



Environment-Sensitive Ectodomain Shedding of Epithin/PRSS14 Increases Metastatic Potential of Breast Cancer Cells by Producing CCL2

Jiyoung Jang^{1,3}, Eun Hye Cho^{2,3}, Youngkyung Cho^{1,2}, Binderya Ganzorig², Ki Yeon Kim², Moon Gyo Kim², and Chunggho Kim^{1,*}

¹Department of Life Sciences, Korea University, Seoul 02841, Korea, ²Department of Biological Sciences, Inha University, Incheon 22212, Korea, ³These authors contributed equally to this work.

*Correspondence: chunggho@korea.ac.kr
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Epithin/PRSS14 is a membrane serine protease that plays a key role in tumor progression. The protease exists on the cell surface until its ectodomain shedding, which releases most of the extracellular domain. Previously, we showed that the remaining portion on the membrane undergoes intramembrane proteolysis, which results in the liberation of the intracellular domain and the intracellular domain-mediated gene expression. In this study, we investigated how the intramembrane proteolysis for the nuclear function is initiated. We observed that ectodomain shedding of epithin/PRSS14 in mouse breast cancer 4T1 cells increased depending on environmental conditions and was positively correlated with invasiveness of the cells and their pro-invasive cytokine production. We identified selenite as an environmental factor that can induce ectodomain shedding of the protease and increase C-C motif chemokine ligand 2 (CCL2) secretion in an epithin/PRSS14-dependent manner. Additionally, by demonstrating that the expression of the intracellular domain of epithin/PRSS14 is sufficient to induce CCL2 secretion, we established that epithin/PRSS14-dependent shedding and its subsequent intramembrane proteolysis are responsible for the metastatic conversion of 4T1 cells under these conditions. Consequently, we propose that epithin/PRSS14 can act as an environment-sensing receptor that promotes cancer metastasis by liberating the

intracellular domain bearing transcriptional activity under conditions promoting ectodomain shedding.

Keywords: cancer microenvironment, CCL2, epithin/PRSS14, invasiveness, selenite

INTRODUCTION

Epithin/PRSS14 (also called matriptase or ST14) is a typical type II transmembrane serine protease (Antalis et al., 2010; Kim et al., 1999; Martin and List, 2019). It has a relatively short cytoplasmic tail, a single-spanning transmembrane domain, and an extracellular domain consisting of a SEA (sperm protein, enterokinease, and agrin) domains, two CUB (complement C1r/C1s, Uegf, Bmp1) domains, four LDLRa (low density lipoprotein receptor a) repeats, and a serine protease domain. During its biosynthesis, the peptide bond between Gly149 and Ser150 in the SEA domain is cleaved (Cho et al., 2001) by a self-cleavage mechanism (Levitin et al., 2005), and the two resulting chains are held together presumably by hydrogen bonds formed between the β strands flanking the cleavage site (Cho et al., 2017). This two-chain form localizes on the cell surface, where it can be released as a soluble form by TACE/ADAM17-mediated ectodomain shedding (Cho et

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al., 2017; Kim et al., 2005; Lee et al., 2014).

Epithin/PRSS14 is known to play important roles in many human epithelial cancers (Ha et al., 2014; Martin and List, 2019; Oberst et al., 2001), and its high expression is correlated with poor survival of cancer patients (Kang et al., 2003; Oberst et al., 2002; Saleem et al., 2006). For example, in esophageal squamous cell carcinoma, post-surgery patients with high expression of the protease showed extremely poor survival (Ha et al., 2014). In estrogen receptor-negative or triple-negative (negative for estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2) breast cancer patients, high expression of the protease is also a parameter for extremely poor survival (Kim et al., 2016). Consistently, in animal models, forced expression of epithin/PRSS14 can produce spontaneous carcinoma (List et al., 2005) and facilitate tumorigenesis by providing a more inflammatory environment (Sales et al., 2015). Conversely, when its expression is genetically diminished, breast cancer formation is largely abolished (Zoratti et al., 2015). Many related studies have univocally suggested the critical role of the protease in tumorigenesis and cancer metastasis (Ihara et al., 2002; Jin et al., 2006; Kim et al., 2011).

Because epithin/PRSS14 has a serine protease domain in its extracellular domain, its serine protease activity is considered the key determinant that governs its cancer-promoting roles. Many studies have shown the important role of protease activity of epithin/PRSS14 in cancer metastasis (Kim et al., 2011; Ko et al., 2017; Tsai et al., 2014). However, our recent observations suggest that the intracellular domain of epithin/PRSS14 might also play a role in tumor progression through its nuclear functions (Cho et al., 2020). The study showed that the intracellular domain of epithin/PRSS14, when cleaved from the plasma membrane by regulated intramembrane proteolysis, can enhance the expression of pro-metastatic genes, such as matrix metalloproteases, cytokines, and growth factors (Cho et al., 2020). Because the generation of the soluble intracellular domain is preceded by its ectodomain shedding, as observed in other substrates of regulated intramembrane proteolysis (Kuhnle et al., 2019), our findings led us to hypothesize that epithin/PRSS14 could be considered a cell surface receptor *per se* controlling metastatic gene expression and its activation could be induced by any environmental cue promoting its ectodomain shedding.

In this study, we tested this hypothesis by investigating various conditions that might result in epithin/PRSS14 ectodomain shedding. We observed different invasive behaviors of 4T1 mouse breast cancer cells under different cell culture conditions. The invasiveness of the cells was positively linked to the degree of epithin/PRSS14 ectodomain shedding and the expression of metastatic chemokines, such as C-C motif chemokine ligand 2 (CCL2). Sodium selenite has been identified as one of the environmental cues governing such differences. We found that selenite can induce ectodomain shedding of epithin/PRSS14, possibly by oxidizing the cellular environment, which triggers the intracellular function of epithin/PRSS14. These results suggest a unique mechanism by which the membrane protease could exert its cancer-promoting role by acting as an environment-sensing receptor.

MATERIALS AND METHODS

Cell culture

4T1 mouse breast cancer cells were obtained from the American Type Culture Collection (ATCC) (CRL-2539TM). 4T1-EpiKD cells, a subline of 4T1 cells in which epithin/PRSS14 was knocked down by stably incorporating shRNA, have been previously described (Kim et al., 2011). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (LM001-05; Welgene, Korea) supplemented with 10% fetal bovine serum (FBS) (S001-07; Welgene), 1X penicillin-streptomycin (LS202-02; Welgene), and 4 mM L-glutamine (LS002-01; Welgene). DMEM was replaced with RPMI1640 (RPMI, LM011-01; Welgene) or Iscove's modified Dulbecco's medium (IMDM) (LM004-01; Welgene) when needed. 4T1-I cells were generated by growing 4T1 cells in IMDM for 6 weeks. For transient transfection, Polyjet (SL100688; SignaGen, USA) was used according to the manufacturer's instructions.

Mice

For the orthotopic tumor model, 4T1 and 4T1-EpiKD cells cultured in DMEM for approximately 6 weeks were used. Under anesthesia with Avertin, 1×10^5 cells in 50 μ l were injected into the mammary fat pad of 8-week-old female BALB/c mice. Each group comprised five mice, and the whole tumors were dissected 26 days after injection. Then, they were analyzed by flow cytometry for Sca-1 expression. For the tail-vein metastasis model, a sample of 5×10^5 cells in 100 μ l phosphate-buffered saline (LB001-02; Welgene) was injected intravenously in 8-week-old BALB/c female mice. Mice were monitored for survival analysis. Animals used in this study were maintained in the Laboratory of Molecular and Cellular Immunology Animal Facility at Inha University under the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023).

Western blots

To analyze epithin/PRSS14 shedding and/or CCL2 secretion, cells were cultured in serum-free media (DMEM, IMDM, or RPMI) and incubated overnight for 4 h, as indicated in the figure legends. When needed, 1 μ M phorbol 12-myristate 13-acetate (PMA), various concentration of Na_2SeO_3 (214485; Sigma, USA), 3 mM N-acetylcysteine (A9165; Sigma), or 0.2 μ g/ml lipopolysaccharide (LPS) was used. Proteins in the media were precipitated by adding 10% TCA (T0699; Sigma) before being subjected to SDS-PAGE and western blotting. Anti-MCP1 (CCL2) (MA517040; Thermo Fisher Scientific, USA), anti-FLAG (F1804; Sigma), anti- β tubulin, and anti-GAPDH (sc-32233; Santa Cruz Biotechnology, USA) antibodies were purchased, and mAb5 against epithin/PRSS14 was previously described (Cho et al., 2001).

Flow cytometry

Cells (1×10^6) cultured for approximately 6 weeks in specific media were used for population heterogeneity testing. For orthotopically implanted tumor cell analysis, dissected tumors were chopped with scissors, followed by treatment with collagenase/dispase (269638; Roche, Switzerland) to suspend as single cells. For staining, 2×10^5 tumor cells were

incubated with 2.4G2 (Kim et al., 2015) to block the Fc receptor and then stained with biotin-conjugated anti-mouse Ly-6A/E (stem cell antigen-1, Sca-1) (108103; BioLegend, USA), followed by streptavidin PE-Cy7 conjugated (#SA1012; Invitrogen, USA). After surface staining, dead cells were excluded by staining with Fixable Viability Dye eFluor® 455UV (65-0868; eBioscience, USA). Data were collected using BD FACSAria and analyzed by FlowJo. For the CCL2 promoter assay, CCL2-green fluorescence protein (GFP) reporter construct (MPRM41761-PF02; GeneCopoeia, USA) was co-transfected into 4T1 cells with tdTomato cDNA as a transfection marker, and the mean fluorescence of GFP intensities from cells with moderate level of tdTomato expression was measured using BD Accuri™ C6 Plus Flow Cytometer.

RNA sequencing (RNA-seq) and transcriptome analysis

RNA of 4T1 sublines grown in each of the three media with a 6-week passage were extracted with TRIzol (15596026; Invitrogen, USA) according to the manufacturer's instructions. The following RNA-seq process was conducted by Macrogen (Korea). DNA-eliminated RNA was used for RNA-seq by Illumina HiSeq with paired-end sequencing. Trimmed reads were aligned to a mouse reference genome mm10 using TopHat (v2.0.13) (Kim et al., 2013), and transcriptome assembly was performed using Cufflinks (v2.2.1) (Trapnell et al., 2012). Using calculated FPKM (fragments per kilobases of transcript per million mapped reads) values, expression levels from reads were extracted and compared between different conditions. To examine statistical relationships, correlation coefficients were calculated by comparing every pair of transcript using the corrplot R package (Wei et al., 2017).

Invasion assay

To analyze the invasion of the cells of the 6-week culture, the cells were deprived of serum for 2 h and dissociated with enzyme-free dissociation buffer (13151014; Thermo Fisher Scientific). The resulting 3×10^4 cells in serum-free media were seeded in the upper part of a Transwell chamber (3422; Corning, USA) and were incubated for 2 days with 10% FBS in the lower chamber. For the analysis of CCL2 response, 2×10^5 cells in DMEM containing 1% FBS were seeded in the upper chamber, treated with the vehicle, 400 ng/ml CCL2 (479-JE-010/CF; R&D System, USA) or 20 ng/ml transforming growth factor β (TGF- β) (100-21; PeproTech, USA), and incubated overnight. After the cells attached to the upper chamber were removed, cells that migrated to the opposite side were fixed with 100% methanol for 20 min, stained with hematoxylin for 5 min, and visualized under a microscope (BX50; Olympus, Japan). Images of three different fields per duplicated sample were taken, and the area of migrated cells was analyzed using ImageJ software (National Institute of Health, USA).

Statistical analysis

Statistical analyses were done by using GraphPad Prism 5 (GraphPad Software, USA). Data are presented as mean \pm SEM with the two-tailed Student's *t*-test for comparing the means of 2 groups or one-way ANOVA followed by Tukey's multiple comparison test for comparing the means among

more than 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

Environment-sensitive tumorigenic potential of 4T1 cells depends on epithin/PRSS14

Epithin/Prss14 has been implicated in tumor progression (Ihara et al., 2002; Jin et al., 2006; Kim et al., 2011). To understand the underlying mechanism involved in tumor progression, we transplanted a mouse breast cancer cell line, 4T1, into the mammary fat pad of the orthotopic mouse strain, BALB/c (Aslakson and Miller, 1992). After 26 days, the resulting tumors were collected and analyzed for their surface expression of cancer stem cell markers, Sca-1 (encoded by Ly6a), by flow cytometry. Intriguingly, 4T1 cells from the tumor mass were almost all Sca-1⁺ (Fig. 1A), whereas 4T1-EpiKD cells showed reduced Sca-1 surface expression, suggesting that epithin/PRSS14 may increase and/or maintain cancer stemness. In the metastasis assay performed by tail-vein injection of 4T1 cells, epithin/PRSS14 in the cell line also resulted in poor survival of the injected mice (Fig. 1B). These results indicate that, in agreement with other studies (Ihara et al., 2002; Jin et al., 2006), the expression of epithin/PRSS14 in cancer cells could increase tumorigenesis.

Despite the clear epithin/PRSS14-dependent tumorigenic properties of 4T1 cells, Sca-1 expression and metastatic properties were barely observed in cells under standard *in vitro* culture conditions, DMEM supplemented with 10% FBS (Fig. 1C). To get a hint of the *in vivo* environmental factors that can convert 4T1 cells into cells with more tumorigenic properties, we attempted to change the cell culture conditions using other media, including IMDM and RPMI media. Interestingly, the Sca-1 positive-population increased up to 2-fold in 4T1 cells maintained in IMDM for 6 weeks (4T1-I cells) compared to in the cells maintained in DMEM (Fig. 1C). Additionally, the 4T1-I cells lost their cell-to-cell contacts with visible spaces between them and exhibited a more scattered pattern as the shapes of the hybrid EMT state (Pastushenko and Blanpain, 2019) rather than the epithelial state (Fig. 1D). In contrast, these changes were not observed in epithin/PRSS14 knockdown cells (Figs. 1C and 1D, right panels), suggesting that epithin/PRSS14 is involved in such conversion. Notably, 4T1 cells cultured in RPMI for 6 weeks did not show any difference from the cells normally kept in DMEM in terms of Sca-1 surface expression (Fig. 1C) and morphology (Fig. 1D). In agreement with their scattering phenotypes, the migration rate of 4T1-I cells was largely increased in an epithin/PRSS14-dependent manner in the serum gradient-induced Transwell migration assay (Figs. 1E and 1F). In the metastasis assay (as shown in Fig. 1B), however, 4T1-I cells did not show significant difference in survival of mice from those kept in DMEM or RPMI (not shown), suggesting the existence of strong tumorigenic environmental factors *in vivo* that masks their *in vitro* differences. Together, these results showed that 4T1 cells maintained in IMDM could acquire greater tumorigenic potential than those maintained in DMEM or RPMI in an epithin-dependent manner.

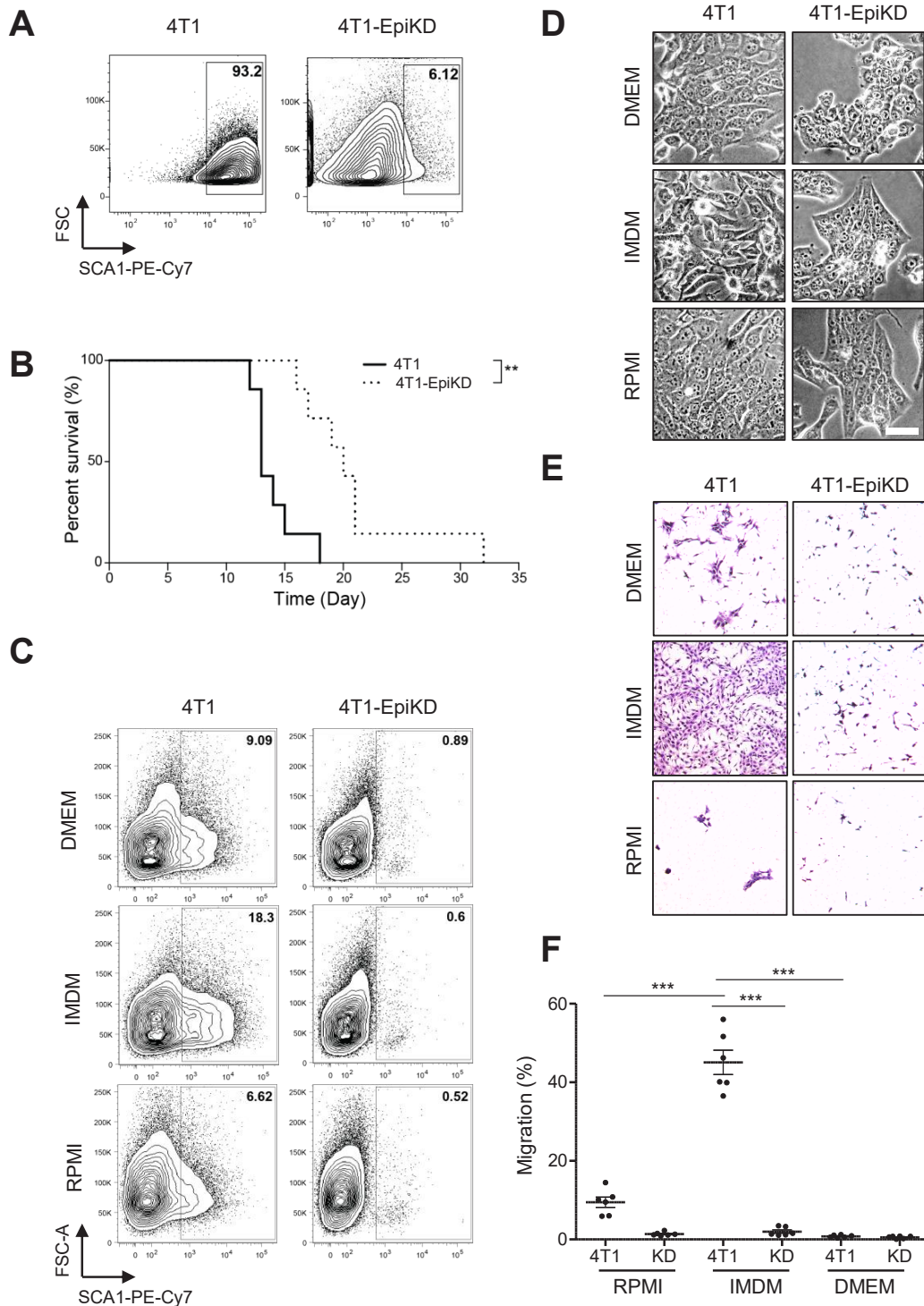


Fig. 1. Epithin/PRSS14 increases tumorigenic potential in an environment-dependent manner. (A) Representative flow cytometry results of primary tumors from orthotopic implantation of 4T1 and epithin/PRSS14-knocked down 4T1 (4T1-EpiKD) cells. Forward scatter (FSC) and Sca-1 levels are shown. The numbers indicate the mean of Sca-1 fluorescence in the region indicated by a box. (B) BALB/c mice in each group were injected with 5×10^5 of 4T1 or 4T1-EpiKD cells through the tail vein and their percent survival was presented ($n = 7$). Survival rate between different conditions was compared using the log-rank (Mantel-Cox) test. $**P < 0.01$. (C) 4T1 and 4T1-EpiKD cell lines grown in different media were analyzed as in (A). (D) Phase contrast images of 4T1 and 4T1-EpiKD cells grown for 6 weeks in DMEM, IMDM, or RPMI are shown. Scale bar = 50 μm . (E) Cells described in (D) were analyzed for their migration abilities using a Transwell migration assay, and the representative results are shown. (F) Migration rates in (E) were quantified by measuring the area covered with migrated cells divided by the total area. Data are presented as mean \pm SE ($n = 6$). $***P < 0.001$ (Tukey's multiple comparison test).

Epithin/PRSS14 ectodomain shedding is correlated with increased expression of cytokine

Because the changes in tumorigenic properties observed under different media conditions depended on epithin/PRSS14, we investigated two major biochemically distinct behaviors of epithin/PRSS14, ectodomain shedding and proteolytic activation, under these conditions. The ectodomain shedding of epithin/PRSS14 is mediated by TACE, which cleaves the region between its SEA and CUB domains (Cho et al., 2017; Lee et al., 2014), whereas its proteolytic activation depends on proteolytic cleavage of the protease activation site located at the N-terminus of the serine protease domain (Kim et al., 2005) (Fig. 2A). Thus, by using western blotting with mAb5 against the C-terminal end of epithin/PRSS14 (Cho et al., 2001), we measured the degree of ectodomain shedding and activation of epithin/PRSS14 in their medium at ~95 kDa and ~30 kDa, respectively. In the assay, ectodomain shedding and proteolytic activation of epithin/PRSS14 increased in 4T1 cells cultured in IMDM compared to that in the cells maintained in DMEM or RPMI (Fig. 2B).

To determine whether changes in gene expression were also involved in the tumorigenic conversion of 4T1 cells (Figs. 1C and 1E), we analyzed the mRNA expression profiles of 4T1-I cells using RNA-seq. In the dataset with *P* values < 0.05, the expression levels of 56 and 170 genes were changed in 4T1-I cells more than 2-fold compared to cells maintained in RPMI and DMEM, respectively (Fig. 2C), with a total of 40 genes common (Fig. 2D). Gene ontology analysis showed that these 40 genes were mainly involved in the immune response and oxidation-reduction processes (Fig. 2E). Notably, several cytokines, such as CXCL5, interleukin 1 α , and CCL2 (Fig. 2D), which are known to regulate inflammation as well as cancer metastasis (Bonapace et al., 2014; Chae et al., 2021; Mantovani et al., 2018; Mao et al., 2020), increased more than ~16-fold, explaining the highly invasive phenotype of 4T1 cells cultured in IMDM (Fig. 1E).

Selenite induces epithin/PRSS14 ectodomain shedding and CCL2 chemokine secretion

Having different invasive phenotypes under different media conditions, we investigated their ingredients in detail and found that sodium selenite is included only in IMDM at a concentration of 0.017 mg/L. When the same concentration of sodium selenite was added to 4T1 cells maintained in DMEM, we observed an increase in epithin/PRSS14 ectodomain shedding (Fig. 3A), and the shedding was more evident and significant at higher doses (Fig. 3B). This suggests that the presence of selenite in the cellular environment can induce epithin/PRSS14 ectodomain shedding, which is further enhanced by other environmental differences in the IMDM condition.

We next examined whether treatment with selenite could induce a similar change in gene expression patterns detected in the RNA-seq analysis, by measuring the secretion of CCL2, one of the chemokines highly expressed under the IMDM condition (Fig. 2D), as a representative example. Indeed, CCL2 secretion from 4T1 cells was increased when the cells were treated with selenite or incubated under IMDM (Fig. 3C). Selenite significantly increased the secretion of CCL2

(Figs. 3D and 3E) as efficiently as LPS, which is known to induce CCL2 expression in various cell types, including 4T1 cells (Yoshimura et al., 2013). Importantly, selenite-induced CCL2 secretion was completely inhibited in epithin/PRSS14 knock-downed cells (Figs. 3F and 3G). These results clearly showed that epithin/PRSS14 is an essential prerequisite for the cellular response to selenite in the secretion of CCL2. We also note that selenite-induced epithin/PRSS14 ectodomain shedding and CCL2 secretion were attenuated by the antioxidant chemical N-acetylcysteine (Fig. 3H), suggesting that the action of selenite is possibly dependent on the production of reactive oxygen species.

Intracellular domain of epithin/PRSS14 induces the expression of CCL2

Previously, we described that ectodomain shedding of epithin/PRSS14 could induce regulated intramembrane proteolysis, which results in the release of the intracellular domain of the protease with transcriptional activity (Cho et al., 2020). Because the epithin/PRSS14-dependent and selenite-induced CCL2 expression (Fig. 2) and secretion (Fig. 3) are correlated with epithin/PRSS14 ectodomain shedding, we hypothesized that the transcription activity of epithin/PRSS14 could be involved in increased CCL2 expression. To test this hypothesis, we first inhibited epithin/PRSS14 ectodomain shedding and analyzed its consequence. When an inhibitor of TACE, an enzyme responsible for epithin/PRSS14 ectodomain shedding (Cho et al., 2020), was treated, the selenite-induced CCL2 expression was inhibited (Fig. 4A). Next, we transiently transfected the epithin/PRSS14 intracellular domain (EICD) in 4T1-EpiKD cells and measured the secretion of CCL2. Consistent with the hypothesis, the medium from EICD-transfected cells contained significantly higher CCL2 secretion than from MOCK cells (Figs. 4B and 4C). Moreover, transfection of EICD in 4T1-EpiKD, although weak but significantly, induced the expression of GFP from CCL2-GFP reporter construct in which the GFP gene is under control of ~1.5 kb-long 5' untranslated region of CCL2 (Fig. 4D). Selenite treatment in 4T1 cells, but not in 4T1-EpiKD cells, also increased the CCL2 promoter activity (Fig. 4E). Lastly, selenite treatment in 4T1 cells induced the characteristic nuclear staining of epithin/PRSS14 (Fig. 4F). These results suggest that the ectodomain shedding-induced intramembrane proteolysis is responsible for selenite-induced CCL2 secretion presumably through the nuclear function of EICD. Finally, we tested the consequences of increased CCL2 secretion in 4T1 cells. When 4T1 cells grown in the Transwell chamber were treated with 400 ng/ml CCL2, their migration rate to the opposite site was greatly increased (Figs. 4G and 4H). Similarly, we observed selenite treatment also can increase the migration rate, which was suppressed by blocking epithin/PRSS14 shedding (Figs. 4I and 4J). Taken together, we concluded that environmental conditions that induce ectodomain shedding of epithin/PRSS14 could enhance the invasiveness of cancer cells by increasing the expression and secretion of pro-invasive soluble factors.

DISCUSSION

In this study, we showed that the presence of selenite in the

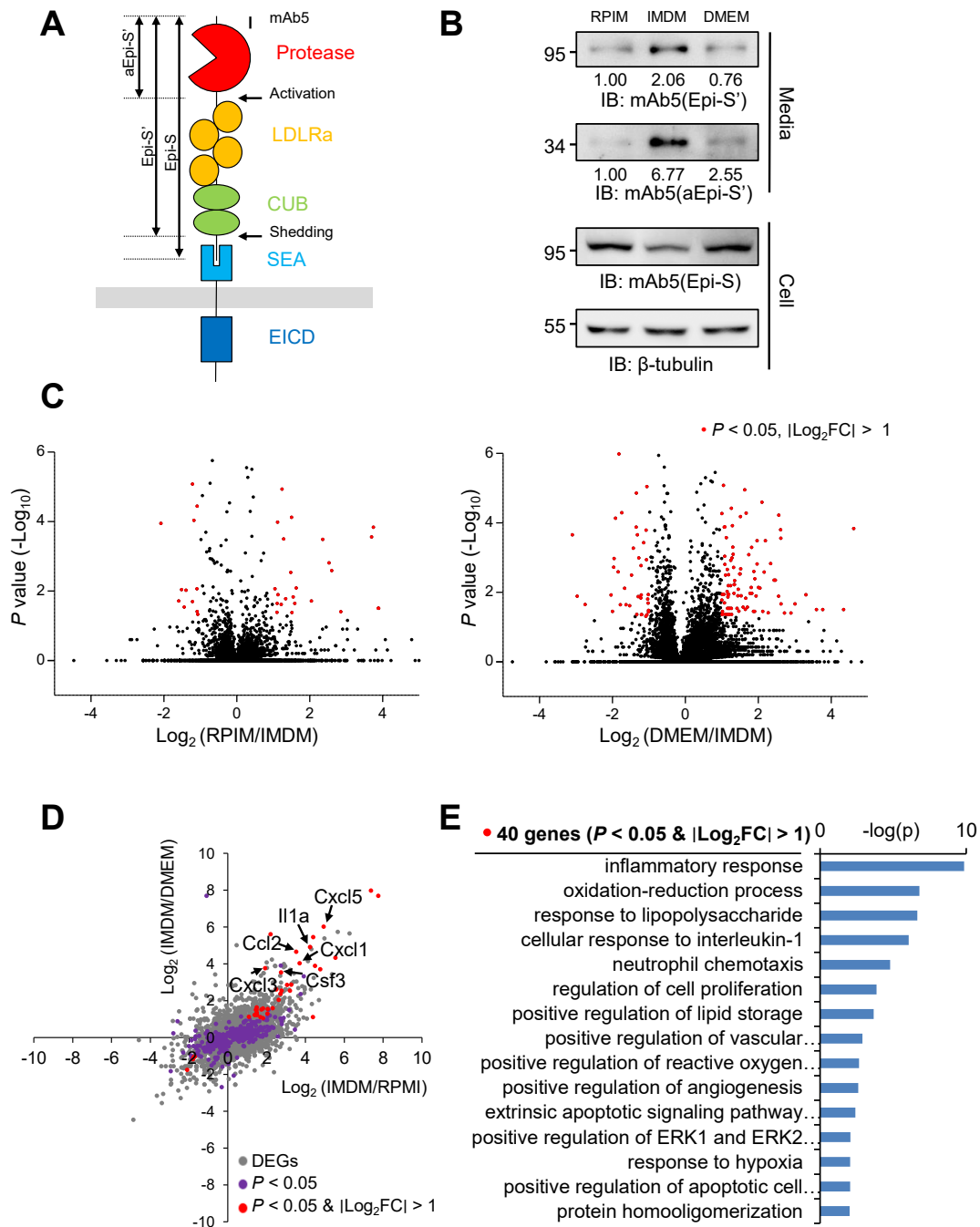


Fig. 2. Epithin/PRSS14 ectodomain shedding and cytokine expression in 4T1 cells depends on the cellular environment. (A) A schematic diagram of epithin/PRSS14 domain structure is illustrated. Cleavages sites for proteolytic activation and ectodomain shedding are indicated. Possible fragments (Epi-S, Epi-S', aEpi-S') of epithin/PRSS14 generated by cleavage are shown. The epitope of monoclonal antibody mAb5 is indicated. SEA, sperm protein, enterokinase, and agrin domains; CUB, complement C1r/C1s, Uegf, Bmp1 domain; LDLRa, low-density lipoprotein receptor a repeat. (B) Media from 4T1 cells incubated under various conditions as indicated for 4 h were analyzed for ectodomain shedding and proteolytic activation of epithin/PRSS14 (top panels). The degree of epithin/PRSS14 and β -tubulin (loading control) in these cells are also shown (bottom panels). IB, immunoblot. (C) Expression of genes in 4T1 cells under various media conditions was analyzed using RNA-seq, and volcano plots for differentially expressed genes (DEGs) between 4T1 cells grown in RPMI and IMDM (left) and between those cultured in DMEM and IMDM (right) are shown. Red dots indicate DEGs with $P < 0.05$ and fold change greater than 2. (D) Gene expression in 4T1 cells cultured in IMDM was compared to in the cells cultured in DMEM (y axis) and RPMI (x axis). DEGs with $P < 0.05$ are in purple, and DEGs with fold change greater than 2 are in red. Representative genes are indicated with arrows. (E) Ontology analysis of 40 genes showing more than 2-fold change in the cells cultured in IMDM compared to the cells cultured in RPMI and DMEM. The top 15 ranked GO terms are indicated with $-\log(P)$ value.

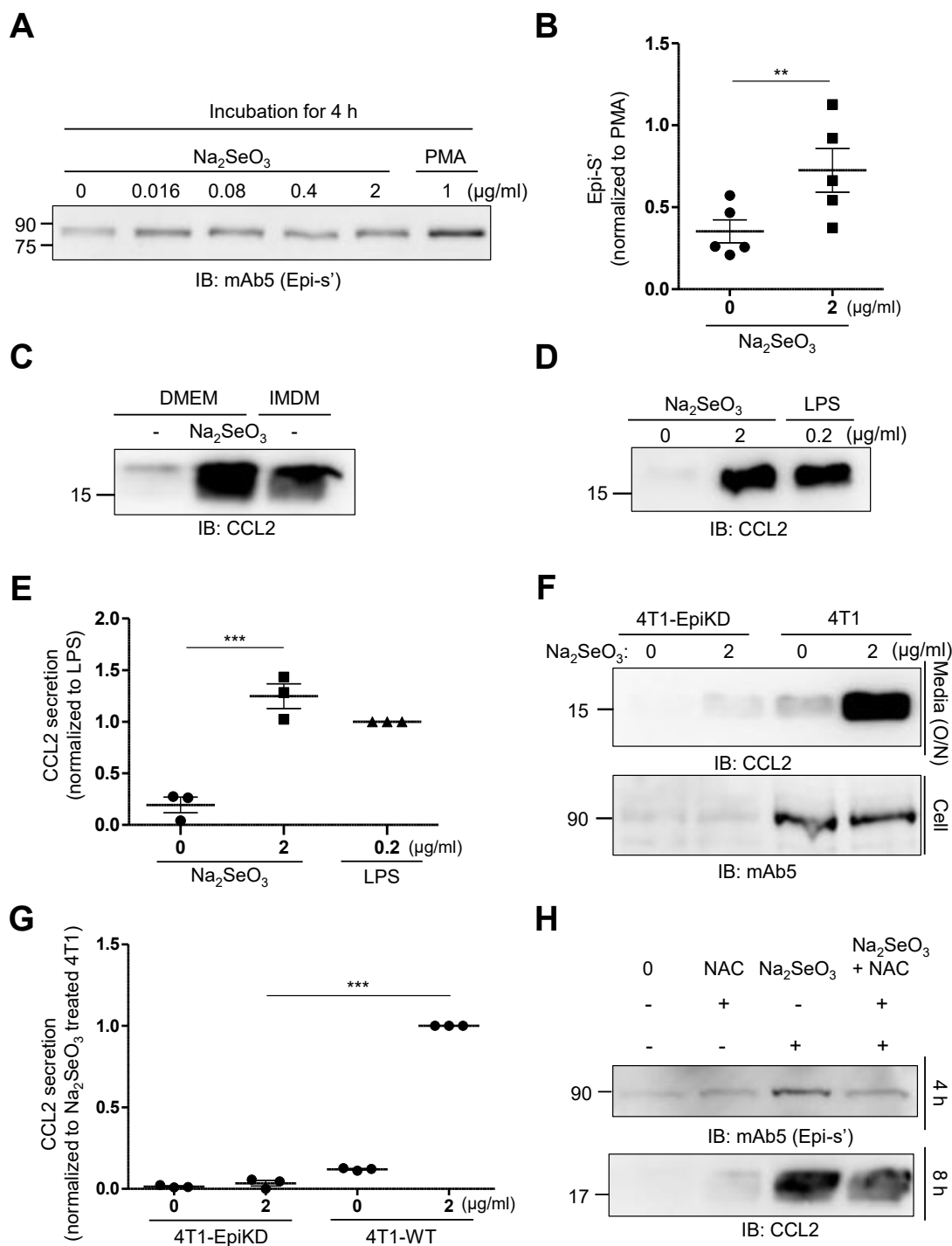


Fig. 3. Sodium selenite induces epithin/PRSS14 shedding and CCL2 secretion. (A) 4T1 cells were treated with increasing concentrations of selenite for 4 h and epithin/PRSS14 shedding was analyzed. PMA treatment was used as a positive control. IB, immunoblot. (B) The degrees of epithin/PRSS14 shedding in cells treated with 0 and 2 µg/ml selenite were normalized against that in PMA-treated cells and are shown in scatter plots. Data are presented as mean ± SE. ***P* < 0.01 (Student's *t*-test, *n* = 5). (C) 4T1 cells grown under DMEM or IMDM condition were treated with sodium selenite for overnight. The degree of CCL2 release was measured by western blot. (D and E) The degree of CCL2 secretion induced by selenite compared to lipopolysaccharide (LPS) was analyzed and quantified as in (B). ****P* < 0.001 (Tukey's multiple comparison test). (F) The degrees of selenite-induced CCL2 secretion in 4T1 and 4T1-EpiKD cells were analyzed. (G) Quantification analysis of CCL2 secretion in (F) normalized cells against that from selenite-treated 4T1 cells is shown. ****P* < 0.001 (Tukey's multiple comparison test, *n* = 3). (H) 4T1 cells treated with selenite and/or N-acetylcysteine (NAC) for indicated times were analyzed for their epithin/PRSS14 shedding and CCL2 secretion.

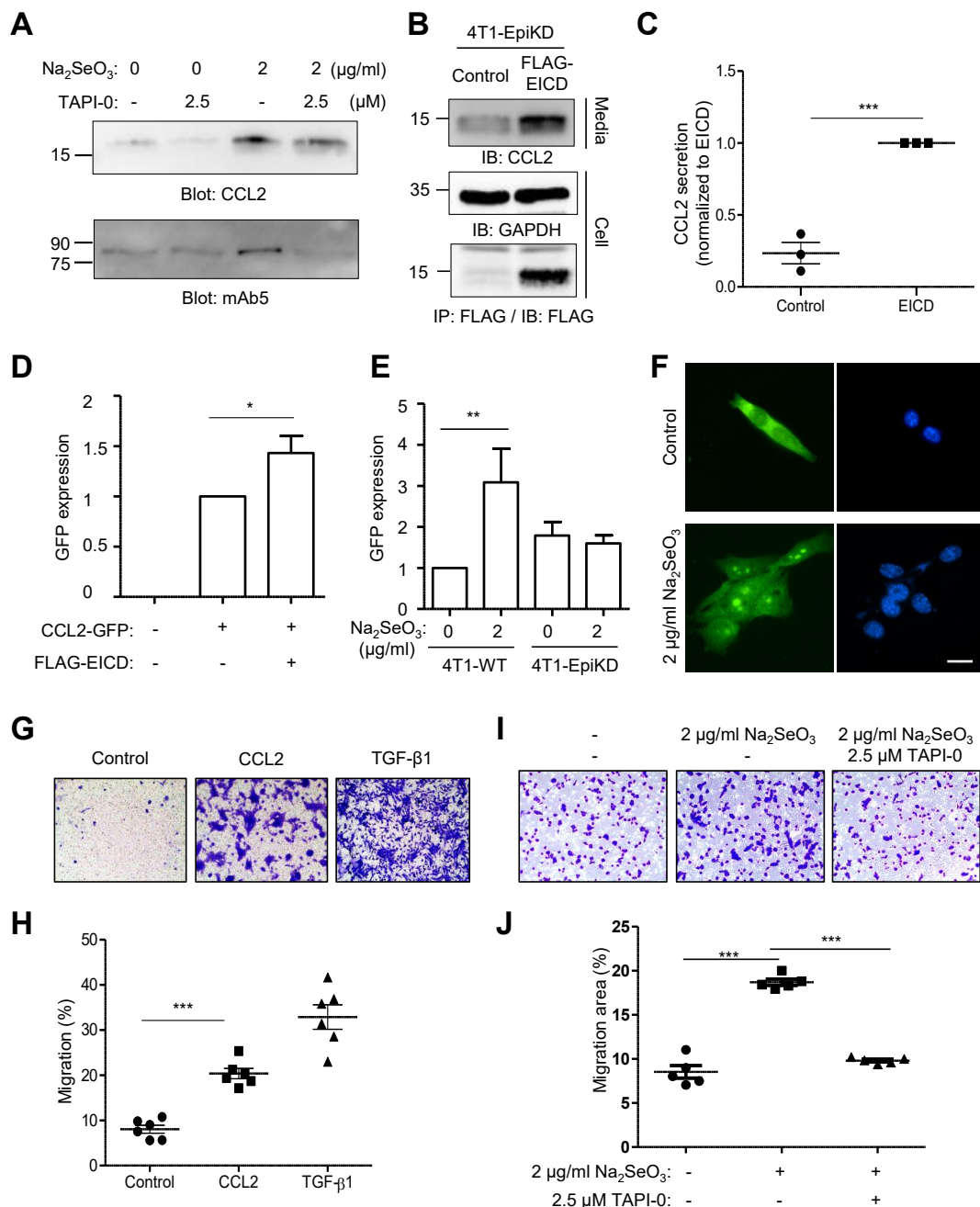


Fig. 4. The EICD induces secretion of pro-invasive cytokine. (A) Serum starved 4T1 cells were treated with selenite and/or TAPI-0 and incubated for 4 h. CCL2 release and Epithin/PRSS14 shedding were measured. (B) 4T1-EpiKD cells were transfected with cDNA encoding FLAG-tagged EICD (FLAG-EICD), and their CCL2 secretion and FLAG-EICD expression were analyzed. The GAPDH blot is shown as a loading control. IB, immunoblot; IP, immunoprecipitation. (C) Quantification of EICD-induced CCL2 secretion is shown. $***P < 0.001$ (Tukey's multiple comparison test, $n = 3$). (D) 4T1-EpiKD cells were transfected with CCL2-GFP reporter and tdTomato as a transfection marker, together with FLAG-EICD or not. GFP expression level in the cells with moderate expression level of tdTomato was measured and normalized by that from control vector transfected cells. $*P < 0.05$ (Tukey's multiple comparison test, $n = 3$). (E) 4T1 and 4T1-EpiKD cells were transfected with CCL2-GFP reporter and TdTomato. Cells were treated with 2 $\mu\text{g/ml}$ selenite, and their GFP expression levels were measured as in (D). GFP expression was normalized against that from non-treated 4T1 cells. $**P < 0.01$ (Tukey's multiple comparison test, $n = 3$). (F) 4T1 cells were treated with selenite, and stained with anti-N antibody against the intracellular domain of epithin/PRSS14 (green). Nucleus was stained with DAPI (blue). Scale bar = 20 μm . (G) 4T1 cells treated with 400 ng/ml CCL2 or 50 ng/ml TGF- β 1 were analyzed for their migration using a Transwell chamber as in Fig. 1E, and representative images are shown. (H) The degree of cell migration in (C) is shown as in Fig. 1F. $***P < 0.001$ (Tukey's multiple comparison test, $n = 6$). (I and J) Effects of selenite and TAPI-0 treatment were analyzed as in (G) and (H). $***P < 0.001$ (Tukey's multiple comparison test, $n = 6$).

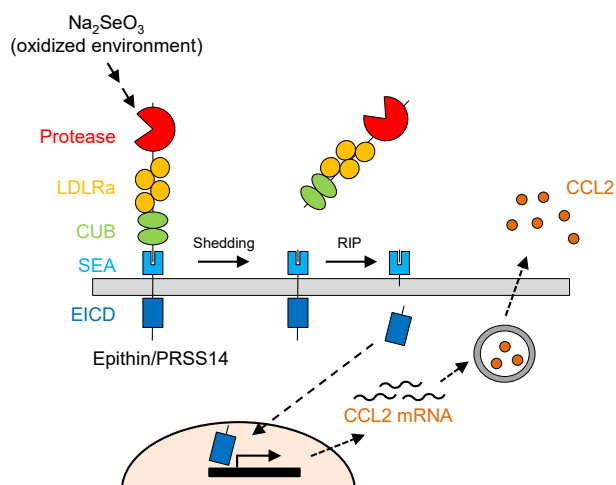


Fig. 5. A hypothetical model on the receptor function of epithin/PRSS14. Upon oxidizing condition, epithin/PRSS14 ectodomain shedding followed by regulated intramembrane proteolysis (RIP) is initiated. The resulting EICD may enhance CCL2 promoter activity.

cell culture environment induces ectodomain shedding of epithin/PRSS14 and the expression of CCL2 in an epithin/PRSS14-dependent manner (Fig. 5). We also showed that expression of the intracellular domain of epithin/PRSS14, which could be generated by intramembrane proteolysis upon its ectodomain shedding (Cho et al., 2020), induces the expression of CCL2; thus, linking the environment-sensitive ectodomain shedding to the expression of soluble pro-invasive factors (Fig. 5). Because intramembrane proteolysis of epithin/PRSS14 is the spontaneous follow-up event of ectodomain shedding (Cho et al., 2020; Lal and Caplan, 2011), the membrane protease could be considered a cell surface receptor that, when activated by conditions promoting its shedding, regulates gene expression. Thus, known inducers of its shedding, including sphingosine-1-phosphate (Benaud et al., 2001; 2002), TGF- β (Lee et al., 2014), metal ions (Wang et al., 2014), hypoxia (Kim et al., 2010) and its own proteolytic activation (Cho et al., 2005; Tseng et al., 2017), could act as agonists of the extraordinary receptor.

What would be the benefit of coupling the transcriptional function of epithin/PRSS14 with its ectodomain shedding? In this study, we found that the expression of cytokines with pro-metastatic functions was predominantly increased in the shedding-prone condition in breast cancer cells (Fig. 2D). Consistently, our previous analysis of thymoma cells also revealed that overexpression of the EICD increased the expression of cytokines with functions related to cell migration and invasion (Cho et al., 2020), showing that the expression of such soluble factors is a common consequence of epithin/PRSS14 ectodomain shedding in these cancer cells. Considering that the released epithin/PRSS14 ectodomain bearing the protease activity could degrade the extracellular matrix and thereby make a path at the extracellular environmental level (Jin et al., 2006), simultaneous secretion of pro-invasive cytokines appears to functionally complement the proteo-

lytic roles by stimulating the cancer cells in an autocrine or paracrine manner and increasing their mobility at the cellular level, as shown in Fig. 4G. Therefore, we believe that these functionally related dual roles of epithin/PRSS14 are the reason why epithin/PRSS14-positive cancers are more aggressive (Ihara et al., 2002; Jin et al., 2006; Kim et al., 2011; List et al., 2005) and have poor prognoses (Ha et al., 2014; Kim et al., 2016; Oberst et al., 2002; Saleem et al., 2006).

Although selenium itself is an essential trace mineral that is incorporated into the active site of selenoenzymes alleviating oxidative stress (Rayman, 2000), selenium compounds, such as sodium selenite, act as a pro-oxidant instead. Thus, they generate reactive oxygen species during the reactions with thiols in the cells (Kursvietiene et al., 2020). Indeed, the selenite-induced epithin/PRSS14 shedding seems to be caused by the pro-oxidative property of selenite because treatment with N-acetylcysteine could largely inhibit selenite-induced shedding (Fig. 3H). Interestingly, oxidative stress is known to activate TACE/ADAM17, the enzyme responsible for epithin/PRSS14 ectodomain shedding (Brill et al., 2009). This suggests that IMDM and/or selenite may exert their effects by providing an oxidative environment which results in increased TACE/ADAM17 activity for the ectodomain shedding of epithin/PRSS14. If this is the case, the well-known oxidative condition found in cancer (Szatrowski and Nathan, 1991) may induce ectodomain shedding of epithin/PRSS14 and subsequently activate its transcriptional function. Thus, it would be of interest to test whether the pro-metastatic effects of oxidative conditions (Aggarwal et al., 2019; Liao et al., 2019) could be explained, at least in part, by the environment-sensitive ectodomain shedding of epithin/PRSS14.

In conclusion, this study showed an extraordinary method by which cancer cells interpret and respond to their extracellular environment by utilizing the environment-sensing function of epithin/PRSS14. We believe that this research will provide a basis for further studies on the molecular mechanism linking oxidative environmental conditions and epithin/PRSS14 ectodomain shedding in cancer, which would provide not only a better understanding of cancer metastasis but also competitive ways to prevent the disease by blocking the dual functions of epithin/PRSS14.

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AUTHOR CONTRIBUTIONS

E.H.C. and M.G.K. designed the project. J.J., E.H.C., Y.C., B.G., and K.Y.K. performed the experiments. J.J., E.H.C., Y.C., M.G.K., and C.K. analyzed the data. J.J., E.H.C., and M.G.K. wrote the paper, which was edited by C.K.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Jiyoung Jang <https://orcid.org/0000-0002-7462-1235>

Eun Hye Cho <https://orcid.org/0000-0001-7752-4441>
 Youngkyung Cho <https://orcid.org/0000-0002-8677-6162>
 Binderya Ganzorig <https://orcid.org/0000-0001-8135-4318>
 Ki Yeon Kim <https://orcid.org/0000-0002-8575-7680>
 Moon Gyo Kim <https://orcid.org/0000-0002-3012-953X>
 Chunggho Kim <https://orcid.org/0000-0003-4343-6333>

REFERENCES

- Aggarwal, V., Tuli, H.S., Varol, A., Thakral, F., Yerer, M.B., Sak, K., Varol, M., Jain, A., Khan, M.A., and Sethi, G. (2019). Role of reactive oxygen species in cancer progression: molecular mechanisms and recent advancements. *Biomolecules* 9, 735.
- Antalis, T.M., Buzza, M.S., Hodge, K.M., Hooper, J.D., and Netzel-Arnett, S. (2010). The cutting edge: membrane-anchored serine protease activities in the pericellular microenvironment. *Biochem. J.* 428, 325-346.
- Aslakson, C.J. and Miller, F.R. (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. *Cancer Res.* 52, 1399-1405.
- Benaud, C., Dickson, R.B., and Lin, C.Y. (2001). Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor. *Eur. J. Biochem.* 268, 1439-1447.
- Benaud, C., Oberst, M., Hobson, J.P., Spiegel, S., Dickson, R.B., and Lin, C.Y. (2002). Sphingosine 1-phosphate, present in serum-derived lipoproteins, activates matriptase. *J. Biol. Chem.* 277, 10539-10546.
- Bonapace, L., Coissieux, M.M., Wyckoff, J., Mertz, K.D., Varga, Z., Junt, T., and Bentires-Alj, M. (2014). Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. *Nature* 515, 130-133.
- Brill, A., Chauhan, A.K., Canault, M., Walsh, M.T., Bergmeier, W., and Wagner, D.D. (2009). Oxidative stress activates ADAM17/TACE and induces its target receptor shedding in platelets in a p38-dependent fashion. *Cardiovasc. Res.* 84, 137-144.
- Chae, D.S., Han, S., Lee, M.K., and Kim, S.W. (2021). Genome edited Sirt1-Overexpressing human mesenchymal stem cells exhibit therapeutic effects in treating collagen-induced arthritis. *Mol. Cells* 44, 245-253.
- Cho, E.G., Kim, M.G., Kim, C., Kim, S.R., Seong, I.S., Chung, C., Schwartz, R.H., and Park, D. (2001). N-terminal processing is essential for release of epithin, a mouse type II membrane serine protease. *J. Biol. Chem.* 276, 44581-44589.
- Cho, E.G., Schwartz, R.H., and Kim, M.G. (2005). Shedding of membrane epithin is blocked without LDLRA4 and its protease activation site. *Biochem. Biophys. Res. Commun.* 327, 328-334.
- Cho, Y., Kim, S.B., Kim, J., Pham, A.V.Q., Yoon, M.J., Park, J.H., Hwang, K.T., Park, D., Cho, Y., Kim, M.G., et al. (2020). Intramembrane proteolysis of an extracellular serine protease, epithin/PRSS14, enables its intracellular nuclear function. *BMC Biol.* 18, 60.
- Cho, Y., Park, D., and Kim, C. (2017). Disruption of TACE-filamin interaction can inhibit TACE-mediated ectodomain shedding. *Biochem. Biophys. Res. Commun.* 490, 997-1003.
- Ha, S.Y., Kim, K.Y., Lee, N.K., Kim, M.G., and Kim, S.H. (2014). Overexpression of matriptase correlates with poor prognosis in esophageal squamous cell carcinoma. *Virchows Arch.* 464, 19-27.
- Ihara, S., Miyoshi, E., Ko, J.H., Murata, K., Nakahara, S., Honke, K., Dickson, R.B., Lin, C.Y., and Taniguchi, N. (2002). Prometastatic effect of N-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding beta 1-6 GlcNAc branching. *J. Biol. Chem.* 277, 16960-16967.
- Jin, X., Yagi, M., Akiyama, N., Hirosaki, T., Higashi, S., Lin, C.Y., Dickson, R.B., Kitamura, H., and Miyazaki, K. (2006). Matriptase activates stromelysin (MMP-3) and promotes tumor growth and angiogenesis. *Cancer Sci.* 97, 1327-1334.
- Kang, J.Y., Dolled-Filhart, M., Ocal, I.T., Singh, B., Lin, C.Y., Dickson, R.B., Rimm, D.L., and Camp, R.L. (2003). Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer. *Cancer Res.* 63, 1101-1105.
- Kim, C., Cho, Y., Kang, C.H., Kim, M.G., Lee, H., Cho, E.G., and Park, D. (2005). Filamin is essential for shedding of the transmembrane serine protease, epithin. *EMBO Rep.* 6, 1045-1051.
- Kim, C., Lee, H.S., Lee, D., Lee, S.D., Cho, E.G., Yang, S.J., Kim, S.B., Park, D., and Kim, M.G. (2011). Epithin/PRSS14 proteolytically regulates angiotensin receptor Tie2 during transendothelial migration. *Blood* 117, 1415-1424.
- Kim, D., Perlea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* 14, R36.
- Kim, K.Y., Lee, G., Yoon, M., Cho, E.H., Park, C.S., and Kim, M.G. (2015). Expression analyses revealed thymic stromal co-transporter/Slc46A2 is in stem cell populations and is a putative tumor suppressor. *Mol. Cells* 38, 548-561.
- Kim, M.G., Chen, C., Lyu, M.S., Cho, E.G., Park, D., Kozak, C., and Schwartz, R.H. (1999). Cloning and chromosomal mapping of a gene isolated from thymic stromal cells encoding a new mouse type II membrane serine protease, epithin, containing four LDL receptor modules and two CUB domains. *Immunogenetics* 49, 420-428.
- Kim, S., Yang, J.W., Kim, C., and Kim, M.G. (2016). Impact of suppression of tumorigenicity 14 (ST14)/serine protease 14 (Prss14) expression analysis on the prognosis and management of estrogen receptor negative breast cancer. *Oncotarget* 7, 34643-34663.
- Kim, S.B., Lee, D., Jeong, J.W., Kim, C., Park, D., and Kim, M.G. (2010). Soluble epithin/PRSS14 secreted from cancer cells contains active angiogenic potential. *Mol. Cells* 29, 617-623.
- Ko, C.J., Lan, S.W., Lu, Y.C., Cheng, T.S., Lai, P.F., Tsai, C.H., Hsu, T.W., Lin, H.Y., Shyu, H.Y., Wu, S.R., et al. (2017). Inhibition of cyclooxygenase-2-mediated matriptase activation contributes to the suppression of prostate cancer cell motility and metastasis. *Oncogene* 36, 4597-4609.
- Kuhnle, N., Dederer, V., and Lemberg, M.K. (2019). Intramembrane proteolysis at a glance: from signalling to protein degradation. *J. Cell Sci.* 132, jcs217745.
- Kursvietiene, L., Mongirdiene, A., Bernatoniene, J., Sulinskiene, J., and Stanviciene, I. (2020). Selenium anticancer properties and impact on cellular redox status. *Antioxidants (Basel)* 9, 80.
- Lal, M. and Caplan, M. (2011). Regulated intramembrane proteolysis: signaling pathways and biological functions. *Physiology (Bethesda)* 26, 34-44.
- Lee, H.S., Park, B.M., Cho, Y., Kim, S., Kim, C., Kim, M.G., and Park, D. (2014). Shedding of epithin/PRSS14 is induced by TGF-beta and mediated by tumor necrosis factor-alpha converting enzyme. *Biochem. Biophys. Res. Commun.* 452, 1084-1090.
- Levitin, F., Stern, O., Weiss, M., Gil-Henn, C., Ziv, R., Prokocimer, Z., Smorodinsky, N.I., Rubinstein, D.B., and Wreschner, D.H. (2005). The MUC1 SEA module is a self-cleaving domain. *J. Biol. Chem.* 280, 33374-33386.
- Liao, Z., Chua, D., and Tan, N.S. (2019). Reactive oxygen species: a volatile driver of field cancerization and metastasis. *Mol. Cancer* 18, 65.
- List, K., Szabo, R., Molinolo, A., Sriuranpong, V., Redeye, V., Murdock, T., Burke, B., Nielsen, B.S., Gutkind, J.S., and Bugge, T.H. (2005). Deregulated matriptase causes ras-independent multistage carcinogenesis and promotes ras-mediated malignant transformation. *Genes Dev.* 19, 1934-1950.
- Mantovani, A., Barajon, I., and Garlanda, C. (2018). IL-1 and IL-1 regulatory pathways in cancer progression and therapy. *Immunol. Rev.* 281, 57-61.
- Mao, Z., Zhang, J., Shi, Y., Li, W., Shi, H., Ji, R., Mao, F., Qian, H., Xu, W.,

- and Zhang, X. (2020). CXCL5 promotes gastric cancer metastasis by inducing epithelial-mesenchymal transition and activating neutrophils. *Oncogenesis* *9*, 63.
- Martin, C.E. and List, K. (2019). Cell surface-anchored serine proteases in cancer progression and metastasis. *Cancer Metastasis Rev.* *38*, 357-387.
- Oberst, M., Anders, J., Xie, B., Singh, B., Ossandon, M., Johnson, M., Dickson, R.B., and Lin, C.Y. (2001). Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. *Am. J. Pathol.* *158*, 1301-1311.
- Oberst, M.D., Johnson, M.D., Dickson, R.B., Lin, C.Y., Singh, B., Stewart, M., Williams, A., al-Nafussi, A., Smyth, J.F., Gabra, H., et al. (2002). Expression of the serine protease matriptase and its inhibitor HAI-1 in epithelial ovarian cancer: correlation with clinical outcome and tumor clinicopathological parameters. *Clin. Cancer Res.* *8*, 1101-1107.
- Pastushenko, I. and Blanpain, C. (2019). EMT transition states during tumor progression and metastasis. *Trends Cell Biol.* *29*, 212-226.
- Rayman, M.P. (2000). The importance of selenium to human health. *Lancet* *356*, 233-241.
- Saleem, M., Adhami, V.M., Zhong, W., Longley, B.J., Lin, C.Y., Dickson, R.B., Reagan-Shaw, S., Jarrard, D.F., and Mukhtar, H. (2006). A novel biomarker for staging human prostate adenocarcinoma: overexpression of matriptase with concomitant loss of its inhibitor, hepatocyte growth factor activator inhibitor-1. *Cancer Epidemiol. Biomarkers Prev.* *15*, 217-227.
- Sales, K.U., Friis, S., Konkkel, J.E., Godiksen, S., Hatakeyama, M., Hansen, K.K., Rogatto, S.R., Szabo, R., Vogel, L.K., Chen, W., et al. (2015). Non-hematopoietic PAR-2 is essential for matriptase-driven pre-malignant progression and potentiation of ras-mediated squamous cell carcinogenesis. *Oncogene* *34*, 346-356.
- Szatrowski, T.P. and Nathan, C.F. (1991). Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res.* *51*, 794-798.
- Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D.R., Pimentel, H., Salzberg, S.L., Rinn, J.L., and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* *7*, 562-578.
- Tsai, C.H., Teng, C.H., Tu, Y.T., Cheng, T.S., Wu, S.R., Ko, C.J., Shyu, H.Y., Lan, S.W., Huang, H.P., Tzeng, S.F., et al. (2014). HAI-2 suppresses the invasive growth and metastasis of prostate cancer through regulation of matriptase. *Oncogene* *33*, 4643-4652.
- Tseng, C.C., Jia, B., Barndt, R., Gu, Y., Chen, C.Y., Tseng, I.C., Su, S.F., Wang, J.K., Johnson, M.D., and Lin, C.Y. (2017). Matriptase shedding is closely coupled with matriptase zymogen activation and requires de novo proteolytic cleavage likely involving its own activity. *PLoS One* *12*, e0183507.
- Wang, J.K., Teng, I.J., Lo, T.J., Moore, S., Yeo, Y.H., Teng, Y.C., Kaul, M., Chen, C.C., Zuo, A.H., Chou, F.P., et al. (2014). Matriptase autoactivation is tightly regulated by the cellular chemical environments. *PLoS One* *9*, e93899.
- Wei, T., Simk, V., Levy, M., Xie, Y., Jin, Y., and Zemla, J. (2017). R package "corrplot": Visualization of a Correlation Matrix (Version 0.84).
- Yoshimura, T., Howard, O.M., Ito, T., Kuwabara, M., Matsukawa, A., Chen, K., Liu, Y., Liu, M., Oppenheim, J.J., and Wang, J.M. (2013). Monocyte chemoattractant protein-1/CCL2 produced by stromal cells promotes lung metastasis of 4T1 murine breast cancer cells. *PLoS One* *8*, e58791.
- Zoratti, G.L., Tanabe, L.M., Varela, F.A., Murray, A.S., Bergum, C., Colombo, E., Lang, J.E., Molinolo, A.A., Leduc, R., Marsault, E., et al. (2015). Targeting matriptase in breast cancer abrogates tumour progression via impairment of stromal-epithelial growth factor signalling. *Nat. Commun.* *6*, 6776.