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TMPRSS2, a novel membrane-anchored mediator in cancer pain

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Abstract

More than half of all cancer patients will suffer significant pain during the course of their disease. The strategic localization of TMPRSS2, a membrane-bound serine protease, on the cancer cell surface may allow it to mediate signal transduction between the cancer cell and its extracellular environment. Here we show TMPRSS2 expression is not only dramatically increased in the primary cancers of patients but TMPRSS2-immunopositivity is also directly correlated with cancer pain severity in these patients. TMPRSS2 induced proteolytic activity, activated trigeminal neurons, and produced marked mechanical hyperalgesia when administered into the hindpaw of wild-type mice but not in PAR2-deficient mice. Co-culture of human cancer cells with murine trigeminal neurons demonstrated co-localization of TMPRSS2 with PAR2. These results point to a novel role for a cell membrane-anchored mediator in cancer pain, as well as pain in general.

INTRODUCTION

Cancer patients suffer from ongoing and breakthrough pain during the course of their disease. Despite advances in cancer biology, mediators involved in the generation of cancer pain remain poorly understood. *TMPRSS2* (*transmembrane protease, serine 2*) is a gene that encodes a type II transmembrane serine protease (TTSP) [11,17,20]. Members of the TTSP family share common protein structures including a transmembrane domain at the N-terminus that anchors a canonical serine protease domain to the plasma membrane [8,18,24]. Various TTSPs are consistently overexpressed in different cancers [17–18,25], suggesting their potential as biomarkers and possible targets for anti-cancer therapy.

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TMPRSS2 has gained interest owing to its highly localized expression in prostate cancer cells and its role in carcinogenesis [1,17,25–26]. *TMPRSS2* was demonstrated to be up-regulated by androgenic hormones in prostate cancer cells and down-regulated in androgen-independent prostate cancer tissue [1]. The *TMPRSS2*-encoded serine protease is expressed as a **70 kDa** full-length form and a cleaved **32 kDa** protease domain. The protease domain of this protein is thought to be cleaved and secreted into cell media after autocleavage [1]. We speculate the strategic localization of TMPRSS2 to the cancer cell surface may allow this unique serine protease to mediate signal transduction between the cancer cell and its extracellular environment, and to regulate different cellular responses including pain. Administration of serine proteases such as trypsin or mast cell tryptase into peripheral tissues produces nocifensive behaviors, neurogenic inflammation and spinal Fos up-regulation in a protease-activated receptor 2 (PAR2)-dependent manner [6–7,10,12,21–22,27]. We recently demonstrated a novel role for trypsin and tryptase in cancer pain [15]. However, the role of a transmembrane serine protease such as TMPRSS2 in cancer pain, and pain in general, is not known. Here we show evidence for a crucial role for TMPRSS2 in the pathogenesis of cancer pain.

METHODS

TMPRSS2 in human head and neck cancer patients

UCSF Oral Cancer Pain Questionnaire—Patients referred to the UCSF Department of Oral & Maxillofacial Surgery were administered the UCSF Oral Cancer Pain Questionnaire at the time of their initial visit before any treatment was performed. This questionnaire consists of eight 100 mm visual analog scale (VAS) questions that assess spontaneous pain intensity, function-related pain intensity, and quality of pain [5,16]. The pain score for each patient was calculated as the average of the eight VAS ratings. Participation in the study was excluded if subjects had a diagnosed psychiatric condition, addiction to pain medications or recreational drugs, or had taken pain medications in the previous six months. The research protocol was approved by the UCSF Committee on Human Research.

Quantification of TMPRSS2 immunoreactivity—After administration of the pain questionnaire, all patients underwent a biopsy procedure. Normal oral mucosa patients (n=5) were healthy volunteers undergoing dental procedures such as third molar extraction; head and neck cancer patients (n=10) had biopsy-proven squamous cell carcinoma (SCC).

Immunohistochemistry for TMPRSS2 was performed on the biopsy specimens collected from the patients who completed the UCSF Oral Cancer Pain Questionnaire (above). Eight μm sections of formalin-fixed, paraffin-embedded tissue specimens were deparaffinized by standard immunohistochemical techniques. Microwave antigen unmasking was performed using Dako Antigen Retrieval Solution (Dako, Carpinteria, CA). Sections were then incubated with the primary goat polyclonal anti-TMPRSS2 goat antibody (N-13, Santa Cruz Biotechnology, Santa Cruz, CA) (0.4 $\mu\text{g}/\text{mL}$) at room temperature for 2 hours.

Immunoreactions were visualized with Vector NovaRED substrate kit (Vector Laboratories, Peterborough, UK), and sections were counterstained with Hematoxylin QS. Tissue immunoreactivity was visualized on a Nikon Eclipse E600 microscope. As a pre-absorption

control for TMPRSS2, primary antibody was pre-incubated with 10-fold excess immunogenic peptide (sc-19686 P) for 24 hours at 4°C before staining commenced. Incubation with the primary antibody was completely omitted for the negative control. TMPRSS2-immunoreactivity of individual cells was categorized as either positive or negative; negative expression denoted complete absence of TMPRSS2 immunoreactivity. To avoid observer bias, a second investigator reviewed the specimens and recorded his findings independently. The immunoreactivity score for each patient was calculated as the percentage of total cells examined that were positive for TMPRSS2.

TMPRSS2 in human cancer cell lines

TMPRSS2 expression in human melanoma (WM164) and breast cancer (MCF-7) cell lines was compared to human head and neck SCC (HSC-3 and SCC-9) cell lines. The human prostate cancer (LNCaP) cell line was used as the positive control. The cell lines were cultured to confluence and washed to remove unattached cells prior to use in the assays described below.

TMPRSS2 immunoreactivity—The above human cancer cells were grown on cover slips at 37°C overnight. After the growth media was removed, the cells were washed with PBS and fixed in cold acetone for 10 minutes. Incubation with the primary goat polyclonal anti-TMPRSS2 goat antibody (N-13, Santa Cruz Biotechnology, Santa Cruz, CA) (0.4 µg/mL) was performed for 2 hours followed by incubation with the secondary anti-goat FITC-conjugated antibody (7.5 µg/mL, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour; both incubations were at room temperature. Cover slips were mounted on slides in Gel-Mount (Biomedica Corp., Foster City, CA) and visualized on a Nikon Eclipse E600 microscope using epifluorescence. Images from each cell line were photographed using the 10× objective and RT Spot camera and software (Diagnostics Instruments Inc., Sterling Heights, MI), and TMPRSS2-immunoreactivity quantification performed with CellProfiler (Broad Institute Imaging Platform) [3] by an observer blinded to the experimental groups. The image intensity from each image was rescaled from 0 to 1 by dividing all pixels in the image by the maximum possible intensity value. The intensity of TMPRSS2-immunofluorescence, as represented by mean intensity units (average pixel intensity within a cell), was measured in at least 375 cells per cell line. Only cells that did not overlap with other cells and had a visible nucleus were used for image analysis. Controls for TMPRSS2 included the replacement of the primary antibody with similarly diluted normal goat serum, omission of the primary antibody, omission of both the primary and secondary antibodies, and pre-incubation of primary antibody with 10-fold excess TMPRSS2 blocking peptide (sc-19686 P) overnight at 4°C.

TMPRSS2 Western blot—We used Western blot to confirm TMPRSS2 expression. HSC-3, SCC-9, LNCaP, WM164 and MCF-7 cells were lysed in Nonidet P-40 lysis buffer. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitro-cellulose membrane (Micron Separation Inc., Westborough, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membranes were developed using ECL Chemiluminescence Kit (Amersham) and images obtained with Bio-Rad Molecular

Imager® ChemiDoc™ XRS™+ System. The blots were quantified and assigned relative value units (RVU) using Image Lab Software (<http://www.biorad.com/webroot/web/pdf/lsr/literature/10017218.pdf>).

TMPRSS2 ELISA—The supernatant levels of TMPRSS2 in human head and neck cancer cells (HSC-3) incubated for 0, 36 and 72 hours were detected in triplicate using the human TMPRSS2 enzyme-linked immunosorbent assay kit (USCN Life Science Inc, Wuhan, Hubei, China) and microplate absorbance reader at 450 nm (Model 680, Bio-Rad Laboratories), according to the manufacturer's specifications. The minimum detectable dose of human TMPRSS2 for this assay was less than 0.039 ng/mL.

TMPRSS2 proteolytic activity

Protease activity levels of increasing doses of TMPRSS2 human recombinant protein (0, 3 and 7 µg; Abnova, Taipei, Taiwan) were determined using a PDQ Protease Assay Kit (Athena Enzyme Systems) and microplate absorbance reader (Model 680, Bio-Rad Laboratories). To confirm that TMPRSS2 was functioning as a serine protease, it was pre-incubated with either a serine protease inhibitor (FUT-175, 50 µg/ml, BD Biosciences, San Jose, CA) or matrix metalloprotease inhibitor (GM 6001, 100nM, Sigma-Aldrich, St. Louis, MO) for 30 minutes prior to performing the protease assay. The concentrations of FUT-175 and GM 6001 were based on their inhibitory efficacy in previous studies [15]. Standard trypsin reaction buffer (10mM Tris-HCl, pH 8.0, 400 µl) and TMPRSS2 test solution (0, 3, and 7 µg TMPRSS2, 100 µl) were added to vials containing the substrate matrix, the vials sealed tightly, and incubated at 37°C for 3 hours. To stop the reaction, 500 µl of 0.2N NaOH was added to each vial. The absorbance at 450 nm of the aqueous phase was measured spectrophotometrically. The proteolytic activity of TMPRSS2 was standardized to the activity generated by known concentrations of trypsin.

TMPRSS2 behavioral testing

Animals—Adult PAR2-deficient (PAR2^{-/-}) mice (6–8 weeks old), originally from Jackson Laboratory (Bar Harbor, ME), were established on a C57BL/6 background together with their wild-type littermates at the UCSF Laboratory Animal Resource Center as previously described [15]. All experiments were performed on adult female PAR2-deficient mice or their age- and sex-matched wild-type (PAR2^{+/+}) littermates. Estrous cycles were not monitored. Mice were housed in a temperature-controlled room on a 12:12 hour light cycle (07:00–19:00 light), with unrestricted access to food and water. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of IASP [29] and were approved by the UCSF Institutional Animal Care and Use Committee.

Nociceptive threshold testing—Testing was performed by an observer blinded to the experimental groups during the afternoon portion of the circadian cycle, between 14:00 and 17:00. Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. The cage consists of 6 cubicles and allows testing 6 mice per session. After placement in the test environment, mice were acclimatized to the test environment for one hour prior to testing. An electronic von Frey anesthesiometer (2390 series, IITC Instruments, Woodland Hills, CA) was used to elicit paw withdrawal. A positive response was defined as

sharp withdrawal and immediate flinching upon application of an increasing force with the electronic von Frey rigid probe tip. Withdrawal threshold was defined as the force (g) that was sufficient to evoke a positive response. Test stimuli were presented three times at one minute intervals to allow resolution of previous stimulus. Baseline paw withdrawal threshold was defined as the mean of the three readings taken before injection of the test agent.

Drug administration—Mice were briefly anesthetized with isoflurane (Summit Medical Equipment Company, Bend, OR) for drug injection with a 25-gauge, 5/8 inch long needle (Becton Dickinson & Co., Franklin Lakes, NJ) placed subdermally into the plantar surface of the right hind paw. All injections were 50 μ l in volume.

Murine trigeminal ganglion and human SCC non-contact co-culture

Trigeminal ganglia were dissected aseptically from adult female wild-type C57BL/6 mice (6–8 weeks old, Jackson Laboratory) and prepared as previously described [15]. Cells were plated on poly-D-lysine-coated glass coverslips and cultured in DMEM/F-12 (1:1) medium (Invitrogen Corp.) supplemented with 10% fetal bovine serum for 48 hours prior to co-culture with human SCC (HSC-3). For the non-contact co-culture, a coverslip with pre-plated murine trigeminal neurons was combined in one non-treated culture dish with a coverslip of pre-plated human SCC cells. Cells were maintained in 3 ml of fresh DMEM/F-12 at 37°C in a humidified atmosphere of 5% CO₂. No physical contact occurred between neurons and SCC cells during this time.

Following 48 hours of non-contact co-culture, the trigeminal neurons were prepared with the same methodology as with the above human cancer cell line immunofluorescence study. The murine trigeminal neurons were incubated with the primary rabbit polyclonal anti-PAR2 antibody (0.4 μ g/mL H-99, Santa Cruz Biotechnology) and the primary goat polyclonal anti-TMPRSS2 goat antibody (0.4 μ g/mL N-13, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 hours followed by incubation with the secondary anti-rabbit Texas Red-conjugated antibody (7.5 μ g/mL, Jackson ImmunoResearch Laboratories) and the secondary anti-goat FITC-conjugated antibody (7.5 μ g/mL, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour at room temperature. The cover slips mounted on slides in Gel-Mount (Biomedica Corp.) and visualized on a Nikon Eclipse E600 microscope using epifluorescence. Controls for PAR2 included the replacement of the primary antibody with similarly diluted normal rabbit serum, omission of the primary antibody and the omission of both primary and secondary antibodies. In addition, as a negative control, incubation of trigeminal neurons cultured from adult female PAR2-deficient (PAR2^{-/-}) littermate mice (6–8 weeks old, Jackson Laboratory) with H-99 failed to demonstrate PAR2-immunoreactivity.

Calcium imaging methods

Trigeminal neurons were cultured on glass coverslips as previously described above, washed with Hank's Balanced Salt Solution (HBSS) and loaded for 45–60 minutes in 5 μ M fura 2-AM in HBSS. Cultures were removed from fura 2-AM loading solution and placed into HBSS for at least 15 minutes before calcium imaging. Fura 2 fluorescence was observed on a Nikon Eclipse Ti microscope with a 20 $\times$ objective and alternating excitation between 340

and 380 nm by a 75-watt xenon bulb. Images of emitted fluorescence at 510 nm were recorded by an intensified charge-coupled device camera (Retiga digital camera, QImaging) and simultaneously displayed on a color monitor. The imaging system was under software control (NIS-Elements, Nikon) and collected a ratio approximately every 0.5 seconds. A typical experiment consisted of 30 seconds recording in HBSS to determine the baseline 340/380 ratio (typically 0.5 for trigeminal neuron cultures) followed by a 10 second wash to introduce serine protease (TMPRSS2) or PAR2 agonist (2-at-LIGRL). TMPRSS2 (Abnova, Taipei, Taiwan) was concentrated using an Amicon Ultra-2 Centrifugal Filter Unit with Ultracel-50 membrane (EMD Millipore) according to the manufacturer's instructions, immediately prior to use in calcium imaging experiments. Neurons were then monitored for an additional 3 minutes to determine the calcium response and followed by a high KCl (75 mM) wash as a positive control.

Statistical analysis

Data are reported as mean $\pm$ s.e.m. The alpha level was set at 0.05. The following statistical tests were employed as appropriate: one-way ANOVA with Scheffé post-hocs, Pearson's correlation, one-way repeated measures ANOVA, two-way repeated measures ANOVA with one between-subjects factor (group) and one within-subjects factor (time). If the group $\times$ time interaction was significant, one-way ANOVAs with Scheffé post hocs were performed. In cases involving multiple comparisons, the alpha level was adjusted by dividing 0.05 by the number of comparisons.

RESULTS

Elevated levels of TMPRSS2 in cancer patients with significant pain at the time of initial diagnosis

To investigate the relationship of TMPRSS2 to clinical pain, biopsy specimens were collected from patients with oral SCC and non-cancer patients as controls. All patients were seen in the UCSF Department of Oral & Maxillofacial Surgery and were administered the UCSF Oral Cancer Pain Questionnaire on the same day as the biopsy [5,13]. Immunocytochemistry for TMPRSS2 was performed on all biopsy specimens. In agreement with our previous studies, oral cancer patients reported significantly greater spontaneous and function-related pain in comparison to normal non-cancer patients [5,13,16] (Figure 1). Pain scores and TMPRSS2 immunoreactivity were highly correlated for SCC patients (Figure 1), suggesting that the proportion of TMPRSS2 positive cells predicted the severity of cancer pain. Non-cancer patients demonstrated neither pain nor significant TMPRSS2 immunoreactivity.

Elevated TMPRSS2 expression in painful cancer cell lines

The reported prevalence of cancer pain is highest for head and neck and prostate cancers [9]. We hypothesize that if cancer pain intensity is mediated by TMPRSS2, then TMPRSS2 expression might be greater in head and neck and prostate cancer cell lines than in cell lines derived from less painful cancers, such as melanoma and breast cancers [23]. To test this hypothesis five human cancer cell lines were cultured and examined for TMPRSS2 expression using immunohistochemical techniques. These five cancer cell lines included two

from head and neck cancer (HSC-3 and SCC-9), and one each from melanoma (WM164), breast cancer (MCF-7) and the prostate cancer (LNCaP) line as a positive control (Figure 2). The greatest TMPRSS2 immunoreactivity was observed with the HSC-3, followed closely by the SCC-9 and LNCaP cell lines. All three showed significantly greater immunofluorescence than either WM164 or MCF-7, which were not significantly different from each other. Consistent with this, Western blotting showed comparable levels of TMPRSS2 in HSC-3 and SCC-9 human cancer cells relative to LNCaP (Figure 3a). TMPRSS2 is also released in significant levels into the cancer microenvironment, as detected by ELISA in the supernatant of HSC-3 cancer cells over a 72 hour time period (Figure 3b).

TMPRSS2 induces proteolytic activity, activates peripheral neurons, and co-localizes with PAR2 receptors on peripheral neurons

Because TMPRSS2-induced pain likely rests on its ability to activate PAR2 receptors through proteolysis, we measured the proteolytic activity of TMPRSS2 (Figure 4a). TMPRSS2 protein readily induced dose-dependent proteolysis that could be blocked by pre-incubation with the serine protease inhibitor FUT-175, consistent with its role as a functional serine protease. Next we tested whether TMPRSS2 alone can activate murine trigeminal ganglion neurons using single cell level calcium imaging. TMPRSS2 readily induced calcium influx in trigeminal neurons (Figure 4b). Further quantification of responsive cells from multiple independent experiments demonstrated that TMPRSS2 activates significantly more trigeminal neurons than the highly potent PAR2 agonist 2-at-LIGRL (63.8% vs. 24.4%). To investigate whether TMPRSS2 produced and released by head and neck cancer cells can co-localize with neuronal PAR2 receptors, murine trigeminal ganglion neurons were co-cultured with HSC-3 cells in non-contact conditions [15] for 48 hours. Trigeminal neurons showed significant co-localized immunoreactivity for TMPRSS2 and PAR2 receptors (Figure 4c).

TMPRSS2 induces pain in a PAR2-dependent manner

To determine if TMPRSS2 induces pain mediated by PAR2 receptors, five groups of mice were tested (n=5/group). Wild-type mice were administered either TMPRSS2 (4.5 µg or 9 µg), TMPRSS2 (9 µg) pre-incubated with FUT-175 for thirty minutes, or vehicle control; one group of PAR2^{-/-} mice was administered TMPRSS2 (9 µg). Both wild-type groups that received TMPRSS2 alone demonstrated spontaneous nocifensive behaviors (licking, shaking) of the injected hindpaw that lasted a few minutes (data not shown) and significant allodynia that began 10 minutes post-administration and lasted until 45 minutes post-administration (Fig. 5); none of the other groups showed any allodynic effect. These results indicate that TMPRSS2 induced spontaneous pain and allodynia in mice similar to that shown in humans (Figure 1) by acting as a serine protease at PAR2 receptors.

DISCUSSION

This is the first study to show a role for a membrane-bound mediator in cancer pain, and pain in general. TMPRSS2 is highly expressed in prostate cancers and is well known for its role in prostate carcinogenesis [2]. Here we report new insight into the role of TMPRSS2 in

cancer pain: TMPRSS2 is markedly elevated in head and neck cancer patients with levels correlating with pain severity, is produced and released by human head and neck cancer cells, induces dose-dependent proteolytic activity and neuronal activation, and pain via a PAR2-dependent mechanism.

The prevailing hypothesis to explain cancer pain posits that cancers generate and secrete mediators, which sensitize and activate primary afferent nociceptors in the cancer microenvironment. While a variety of pain mediators such as ATP, bradykinin, cytokines, chemokines, nerve growth factor, and vascular factors such as endothelin 1 may be released into the cancer microenvironment to excite or sensitize nociceptive primary afferents, little work has focused on the role of proteases [19]. We recently showed an important role for serine proteases in cancer-induced mechanical allodynia [15]. We now demonstrate that TMPRSS2 is a functional serine protease that plays a critical role in cancer-induced proteolysis, spontaneous pain, and mechanical allodynia. The onset of significant PAR2-dependent mechanical allodynia induced by TMPRSS2 is in agreement with the onset of mechanical allodynia evoked by injection of trypsin, also a serine protease and PAR2 agonist, into the mouse hindpaw shown previously [21]. However, in contrast to trypsin, TMPRSS2 as a membrane-bound protease that can be shed from the cell surface, may exert its nociceptive effects via intimate cancer cell surface-nociceptive afferent ending contact, as well as via release from cancer cells to affect more distant primary afferents. Thus, in addition to being released via cancer cell death and autolysis, the protease domain of TMPRSS2 may be cleaved and secreted into cell media after autocleavage [1]. Mutational inactivation of the TMPRSS2 protease revealed that the cleavage of the protease is a consequence of its own catalytic activity, suggesting that TMPRSS2 may be its own substrate [1]. Alternatively, TMPRSS2 may activate a secondary protease that then cleaves the TMPRSS2 protease. Both interpretations require an active TMPRSS2 protease as we have shown in the present study. Since human prostate cells are not known to release significant amounts of TMPRSS2 into the cancer microenvironment[4], our findings that TMPRSS2 is released by head and neck cancers suggest that the nociceptive effects of TMPRSS2 may be more far-reaching in head and neck cancer.

TMPPRSS2 has been shown to activate PAR2 on prostate cancer cells [28]. We now show TMPRSS2 activates trigeminal neurons and contribute to both spontaneous pain and mechanical allodynia. Our non-contact co-culture demonstrating co-localization of TMPRSS2 with PAR2 in trigeminal neurons suggests TMPRSS2 released from cancer cells co-localizes with peripheral PAR2 on sensory neurons supplying the cancer microenvironment. Alternatively, since it does not establish that the TMPRSS2 is of non-neuronal origin, it is possible that the co-culture method released growth factors from cancer cells that induced expression of TMPRSS2 in neurons. However, whether the source of TMPRSS2 is directly or indirectly from cancer cells, TMPRSS2 is available to activate PAR2 on neurons. PAR2 up-regulation on peripheral nociceptive afferents innervating the cancer microenvironment contributes to the transition to chronic cancer pain [14]. The inhibitory effect of a serine protease inhibitor on TMPRSS2-induced proteolysis and mechanical allodynia in the present study confirms the importance of serine protease activity in cancer pain. TMPRSS2 may not only facilitate and perpetuate tissue damage and invasion during carcinogenesis but may also contribute to cancer-induced pain by direct effects on

PAR2 located on primary afferents. Since we have provided the first evidence that TMPRSS2 not only contributes to cancer pain but its effects are reversible with serine protease inhibition, the targeting of TMPRSS2 that is highly localized in the cancer microenvironment may be a novel approach to the treatment of cancer pain, and minimize any possible untoward treatment-related systemic side effects.

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Figure 1. TMPRSS2 expression correlates with cancer pain intensity in patients

a) Representative image of heavy TMPRSS2 immunoreactivity (dark brown) in cancer cell cytoplasm and membrane of tissue biopsies from head and neck cancer patients. The hematoxylin counterstain (stains nuclear material blue) readily demonstrates the abnormal, enlarged, invasive squamous carcinoma cells. Horizontal white bar = 100 μ m.

b) Representative image showing lack of TMPRSS2 immunoreactivity in tissue biopsies of normal oral mucosa from non-cancer patients. The hematoxylin counterstain (stains nuclear material blue) readily demonstrates the normal, organized architecture of healthy oral epithelium. Horizontal white bar = 100 μ m.

c) *Left panel*, head and neck cancer patients with heavy TMPRSS2 immunoreactivity (a) have clinically significant spontaneous and function-related pain in contrast to pain-free normal control patients ($*p < 0.001$, RM ANOVA). *Right panel*, average VAS pain intensity plotted against the number of TMPRSS2-positive cells (shown as percent of total cells) for each of the 10 head and neck cancer patients included in the study. Note the high predictability of pain intensity based on the proportion of cells positive for TMPRSS2 (Pearson correlation = 0.924; $p < 0.001$). Number of cells counted was 484 ± 16.59 for head

and neck cancer patients and 461 ± 18.04 for normal controls (mean $\pm$ s.e.m.). The total pain scores for all normal control patients was zero and the total number of TMPRSS2-positive cells was six (i.e., about one cell per patient).

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Figure 2. TMPRSS2 expression greater in more painful cancer cell lines

a) The prevalence of cancer pain in head and neck and prostate cancers is known to be higher than melanoma and breast cancers[9]. Human head and neck cancer cell lines, HSC-3 and SCC-9, show comparable or greater levels of TMPRSS2 immunoreactivity than the human prostate cancer cell line LNCaP. All three show significantly greater immunoreactivity than either the human melanoma (WM164) or breast cancer (MCF-7) cell lines. A one-way ANOVA showed significant differences between the groups ($F_{2,2158}=207.861$; $p<0.001$). Scheffé post hocs showed that HSC-3 (n=404), SCC-9 (n=527),

and LNCaP (n=419) each showed significantly greater relative intensity than either MCF-7 (n=437) or WM164 (n=376), $p<0.001$ in all cases. In fact, HSC-3 showed greater intensity than the positive control LNCaP ($p=0.001$). MCF-7 and WM164 were not significantly different from each other ($p=0.979$). Symbols indicate statistical significance: different symbols are statistically different; the same symbols are not.

b–g) Representative images of TMPRSS2 immunoreactivity in LNCaP preabsorption control, LNCaP, HSC-3, SCC-9, WM164, and MCF-7 cell lines, respectively. Horizontal white bar = 100 μm .

Figure 3. TMPRSS2 is produced and released by cancer cells into the microenvironment
 a) Western blot analysis of TMPRSS2 expression in human head and neck cancer cell lines (SCC-9 and HSC-3) show comparable or greater levels of TMPRSS2 expression than the human prostate cancer cell line (LNCaP). All three show greater expression than the human melanoma (WM164) and breast cancer (MCF-7) cell lines. Image analysis also indicates that the cancer cell lines appear to produce greater amounts of the serine protease domain (32 kDa) than the full-length TMPRSS2 protein (70 kDa). GAPDH was used as a loading control. RVU = relative value units.

b) TMPRSS2 is released by the head and neck cancer cell line HSC-3 at increasing levels into the supernatant media over a 72 hour time period as detected by ELISA. A one-way repeated measures ANOVA showed a significant main effect of time ($F_{2,4}=16.079; p=0.002$).

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Figure 4. TMPRSS2 is a functional protease that activates trigeminal neurons

a) TMPRSS2 human recombinant protein induced dose-dependent proteolytic activity that was blocked by the serine protease inhibitor FUT-175 ($F_{3,12}=850.086$; $p<0.001$). Scheffé post hoc tests showed that the activity of the 7 µg (n=4) sample** was significantly greater than that of the 3 µg (n=4) sample* ($p<0.001$) and that both were significantly greater than either the vehicle (n=4) or the FUT-175 (n=4) samples ($p<0.001$ in each case). The vehicle and FUT-175 samples were not significantly different from each other ($p=0.988$). Similarly,

the 7 μ g (n=4) and matrix metalloprotease inhibitor GM 6001 (n=4) samples were not significantly different from each other ($p=0.988$).

b) Representative tracing (left panel) of TMPRSS2 human recombinant protein-induced calcium influx in cultured trigeminal neurons from multiple independent experiments (n=5). TMPRSS2-induced activation in a significantly greater percentage of trigeminal neurons than the PAR2 agonist 2-at-LIGRL ($p<0.0001$, right panel).

c) Murine trigeminal neurons were co-cultured with human cancer (HSC-3) cells in non-contact conditions for 48 hours. Representative images showing TMPRSS2 (*left panel*), PAR2 (*middle panel*), and merged (*right panel*) immunofluorescence.

Figure 5. TMPRSS2 induces pain in a PAR2-dependent manner

Allodynic effect of TMPRSS2 following subdermal administration into the plantar surface of the right hind paw. A two-way repeated measures ANOVA showed a significant group $\times$ time interaction ($F_{28,140}=1997.704$; $p<0.001$) and a significant main effect of group ($F_{4,20}=25.146$; $p<0.001$). Based on the significant interaction, one-way ANOVAs were performed for each time point followed by Scheffé post-hoc tests to determine the basis of the interaction. For the five minute time point $F_{4,10}=1955.995$; $p=0.012$; 10 minutes: $F_{4,10}=3857.80$, $p<0.001$ *; 15 minutes: $F_{4,10}=4427.423$, $p<0.001$ *; 30 minutes: $F_{4,10}=5588.449$, $p<0.001$ *; 45 minutes: $F_{4,10}=4860.896$, $p<0.001$ *; 60 minutes: $F_{4,10}=138.797$, $p=0.773$; 90 minutes: $F_{4,10}=22.969$, $p=0.859$. Asterisks (*) indicate time points showing significance in the figure. The two wild-type groups receiving TMPRSS2 alone were significantly different from the other three groups but not significantly different from each other at each time point showing significant differences. There were no significant differences among the other three groups (i.e., vehicle control, TMPRSS2 in PAR2^{-/-} mice, and TMPRSS2 combined with FUT-175). Also, there were no differences in baseline

withdrawal-thresholds: PBS = 3.86 ± 0.21 g, 4.5 μ g TMRSS2 = 4.33 ± 0.31 g, 9 μ g TMRSS2 = 4.21 ± 0.32 g, TMRSS2 with FUT-175 = 4.19 ± 0.29 g, and PAR2^{-/-} 4.06 ± 0.56 g. N=5 for all groups. Abbreviation: PWT = paw withdrawal threshold.