$  to  $+621$  nucleotide (nt) region relative to the transcription start site of MDA-9/Syntenin were identified. We designed two sets of nonoverlapping bisulfite sequencing primers within these clustered CpG islands region. The first primer set includes forward 5'-GTATATAGTAGT-GAGTGGGTGGTA-3' and reverse 5'-TAACCTCTCACC-TAACCCACAAA-3'; the second primer set includes forward 5'-TTTGTGGGTAGGTGAGAGGTTA-3' and reverse 5'-AACCAAAAACCTCTTTCTTATATC-3'. Bisulfite-treated DNA was analyzed by touch-down PCR using the following conditions: 95°C for 5 minutes, followed by cycles consisting of a 30-second denaturation step at 95°C, holding at an annealing temperature for 30 seconds, extension at 72°C for 1 minute, with a final extension at 72°C for 5 minutes. The annealing temperature was then gradually decreased as two cycles at 64°C, 62°C, 60°C, 58°C and then 35 cycles at 56°C. The PCR products were purified using the QIAquick 96 PCR Purification Kit (Qiagen), according to the manufacturer's instructions. Purified PCR products were then subjected to direct sequencing (Genewiz Inc.) for the detection of the methylation pattern.

### **Statistical analysis**

Chi-square, Fisher exact, or Student *t* test tests were used when appropriate. Ordinal logistic regression analysis was also conducted to determine the impact of various gene signature alterations alone and in various combinations. All *P* values were two-sided and all confidence intervals were at the 95% level. Computation for all the analysis was conducted using the Statistical Analysis System (SAS).

## **Results**

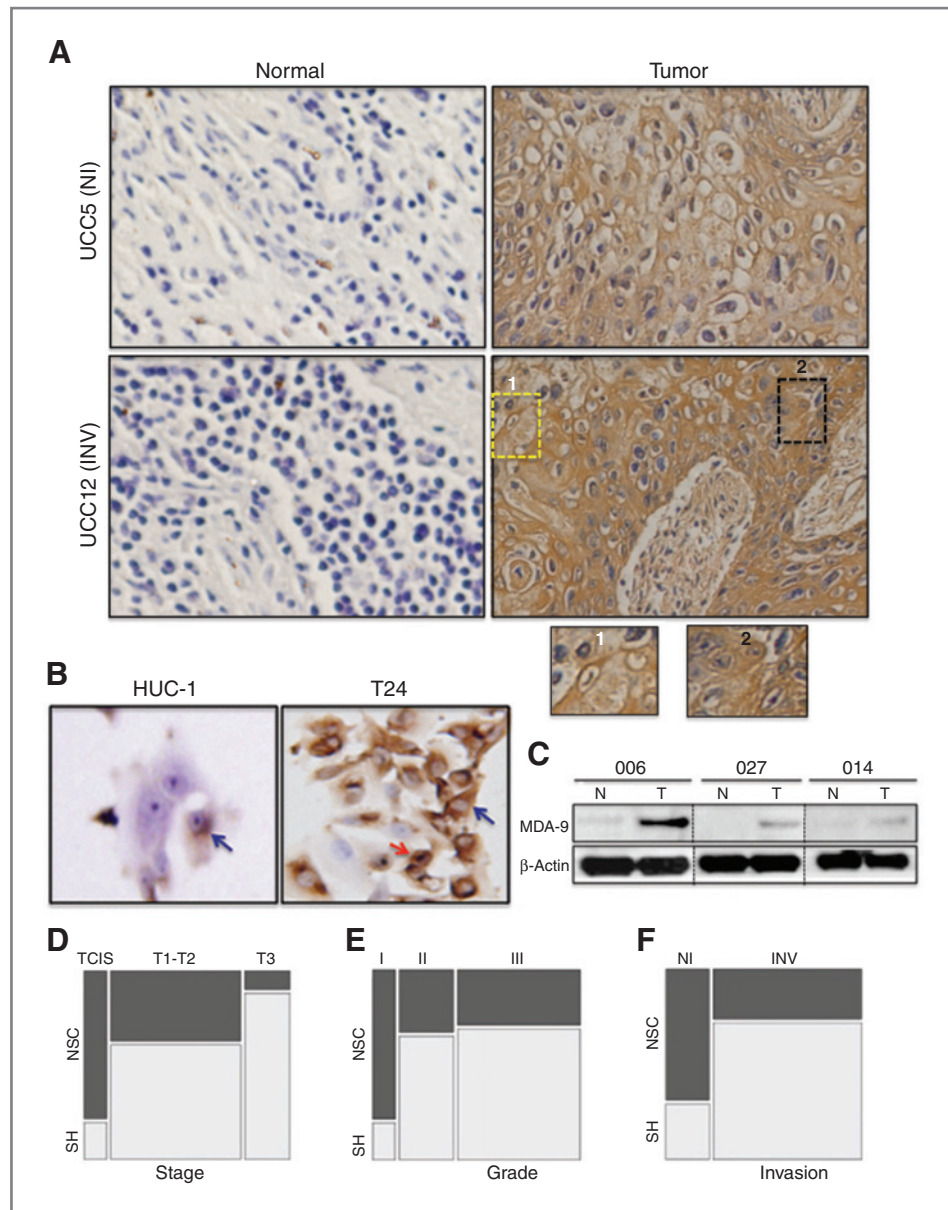
### **MDA-9/Syntenin expression is elevated in UCC and correlates with disease progression**

The expression pattern of MDA-9/Syntenin was examined in 44 primary UCC tumors containing both noninvasive and invasive counterparts. All cases were TCC and had corresponding adjacent normal tissue. Immunohistochemical analysis confirmed significantly higher ( $P = 0.002-0.003$ ) expression of MDA-9/Syntenin in 64% (28 of 44) of the primary tumors (Supplementary Table S1 and Fig. 1A). The noncancer normal individual with reactive hyperplasia (UCC34) did not show increased expression of MDA-9/Syntenin (Supplementary Table S1). Membranous or cytoplasmic expression of MDA-9/Syntenin was evident in both noninvasive and invasive tumors (Fig. 1A, box 1 and inset 1). MDA-9/Syntenin expression was also noticeable in the nucleus of approximately 5% of the majority of the invasive primary tumors (Fig. 1A, box 2 and inset 2). IHC of uroepithelial cell lines also revealed high level of membranous (blue arrow) and nuclear expression (red arrow) of MDA-9/Syntenin in tumorigenic T24 cells compared with a low level or undetectable level of membranous MDA-9/Syntenin expression (blue arrow) in nontumorigenic HUC-1 cells (Fig. 1B). Tumor-specific elevated expression of MDA-9/Syntenin was also confirmed by Western blot analysis in three primary UCC tumors (Fig. 1C). We further compared the expression pattern of MDA-9/Syntenin with different clinical parameters. MDA-9/Syntenin expression was higher in each stage such as TCC *in situ* (TCIS), T1-T2, and T3 compared with their corresponding normal ( $P = 0.002-0.003$ ) and 20% (1 of 5) of TCIS, 62% (18 of 29) of T1-T2, and 90% (9 of 10) of the T3 staged tumors had higher expression of MDA-9/Syntenin. When compared across the stages, grades, and with invasion status, MDA-9/Syntenin expression was significantly associated with progressive stages ( $P = 0.01$ ; Fig. 1D), grades ( $P = 0.03$ ; Fig. 1E), and invasion ( $P = 0.02$ ; Fig. 1F; Supplementary Table S1).

### **MDA-9/Syntenin enhances proliferation and invasion of nontumorigenic uroepithelial HUC-1 cells**

Elevated expression of MDA-9/Syntenin in invasive tumors and its direct association with disease progression suggests a potential role of this gene in invasive progression of UCC. To understand its precise role in UCC proliferation and progression, we stably overexpressed MDA-9/Syntenin in a nontumorigenic uroepithelial HUC-1 cell line, which

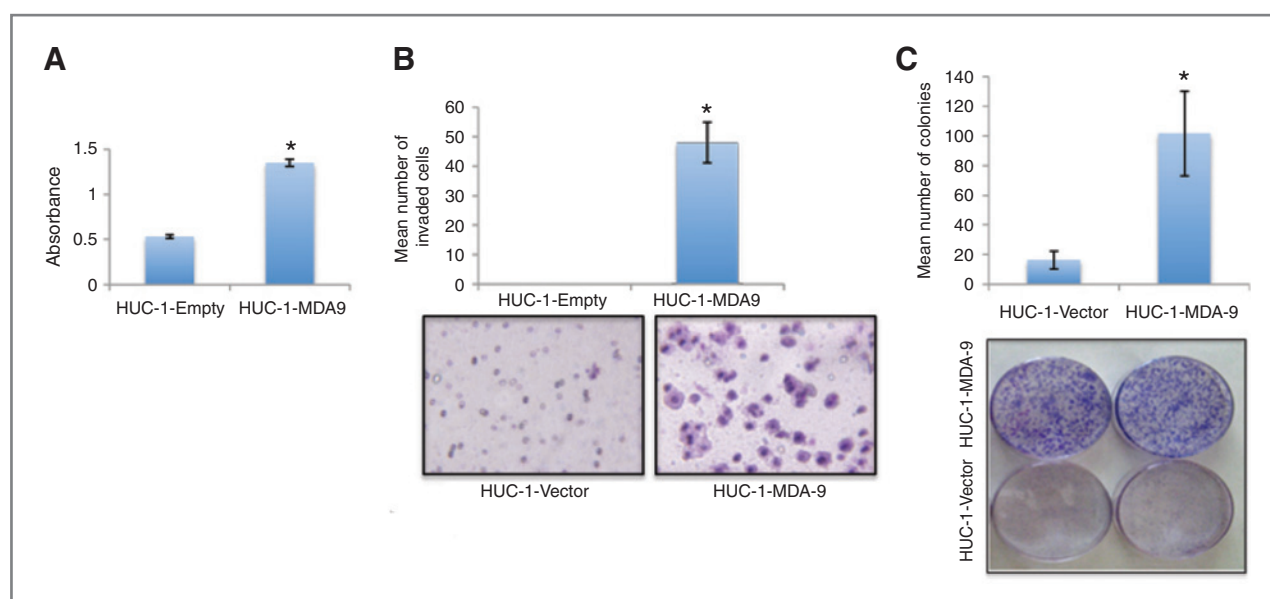
**Figure 1.** Expression pattern of MDA-9/Syntenin in primary UCC tumors and its association with UCC progression. **A**, MDA-9/Syntenin expression was significantly higher ( $P = 0.002$ – $0.003$ ) in primary tumors and was visible in the membrane (box 1 and inset 1) and nucleus (box 2 and inset 2). **B**, markedly elevated levels of MDA-9/Syntenin expression were evident in the membrane (blue arrow) and nucleus (red arrow) in the tumorigenic T24 cells compared with the low level of membranous MDA-9/Syntenin expression in the nontumorigenic HUC-1 cells. **C**, elevated tumor-specific expression of MDA-9/Syntenin confirmed by Western blot analysis in three primary urothelial cell tumors. All cases were TCCs. The expression of MDA-9/Syntenin significantly correlated with stage ( $P = 0.01$ ; **D**), grade ( $P = 0.03$ ; **E**), and invasion status ( $P = 0.02$ ; **F**) of the disease. N, normal; T, corresponding tumor; NI, noninvasive carcinoma; INV, invasive carcinoma; SH, significantly higher; NSC, no significant changes. In all cases, 70% or more tumor cells were positive for MDA-9 expression compared with 10% to 20% positive cells in the corresponding normal. The mean intensity value in tumors (range, 107–213) and corresponding normal (range, 0–50) was represented by single or multiple "+" signs. Representative field values were then compared between matched normal and tumor by student *t* test to infer significance.



expresses low levels of endogenous MDA-9/Syntenin protein (Fig. 1B and Supplementary Fig. S1A). Western blot analysis detected the c-myc-tagged MDA-9/Syntenin protein in the transfected cells (Supplementary Fig. S1B). Because both clones displayed comparatively similar patterns of changes, we carried out all subsequent studies with clone number 7. A significant increase was found in proliferation ( $P = 0.0012$ ; Fig. 2A), invasion ( $P = 0.0001$ ; Fig. 2B), and colony focus formation ( $P = 0.0002$ ; Fig. 2C) in the MDA-9/Syntenin-overexpressed cells compared with empty vector-transfected cells.

In addition to *EGFR* overexpression, EMT was implicated in UCC progression to muscle-invasive disease (6). Because MDA-9/Syntenin overexpression was associated with inva-

sive UCC progression and resulted in increased proliferation and invasion of HUC-1 cells, we examined the expression pattern of *EGFR*, its associated signaling and EMT molecules by Western blot analysis. As shown in Fig. 3A, we observed pronounced upregulation of activated *EGFR* (*Tyr*<sup>845</sup>), *AKT* (*Ser*<sup>473</sup>), *c-Src* (*Tyr*<sup>418</sup>), and *PI3K* (p101) in the MDA-9/Syntenin-transfected cells compared with empty vector-transfected cells. Among the various EMT molecules examined, we observed marked upregulation of  $\beta$ -catenin (*Tyr*<sup>333</sup>), vimentin, and *TCF4* (Fig. 3B). In contrast, we observed discernible downregulation of ZO-1, claudin-1, and E-cadherin in the MDA-9/Syntenin-transfected cells (Fig. 3B). We also observed morphologic changes corresponding to a mesenchymal phenotype in



**Figure 2.** Effect of MDA-9/Syntenin overexpression on nontumorigenic uroepithelial HUC-1 cells. Overexpression of MDA-9/Syntenin in HUC-1 cells results in a significant increase in proliferation ( $P = 0.0012$ ; A), invasion ( $P = 0.0001$ ; B), and colony focus formation ( $P = 0.0002$ ; C) as compared with the empty vector-treated group. Representative photomicrographs showing invasion and colony focus formation, respectively, are shown in (B) and (C). \* indicates level of significance.

the nontumorigenic uroepithelial cells overexpressing MDA-9/Syntenin (Fig. 3C). Thus, forced overexpression of MDA-9/Syntenin significantly increases cellular growth, invasion, and EMT that is accompanied with alterations of key EGFR-signaling and EMT-associated molecules.

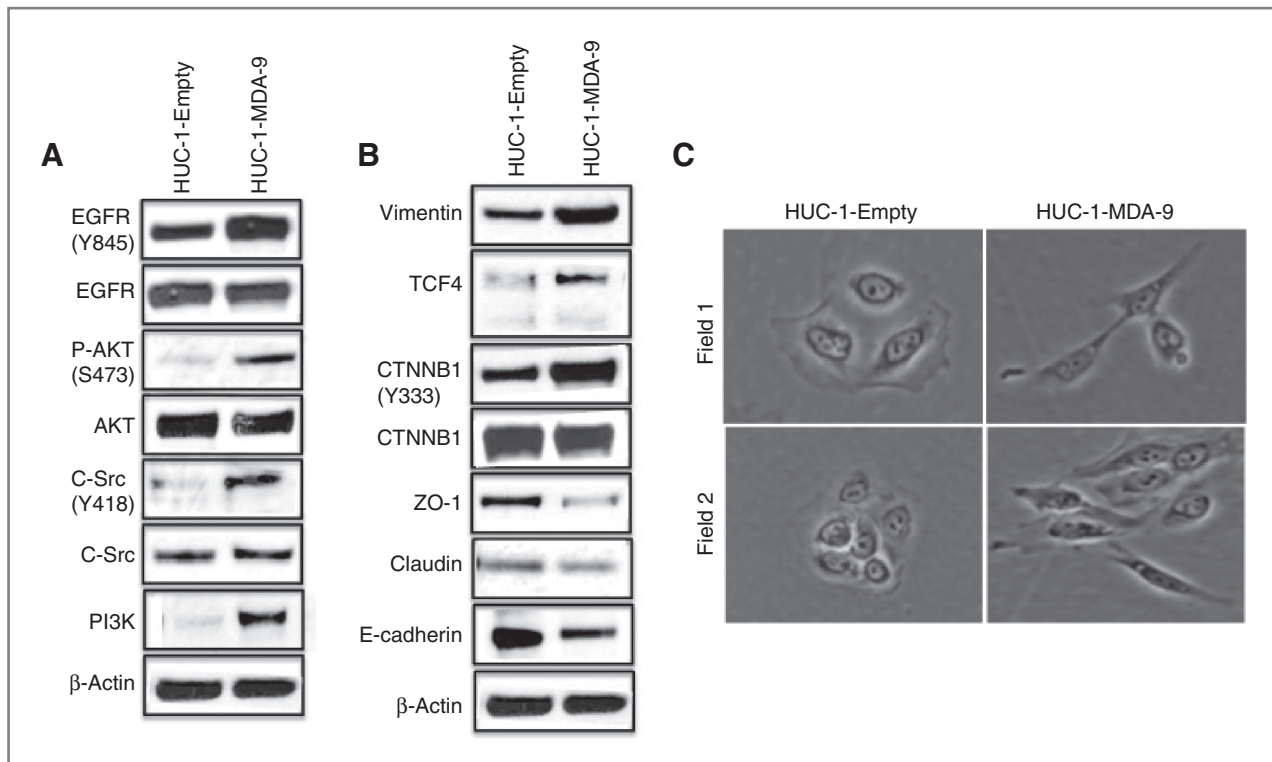
#### Knockdown of MDA-9/Syntenin inhibits cellular growth and invasion by inhibiting EGFR-signaling and key EMT-associated molecules

Because forced overexpression of MDA-9/Syntenin significantly modified cellular growth and altered expression of EGFR- and specific EMT-associated molecules, we examined the impact of knockdown of MDA-9/Syntenin in three UCC TCC cell lines, T24, SCaBER, and SW780. Transfected cells displayed 70% to 80% stable knockdown of MDA-9/Syntenin (Fig. 4A, left) and marked transition from a mesenchymal-to-epithelial phenotype in MDA-9/Syntenin-depleted T24 cells (Fig. 4A, right). Because comparable patterns of changes were observed in different clones, a single clone was selected from each UCC TCC cell line for all subsequent analyses. In the MDA-9/Syntenin knockdown clones from the three UCC TCC cell lines, there was significant inhibition of cellular proliferation (Fig. 4B;  $P = 0.002$ – $0.003$ ) and invasion (Fig. 4C;  $P = 0.001$ – $0.002$ ). Marked downregulation of activated EGFR (Tyr<sup>845</sup>) and its downstream and interacting signaling molecules AKT (Ser<sup>473</sup>), PI3K (p101), and *c-Src* (Tyr<sup>418</sup>; Fig. 4D) were also evident in these clones. In the MDA-9/Syntenin-depleted T24 cells, we observed cytoplasmic/perinuclear localization of EGFR and low level of activated C-Src (Supplementary Fig. S2, white arrows) compared with the control T24 cells exhibiting predominantly membranous and cytoplasmic

localization (Supplementary Fig. S2, yellow arrows). Notably, the membranous expression level of both of these molecules was comparatively low in the MDA-9/Syntenin-depleted cells compared with the control cells (Supplementary Fig. S2, yellow arrows). Notably, a decreased level of total AKT expression was also evident in the MDA-9/Syntenin knockdown T24 cells (Fig. 4D). Of the various EMT molecules analyzed, we observed pronounced downregulation of  $\beta$ -catenin (CTNNB1; Tyr<sup>333</sup>) in all three MDA-9/Syntenin knockdown UCC cell lines (Fig. 4E). Significant downregulation of N-cadherin, Slug, and Snail with increased expression of ZO-1, TCF4, claudin-1, and E-cadherin was observed in all but one of the UCC clones (Fig. 4E). Accordingly, in general, a similar pattern of molecular changes, except for T-cell factor-4 (TCF4), was observed following overexpression or knockdown of MDA-9/Syntenin in the UCC cells.

#### MDA-9/Syntenin interacts and colocalizes with EGFR in UCC cells

Because overexpression or knockdown of MDA-9/Syntenin significantly affected cellular proliferation and EGFR signaling in UCC cells, we examined whether EGFR- and MDA-9/Syntenin colocalize or physically interact. Immunofluorescence analysis was conducted using both EGFR- and MDA-9/Syntenin-specific antibodies. Both membranous and cytoplasmic colocalization of EGFR and MDA-9/Syntenin were detected in the T24 cell line (Fig. 5A, top and Supplementary Fig. S3). We also confirmed colocalization of MDA-9/Syntenin and EGFR in HEK-293 cells transfected with HA-tagged



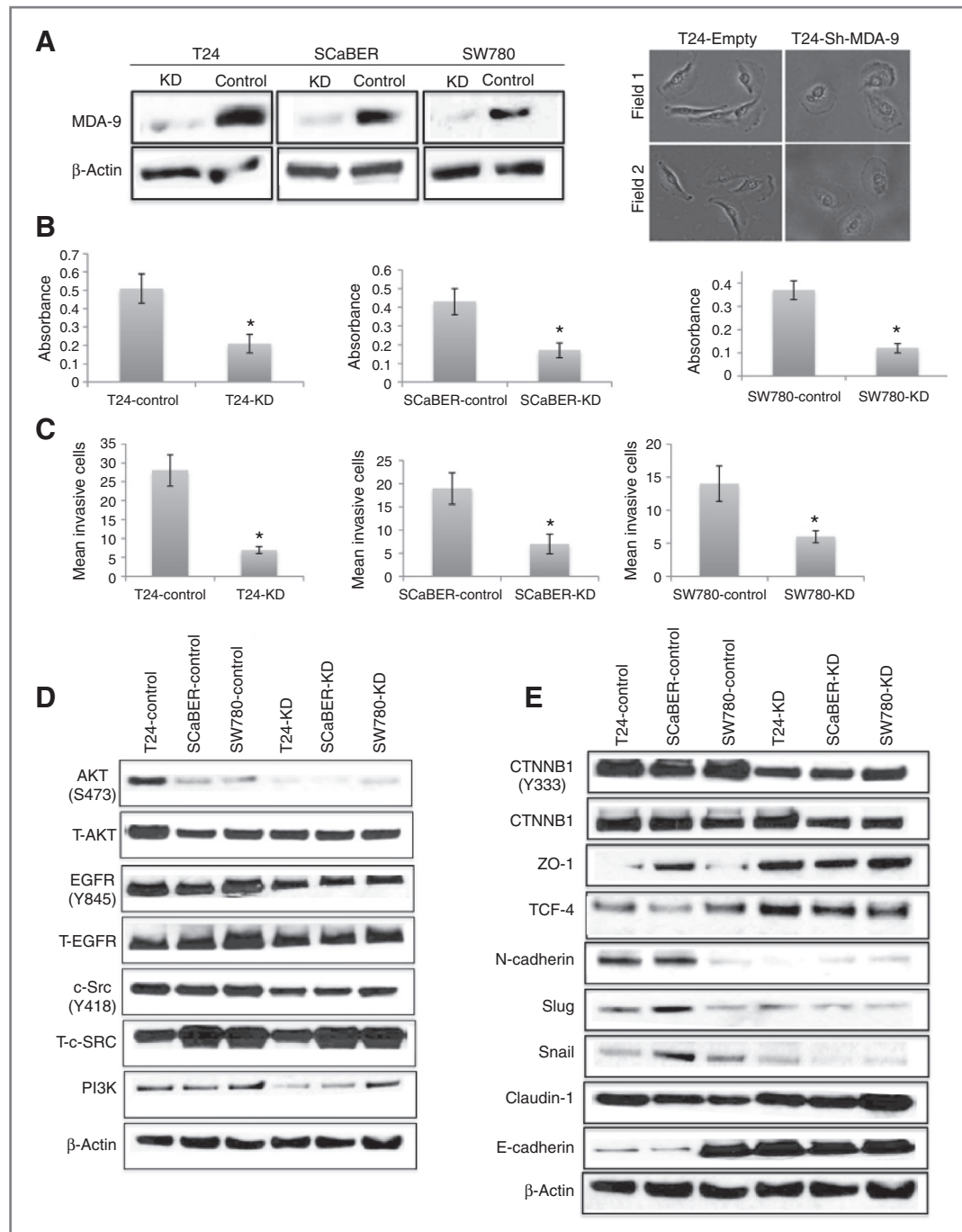
**Figure 3.** Marked upregulation of activated EGFR (Tyr<sup>845</sup>), AKT (Ser<sup>473</sup>), c-Src (Tyr<sup>418</sup>), and PI3K in the wt-MDA-9/Syntenin-transfected cells compared with the empty vector-treated cells (A). Discernible upregulation of  $\beta$ -catenin (CTNNB1; Tyr<sup>333</sup>), vimentin, and *TCF4* was observed in the wt-MDA-9/Syntenin-transfected cells (B). Reduced expression of ZO-1, claudin-1, and E-cadherin was also apparent in the wt-MDA-9/Syntenin-transfected cells (B). HUC-1-Empty: empty vector transfected; HUC-1-MDA-9: wt-MDA-9/Syntenin transfected HUC-1 cells.  $\beta$ -Actin was used as a loading control. Morphologic changes corresponding to EMT phenotype in MDA-9/Syntenin-overexpressing HUC-1 cells at two different microscopic fields. HUC-1-Empty, empty vector transfected; HUC-1-MDA-9, MDA-9 transfected. Magnification,  $\times 200$ .

MDA-9/Syntenin (Fig. 5A, bottom). In parallel, we conducted Co-IP analysis to determine whether MDA-9/Syntenin could pull down EGFR and *vice versa*. As shown in Fig. 5B, MDA-9/Syntenin or EGFR interacted in all the three UCC cell lines. MDA-9/Syntenin could also pull down EGFR from three primary UCC tumors (Fig. 5C) showing elevated levels of MDA-9/Syntenin (Fig. 1C). We further used a HA-tagged wt MDA-9/Syntenin construct and confirmed MDA-9/Syntenin and EGFR interaction in HEK-293 cells (Fig. 5D). MDA-9/Syntenin is known to interact through the PDZ domains (12). To identify the potential binding site(s) of MDA-9/Syntenin necessary to interact with EGFR, we used HA-tagged wt MDA-9/Syntenin and a deletion mutation construct with intact PDZ1/PDZ2 domains but lacking both N- and C-terminal domains (Supplementary Fig. S4). However, MDA-9/Syntenin lacking both N- and C-terminal domains (17 kDa) could not pull down EGFR, suggesting that N- or C-terminal domains of MDA-9/Syntenin contain potential binding sites for EGFR (Fig. 5E). To further show MDA-9/Syntenin and EGFR interactions, we conducted Co-IP and Western blot analysis with both MDA-9/Syntenin and EGFR antibodies in the HEK-293 cells overexpressing wt MDA-9/Syntenin. The C-terminal HA tag was evident in these cells (Fig. 5D). Because

MDA-9/Syntenin antibody uses a C-terminal epitope, the overexpression of MDA-9/Syntenin was also detectable using the MDA-9/Syntenin antibody (Fig. 5F, lane 1). A concomitant and appreciable increase in EGFR expression was also evident in these cells compared with the control cells (lane 2).

#### Coexpression signature of MDA-9/Syntenin, EGFR, AKT, $\beta$ -catenin, E-cadherin, and c-Src in primary UCC tumors

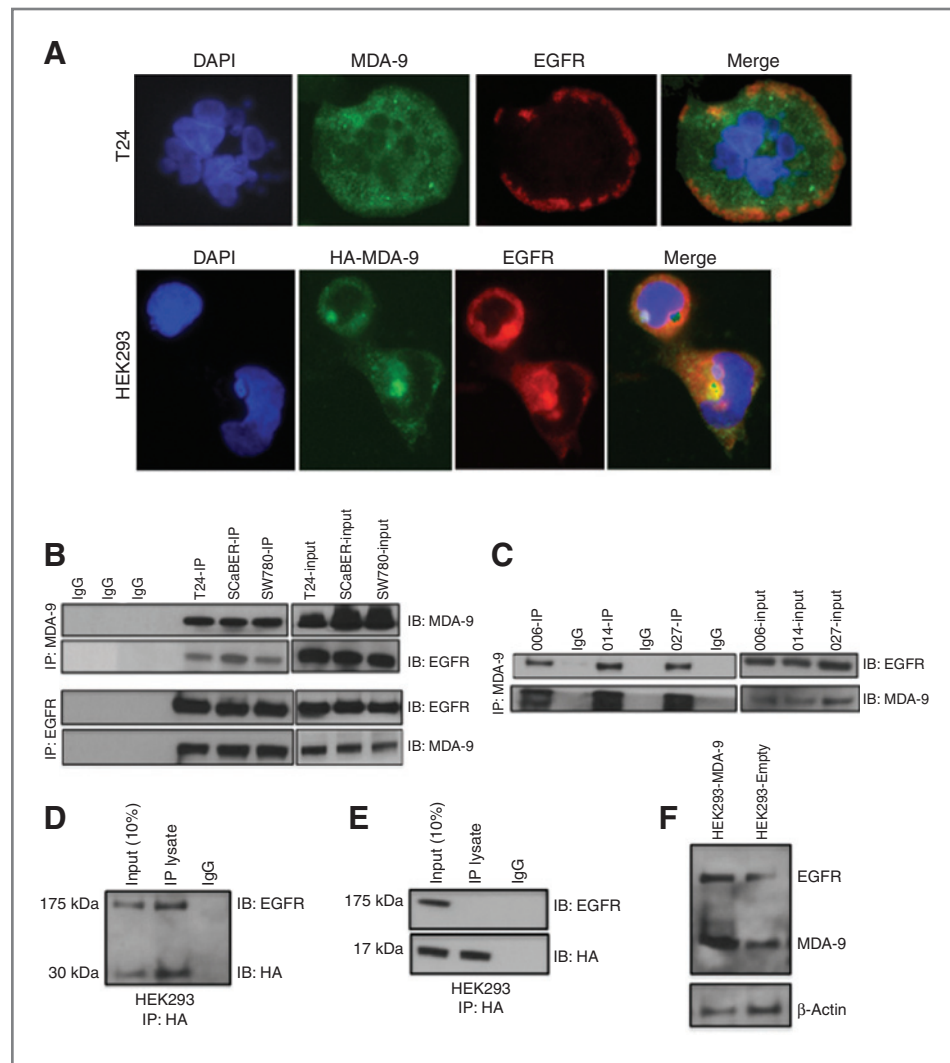
To determine whether the molecular changes observed in the cell lines was recapitulated in human samples, we conducted immunohistochemical analysis with EGFR,  $\beta$ -catenin (CTNNB1; Tyr<sup>333</sup>), AKT (Ser<sup>473</sup>), c-Src (Tyr<sup>418</sup>), and E-cadherin antibodies in the same cohort of 44 primary tumors, described earlier. Analogous to the cell lines, we observed a significantly higher expression of EGFR ( $P = 0.004$ ) in 41% (18 of 44) of the primary UCC tumors (Supplementary Fig. S5 and Supplementary Tables S2 and S3). Significantly higher coexpression of EGFR and MDA-9/Syntenin was observed in 72% (13 of 18) of these primary tumors (Supplementary Tables S2 and S3 and Supplementary Fig. S5). Significantly higher expression of  $\beta$ -catenin (CTNNB1; Tyr<sup>333</sup>;  $P = 0.002$ ), AKT (Ser<sup>473</sup>;  $P = 0.001$ ), and c-Src (Tyr<sup>418</sup>;  $P = 0.002$ ) and lower expression of E-cadherin



**Figure 4.** Impact of knockdown (KD) of MDA-9/Syntenin in UCC cells. **A**, effective knockdown of MDA-9/Syntenin in UCC cells (left). We observed 70% to 80% knockdown of MDA-9/Syntenin in the UCC cell lines. β-Actin was used as a loading control. KD: Sh-MDA-9/Syntenin transfected; Control: control shRNA transfected; right, mesenchymal-to-epithelial transition in MDA-9 knockdown T24 cells at two different fields. T24-Empty, empty vector transfected; T24-ShMDA-9, shMDA-9 transfected. Magnification, ×200. **B** and **C**, effect of knockdown of MDA-9/Syntenin on proliferation and invasion. Significant inhibition of cellular proliferation ( $P = 0.002-0.003$ ) and invasion ( $P = 0.001-0.002$ ) was observed in the MDA-9/Syntenin knockdown cells compared with the empty vector-transfected cells, respectively. **C**, control, control shRNA transfected; knockdown, shMDA-9 transfected. **D**, marked downregulation of activated EGFR (Tyr<sup>845</sup>) and its downstream and interacting signaling molecules including AKT (Ser<sup>473</sup>), c-Src (Tyr<sup>418</sup>), and PI3K (p101; left) following knockdown of MDA-9/Syntenin in UCC cells. **E**, reduced expression of β-catenin (Tyr<sup>333</sup>) was evident in all MDA-9/Syntenin knockdown UCC cells and increased expression of ZO-1 and TCF4 was also evident in all but one MDA-9/Syntenin knockdown cell line compared with control cells (right). Low expression of N-cadherin, Slug, and Snail was also evident in all but one MDA-9/Syntenin knockdown UCC cell line. Markedly higher expression of claudin-1 was observed in all cell lines except for T24 cells. Markedly higher expression of E-cadherin was observed in all cell lines except for SW780. β-Actin was used as a loading control. \* indicates level of significance.



**Figure 5.** Colocalization and interaction of MDA-9/Syntenin with EGFR in UCC cells. **A**, immunofluorescence analysis using EGFR- and MDA-9/Syntenin-specific antibodies showing colocalization of both EGFR and MDA-9/Syntenin in the membrane of T24 cells (top). Colocalization of MDA-9/Syntenin and EGFR was also confirmed in HEK-293 cells transfected with HA-tagged MDA-9/Syntenin (bottom). **B**, reciprocal interaction between MDA-9/Syntenin and EGFR in the three UCC cell lines was detected as shown using either MDA-9/Syntenin (top) or EGFR (bottom) antibody. Whole-cell lysates were immunoprecipitated and immunoblotted with anti-MDA-9/Syntenin or EGFR antibody. **C**, MDA-9/Syntenin pulls down EGFR in three primary UCC tumors showing high levels of MDA-9/Syntenin expression. **D**, confirmation of MDA-9/Syntenin and EGFR interaction in HEK293 cells transfected with HA-tagged MDA-9/Syntenin. Anti-HA antibody successfully pulled down EGFR. **E**, HA-tagged MDA-9/Syntenin lacking both N- and C-terminal domains (17 kDa) fails to immunoprecipitate EGFR in HEK293 cells. **F**, MDA-9/Syntenin overexpression in HEK-293 cells resulted in an marked increase in EGFR expression (lane 1) compared with the control (empty) cells (lane 2). IgG, immunoglobulin G.



( $P = 0.006$ ) was also evident in 43% (19 of 44), 48% (21 of 44), 48% (21 of 44), and 50% (22 of 44) of primary tumors, respectively, compared with their corresponding normal counterparts (Supplementary Tables S2 and S3 and Supplementary Fig. S5). Altered coexpression of  $\beta$ -catenin-Tyr<sup>333</sup>:MDA-9/Syntenin, AKT-Ser<sup>473</sup>:MDA-9/Syntenin, c-Src-Tyr<sup>418</sup>:MDA-9/Syntenin, and E-cadherin:MDA-9/Syntenin was observed in 84% (16 of 19), 62% (13 of 21), 90% (19 of 21), and 77% (17 of 22) of cases, respectively (Supplementary Table S2).

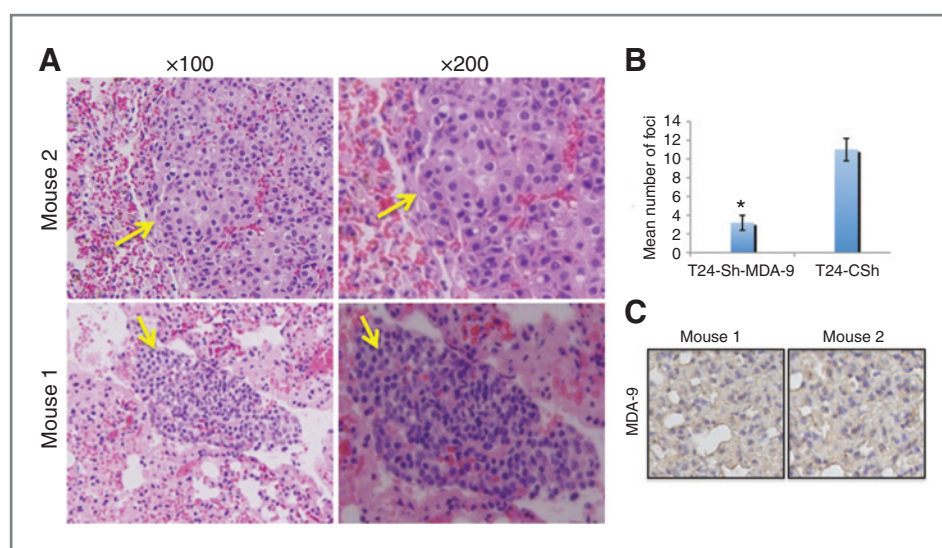
#### A two-gene expression signature is associated with UCC progression

We observed alterations of some key regulatory molecules, such as EGFR,  $\beta$ -catenin (CTNNB1; Tyr<sup>333</sup>), AKT (Ser<sup>473</sup>), c-Src (Tyr<sup>418</sup>), and E-cadherin, following GOF/LOF analysis in the UCC cell lines that was recapitulated in the primary tumors. To determine whether their alteration in concert with MDA-9/Syntenin was associated with disease progression, we conducted a logistic regression model

analysis using these alterations alone and in various combinations with MDA-9/Syntenin overexpression. We observed a significant correlation between MDA-9/Syntenin and EGFR coexpression with clinical stage ( $P = 0.04$ ), but not with grade ( $P = 0.28$ ) or invasion ( $P = 0.05$ ; Supplementary Table S2). A significant correlation was also observed between MDA-9/Syntenin and  $\beta$ -catenin coexpression with clinical stage ( $P = 0.03$ ) as well as with invasion ( $P = 0.04$ ), but not with grade ( $P = 0.29$ ). There was a significant correlation between MDA-9/Syntenin and AKT coexpression with clinical stage ( $P = 0.01$ ), but not with grade ( $P = 0.29$ ) or invasion ( $P = 0.06$ ). No significant association of these gene alterations was evident at a single gene level (except for MDA-9/Syntenin alone) or in a combination of all of the gene changes with any clinical parameter ( $P = 0.92$ – $0.08$ ).

#### MDA-9/Syntenin knockdown UCC cells display reduced metastasis *in vivo*

Both GOF and LOF analysis of MDA-9/Syntenin affected *in vitro* proliferation and invasion potential of UCC



**Figure 6.** Impact of knockdown of MDA-9/Syntenin on *in vivo* metastasis of UCC cells. **A**, effect of knockdown of MDA-9/Syntenin on lung metastases. Lung metastases (left; yellow arrow) of the control shRNA transfected T24 cells was evident at 12 weeks as shown in several mice. **B**, the number of metastatic foci was significantly lower ( $P = 0.0016$ ) in the MDA-9/Syntenin knockdown T24 group compared with the control group. **C**, barely detectable levels of MDA-9/Syntenin expression were observed in the metastatic tumor's surrounding normal lung tissues. Magnification,  $\times 100$  and  $\times 200$ . \* indicates level of significance.

cells. A significant association between MDA-9/Syntenin overexpression and the progression of primary UCC tumors was also found. To examine the impact of MDA-9/Syntenin knockdown *in vivo*, we implanted empty vector-transfected control T24 and MDA-9/Syntenin knockdown T24 cells through the intracardiac injection route into nude mice. As shown in Fig. 6A, lung metastases caused by control T24 cells was evident at 12 weeks in cells that express steady-state levels of MDA-9/Syntenin (Supplementary Fig. S6). The number of metastatic foci was significantly reduced ( $P = 0.0016$ ) in the MDA-9/Syntenin knockdown group compared with the control T24 cells (Fig. 6B). In the surrounding normal lung tissues of the metastasized T24 cells, expression of MDA-9/Syntenin was barely detectable (Fig. 6C).

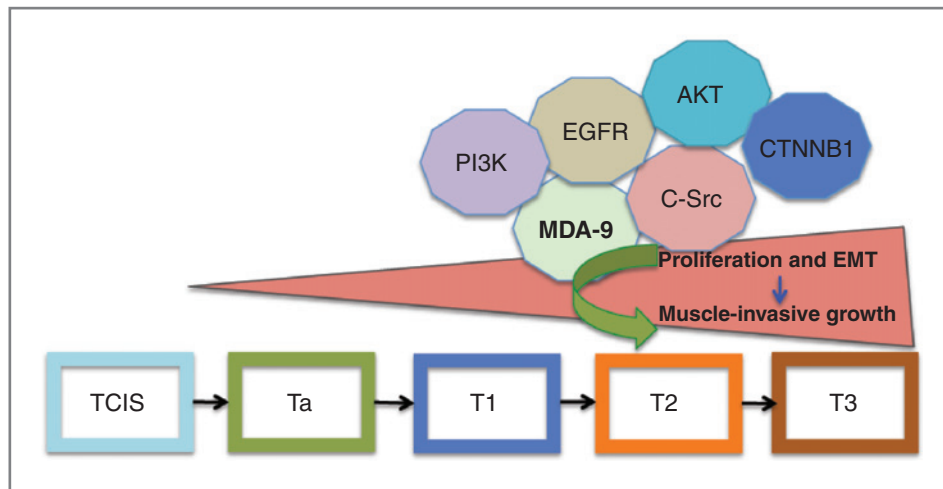
## Discussion

The precise role of MDA-9/Syntenin in UCC progression to muscle-invasive disease and its clinical relevance is mostly unknown. In the present study, progressive increase of MDA-9/Syntenin across the stages, grades, and its association with invasion supports a potential contribution of MDA-9/Syntenin in UCC progression. A progressive increase of *mda-9/syntenin* mRNA from normal urothelium to carcinoma *in situ* to muscle-invasive disease and its association with the development of therapeutic resistance and stable disease are evident in various expression databases (Supplementary Figs. S7 and S8). Notably, MDA-9/Syntenin overexpression was detectable as early as stage I (Table 1), thereby indicating its alteration at the initial stage of progression. Because of the lack of precancer urothelial lesions, we could not assess the true earliest time point of MDA-9/Syntenin overexpression in development of UCC. *In vivo* metastasis of highly invasive T24 cells following MDA-9/Syntenin is strongly inhibited by LOF (knockdown) studies providing functional support for a definitive role of MDA-9/Syntenin in disease progression. Other stud-

ies reported association of MDA-9/Syntenin overexpression with metastatic progression of cutaneous and uveal melanoma patients (13). The nuclear localization of MDA-9/Syntenin in the UCC tumors could be necessary for interaction with several growth-promoting molecules including CTNNB1, c-Src, which were upregulated following MDA-9/Syntenin overexpression. Nuclear localization of MDA-9/Syntenin was also observed in a recent study on uveal melanoma (13).

To define the mechanism underlying increased expression of MDA-9/Syntenin, we conducted FISH analysis. However, no amplification of *MDA-9/Syntenin* was evident in any of these primary tumors (data not shown). Similarly, Gangemi and colleagues (13) did not detect any concomitant amplification of *mda-9/syntenin* in their cohort of 29 uveal melanoma samples, despite significantly higher expression levels of MDA-9/Syntenin. We also conducted promoter methylation analysis hypothesizing hypomethylation as another potential mechanism of MDA-9/Syntenin activation. However, no significant increase in the level of hypomethylation was observed in the UCC cases (data not shown). Thus, overexpression of MDA-9/Syntenin may not be associated with gene amplification or promoter hypomethylation in these tumors. Other potential mechanism(s) including activating mutation, microRNA regulation, changes in promoter expression, and/or alterations in the processing of *mda-9/syntenin* mRNA into protein, may contribute to enhanced MDA-9/Syntenin expression. These possibilities are currently being explored.

Earlier studies showed the MDA-9/Syntenin-mediated activation of AKT and c-Src and MDA-9/Syntenin/c-Src interactions in breast cancer and melanoma cells, respectively (11, 21). When phosphorylated at Tyr<sup>418</sup>, c-Src can phosphorylate EGFR at Tyr<sup>845</sup> and Tyr<sup>1101</sup>, and thereby regulate EGFR functions and tumor progression (22). We observed increased phosphorylation of both c-Src-Tyr<sup>418</sup> and EGFR-Tyr<sup>845</sup> in MDA-9/Syntenin-overexpressing



**Figure 7.** Schematic representation of MDA-9/Syntenin-mediated UCC progression. Increased MDA-9/Syntenin expression during the pathologic progression of UCC (TCIS to T3) and its cross-talk with c-Src (Tyr<sup>418</sup>) can activate EGFR (Tyr<sup>845</sup>). The activated EGFR-PI3K-AKT can provide a continuous proliferation signal to the UCC cells and in turn transactivate  $\beta$ -catenin, a key molecule implicated in cellular growth and metastasis. On the other hand, disruption of adherens junctions and EMT by the simultaneous alteration of key regulatory molecules such as E-cadherin, ZO-1, claudin-1, TCF4, and vimentin may lead to metastatic initiation and progression of UCC to invasive disease.

HUC-1 cells with concomitant increases in phosphoinositide 3-kinase (PI3K) and AKT, which were reversed by knockdown of MDA-9/Syntenin in multiple UCC cell lines. Notably, MDA-9/Syntenin depletion altered localization of EGFR and activated C-Src in the UCC cells, which could be associated with their functional impairment. It is worth noting that in the majority of primary UCC tumors, we could detect simultaneous higher expression of EGFR, c-Src (Tyr<sup>418</sup>), and MDA-9/Syntenin. It seems that MDA-9/Syntenin overexpression activates c-Src, which in turn upregulates EGFR by phosphorylation at Tyr<sup>845</sup> with concomitant activation of PI3K/AKT and resulted in increased proliferation. Colocalization and Co-IP of MDA-9/Syntenin and EGFR in uroepithelial, HEK-293 cells, and primary tumors further support this relationship. Moreover, the association between MDA-9/Syntenin:EGFR and MDA-9/Syntenin:AKT expression with progression implicates EGFR signaling as one of the key signaling pathways required for progression. To our knowledge, this is the first report showing that MDA-9/Syntenin mediates upregulation of cellular proliferation via EGFR signaling in UCC.

EGFR-mediated activation of PI3K/AKT promotes key Wnt signaling molecule CTNNB1 transactivation and invasion (23). Recently, c-Src-mediated phosphorylation of  $\beta$ -catenin at Y<sup>333</sup> upon EGFR activation was implicated in tumorigenesis (24). In another study, MDA-9/Syntenin was shown to colocalize with  $\beta$ -catenin, Syndecan-1, and E-cadherin and coimmunoprecipitated with these proteins (25). We observed activation of CTNNB1 in MDA-9/Syntenin-overexpressing HUC-1 cells with a concomitant increase in EGFR, PI3K, AKT, and c-Src, as described earlier, with reversal of their expression following knockdown of MDA-9/Syntenin. Thus, CTNNB1 in concert with EGFR signaling may also be involved in UCC progression by

physically interacting with each other. The association between MDA-9/Syntenin:CTNNB1 expression and progressive disease stage and invasion of primary UCC tumors strongly supports this assumption. Activation of the Wnt signaling pathway also requires association of CTNNB1 and TCF4, which induces transcription of target genes, such as *c-myc*, *Cyclin D1*, *VEGF*, and *MMP-7* involved in cellular proliferation and invasion (24, 26). We observed a markedly increased TCF4 expression in MDA-9/Syntenin-overexpressing HUC-1 cells in concert with CTNNB1 activation. However, we did not observe the expected decrease of TCF4 in UCC cells following knockdown of MDA-9/Syntenin, rather we observed a moderate increase of TCF4. A recent study showed a tumor suppressor role for TCF4 in colon tumorigenesis (27). Thus, there could be a CTNNB1-independent role of TCF4 in concert with MDA-9/Syntenin to regulate UCC progression.

Alterations of adherens junctions and EMT play a crucial role in the development of muscle-invasive urothelial tumor (6). Progression of EMT correlates with the disintegration of cell-cell adhesion and alteration of various key junctional proteins including E-cadherin, ZO-1, and claudin-1 (28). In the EMT, E-cadherin and ZO-1 are among the key molecules associated with the "epithelial phenotype," whereas vimentin, CTNNB1, and N-cadherin with the "mesenchymal phenotype" (29). Selective overexpression of vimentin is observed in various cancers and correlates with increased migration and invasion of cancer cells (30). We observed decreased E-cadherin, ZO-1, and claudin-1 and increased vimentin expression in the MDA-9/Syntenin-overexpressing HUC-1 cells exhibiting characteristic EMT phenotype. Noticeably, reversal of the mesenchymal-to-epithelial transition phenotype was associated with increased E-cadherin, claudin-1, and ZO-1 expression and concomitant decrease of Snail, Slug, and N-cadherin

expression was evident following knockdown of MDA-9/Syntenin. The altered pattern of E-cadherin was also recapitulated in UCC tumors further supporting the relationship between MDA-9/Syntenin expression and metastasis through EMT regulation. E-cadherin is well established as a "suppressor of invasion" and is a common target for transcriptional repression in epithelial malignancies and its regulation is considered a pivotal step in the metastasis of various carcinomas (31, 32). In this cascade of events, the transcriptional repressors Snail and Slug are direct repressors of E-cadherin and claudin-1 (32). Along this pathway, induction of aberrant N-cadherin expression and silencing of E-cadherin transcription and an E-cadherin to N-cadherin switch during metastatic progression are also evident in various cancers (33).

In summary, we show a marked alteration of cellular growth and invasion following overexpression or knockdown of MDA-9/Syntenin in uroepithelial cells. These phenotypes correlate with altered expression of EGFR and its downstream signaling partners, necessary for the progression from noninvasive to invasive disease (Fig. 7). Conversely, alteration of CTNNB1 and other key EMT-associated molecules in concert with EGFR results in the disruption of the adherens junctions and leads to muscle-invasive disease (Fig. 7). MDA-9/Syntenin seems to be a fundamental molecule involved in UCC progression and could provide an attractive target to develop novel strategies for therapeutic management and monitoring UCC progression.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** S. Dasgupta, D. Sarkar, P.B. Fisher  
**Development of methodology:** S. Dasgupta, N.D. Mukhopadhyay  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** S. Dasgupta, S.K. Das, C. Shao, P.B. Fisher  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** S. Dasgupta, N.D. Mukhopadhyay, C. Shao, P.B. Fisher  
**Writing, review, and/or revision of the manuscript:** S. Dasgupta, M.E. Menezes, S.K. Das, N.D. Mukhopadhyay, C. Shao, D. Sarkar, P.B. Fisher  
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** S. Dasgupta, M.E. Menezes, L. Emdad, A. Janjic, S. Bhatia  
**Study supervision:** S. Dasgupta, D. Sarkar, P.B. Fisher

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