Original Article

Dexmedetomidine promotes breast cancer cell migration through Rab11-mediated secretion of exosomal TMPRSS2

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Background: Dexmedetomidine (DEX), a highly selective α2-adrenergic receptor agonist, has been reported to increase the malignancy of breast cancer cells *in vitro* and stimulate tumor growth in mice. Transmembrane protease serine 2 (TMPRSS2) demonstrates proteolytic activity, resulting in degradation of the extracellular matrix (ECM). This study investigated whether and how TMPRSS2 regulates migration of DEX-treated breast cancer cells.

Methods: Breast cancer cell lines MCF-7 and MDA-MB-231 were treated with DEX and scratch assay was performed. Expressions of TMPRSS2, α2-adrenergic receptor, phospho-STAT3^{Tyr705}, Rab11, and ECM components were assessed using real-time polymerase chain reaction (real-time PCR), Western blotting, and immunofluorescence staining. ELISA and ultracentrifugation were used to quantify secreted exosomal proteins. Knockdown assay was used to inhibit the expression of TMPRSS2 and Rab11.

Results: DEX significantly increased the migration of MCF-7 and MDA-MB-231, which was accompanied by the upregulation and colocalization of TMPRSS2 and α2-adrenergic receptor. Nuclear phospho-STAT3^{Tyr705} was increased dramatically following DEX treatment, and TMPRSS2 upregulation was significantly suppressed by the STAT3 inhibitor WP1066. Meanwhile, TMPRSS2 knockdown decreased DEX-induced cellular migration. TMPRSS2 and Rab11 were significantly detected in the media and the isolated exosomes from DEX-treated cells, and their colocalization was also revealed. Rab11 knockdown prevented exosomal TMPRSS2 from increasing in DEX-treated cells. In normal cultured MDA-MB-231, migration was increased by Rab11-positive exosomes isolated from DEX-treated MCF-7. Moreover, transmission electron microscopy showed that Rab11-positive exosomes enriched more components than Rab11-negative exosomes. Additionally, a reduction in ECM components fibronectin, collagen IV, matrix metallopeptidase 16, and Tenascin C was detected after DEX treatment, but was prohibited when TMPRSS2 or Rab11 were knocked down.

Conclusions: This study provides evidence that DEX upregulates TMPRSS2 expression via the activation of α2-adrenergic receptor/STAT3 signaling and promotes TMPRSS2 secretion in exosomes through Rab11, thus resulting in degradation of the ECM, which is responsible for DEX-induced migration of breast cancer cells.

Keywords: Dexmedetomidine (DEX); transmembrane protease serine 2 (TMPRSS2); exosome; Rab11; breast cancer; cellular migration

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Introduction

Breast cancer is one of the most common types of cancer affecting women worldwide (1). Surgery is currently the primary approach in breast cancer treatment. However, owing to the high rates of recurrence and metastasis, some patients with breast cancer still have a poor prognosis after treatment (1). Dexmedetomidine (DEX) is a new generation α2-adrenergic receptor agonist that is highly selective and serves as a sedative and an adjuvant to anesthetic strategies in the perioperative context (2). By binding to α2-adrenergic receptors, DEX plays an important role in various biological processes (3). In tumors of a more malignant phenotype characteristic, α2-adrenergic receptor is overexpressed and, in breast cancer, is associated with relapse and poor clinical outcome (4,5). Recent studies showed that DEX increased the proliferation, migration and invasion ability of breast cancer cells *in vitro*, possibly by regulating the α2-adrenergic receptor/Erk1/2 signaling pathway (6). An *in vivo* study showed that DEX mainly increased tumor-cell retention and metastasis in mammary adenocarcinoma in rats via α2-adrenergic receptors (7). Interestingly, a prospective randomized clinical study that used breast cancer cell line MCF-7 showed that the postoperative serum of patients who received 2 μg/kg DEX for two hours during surgery but did not receive saline, showed significantly higher proliferation, migration, and invasion compared to serum taken preoperatively (8). This study provides indirect evidence indicative of the possibility of deleterious effects of the perioperative utilization of DEX in the prognosis of breast cancer. Nevertheless, the underlying mechanisms by which DEX promotes breast cancer cell migration remain elusive.

Type II transmembrane serine protease (TTSP) plays a key role in tumor growth, invasion, and metastasis (9). There is a significant association between genetic variants of *TTSP* genes and the risk and prognosis of breast cancer (10). A member of the TTSP subfamily, transmembrane protease serine 2 (TMPRSS2) is regulated by androgens and is strongly related to prostate cancer (11). In addition, a correlation exists between TMPRSS2 protein levels and the progression of prostate cancer (12). By activating matriptase and disrupting the extracellular matrix (ECM), TMPRSS2 can promote the growth, invasion, and metastasis of prostate cancer cells *in vitro* (13). TMPRSS2 is expressed as a 70-kDa full-length protein and a 32-kDa cleaved protease domain. The cleaved protease domain of TMPRSS2 is thought to be secreted into cell media after autocleavage (14). Notably, the expression of TMPRSS2 in both its full-length and secreted forms was detected in breast cancer cell line MCF-7 (15). Meanwhile, the influence of TMPRSS2 on the migration of DEX-treated breast cancer cells is still unclear. We hypothesized that DEX may regulate the migration of breast cancer cells through the upregulation of TMPRSS2.

Methods

Antibodies

The following commercial primary antibodies were obtained: rabbit monoclonal anti-TMPRSS2, rabbit anti-heat shock protein 70 (Hsp70), rabbit anti-a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), rabbit anti-EEA1, rabbit anti-collagen IV, rabbit anti-matrix metallopeptidase 16 (MMP16) and rabbit anti-tenascin C antibodies from Abcam (Cambridge, MA, USA); rabbit anti-α2-adrenergic receptor and mouse anti-Hsp90 antibodies from Sigma-Aldrich (St. Louis, MO, USA); mouse anti-signal transducer and activator of transcription 3 (STAT3), mouse anti-GAPDH, mouse anti-histone H3, rabbit anti-Rab35, rabbit anti-fibronectin, rabbit anti-phospho-STAT3^{Tyr705}, rabbit anti-Rab7, rabbit anti-Rab4 and rabbit anti-Rab11 antibodies from Cell Signaling (Danvers, MA, USA); mouse anti-Rab11 antibody from BD Biosciences (San Jose, CA, USA).

Cell culture and treatment

Human breast adenocarcinoma cell lines MCF-7 and MDA-MB-231 were obtained from ATCC (ATCC, Manassas, VA, USA) and maintained and stored at the College of Life Science of Northeast Agricultural University (Harbin, China). The MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (cat. no. 16000–044; Gibco/Thermo Fisher Scientific) and 1% penicillin-streptomycin (cat. no. 15140–122; Gibco/Thermo Fisher Scientific) at 37 ℃ in a humidified atmosphere of 5% CO_2.

Cells were treated with DEX (Sigma-Aldrich) at different concentrations for various periods of time (0, 12, 24 and 48 h). The concentrations of DEX ranged from 0.01 to 1 μM, based on previous studies (6,16-18). In some cases, the cells were pretreated for 1 h with STAT3 inhibitor III (WP1066, cat. no. 573097; EMD Millipore, Kankakee, IL, USA) before DEX was added. They were subsequently incubated for the

indicated time periods with or without WP1066.

Knockdown assay

Two validated commercial siRNAs specifically targeted against different coding sites of human TMPRSS2 were used to knockdown TMPRSS2 expression (siTMP-a, cat. no. SASI_Hs01_00072210; siTMP-b, cat. no. SASI_Hs01_00072211; Sigma-Aldrich). Simultaneously, Rab11a and Rab11b isoforms were knocked down using siRNAs targeted against human Rab11a (5'-ugucagacagacgcgaaaa-3') and Rab11b (5'-gcaccugaccuaugagaac-3'), respectively. MISSION® siRNA Universal Negative Control #1 (siCTL, cat. no. SIC-001; Sigma-Aldrich) was used as the control. The siRNAs were introduced at the final concentration of 20 nM with RNAiMAX (cat. no. 13778-075; Invitrogen/Thermo Fisher Scientific). After 48 h, the cells were harvested.

Reverse transcription and real time polymerase chain reaction (PCR)

Total RNA was extracted using Trizol reagent (cat. no. 15596026; Invitrogen) according to the manufacturer's instructions. SuperScript™ III First-Strand Synthesis Kit (cat. no. 11752-050; Invitrogen) was used to reverse 2 μg of RNA to the complementary DNA (cDNA). The PCR reaction included 1 × SYBR Green PCR Master Mix (cat. no. 1725270; Bio-Rad, Hercules, CA, USA), 2 μL of cDNA, and 0.2 μM of the specific primer pairs. The primers for TMPRSS2 are forward 5'-gtgacagtggagggcctctgg-3' and reverse 5'-cgtcaaggacgaagaccatgt-3', respectively. The primers for GAPDH are forward 5'-tgtgtccgtcgtggatctga-3' and reverse 5'-cctgcttcaccaccttcttga-3', respectively. Real time PCR was performed on a 7500 fast Real-Time PCR system (Applied Biosystems/Thermo Fisher Scientific) with a two-step PCR program: 1 cycle of 10 min at 95 ℃; 40 cycles of 15 sec at 95 ℃, 45 sec at 56 ℃, 30 sec at 72 ℃. The relative expression levels of TMPRSS2 were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the house-keeping gene GAPDH. The fold change over the controls was presented and compared.

Scratch assay

The migration ability of the MCF-7 and MDA-MAB-231 cells was assessed by performing scratch assay, as described in the literature. Cells were seeded in 6-well plates and cultured when they reached 90% confluence. A scratch or wound was made by scraping a 200 μL sterile plastic pipette tip across the surface of the cell layers. The cells were rinsed with media three times, and then maintained in a fresh serum free medium for 24 or 48 h. The wound was photographed in three randomly selected fields under a phase contrast microscope (Olympus, Tokyo, Japan) at 0 h and at 48 h after the scratch. The experiment was carried out in triplicate, and the migration rate was calculated and compared between different conditions.

ELISA assay

Cell culture media were collected and centrifuged for 10 min at 12,000 rpm at 4 ℃. The supernatant was collected and 100 μL was used for measurement of TMPRSS2 with Human TMPRSS2 ELISA Kit (cat. no. GWB-KBBFP3; Genway Biotech Inc., San Diego, CA, USA) according to the manufacturer's protocol.

Ultracentrifugation: isolation of exosomes

Exosomes were isolated from the cell culture media. Briefly, the media were harvested from treated MCF-7 cells, and then centrifuged in a Beckman Coulter Allegra X-15R centrifuge at 3,000 g at 4 ℃ for 20 min to remove any detached cells. The supernatant was collected and centrifuged in a Beckman Coulter Optima L-80XP Ultracentrifuge at 10,000 g at 4 ℃ for 90 min with a Type 50.2 Ti rotor to remove contaminating apoptotic bodies, microvesicles, and cell debris. The clarified media were then centrifuged in a Beckman Coulter Optima L-80XP Ultracentrifuge at 120,000 g at 4 ℃ for 120 min with a Type 50.2 Ti rotor to pellet exosomes. The supernatant was carefully removed, and the crude exosome-containing pellets were resuspended in ice-cold PBS.

For transmission electron microscopy, the samples were fixed with 4% glutaraldehyde-formalin, and then sent to Northeast Agriculture University Microscopy Research Laboratory Core (Harbin, China) for preparation. The exosomes were examined and imaged with a JEOL 1010 electron microscope (Peabody, MA, USA).

Western blot

Cells were lysed in RIPA buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40] supplemented with 1×

protease and phosphatase inhibitor (cat. no. 78441; Thermo Fisher Scientific). After centrifugation at 4 ℃ for 20 min, the supernatant was carefully collected and quantitated, before being stored at –80 ℃ for further analysis. Nuclear protein was isolated using the Nuclear Extraction Kit (cat. no. ab113474; Abcam). Equal amounts of proteins and exosomes were loaded onto 7.5 or 12.5% SDS polyacrylamide gel, and then transferred to a nitrocellulose membrane (cat. no. 88025; Thermo Fisher Scientific). The membrane was blocked for 1 h in 5% nonfat milk or bovine serum albumin, and then incubation with the indicated primary antibodies took place over-night at 4 ℃. After incubation with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (cat. no. G-21234 and G-21040; Invitrogen) for 1 h at room temperature, the signals were detected using ECL Western Blotting Substrate (cat. no. 32109; Pierce/Thermo Fisher Scientific). To remove the primary antibodies for reblotting with the other primary antibody, Stripping Buffer for Western blot (Thermo Fisher Scientific) was applied to some blots. The specific bands were quantitated using Image J (Version 1.51s; National Institute of Health, Rockville, MD, USA).

Indirect immunofluorescence

Cells were cultured on cover slides and treated as described in the literature. The cells were then fixed in 4% paraformaldehyde for 15 min at room temperature, washed 3 times, and then permeabilized with 0.1% Triton X-100 for 10 min. Non-specific staining was blocked with 10% goat serum. The fixed cells were then incubated with rabbit anti-TMPRSS2 antibody (1:100) alone or rabbit anti-TMPRSS2 (1:100) and mouse anti-Rab11 (1:100) antibodies overnight at 4 ℃. They were then incubated with the secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:2,000; cat. no. A27034, Invitrogen) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:2,000; cat. no. A11005, Invitrogen), for 1 h at room temperature. The secondary antibodies alone were used as the blank control. The cover slides were mounted with ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI; cat. no. P36935, Invitrogen), and images were captured with Zeiss LSM 710 laser scanning confocal system equipped with a *63x* oil immersion objective lens (Zeiss, Beijing, China).

Statistical analysis

Data are shown as mean ± SD. Differences between two groups were compared with unpaired *t*-test and the Mann-Whitney test. One-way ANOVA with the Tukey test and two-way ANOVA with the Sidak correction were used to draw multiple comparisons, respectively. GraphPad Prism 6.0 (La Jolla, CA, USA) was used to perform statistical analysis. Statistical difference was indicated when $P<0.05$.

Results

TMPRSS2 is upregulated in DEX-treated breast cancer cells

TMPRSS2 is expressed in various human cancer cells, particularly in cells from cancers such as prostate, head, and neck cancer (15). In this study, we hypothesized that if TMPRSS2 is expressed in cultured breast cancer cells *in vitro*, the regulation of TMPRSS2 by DEX may in turn be demonstrated to regulate the migration and metastasis of breast cancer cells. To test our hypothesis, the expression of TMPRSS2 was firstly assessed in cultured MCF-7 and MDA-MB-231 cells, two of the most commonly used human breast cancer cell lines (19). The results from real-time PCR demonstrated that compared to the non-treated cells, the mRNA levels of TMPRSS2 were significantly upregulated in both a time- and dose-dependent manner in the DEX-treated MCF-7 and MDA-MB-231 cells (*Figure 1A*, *Figure S1*). Similar results were found with Western blot assay, which demonstrated a significant increase of TMPRSS2 at the protein levels by DEX (*Figure 1B*). Image analysis identified two specific bands, with one close to 75 kDa and the other between 37 and 25 kDa (*Figure 1B*). As previously mentioned, TMPRSS2, a transmembrane serine protease, is expressed in two forms, as a full-length protein and as a cleaved protease domain; which are recognized by 70- and 32-kDa, respectively (14). In our study, an increase in TMPRSS2 in both its full-length and cleaved protease domain forms was detected in DEX-treated MCF-7 and MDA-MB-231 cells (*Figure 1B*). Notably, we detected higher levels of TMPRSS2 in the non-treated MDA-MB-231 cells than in the non-treated MCF-7 cells (*Figure 1A*,*B*). It has been reported that the migration rate of MCF-7 is lower than that of MDA-MB-231 (19,20). Although TMPRSS2 was induced by DEX in both the

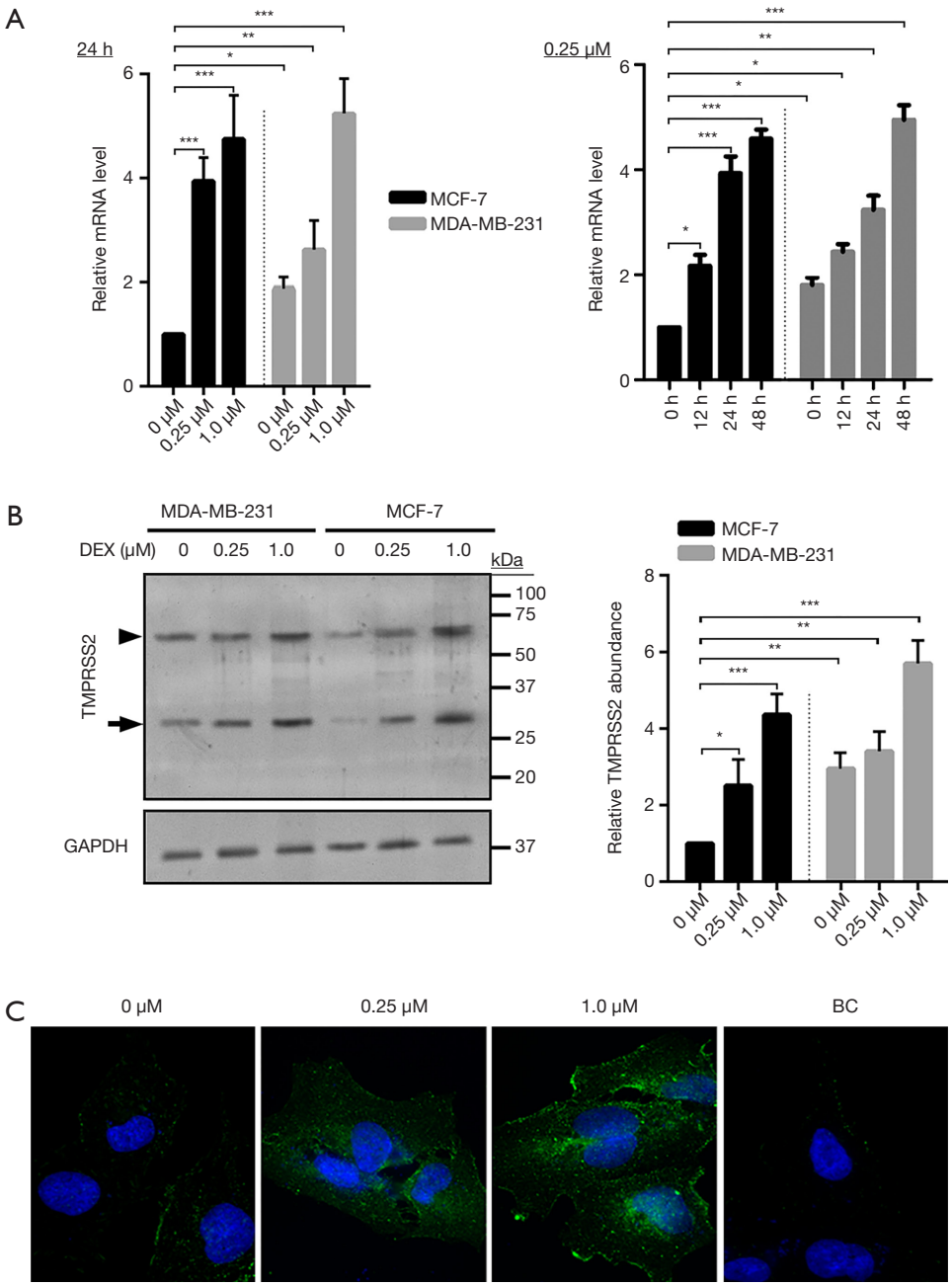


Figure 1 TMPRSS2 is upregulated in dexmedetomidine-treated breast cancer cells. MCF-7 and MDA-MB-231 cell lines were treated with dexmedetomidine (DEX). (A) Real-time PCR was performed for the mRNA expressions of TMPRSS2, showing a dose- and time-dependent increase of TMPRSS2 following DEX treatment. (B) Total cellular protein was extracted, and Western blot was performed for the protein expressions of TMPRSS2. Both full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2 were detected, showing that DEX induced a dose-dependent increase in the total TMPRSS2. Data are presented as the mean ± SD. The experiment was carried out independently in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001. (C) Indirect immunofluorescence confocal microscopy was performed to image TMPRSS2 (green color) and the nuclei (blue color). The blank control in the presence of only the secondary antibody was included while the staining was performed. Magnification ×63 for all panels.

MCF-7 and MDA-MB-231 cells, MCF-7 was selected and used in the following experiments due to having a relatively low basal level of TMPRSS2. Certainly, some key findings that obtained from MCF-7 were further validated in MDA-MB-231, as shown in the supplementary figures. To establish the distribution of TMPRSS2 in the DEX-treated MCF-7 cells, we did indirect immunofluorescence staining with the specific anti-TMPRSS2 antibody. The intensity of the TMPRSS2 signal was remarkably increased 48 h following DEX treatment, particularly in the 1 μM DEX-treated cells (*Figure 1C*). We revealed that TMPRSS2 was predominantly localized in the cell peripheral membrane. In the cytoplasmic region, some vesicles of TMPRSS2 were also revealed (*Figure 1C*).

Increased migration is mediated by TMPRSS2 in DEX-treated breast cancer cells

To explore the effects of DEX on the migration of breast cancer cells, scratch assay was carried out. We found that DEX increased the migration rate of MCF-7 and MDA-MB-231 in a dose-dependent manner (*Figure 2A*, *Figure S2A*). To test if TMPRSS2 involved DEX-induced increase in cellular migration, we knocked down TMPRSS2 expression with two different siRNAs specifically targeted against the human *TMPRSS2* gene. In comparison with the control siRNAs, siTMP-a and especially siTMP-b, significantly decreased the protein levels of TMPRSS2 in DEX-treated MCF-7 (*Figure 2B*). A scratch assay was then carried out with DEX-treated MCF-7 that expressed siTMP-b. Our results showed that the increased migration rate was suppressed significantly in DEX-treated MCF-7 with TMPRSS2 knockdown (*Figure 2C*). The knockdown of TMPRSS2 was also discovered to significantly inhibit the cellular migration of DEX-treated MDA-MB-231 (*Figure S2B*,*C*). These findings indicate that DEX increases the migration of breast cancer cells *in vitro* by upregulating TMPRSS2.

Activation of the α2-adrenergic receptor/STAT3 signaling is responsible for the induction of TMPRSS2 expressions in DEX-treated MCF-7

DEX, an α2-adrenergic agonist, induces downstream signaling via the activation of α2-adrenergic receptors (2,3). There are three subtypes of Alpha 2-adrenergic receptor, and it has been shown each of them presents in MCF-7 cells (21). It was recently reported that alpha 2A subtype overexpression was associated with a better disease-free survival in patients with luminal tumors, while the overexpression of alpha 2C subtype was associated with worse prognosis for breast cancer patients with basal-like tumors (4,22). Nevertheless, the role of the alpha 2B subtype has been the subject of fewer studies on the pathogenesis of breast cancer. This study focused on the alpha 2B subtype by using the antibody ABN1481, which highly and specifically targets the alpha 2B subtype. In the current study, the expression of α2-adrenergic receptor was significantly increased at 48 h in 1 μM of DEX-treated MCF-7 (*Figure 3A*). Indirect immunofluorescence also revealed obvious colocalization of TMPRSS2 and α2-adrenergic receptors in the DEX-treated cells (*Figure 3B*). In addition, we found the levels of the phosphorylated $STAT3^{Tyr705}$ in the nuclear fractions to be significantly increased at 24 and 48 h after DEX treatment (*Figure 3C*), suggesting that the STAT3 signaling pathway is activated. To investigate the role of the STAT3 signaling pathway in breast cancer, its specific inhibitor WP1006 was applied in this study. Our data showed that WP1006 at 5 and 10 μM dramatically inhibited DEX-induced elevation of the phospho-$STAT3^{Tyr705}$ (*Figure 3D*). Moreover, the administrated WP1006 significantly suppressed DEX-induced upregulation of both full-length TMPRSS2 and its cleaved protease domain (*Figure 3E*). These results suggest that DEX upregulates the expression of TMPRSS2 possibly by activating the α2-adrenergic receptor/STAT3 signaling pathway.

DEX increases the secretion of the TMPRSS2 cleaved protease domain via Rab11 in breast cancer cells

The cleaved protease domain of TMPRSS2 has been found to be secreted into the cell media after autocleavage (14). We thus assessed the level of TMPRSS2 in the culture media from DEX-treated MCF-7 cells. The data from the ELISA assay showed that DEX induced a significant dose- and time- dependent increase of TMPRSS2 in the media (*Figure 4A*), evidencing that TMPRSS2 was secreted and present in the cultured media following treatment with DEX. We also isolated exosomes from the cultured media, and tested for the presence of TMPRSS2. Increases in exosomal markers such as a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and heat shock protein 70 (Hsp70) were detected in the isolated exosomes by Western blot assay (*Figure 4B*), suggesting that the exosomes had been successfully isolated

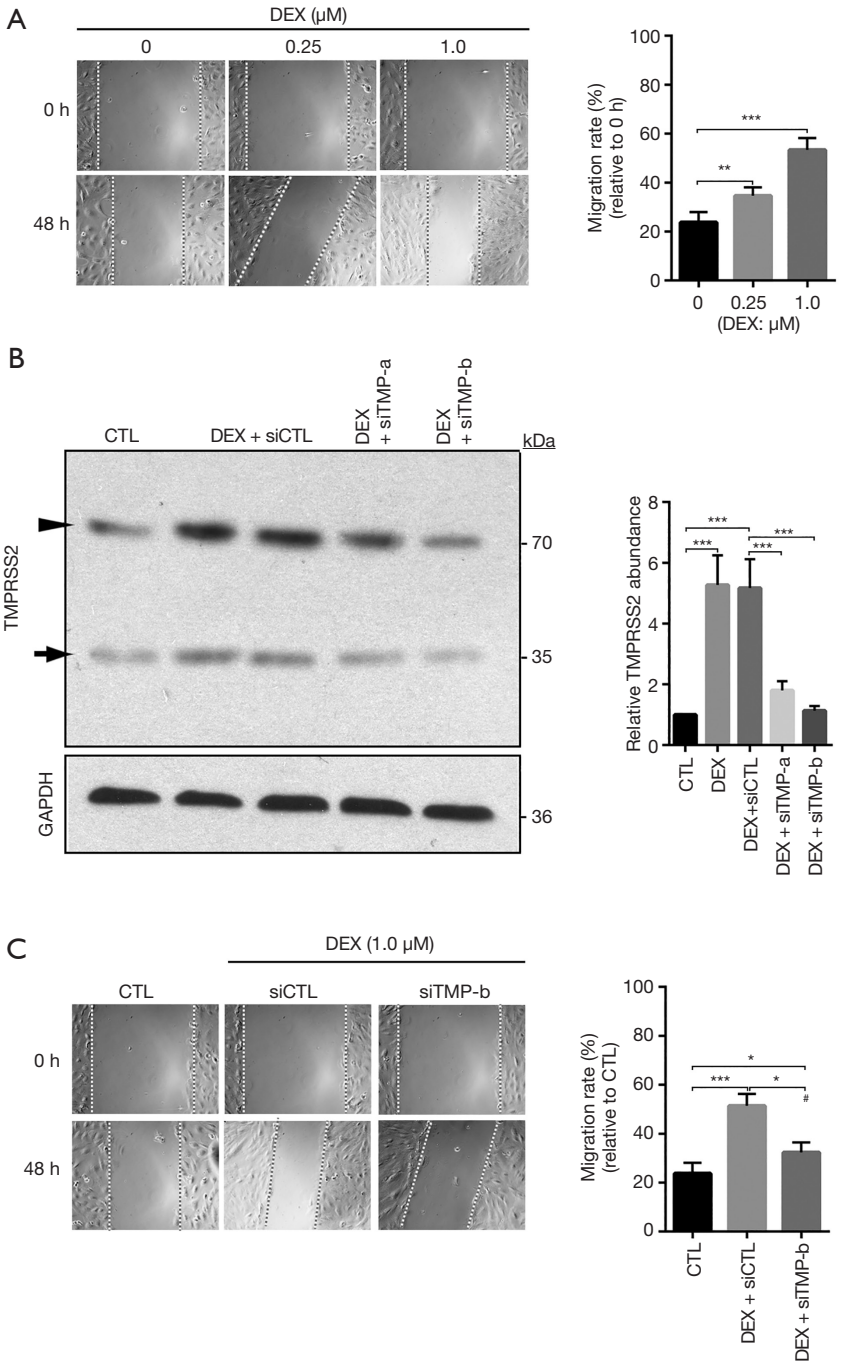


Figure 2 Dexmedetomidine increases migration in the cultured MCF-7 through TMPRSS2. (A) MCF-7 was treated for 48 h with dexmedetomidine (DEX) and scratch assay was performed. DEX increased migration rate of MCF-7 in a dose-dependent manner. Magnification ×20 for all panels. (B) Two unique siRNAs (siTMP-a and siTMP-b) were respectively introduced to MCF-7 to knock down the expression of TMPRSS2. After 6 h, the media were replaced with 1 μM of DEX and then incubated for 48 h. The non-treated cells were used as controls (CTL). TMPRSS2 protein levels were measured by using Western blot assay. Both full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2 were detected, showing a significant reduction in the siRNA-TMPRSS2, but not the control siRNAs. (C) Scratch assay was performed in DEX-treated MCF-7 that expressed siTMP-b. DEX-induced increase of migration was prevented by the siTMP-b. Magnification × 20 for all panels. Data are presented as mean ± SD. The experiment was carried out independently in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; #, P<0.05.

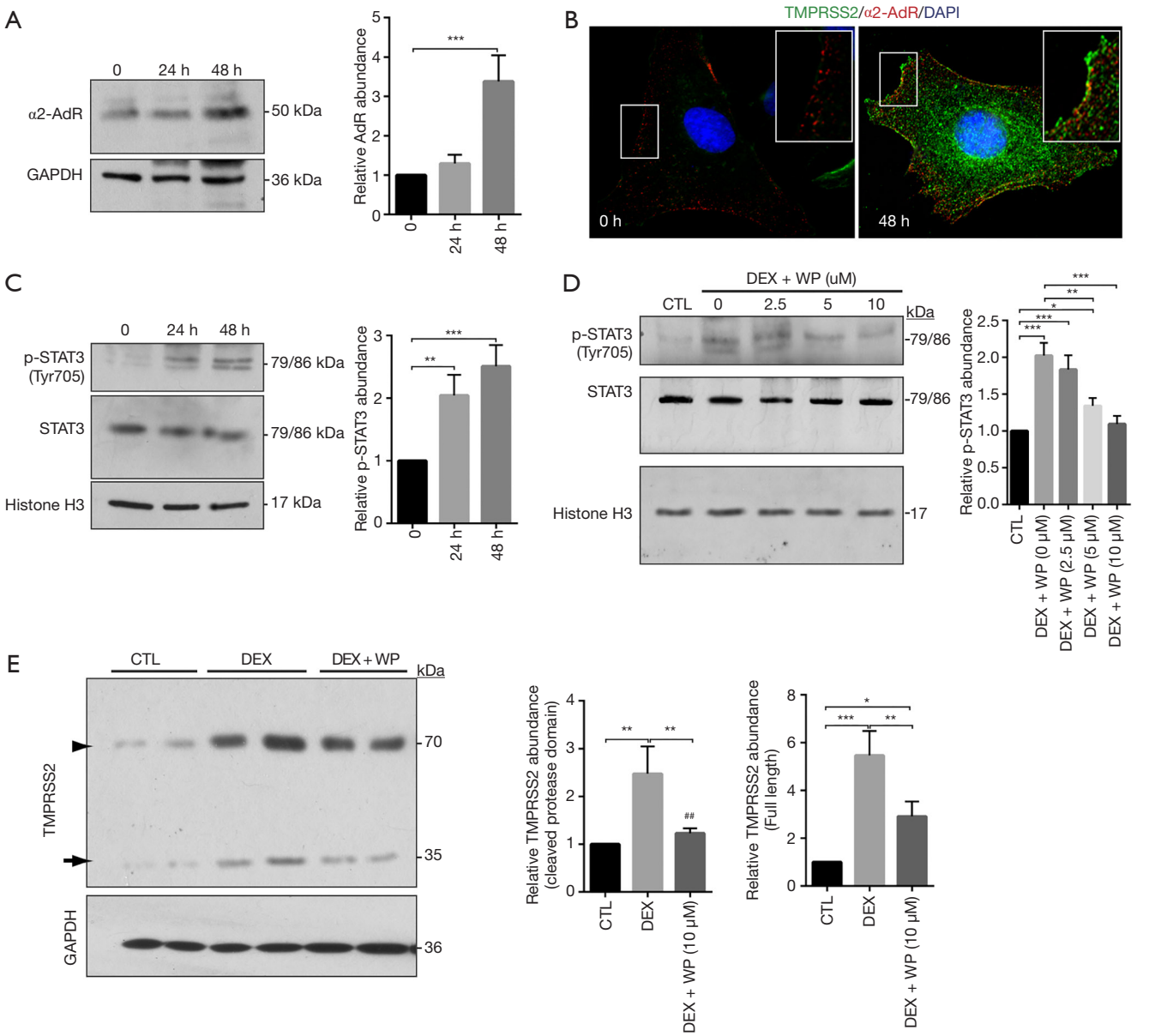
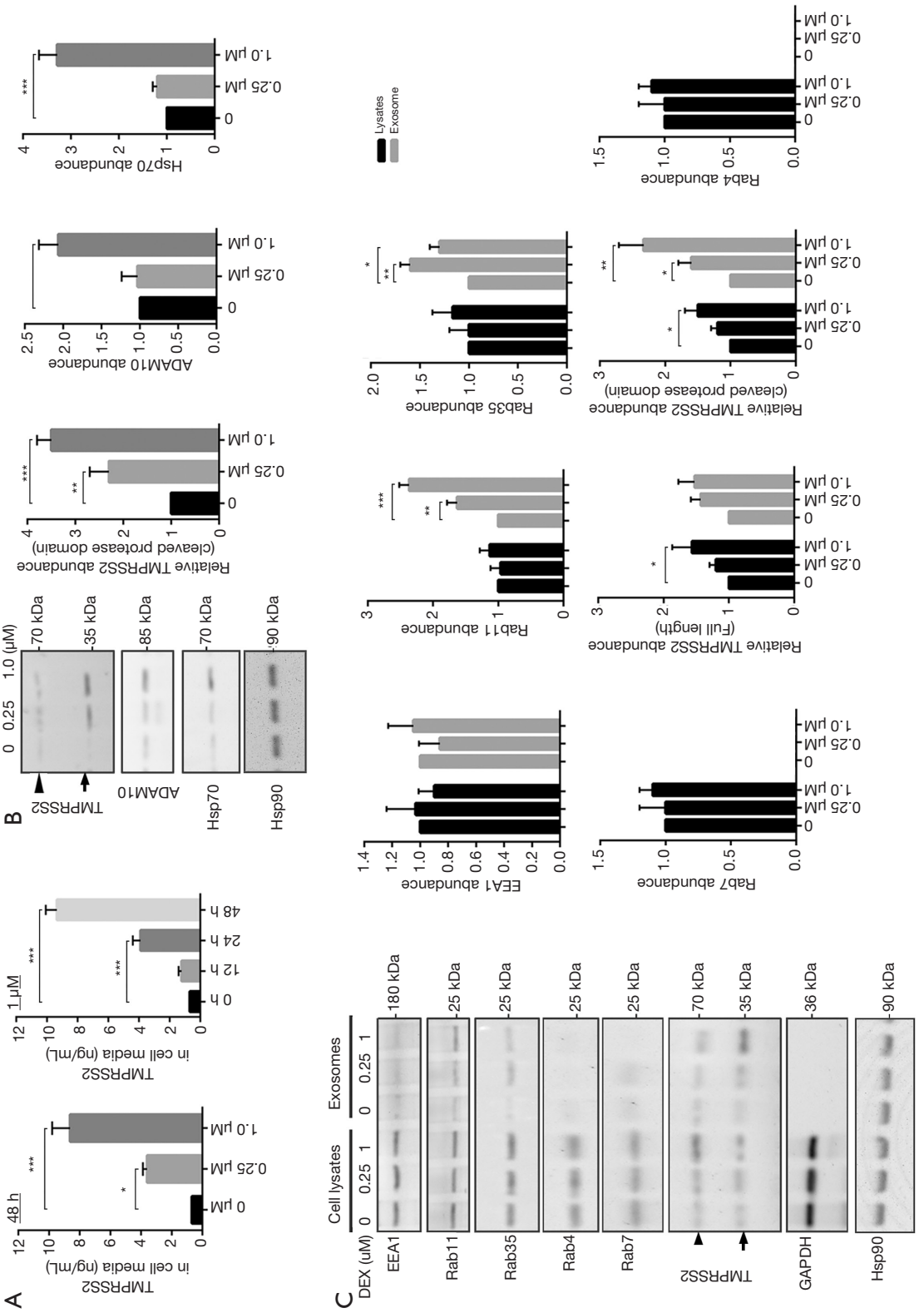


Figure 3 Dexmedetomidine increases TMPRSS2 expressions through the α2-adrenergic receptor/STAT3 pathway in the cultured MCF-7. MCF-7 was treated with 1 μM of dexmedetomidine (DEX) for the indicated time periods. (A) Total cellular protein was extracted, and Western blot assay was performed to detect the expression of α2-adrenergic receptor (α2-AdR). (B) Indirect immunofluorescence confocal microscopy was used to image TMPRSS2 (green color), α2-adrenergic receptor (red color), and the nuclei (blue color). A high-power view of the selected area was obtained. Magnification ×63 for all panels. (C) The nuclear fractions were extracted, and Western blot assay was performed for phospho-STAT3^{Tyr705} and total STAT3. The nuclear protein histone H3 was used as the loading control. (D) MCF-7 was pretreated for 1 h with different concentrations of the STAT3 inhibitor (WP1066) followed by incubation for 48 h with 1 μM of DEX. The nuclear fractions were extracted, and Western blot assay was performed to validate the inhibition of STAT3 signaling. (E) MCF-7 was pretreated for 1 h with 10 μM of WP1066 followed by incubation for 48 h with 1 μM of DEX. Total cellular protein was extracted, and Western blot assay was performed to detect the expressions of full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2. Data are presented as the mean ± SD. The experiment was conducted independently in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001; ##, P<0.01.



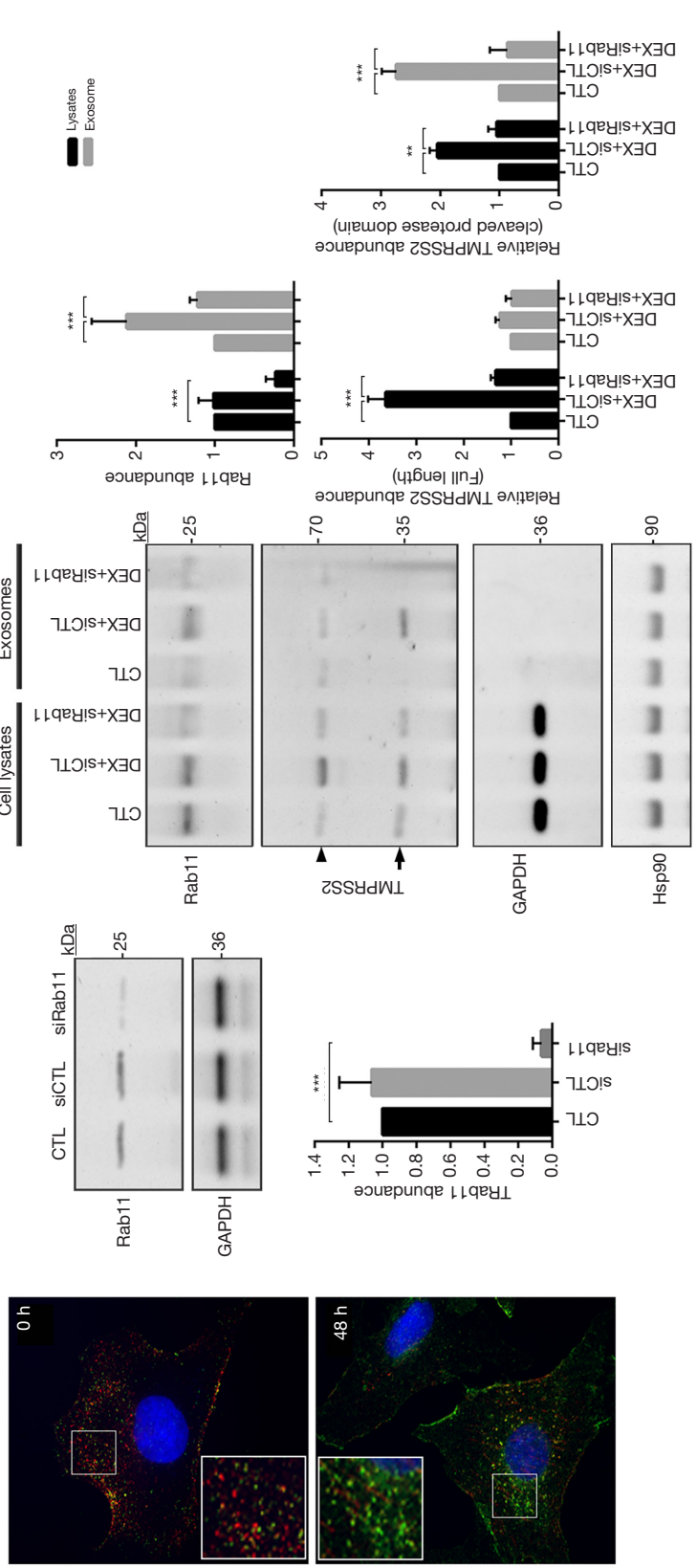


Figure 4 The secretion of the exosomal TMPRSS2 is mediated by Rab11 in dexmedetomidine-treated MCF-7. (A) MCF-7 was treated with dexmedetomidine (DEX) for the indicated time periods and concentrations. The culture media were collected, and the secreted TMPRSS2 was measured by ELISA. (B) MCF-7 was treated for 48 h with DEX at the indicated concentrations. Exosomes were isolated from the culture media using sequential centrifugation methods, and Western blot assay was performed to detect the expression of full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2 as well as the exosomal markers ADAM10, Hsp70, and Hsp90. Hsp90 was used as the loading control for quantification analysis of exosomal proteins. (C) MCF-7 was treated for 48 h with DEX at the indicated concentrations. Total cellular protein was extracted, and the exosomes were isolated from the culture media. Western blot assay was performed for the expressions of TMPRSS2 as well as multiple vesicle markers EEA1, Rab7, Rab4, Rab11, and Rab35. (D) Indirect immunofluorescence confocal microscopy was performed to image TMPRSS2 (green color), Rab11 (red color), and the nuclei (blue color) in 1 μM of DEX-treated MCF-7. A high-power view of the selected area was obtained. Magnification ×63 for all panels. (E) Rab11 was knocked down using the siRNAs specifically against human Rab11 (siRab11). The control siRNAs (siCTL) do not target against any human genes. 48 h after transfection, total cellular protein was extracted, and Western blot assay was performed to validate Rab11 knockdown. (F) Rab11 was knocked down in 1 μM of DEX-treated MCF-7. Total cellular protein was extracted, and the exosomes were isolated from the culture media. Western blotting was performed for the expressions of TMPRSS2 and Rab11. Data are presented as the mean ± SD. The experiment was conducted independently in triplicate. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

and obtained. In our study, Hsp90, another exosomal marker revealed no significant difference in the isolated exosomal fractions of the DEX-treated cells when compared with the controls. Hsp90 was therefore used as the loading control for the quantification of exosomal proteins (*Figure 4B*). As expected, a dose-dependent increase of the TMPRSS2 cleaved protease domain was significantly detected in the isolated exosomes from the cultured media of DEX-treated MCF-7 (*Figure 4B*).

To reveal the mechanisms by which TMPRSS2 was secreted, we analyzed the levels of multiple vesicle-related markers, including the early endosome antigen (EEA1) and the late endosome (Rab7) markers as well as the recycling endosomal markers (Rab4 and Rab11) and the exosomal markers (Rab27 and Rab35). In the isolated exosomes, we detected a significant increase of Rab11 and Rab35, rather than EEA1, Rab4, and Rab7 (*Figure 4C*). Consistently, increases in full-length TMPRSS2 were mainly detected in total cell lysates, whereas increases of the TMPRSS2 cleaved protease domain were significantly detected in the exosomal fractions in DEX-treated MCF-7 cells (*Figure 4C*). The TMPRSS2 cleaved protease domain and Rab11 were also detected in DEX-treated MDA-MB-231 cells (*Figure S3A*). Rab11 acts in conjunction with a protein complex known as the exocyst to mediate terminal steps in cargo transport by recycling endosomes to cell-cell junctions (23). We thus performed indirect immunofluorescence to image the location of TMPRSS2 and Rab11. Obvious colocalization of TMPRSS2 and Rab11 was observed in the DEX-treated MCF-7 cells. This provides further evidence of the presence of TMPRSS2 in Rab11-positive exosomes (*Figure 4D*), indicating that Rab11 could play an important role in mediating the secretion of the TMPRSS2 cleaved protease domain. Concurrently, Rab11 was knocked down using the siRNAs specifically targeted to the two isoforms of human Rab11 (Rab11a and Rab11b) in cultured MCF-7 and MDA-MB-231 (*Figure 4E*, *Figure S3B)*. DEX-induced increase of the TMPRSS2 cleaved protease domain in the exosomes was dramatically inhibited by siRab11 **(***Figure 4F, Figure S3B*). These data suggest that Rab11 mediates DEX-induced secretion of the cleaved protease domain of TMPRSS2. In addition, increases of full-length TMPRSS2 and the TMPRSS2 cleaved protease domain in total cellular lysates were also significantly prevented by siRab11 (*Figure 4F, Figure S3B*), suggesting that overproduced TMPRSS2 may undergo degradation, most likely as a result of the blockage of the secretion pathway of TMPRSS2 caused by Rab11 deficiency.

Rab11 increases the migration of DEX-treated human breast cancer cells

Our data demonstrated the mediating effect of Rab11 on the secretion of TMPRSS2 into exosomes following treatment with DEX treatment. To further assess the regulatory role of Rab11 and exosomal TMPRSS2 in the migration of DEX-treated MCF-7, we investigated the effects of Rab11 knockdown on the migration rate in DEX-treated MCF-7 cells. In comparison with the control siRNAs, the increase in the migration rate of the DEX-treated MCF-7 cells was remarkably reduced by siRab11 (*Figure 5A*). As described above, Rab11 was mainly present in the exosomes of the DEX-treated MCF-7 cells. To further elucidate the role of exosomal Rab11, we isolated Rab11-positive or negative exosomes from DEX-treated MCF-7 that expressed the control siRNA or siRab11, respectively, and then applied them to the other normally cultured MDA-MB-231 cell line. Firstly, we imaged the exosomes with a transmission electron microscope, finding that the Rab11-positive exosomes appeared to be much darker than the Rab11-negative exosomes (*Figure 5B*), which was indicative that more components in the exosomes were enriched by Rab11. Then, we investigated the effects of the Rab11-positive and negative exosomes on cellular migration in normally cultured MDA-MB-231. We found that compared to the non-treated cells, migration was significantly increased by the Rab11-positive exosomes, but not by the Rab11-negative exosomes (*Figure 5C*). These findings indicate that Rab11 that is present in exosomes play a critical role in the DEX-induced migration of breast cancer cell *in vitro*.

DEX promotes the ECM degradation through TMPRSS2 in the cultured breast cancer cells

Degradation of the ECM promotes migration in various types of cells, such as prostate cancer cells (13). We assessed the alterations of several ECM proteins in DEX-treated MCF-7 and MDA-MB-231 cell lines. As expected, the protein levels of fibronectin, collagen IV, matrix metallopeptidase 16, and tenascin C were significantly downregulated by DEX (*Figure 6A*, *Figure S3C*). Moreover, the knockdown of Rab11 or TMPRSS2 remarkably inhibited DEX-induced downregulation of the ECM components (*Figure 6B*, *Figure S3C*). Therefore, our findings demonstrate that TMPRSS2 expression

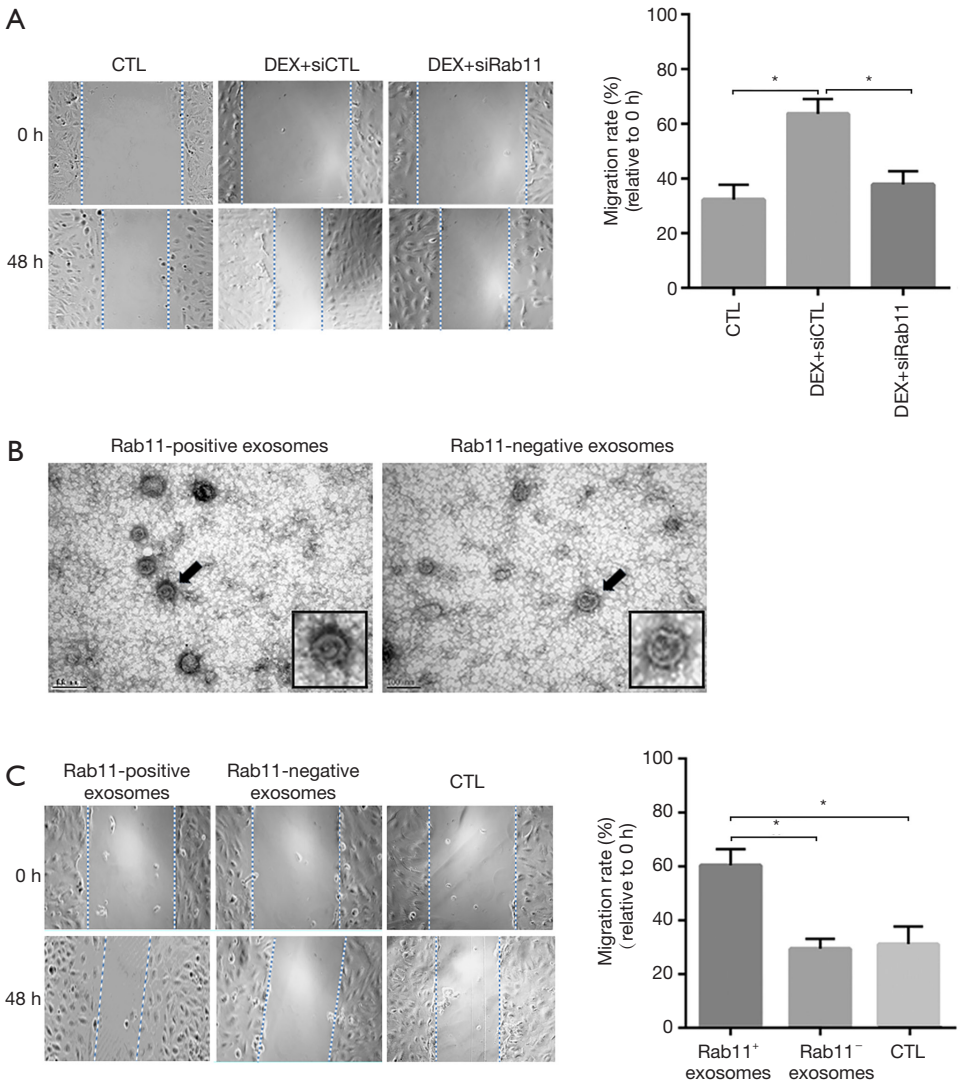


Figure 5 Rab11 involves the increase of migration of dexmedetomidine-treated MCF-7. Rab11 was knocked down using the specific siRNAs against human Rab11 (siRab11). Six hours after transfection, dexmedetomidine (DEX) was added at the final concentration of 1 μM and incubated for 48 h. (A) Scratch assay was performed, and the migration rate was calculated and compared. The non-treated cells were used as the controls (CTL). Magnification ×20 for all panels. (B) Rab11-positive and negative exosomes were isolated from DEX-treated MCF-7 that expressed siCTL and siRab11, respectively. Transmission electron microscopy was performed to capture images of the exosomes, and a high-power view was obtained of the arrow-indicated exosomes. Magnification bar =100 nm. (C) Normally cultured MDA-MB-231 cells were treated for 48 h with the Rab11-positive and negative exosomes, respectively. The non-treated cells were used as the controls (CTL). Scratch assay was performed and the migration rate was calculated and compared. Magnification ×20 for all panels. Data are presented as the mean ± SD. The experiment was independently conducted in triplicate. *, $P<0.05$.

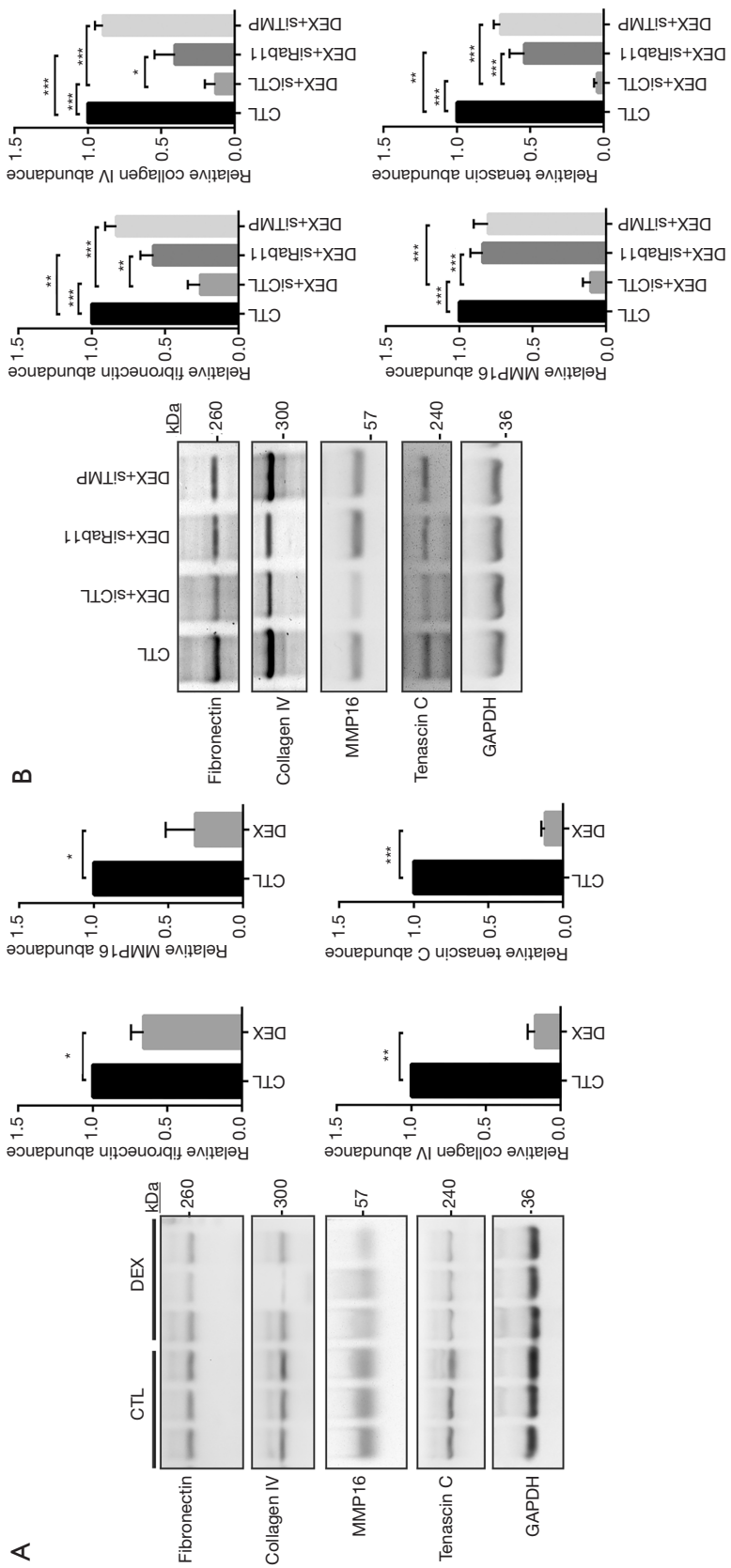


Figure 6 Dexmedetomidine promotes the degradation of the extracellular matrix through Rab 11 and TMPRSS2 in the cultured MCF-7. (A) MCF-7 was cultured for 48 h in the presence of 1 μM of dexmedetomidine (DEX). Total cellular protein was extracted, and Western blot assay was performed to detect the expression of the extracellular matrix components fibronectin, collagen IV, matrix metallopeptidase 16 (MMP16), and tenascin C. (B) Rab11 and TMPRSS2 were knocked down using the specific siRNAs targeted against human Rab11 and TMPRSS2, respectively. Six hours after transfection, 1 μM of DEX was added and incubated for 48 h. Total cellular protein was extracted, and Western blot assay was performed to detect the expression of the extracellular matrix components. The reduction of fibronectin, collagen IV, matrix metallopeptidase 16 (MMP16), and tenascin C was significantly prevented by siRab11 and siTMPRSS2. Data are presented as the mean ± SD. The experiment was independently carried out in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001.

and secretion is elevated by DEX, thus inducing ECM degradation, which is responsible for the increase in breast cancer cell migration *in vitro*.

Discussion

DEX, a lipophilic α2 adrenergic agonist, has been widely used in surgery as an anesthetic to effectively reduce stress reaction and systemic inflammation and maintain the normal function of the cardiovascular system (16). TMPRSS2 has been found to be upregulated by androgenic hormones in prostate cancer cells and downregulated in androgen-independent prostate cancer tissue (14). In patients with prostate cancer, TMPRSS2 has been identified as a key driver of metastasis and a marker for poor prognosis (12-14). The combined effects of TTSP variants and their related genes, including *TMPRSS2*, were reported in relation to risk and patient outcome in breast cancer (10). Thus, we hypothesized that DEX may promote breast cancer cell migration and metastasis *in vitro* by regulating TMPRSS2 and its downstream signaling.

TMPRSS2 belongs to the serine protease family and contains a type II transmembrane domain, a receptor class A domain, a scavenger receptor cysteine-rich domain, and a protease domain (24). Serine proteases are known for their involvement in many physiological and pathological processes, and the protease domain of TMPRSS2 is thought to be cleaved and secreted into cell media after autocleavage (14). In this study, increases in both full-length TMPRSS2 and its cleaved protease domain were detected in two widely used breast cancer cell lines, MCF-7 and MDA-MB-231, following treatment with DEX. Consistently, it has been reported that the full-length TMPRSS2 and its cleaved protease domain were both present in cultured MCF-7, albeit with a lower level relative to head and neck cancer cells HSC-3 and SCC-9 as well as prostate cancer cells LNCaP (15). In this study, we also investigated the mechanism by which TMPRSS2 expression is induced by DEX. Firstly, we detected elevated expressions of α2-adrenergic receptors and the colocalization of α2-adrenergic receptors with TMPRSS2 along the cell membrane in DEX-treated MCF-7. The expression of TMPRSS2 has been reported to be significantly reduced by galiellalactone, a small-molecule STAT3 inhibitor, in both prostate and breast cancer cells (25). Moreover, galiellalactone has also been observed to inhibit the growth of triple-negative breast cancer cell lines (26). In our study, hosphor-STAT3 in the tyrosine 705 residue was detected to be elevated in the nuclear fractions of DEX-treated MCF-7. Interestingly, the inhibition of STAT3 by WP1006 significantly reduced DEX-mediated upregulation of TMPRSS2. Therefore, our data demonstrate that DEX can elevate the expression of TMPRSS2 partly through the activation of the α2-adrenergic receptor/STAT3 signaling pathway.

TMPRSS2 was detected in the supernatant media of the cultured HSC-3 cells by ELISA over a 72-h period (15), which suggests that TMPRSS2 can be released into the cancer microenvironment. Here, the elevated TMPRSS2 was also revealed by ELISA in the cultured media in DEX-treated MCF-7 cells, indicating that TMPRSS2 was produced, cleaved, and secreted into the media following DEX treatment. TMPRSS2 can induce proteolytic activity, resulting in the degradation of the components of the ECM (13). In this study, the degradation of ECM components such as fibronectin, collagen IV, matrix metallopeptidase 16, and tenascin C was detected in the DEX-treated MCF-7 and MDA-MB-231 cells, but not in the cells with knocked-down TMPRSS2. These findings suggest that TMPRSS2 may play an important role in DEX-induced migration of breast cancer cells, at least partly, through the degradation of the components of the ECM. Therefore, we assessed the migration rate of MCF-7 and MDA-MB-231 cells following DEX treatment. In our study, a dose-dependent increased migration rate was detected following DEX treatment over 24- and 48-h periods. As expected, the knockdown of TMPRSS2 significantly decreased DEX-induced migration, providing evidence that DEX can increase breast cancer cell migration by upregulating TMPRSS2. In the current study, a higher basal expression level of TMPRSS2 was detected in MDA-MB-231 than in MCF-7, which may be an important factor to explain why the migration rate of MDA-MB-231 is higher than MCF-7 under normal conditions (19,20).

We detected elevated TMPRSS2 in the cultured media of DEX-treated MCF-7, which is indicative of the presence of TMPRSS2 in the extracellular vesicles. Exosomes, one of the most notable populations of extracellular vesicle, are released by viable cells under normal conditions (27). TMPRSS2 has previously been detected in extracellular vesicles isolated from the prostate cancer cell LNCaP (28,29). Thus, we isolated the exosomes from the cultured media of DEX-treated MCF-7 and MDA-MB-231, and found a dramatic increase in the cleaved protease domain of TMPRSS2. To reveal the potential pathway responsible for TMPRSS2 secretion, we analyzed the levels of several extracellular vesical markers in the isolated exosomes. A

significant increase of Rab11 was detected in relation to Rab35, whereas the early endosomal marker EEA1, the recycling endosomal marker Rab4, and the late endosomal marker Rab7 were not detected in the isolated exosomes. In addition, the colocalization of TMPRSS2 and Rab11 was clearly revealed in the DEX-treated MCF-7 cells. Similarly, Rab11 has also been identified in exosomes isolated from prostate cancer cell line PC346 (29). Rab11, a member of the Rab family within the GTPase superfamily, is associated with both the constitutive and regulated secretory pathways, and may be involved in protein transport (30). Here, the two isoforms of Rab11, Rab11a and Rab11b, were simultaneously knocked down using the siRNAs specifically targeted against human Rab11a and Rab11b. The Rab11 knockdown reduced the abundance of the cleaved protease domain of TMPRSS2 in the exosomal fractions isolated from the DEX-treated MCF-7 and MDA-MB-231 cells, which indicates that Rab11 mediates the secretion of cleaved TMPRSS2 into the culture media of breast cancer cells following DEX treatment *in vitro*. In mitotic cells, the localization of TMPRSS2 to recycling endosomes in interphase and their transport to the intercellular bridge during cytokinesis depend on Rab11 (31). These findings demonstrate the crucial role Rab11 plays in the regulation of endocytic and recycling events. Moreover, the DEX-induced degradation of the ECM components was remarkably prevented by the knockdown of Rab11 in the two breast cancer cell lines. It is further supported by the observation that compared to Rab11-negative exosomes, the application of Rab11-positive exosomes isolated from DEX-treated MCF-7 significantly increased migration in normally cultured MDA-MB-231. Moreover, transmission electronic microscopy shows that the components of the Rab11-positive exosomes were likely to be much richer than those of the Rab11-negative exosomes. The role of exosomes in the migration of breast cancer cells should be further investigated.

Conclusions

We demonstrated that DEX increases migration in breast cancer cells *in vitro* through TMPRSS2-mediated degradation of ECM components. We also showed that DEX upregulates the expression of TMPRSS2 through the activation of the α2-adrenergic receptor/STAT3 signaling pathway, and increases the secretion of the cleaved protease domain of TMPRSS2 through exosomal Rab11. Our findings provide *in vitro* evidence of the deleterious effects of DEX. More *in vivo* research to assess the influence of the perioperative DEX application on the outcomes of breast cancer, particularly studies of a clinical nature, is required.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm.2020.04.28). Dr. MC reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study; Dr. XS reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study; Dr. XH reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study; Dr. XW reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study; Dr. PZ reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study; Dr. GW reports grants from the National Nature Science Foundation of China, grants from Haiyan Foundation of Harbin Medical University Cancer Hospital, during the conduct of the study.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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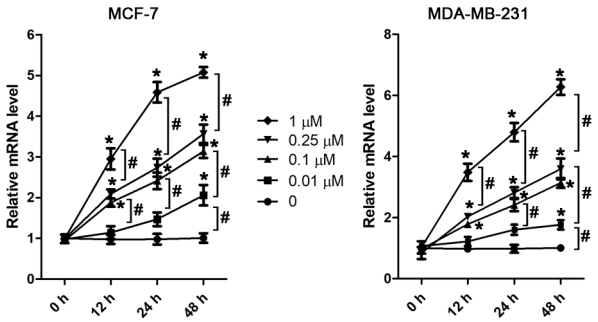

Figure S1 TMPRSS2 is upregulated in dexmedetomidine-treated breast cancer cells. MCF-7 and MDA-MB-231 were treated with dexmedetomidine (DEX) for the time periods and at the concentrations indicated. Real-time PCR was performed for the mRNA expression of TMPRSS2, showing a dose- and time-dependent increase of TMPRSS2 following DEX treatment. Data are presented as the mean ± SD. The experiment was independently carried out in triplicate. *, P<0.05 *vs.* 0 h; #, P<0.05 as the indicated comparison.

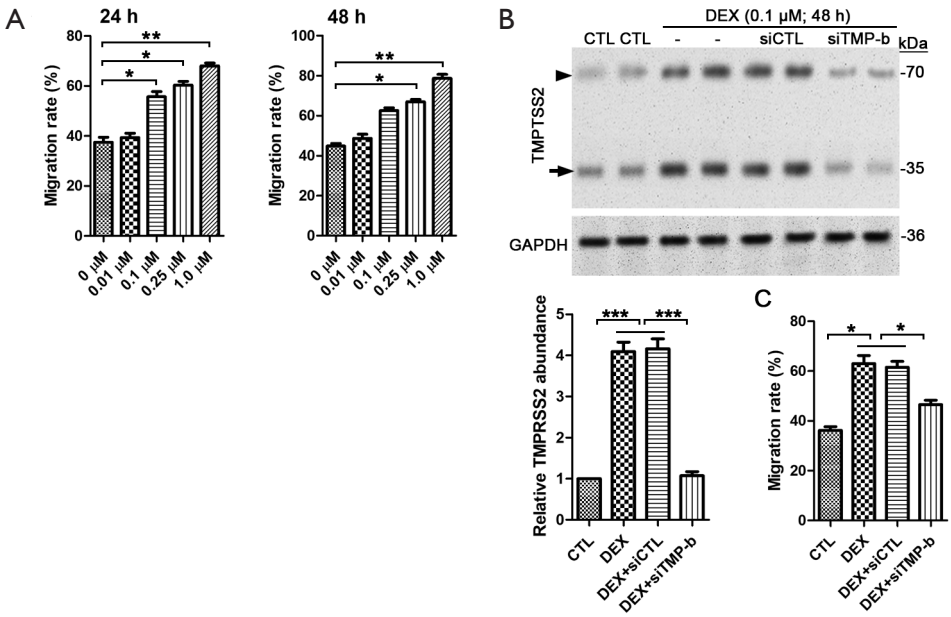

Figure S2 TMPRSS2 mediates the dexmedetomidine-induced increase in migration in the cultured MDA-MB-231. (A) MDA-MB-231 was treated with dexmedetomidine (DEX) for the time periods and at the concentrations indicated. Scratch assay was then performed. DEX increased the migration of MDA-MB-231 in a dose-dependent manner. (B) The specific siRNAs (siTMP-b) were introduced to knock down the expression of TMPRSS2. Six hours later, the media were replaced with 0.1 μM of DEX and then incubated for 48 h. The non-treated cells were used as the controls (CTL). TMPRSS2 protein levels were measured by Western blot assay. The expressions of both full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2 were reduced by the siTMP-b, but not the siCTL. (C) Scratch assay was performed on DEX-treated MDA-MB-231 that expressed siTMP-b. The dexmedetomidine-induced increase in the migration rate was prevented by siTMP-b. Data are presented as the mean ± SD. The experiment was independently carried out in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001.

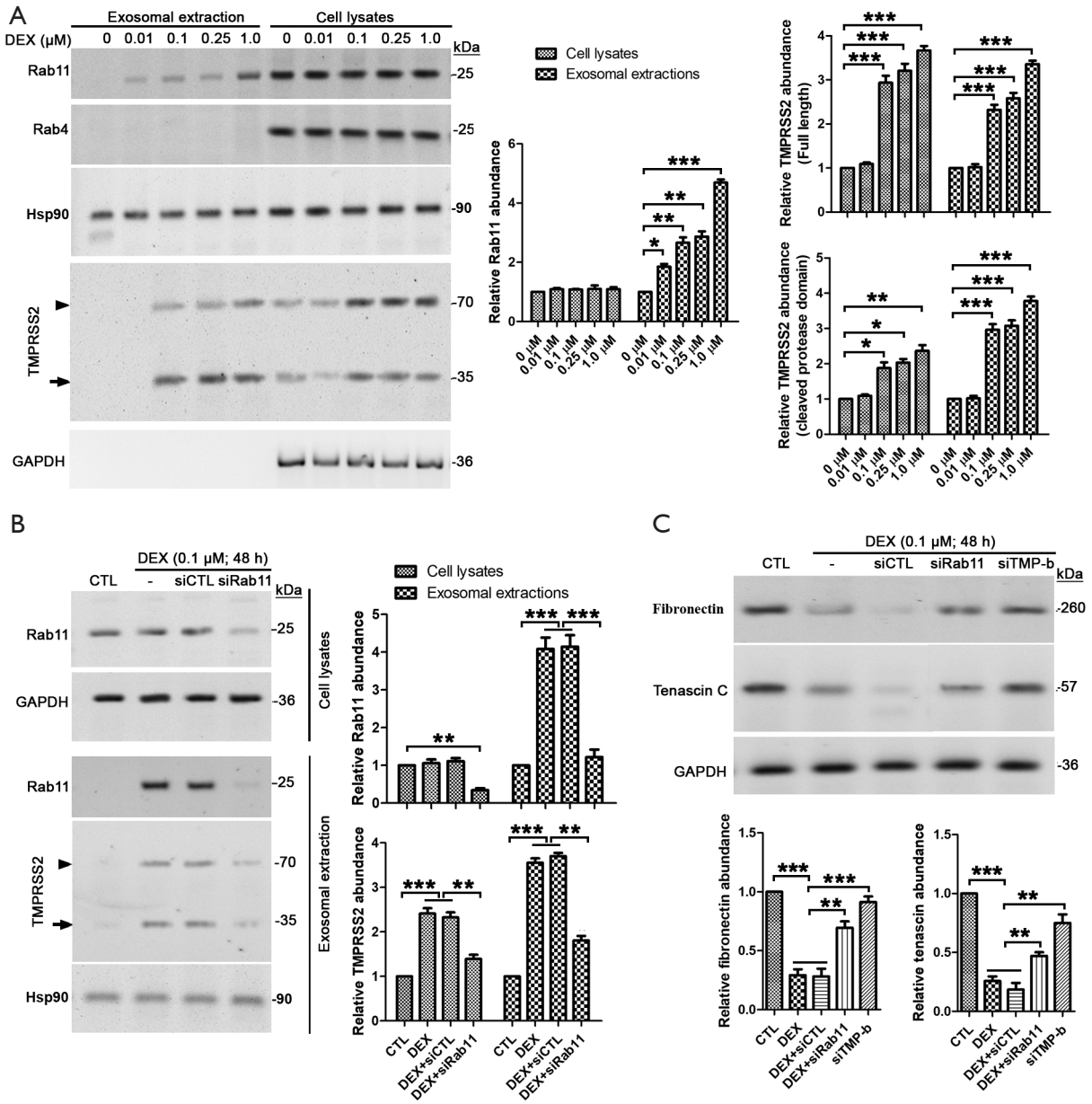

Figure S3 Rab11 mediates the secretion of TMPRSS2 and the degradation of extracellular matrix components in dexmedetomidine-treated MDA-MB-231. (A) MDA-MB-231 was treated for 48 h with dexmedetomidine (DEX) as the indicated concentrations. Exosomes were isolated from the culture media using sequential centrifugation methods, and Western blot assay was performed to detect the expressions of both full-length (arrowhead) and the cleaved protease domain (arrow) of TMPRSS2, as well as Rab11, Rab4, and Hsp90. (B) Rab11 was knocked down using the siRNAs specifically against human Rab11 (siRab11). Six hours after transfection, 0.1 μM of DEX was added and incubated for 48 h. Total cellular protein and the exosomal fractions were extracted, and Western blot assay was performed to quantify the abundance of Rab11 and TMPRSS2 relative to Hsp90. (C) Rab11 and TMPRSS2 were knocked down using the specific siRNAs targeted against human Rab11 and TMPRSS2, respectively. Six hours after transfection, 0.1 μM of DEX was added and incubated for 48 h. Total cellular protein was extracted, and Western blot assay was performed to detect the expressions of fibronectin and tenascin C. Data are presented as mean ± SD. The experiment was independently carried out in triplicate. *, P<0.05; **, P<0.01; ***, P<0.001.