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TMPRSS4 induces cancer cell invasion through pro-uPA processing

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ABSTRACT

TMPRSS4 is a novel type II transmembrane serine protease that is highly expressed on the cell surface in pancreatic, thyroid, colon, and other cancer tissues. Previously, we demonstrated that TMPRSS4 mediates cancer cell invasion, epithelial-mesenchymal transition, and metastasis and that increased TMPRSS4 expression correlates with colorectal cancer progression. We also demonstrated that TMPRSS4 upregulates urokinase-type plasminogen activator (uPA) gene expression to induce cancer cell invasion. However, it remains unknown how proteolytic activity of TMPRSS4 contributes to invasion. In this study, we report that TMPRSS4 directly converted inactive pro-uPA into the active form through its proteolytic activity. Analysis of conditioned medium from cells overexpressing TMPRSS4 demonstrated that the active TMPRSS4 protease domain is released from the cells and is associated with the plasma membrane. Furthermore, TMPRSS4 could increase pro-uPA-mediated invasion in a serine proteolytic activity-dependent manner. These observations suggest that TMPRSS4 is an upstream regulator of pro-uPA activation. This study provides valuable insights into the proteolytic function of TMPRSS4 as well as mechanisms for the control of invasion.

1. Introduction

The dysregulation of proteases is a key hallmark of cancer, and thus proteases have been the subject of numerous cancer studies. Extracellular proteolytic enzymes, including matrix metalloproteinases (MMPs) and serine proteases, contribute to tumor cell invasion and metastasis through both direct proteolytic activity and the regulation of cellular signaling and functions [1–5].

Urokinase-type plasminogen activator (uPA) is a well-known serine protease involved in invasion and metastasis [2,3]. uPA catalyzes the conversion of inactive plasminogen to active plasmin, which can degrade most extracellular proteins and activate MMPs to facilitate invasion [3,6]. uPA is usually derived from stromal cells in cancer tissues. Complexes of uPA and uPAR (uPA receptor; CD87) on the tumor cell surface interact with integrin co-receptors to activate intracellular signaling pathways (e.g., MAPK and JAK/STAT) for cell migration, invasion, proliferation, and survival [7]. Increased levels of uPA correlate with invasive properties and poor prognosis in various cancer types, including breast, lung, stomach, bladder, colon, prostate and ovary [3,8].

The synthesis of uPA is regulated at both the transcriptional and posttranslational levels [3,9]. At the transcriptional level, uPA is induced mainly by the transcription factors AP-1 and Sp1 [10,11]. At the posttranslational level, uPA is produced as an inactive single-chain protein (known as pro-uPA or sc-uPA) that is processed into the active disulfide-linked two-chain form of uPA by a proteolytic event. Pro-uPA processing is catalyzed by plasmin [3]. Other proteases, such as plasma kallikrein, trypsin-like proteases from human ovarian tumors, cathepsins B and L, T cell-associated serine protease, nerve growth factor-gamma, and prostate-specific antigen, have also been reported to activate pro-uPA [3].

Recently, type II transmembrane serine proteases (TTSPs) have been recognized as a new subfamily of serine proteases that have in common an extracellular proteolytic domain, a transmembrane domain, and a short cytoplasmic domain [12–15]. Most TTSPs are overexpressed in various tumors compared to normal tissues, implicating their potential roles in tumor development and progression [15,16]. TMPRSS4, initially referred to as TMPRSS3, is a TTSP that is highly expressed in pancreatic, thyroid, lung, and colorectal cancers [17–20]. Previously, we reported that TMPRSS4 is an important mediator of cell migration, invasion, epithelial-mesenchymal transition (EMT), and metastasis in colon cancer cells, and increased TMPRSS4 expression correlated with colorectal cancer stage progression [19,21]. We also observed that the recombinant TMPRSS4 extracellular domain fusion protein displayed enzyme activity toward the oligopeptide substrate for trypsin-like serine protease (Gln-Ala-Arg) [21], suggesting that TMPRSS4 may

Abbreviations: TTSP, type II transmembrane serine protease; uPA, urokinase-type plasminogen activator; EMT, epithelial-mesenchymal transition; uPAR, uPA receptor.

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have trypsin-like serine protease activity, which is defined by cleavage of synthetic substrates with Arg or Lys in the P1 position. However, it remains unknown which naive/precursor substrates are cleaved by TMPRSS4 to contribute to invasion.

Recently, we demonstrated that TMPRSS4 induced uPA gene expression through JNK signaling activation to induce cancer cell invasion [22]. We also observed that active uPA protein was reduced by TMPRSS4 suppression [22]. Therefore, we hypothesized that TMPRSS4 may play a role in the processing of pro-uPA. In this study, we show that TMPRSS4 directly induces processing of pro-uPA into the active form through proteolytic activity to enhance cancer cell invasion, suggesting that TMPRSS4 is an upstream regulator of invasion through pro-uPA activation.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney 293E (HEK293E) cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in DMEM with 10% FBS at 37 °C/5% CO_2. SW480 colon cancer cells (ATCC) were maintained in RPMI1640 with 10% FBS.

2.2. Plasmid and cDNA constructs

A cDNA encoding wild-type full-length human pro-uPA was provided by the National Genome Information Center (Daejon, Korea). A fragment containing the coding sequence of pro-uPA with a stop codon was subcloned into the expression vector pcDNA3.1/myc-His(−) to produce pcDNA3.1-pro-uPA. The TMPRSS4-expressing construct pCMV-myc-TMPRSS4 was described previously [21]. The active-site-mutant TMPRSS4 constructs were generated from pCMV-myc-TMPRSS4 by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following mutagenesis primers were used: 5′-CATGTACCCCAAAGACAATGCCATCGCCCT CATG-3′ and its complementary strand for D290A; 5′-CTGCCAGGG TGACGCTGGTGGGCCCCTG-3′ and its complementary strand for S387A.

2.3. Transfection with expression vectors

Cells were transfected with pCMV-myc-TMPRSS4 or pcDNA3.1-pro-uPA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 48 h after transfection, the medium was changed to serum-free medium. Conditioned medium was collected at 48 h.

2.4. Immunoblot analysis

Whole-cell lysates prepared using RIPA buffer as described previously [19], and conditioned media were analyzed using the following primary antibodies: anti-uPA, anti-β-actin, and anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-myc antibody (Upstate Biotechnology, Lake Placid, NY); anti-TMPRSS4 (in-house) [22].

2.5. Invasion assay

Invasion assays were performed as described previously [21]. Cells were plated in serum-free medium on Transwell inserts (Costar, Corning, NY) coated with 25 μg of Matrigel (BD Biosciences, San Jose, CA). The underside of the insert was pre-coated with 2 μg of collagen type I (Sigma, St Louis, MO). After incubation for 48 h, the inserts were fixed with 3.7% paraformaldehyde/PBS and stained with 2% crystal violet. The number of cells that had invaded was counted in five representative (×200) fields per insert.

2.6. Cleavage of a synthetic oligopeptide substrate and pro-uPA

HEK293E cells were transfected with myc-tagged TMPRSS4 constructs (wild-type or mutants D290A and S387A) or a pro-uPA construct (pcDNA3.1-pro-uPA) using Lipofectamine 2000. At 48 h after transfection, the media were changed to serum-free media. Conditioned media were collected at 48 h. TMPRSS4 proteins in conditioned medium and whole-cell lysates were analyzed by immunoblot using anti-myc and anti-TMPRSS4 antibodies. To examine the proteolytic activity of the released TMPRSS4 proteins, conditioned medium was incubated with 100 μM Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride (Sigma) in DMEM at 25 °C. The fluorescence resulting from hydrolysis of the peptide substrate was measured at 385/455 nm using a Victor3 plate reader (PerkinElmer, Wellesley, MA). In another experiment, conditioned medium from cells transfected with the TMPRSS4 constructs was incubated with pro-uPA-containing conditioned medium for 16 h at 37 °C, and pro-uPA processing was analyzed by immunoblotting.

A recombinant TMPRSS4 serine protease domain tagged with 6 × His at the C-terminus (pro-form) was prepared as described previously [23], and treated with enterokinase (New England Biolabs, Ipswich, MA) to produce the active form of recombinant TMPRSS4 serine protease. Enterokinase was removed by purifying the recombinant proteins by Ni–NTA affinity chromatography. Proteolytic activity of the recombinant TMPRSS4 serine protease was measured in similar ways; 2–10 μg of the recombinant TMPRSS4 serine protease (pro-form or active form) was incubated with 100 μM Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride in a reaction buffer (100 mM Tris, pH 8.0, 10 mM $CaCl_2$, 1 μM $ZnCl_2$) in the presence or absence of 1 mM AEBSF (Calbiochem, La Jolla, CA) at 25 °C, and fluorescence intensity was measured. The recombinant TMPRSS4 serine protease (0.5, 1, or 2 μg) was incubated with a purified single-chain pro-uPA (100 ng; American diagnostica Inc., Stamford, CT) for 16 h at 37 °C, and pro-uPA processing was analyzed by immunoblotting.

2.7. Statistical analysis

Statistical analyses were performed using Student's *t*-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. TMPRSS4 protease domain was released from the cells and induced pro-uPA processing

Previously, we observed that active uPA protein level was substantially reduced by TMPRSS4 suppression in NCI-H322 cells [22]. Therefore, we hypothesized that TMPRSS4 may play a role in inducing pro-uPA processing. To explore whether TMPRSS4 is involved in the processing of pro-uPA, HEK293E cells were co-transfected with the pro-uPA-expressing and TMPRSS4-expressing vectors. Immunoblot analysis of the conditioned medium demonstrated that pro-uPA processing was increased by wild-type TMPRSS4 but not by the active site mutant D290A (Fig. 1A).

We previously reported that the recombinant extracellular domain of TMPRSS4 displayed proteolytic activity toward the standard peptide substrate for trypsin (Gln-Ala-Arg) and that TMPRSS4 induces invasion in a manner that is partially dependent on MMP(s) but mainly dependent on serine proteolytic activity [19,21]. However, it remains unknown whether TMPRSS4 on the cell surface has endogenous/specific proteolytic activity and which naive/precursor substrate(s) TMPRSS4 can cleave. Therefore, we examined whether the proteolytic activity of native TMPRSS4 is

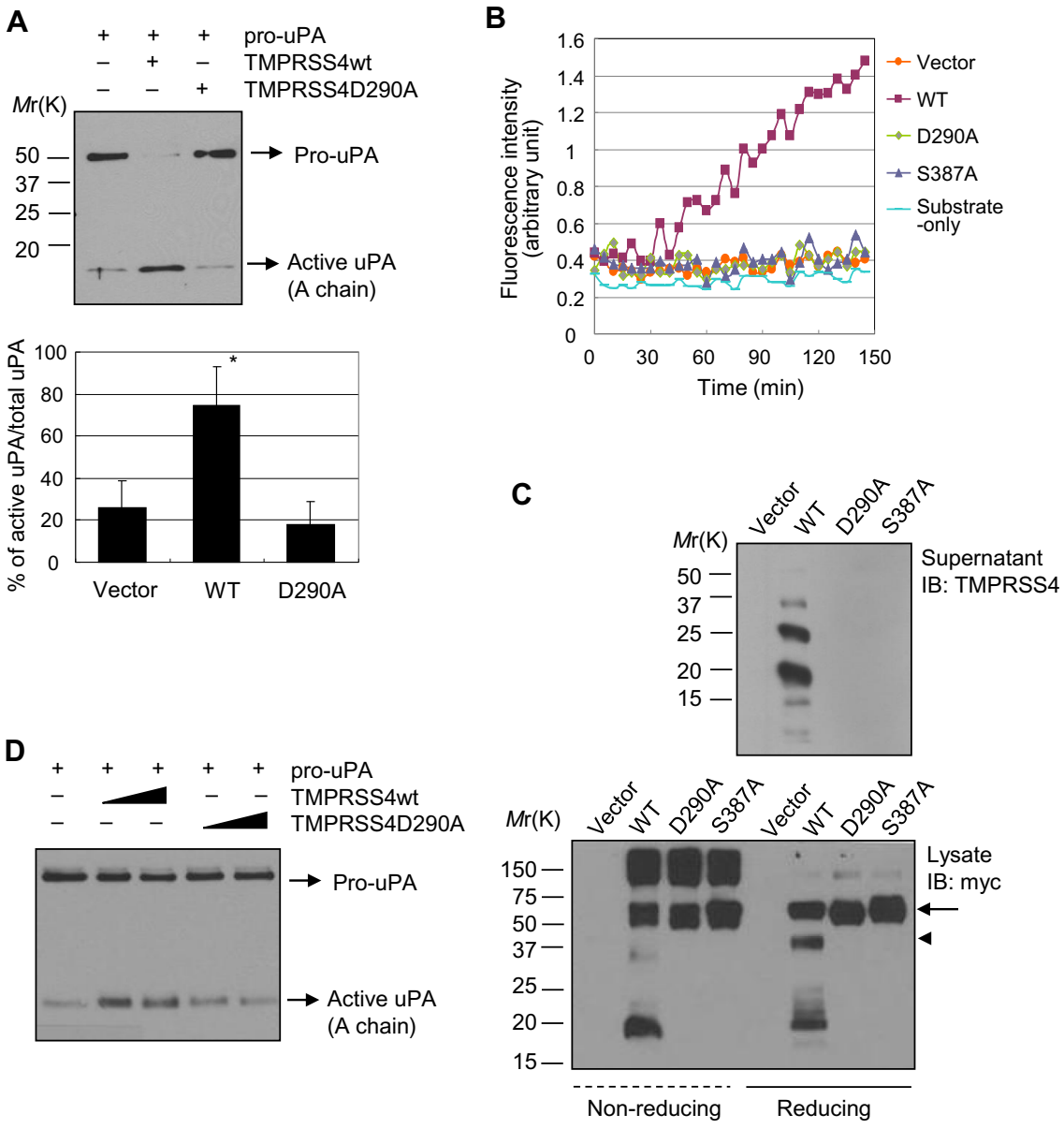

Fig. 1. TMPRSS4 protease domain is released from the cells to induce pro-uPA processing. (A) HEK293E cells were co-transfected with the pro-uPA expression vector and the TMPRSS4 expression vectors (wild-type and D290A mutant) or the corresponding empty vectors for 48 h, and then conditioned medium was collected for 48 h for immunoblot analysis with anti-uPA antibody. The ratio of active uPA to total uPA (pro-uPA + active uPA) was calculated by densitometry, using the average of three independent experiments. Values represent mean ± SD. $^{*}P < 0.05$. (B and C) HEK293E cells were transfected with TMPRSS4 (wild-type, and D290A and S387A mutants) expression vectors or empty vector for 48 h, and then conditioned medium was collected for 48 h. (B) The conditioned media were incubated with 100 μM of the fluorogenic peptide substrate, Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride, at 25 °C. Fluorescence intensity was measured with excitation at 385 nm and emission at 455 nm. (C) The released proteins were analyzed by immunoblotting using anti-TMPRSS4 antibody under reducing conditions. Whole-cell lysates were also analyzed using anti-myc antibody under both reducing and non-reducing conditions. (D) HEK293E cells were transfected with the pro-uPA expression vector for 48 h, and then conditioned medium was collected for 48 h. Conditioned media from cells transfected with TMPRSS4 constructs (wild-type and D290A mutant) from (B and C) were incubated with pro-uPA-containing conditioned medium for 16 h at 37 °C and subjected to immunoblotting with anti-uPA antibody to determine the level of pro-uPA cleavage.

involved in the cleavage of pro-uPA. Conditioned medium from cells transfected with wild-type TMPRSS4 had proteolytic activity toward the fluorogenic peptide substrate, Z-Phe-Arg 7-amido-4-methylcoumarin, whereas conditioned medium from cells transfected with the empty vector or active site residue mutants D290A and S387A only exhibited background activity (Fig. 1B). Immunoblot analysis of conditioned media under reducing conditions using an antibody against the TMPRSS4 protease domain demonstrated that peptide fragments smaller than intact TMPRSS4 (~35, ~27, and ~20 kDa) were released from cells transfected with wild-type TMPRSS4, but no fragments were observed in cells transfected with D290A and S387A mutants (Fig. 1C, upper). Of note, 27 kDa is close to the calculated molecular mass of the active protease domain of TMPRSS4 (25.7 kDa).

Intact TMPRSS4 (approximately 60–70 kDa; Fig. 1C, arrow) and the smaller size fragments (~40 and ~20 kDa) were detected using anti-myc antibody under reducing conditions in lysates from cells transfected with wild-type TMPRSS4 (Fig. 1C, lower right). This result is consistent with our previous observation in SW480 cells [21], confirming the possibility that the TMPRSS4 extracellular domain fragment may be released from cells. Of note, 40 kDa is close to the estimated molecular mass of the myc-tagged N-terminal fragment of TMPRSS4 without the protease domain (33–43 kDa). However, no small fragments were detected from

the D290A and S387 mutant-transfected cells (Fig. 1C, lower right). Together, these observations suggest that these small fragments, derived from the TMPRSS4 extracellular domains containing the protease domain, are shed from the plasma membrane, possibly due to autocleavage.

We previously observed that TMPRSS4 was mainly present in the plasma membrane-enriched fraction [21]. The amount of the small (~40 kDa) N-terminal fragment of TMPRSS4 that was detected by immunoblot analysis was markedly reduced under non-reducing conditions compared with reducing conditions (Fig. 1C, lower; arrowhead). This observation suggests that the TMPRSS4 extracellular domain containing the protease domain may be associated with the plasma membrane.

Furthermore, inactive single-chain pro-uPA (50 kDa) was converted into a smaller, active uPA fragment (~18 kDa) after incubation with conditioned medium from cells transfected with wild-type TMPRSS4 compared with the conditioned medium from the empty vector and D290A mutant-transfected cells (Fig. 1D), confirming that soluble TMPRSS4 protease domain released from cells is involved in the processing of pro-uPA into the active uPA.

3.2. TMPRSS4 directly induced pro-uPA processing via its proteolytic activity

To determine whether pro-uPA processing was directly mediated by the TMPRSS4 serine protease, the TMPRSS4 protease domain (residues 205–437) fused with an enterokinase cleavage sequence at the N-terminus and with a 6 × His tag at the C-terminus (pro-form) was produced in *Escherichia coli* (Fig. 2A). The active form of the recombinant TMPRSS4 serine protease generated by enterokinase treatment displayed proteolytic activity toward the peptide substrate (Phe-Arg), whereas the inactive form of the recombinant TMPRSS4 serine protease (pro-form) only exhibited background activity (Fig. 2A and B). Furthermore, this activity was completely blocked by the serine protease inhibitor AEBSF (Fig. 2B). Purified inactive pro-uPA was directly cleaved into a smaller, active uPA fragment by the active form of the recombinant TMPRSS4 serine protease compared with the pro-form of the recombinant TMPRSS4 serine protease (Fig. 2C). Taken together, these results suggest that active TMPRSS4 protease released from the cell surface is able to cleave pro-uPA to produce active uPA.

3.3. TMPRSS4 enhanced pro-uPA-mediated invasion

We then determined whether TMPRSS4 and uPA could enhance the invasion of cancer cells that normally display a low level of endogenous uPA. SW480 cells were transfected with TMPRSS4 in the presence or absence of the pro-uPA-expressing vector. The combination of TMPRSS4 and pro-uPA enhanced invasion by 3-fold, which was significantly more efficient than TMPRSS4 or pro-uPA alone (Fig. 3A). Immunoblot analysis of conditioned media using an antibody against the TMPRSS4 protease domain demonstrated that the TMPRSS4 serine protease domain fragment (~27 kDa) was released from cells transfected with TMPRSS4, but

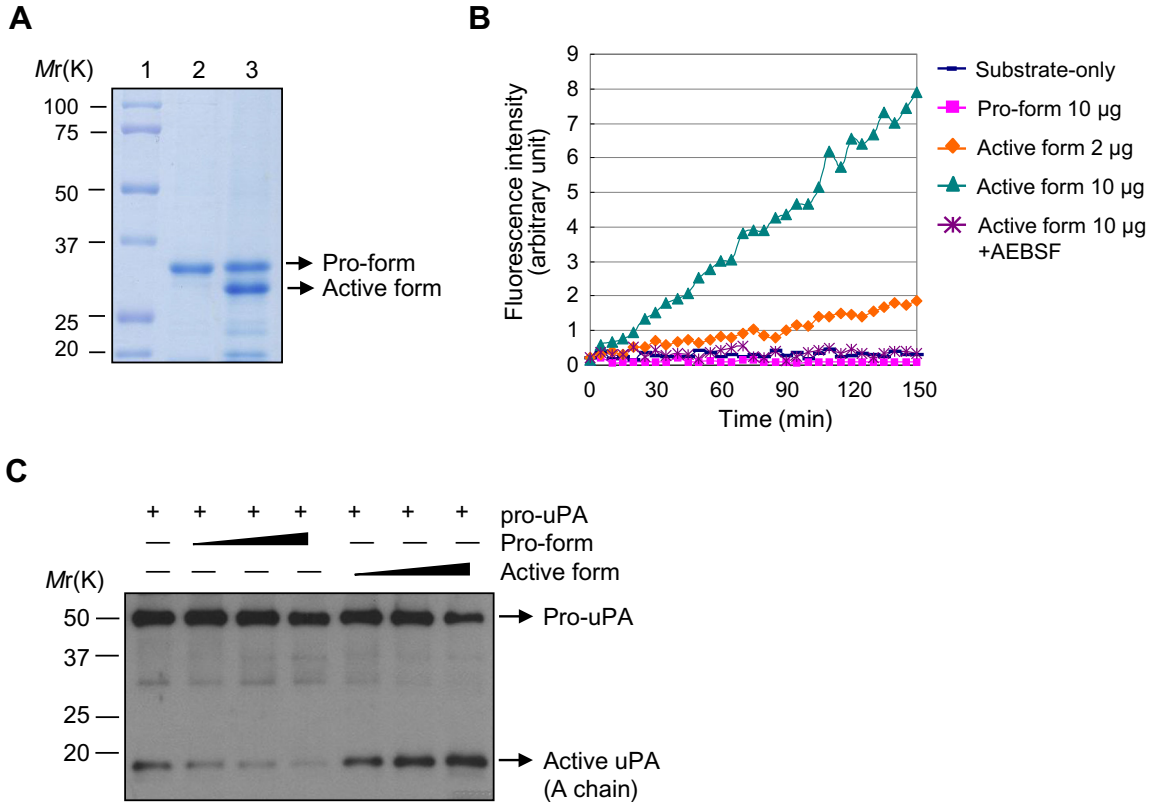

Fig. 2. TMPRSS4 directly cleaved pro-uPA into active uPA. (A) The recombinant TMPRSS4 serine protease domain fused with an enterokinase cleavage sequence and tagged with 6 × His (lane 2) was produced in *Escherichia coli*, purified by Ni–NTA affinity chromatography, and analyzed by SDS–PAGE followed by Coomassie brilliant blue staining. The active form of the recombinant TMPRSS4 serine protease was generated by enterokinase treatment of the recombinant TMPRSS4 serine protease pro-form, and purified by Ni–NTA affinity chromatography (lane 3). Lane 1, size marker. (B) The recombinant TMPRSS4 serine protease (pro-form or active form) was incubated with 100 μM Z-Phe-Arg 7-amido-4-methylcoumarin hydrochloride in a reaction buffer (100 mM Tris, pH 8.0, 10 mM $CaCl_2$, 1 μM $ZnCl_2$) in the presence or absence of 1 mM AEBSF at 25 °C, and fluorescence intensity was measured as in Fig. 1B. (C) The recombinant TMPRSS4 serine protease (pro-form or active form; 0.5, 1, or 2 μg) was incubated with purified pro-uPA (100 ng; American diagnostica Inc.) for 16 h at 37 °C and subjected to immunoblotting with anti-uPA antibody to determine the level of pro-uPA cleavage. SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

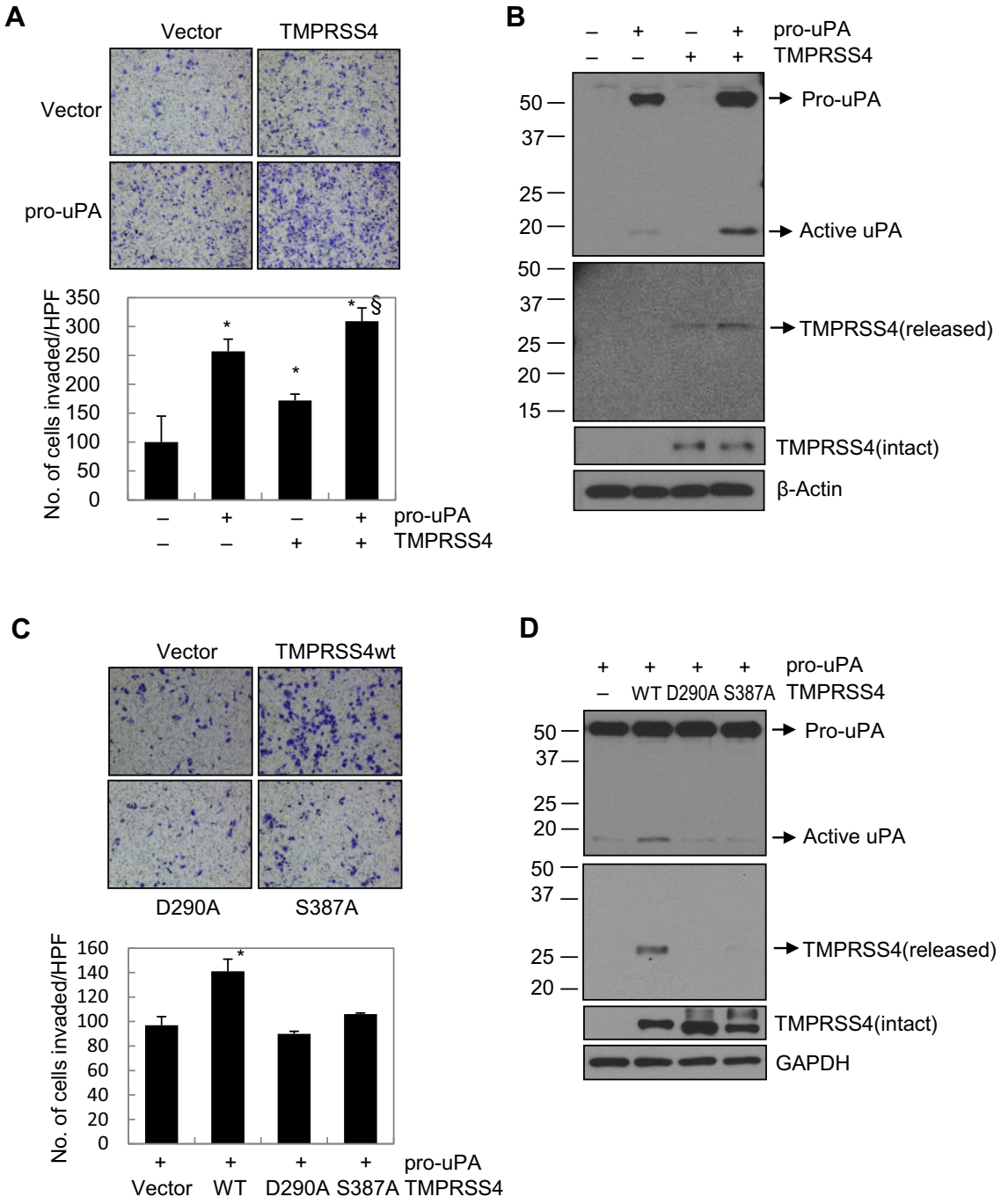

Fig. 3. TMPRSS4 enhanced pro-uPA-mediated invasion. (A and B) Plasmids expressing TMPRSS4 or pro-uPA or the corresponding empty vectors were transfected into SW480 cells for 48 h. (A) Transfected cells (4×10^4) were allowed to invade Matrigel toward collagen type I for 48 h. The number of cells that had invaded was counted in five representative high-power (×200) fields (HPFs) per Transwell insert. Values represent mean ± SD. $^*P < 0.05$, compared with empty vector; $^{\S}P < 0.05$, compared with pro-uPA. (B) Whole-cell lysates and conditioned media from transfected cells were analyzed by immunoblotting. Intact TMPRSS4 was detected with an anti-myc antibody from lysates while released TMPRSS4 fragment was detected with an anti-TMPRSS4 antibody from conditioned media under reducing conditions. β-Actin was used as an internal control. (C and D) SW480 cells were transfected with the TMPRSS4 expression vectors (wild-type and mutants) or the empty vector in the presence of the pro-uPA-expressing vector for 48 h. (C) Invasion assay was performed as in (A) except that 3×10^4 cells were used. Values represent mean ± SD. $^*P < 0.05$, compared with pro-uPA + empty vector. (D) Immunoblot analysis was performed as in (B). GAPDH was used as an internal control.

no fragments were observed from cells transfected with empty vector (Fig. 3B). Furthermore, conversion of pro-uPA to active uPA was enhanced by TMPRSS4 (Fig. 3B). Interestingly, pro-uPA protein level was moderately elevated by TMPRSS4, indicating the possibility that TMPRSS4 may be involved in the stability of pro-uPA/uPA proteins on cancer cell surfaces. In addition, catalytically inactive mutants D290A and S387A did not significantly enhance invasiveness in the presence of pro-uPA as compared with wild-type TMPRSS4 (Fig. 3C), which was consistent with the observation that mutants did not enhance conversion of pro-uPA into active uPA or release of the TMPRSS4 serine protease domain fragment (Fig. 3D). Taken together, these results suggest that TMPRSS4 contributes to pro-uPA/uPA-mediated invasion probably through pro-uPA processing.

4. Discussion

TMPRSS4 is a novel TTSP found at the cell surface that is highly expressed in pancreatic, thyroid, lung, and colorectal cancers. We recently reported that TMPRSS4 induces invasion and EMT of colon cancer cells through upregulation of integrin α5 [19,21]. In addition, TMPRSS4 induces invasion through uPA gene expression in a cell- or context-dependent manner [22]. However, it remains unknown whether TMPRSS4 on the cell surface has endogenous/specific proteolytic activity and which naive substrates are cleaved by TMPRSS4. In this study, we report that TMPRSS4 upregulates uPA by processing pro-uPA into its active form, leading to cancer cell invasion, suggesting that TMPRSS4 is an upstream regulator of invasion. This study is the first to demonstrate the endogenous and specific proteolytic activity of TMPRSS4 on cancer cells, which involves the processing of pro-uPA as a naive substrate. In our system, specific activity of the recombinant TMPRSS4 serine protease produced in *E. coli* appeared to be low, which may be partially due to insufficient protein folding or the lack of potential modification. It may be also possible that TMPRSS4 activates more efficiently uPAR-bound pro-uPA compared with soluble pro-uPA. It could explain the observation that the efficiency of pro-uPA processing induced by TMPRSS4 differs in different cell types (Figs. 1 and 3) although it remains to be examined.

It has been reported that all tested members of the TTSP family display a preference for cleavage of substrates with a basic Arg or Lys residue in the P1 position [24] and pro-uPA was shown to be processed by TTSP members such as matriptase and hepsin other than TMPRSS4 [25–27]. On the other hand, TMPRSS4 can induce pro-uPA transcription [22] whereas matriptase did not elevate uPA gene expression [28]. It is interesting that TMPRSS4 is able to upregulate uPA through dual mechanisms, leading to cancer cell invasion; TMPRSS4 can directly induce uPA transcription in cancer cells themselves and subsequently process inactive pro-uPA into its active form via an autocrine mechanism. It is possible that cancer cells can drastically accelerate the aggressiveness of a malignancy by themselves via the upregulation of uPA by TMPRSS4 at both the transcriptional and posttranslational levels. Together, these results suggest a novel mechanism for the control of invasion.

The observation that cells transfected with wild-type TMPRSS4 displayed soluble protease domain fragments with proteolytic activity toward peptide substrates, whereas cells transfected with the active site mutants (D290A and S387A) did not suggests that the extracellular domains containing the protease domain are shed/released from the cell surface, probably due to autocleavage (autoactivation), which is also associated with pro-uPA processing and invasiveness. Consistent with our observation, mouse channel activating protease 2 (mCAP2; homologue of human TMPRSS4) was reported to be autoactivated in the *Xenopus* oocyte expression system [29]. Therefore, it will be worth isolating naturally-occurring shed form of TMPRSS4 (e.g., active TMPRSS4 protease) from human serum and exploring whether soluble TMPRSS4 can be used as a diagnostic or prognostic biomarker of cancer patients.

We have previously demonstrated that TMPRSS4 induces invasion and EMT through upregulation of integrin α5 and FAK/ERK signaling pathways in SW480 colon cancer cells [19]. In this study, we report that TMPRSS4 could enhance invasion through pro-uPA processing although we previously observed that uPA expression was not induced by TMPRSS4 in SW480 cells [22]. We also observed potential association of TMPRSS4 and uPAR in cancer cells [22], suggesting that a physical association between TMPRSS4 and uPAR might allow TMPRSS4 to gain access to pro-uPA and efficiently cleave it. Together, it might be possible that TMPRSS4-induced integrin α5 expression could mediate uPAR-integrin-TMPRSS4 complex formation to induce pro-uPA processing and intracellular signaling although it remains to be determined.

This study and our previous results [22] suggest that TMPRSS4 is an important upstream regulator of invasion through uPA gene expression and pro-uPA activation, implying that TMPRSS4 may represent a potential target for novel cancer treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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