

Calcium Promotes Human Gastric Cancer via a Novel Coupling of Calcium-Sensing Receptor and TRPV4 Channel



Rui Xie^{1,2}, Jingyu Xu², Yufeng Xiao¹, Jilin Wu³, Hanxing Wan¹, Bo Tang¹, Jingjing Liu¹, Yahan Fan¹, Suming Wang¹, Yuyun Wu¹, Tobias Xiao Dong⁴, Michael X. Zhu^{3,5}, John M. Carethers⁶, Hui Dong^{1,4}, and Shiming Yang¹

Abstract

Although dietary calcium intake has long been recommended for disease prevention, the influence of calcium in development of cancer in the upper gastrointestinal tract has not been explored. Here, we assess the roles of calcium and calcium-sensing receptor (CaSR) in gastric cancer development. CaSR expression was enhanced in gastric cancer specimens, which positively correlated with serum calcium concentrations, tumor progression, poor survival, and male gender in gastric cancer patients. CaSR and transient receptor potential cation channel subfamily V member 4 (TRPV4) were colocalized in gastric cancer cells, and CaSR activation evoked

TRPV4-mediated Ca^{2+} entry. Both CaSR and TRPV4 were involved in Ca^{2+} -induced proliferation, migration, and invasion of gastric cancer cells through a Ca^{2+} /AKT/ β -catenin relay, which occurred only in gastric cancer cells or normal cells overexpressing CaSR. Tumor growth and metastasis of gastric cancer depended on CaSR in nude mice. Overall, our findings indicate that calcium may enhance expression and function of CaSR to potentially promote gastric cancer, and that targeting the novel CaSR/TRPV4/ Ca^{2+} pathway might serve as preventive or therapeutic strategies for gastric cancer. *Cancer Res*; 77(23); 6499–512. ©2017 AACR.

Introduction

Gastric cancer is the second leading cause of cancer-related death worldwide (1). Currently, gastric cancer is difficult to prevent and cure because of the poor understanding of its pathogenesis and difficulty in its early diagnosis. Dietary calcium intake has long been recommended for prevention of several human cancers, including colorectal cancer and pancreatic cancer, as a tumor suppressor therapy likely through activation of calcium-sensing receptor (CaSR; refs. 2, 3). However, whether calcium and CaSR have oncogenic potential or tumor suppressing role in the upper gastrointestinal tract is not known. It is essential to

investigate these important issues because calcium supplement has been widely advocated in our daily life.

CaSR plays critical roles in systemic calcium homeostasis and diverse pathophysiological conditions (4–7), including a pathogenic role in human pulmonary arterial hypertension recently identified by us (8). When stimulated by extracellular calcium ($[\text{Ca}^{2+}]_o$), CaSR acts through at least two G protein pathways (Gq/11 and Gi/o) to regulate cell signaling, including increases in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{cy}$), which can result from Ca^{2+} release from internal stores or Ca^{2+} entry from extracellular space through Ca^{2+} -permeable cation channels. The activation of intracellular Ca^{2+} effectors in turn regulates cellular activities necessary for cell-cycle progression and cell proliferation (5, 9). Abnormal expression and activity of Ca^{2+} signaling proteins have been suggested to contribute to the highly proliferative capacity of several human cancers (7, 9). These include members of the transient receptor potential (TRP) superfamily, which form Ca^{2+} entry channels. For example, TRPC6 and TRPV4 are involved in the development of gastric cancer (10, 11) and inflammatory bowel diseases (12, 13), respectively. However, little is currently known about the pathogenic roles of aberrant Ca^{2+} signaling and TRPV channels in gastric cancer.

In this study, we examined the roles of calcium and CaSR in gastric cancer development and elucidated the underlying molecular mechanisms. Unexpectedly, we found that calcium activates CaSR to promote gastric cancer growth and metastasis, instead of suppressing it as in the cases of colorectal cancer and pancreatic cancer. We further showed that CaSR activation triggers Ca^{2+} entry through TRPV4 channels and the downstream AKT/ β -catenin pathways to facilitate gastric cancer growth. Our results indicate that calcium supplement may be problematic, especially for gastric cancer patients, and also suggest a novel strategy for gastric

¹Department of Gastroenterology, Xinqiao Hospital, Third Military Medical University, Chongqing, China. ²Department of Gastroenterology, Affiliated Hospital to Zunyi Medical College, Zunyi, China. ³Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China. ⁴Department of Medicine, University of California, San Diego, California. ⁵Department of Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, Texas. ⁶Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

R. Xie and J. Xu equally contributed to this article.

Corresponding Authors: Hui Dong, Department of Gastroenterology, Xinqiao Hospital, Third Military Medical University, Chongqing, China. Phone: 86-136-0836-9343; E-mail: h2dong@ucsd.edu; and Shiming Yang, Department of Gastroenterology, Xinqiao Hospital, Third Military Medical University, Chongqing, China. E-mail: shimingyang@yahoo.com

doi: 10.1158/0008-5472.CAN-17-0360

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cancer prevention and therapy via modulation of CaSR-mediated signaling pathway.

Materials and Methods

Ethics statement and human specimens

All animal and clinical studies were approved by the Clinical Research Ethics Committee of the Third Military Medical University, Chongqing, China. Informed consent was obtained for all patients. Primary cancer tissues were collected from 91 patients who underwent curative surgery for gastric cancer at the Department of Surgery, Xinqiao Hospital, The Third Military Medical University, and then were stored at -80°C in liquid nitrogen. Cancer specimens were stratified according to histologic tumor type, grading, staging, gender, patient's age, and tumor size. This study was conducted in accordance with the Helsinki Declaration and the guidelines of the Ethics Committee of Xinqiao Hospital, Third Military Medical University. The study and the informed consent forms were approved by the Institutional Review Board of Xinqiao Hospital, Third Military Medical University.

Reagents and cell culture

All reagents were from Sigma. After dissolved in DMSO, they were diluted in cell culture media with final concentrations of below 0.1%. The rat intestinal epithelial cell (IEC) line IEC-6 was purchased from the Chinese Academy of Sciences in 2011. The human gastric normal epithelial mucosa GES-1 cell line and gastric cancer cell lines MKN45 and SGC-7901 were purchased from the Chinese Academy of Sciences in 2012. All cell lines were kept frozen in liquid nitrogen and after they were thawed, less than 20 of passages were used for 3 months in the present experiments. GES-1 and MKN45 cells were authenticated using short tandem repeat (STR) profiling analysis by Shanghi Meixuan Biological Science and Technology Ltd. SGC-7901 and IEC-6 cells were STR-authenticated by Shanghai Biowing Applied Biotechnology Co. Ltd. The cells lines were grown in RPMI1640, or Iscove's DMEM medium (GIBCO-BRL) supplemented with 10% FCS (GIBCO-BRL) in an incubator with 5% CO_2 at 37°C . IEC-6 cells ($2-4 \times 10^5$) were applied to 12-well Millicell inserts. After they were maintained for 14 days to form a monolayer, transepithelial resistance (TER) was determined with a Millicell-ERS volt ohmmeter (Millipore). The TER of IEC-6 monolayer usually could reach to over 120 Ω .

Immunohistochemistry

The slides with human gastric cancer tissues were incubated with an anti-CaSR monoclonal antibody (1:100 dilution; Abcam). The primary antibodies were detected with biotinylated goat anti-mouse IgG (Vector Laboratories) secondary antibodies. Immunoreactivity was detected using a Horseradish Peroxidase (3', 3'-diaminobenzidine) Kit (BioGenex), followed by counterstaining with hematoxylin, dehydration, and mounting.

Immunofluorescence staining

The cells grown in coverslips were fixed with ice-cold methanol/acetone for 20 minutes, permeabilized for 25 minutes, blocked for 30 minutes, and incubated with primary antibodies against CaSR (1:100 dilution; Thermo) overnight at 4°C . Then the cells were incubated with FITC-IgG fluorescence secondary antibody (1:500 dilution; ZSGB-BIO) at 37°C for 1 hour.

Measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$ by digital Ca^{2+} imaging

The cells grown on coverslips or inserts were loaded with $5 \mu\text{mol/L}$ fura-2 AM in physiologic salt solution (PSS) at 22°C for 50 minutes and then washed in PSS for 30 minutes. Thereafter, cells on coverslips were mounted in a standard perfusion chamber on a Nikon microscope stage. However, cells on inserts were mounted in a special dual perfusion chamber so that the monolayer could be treated with drugs separately from apical or basolateral side of the cells. The ratio of fura-2 fluorescence with excitation at 340 or 380 nm (F340/380) was followed over time and captured with an intensified CCD camera (ICCD200) and a MetaFluor Imaging System (Universal Imaging). PSS used in Ca^{2+} measurement contained the following (in mmol/L): 140 Na^+ , 5 K^+ , 2 Ca^{2+} , 147 Cl^- , 10 HEPES, and 10 glucose, pH 7.4. For the Ca^{2+} -free solution, Ca^{2+} was omitted and 0.5 mmol/L EGTA was added.

Quantitative real-time PCR

Total RNA was extracted by RNAiso plus (Takara). The RNA was reverse transcribed to cDNA using PrimeScript RT-polymerase (Takara). qPCR was performed using cDNA primers specific for CaSR. The gene β -actin was used as an internal control. All the real-time RT-PCR reactions were performed with SYBR Green Supermix (Bio-Rad). The following SYBR green primers were used.

CASR-F: AGGCCGGAGTCTGTGGAATGTA
 CASR-R: CAGCGTCAAGTTGGGAAGAAGG
 β -actin-F: ACCCCGTGCTGCTGACCCGAG
 β -actin-R: TCCCGGCCAGCCAGGTCCA

Western blotting

For immunoprecipitation studies, lysates were incubated with a CaSR antibody for 1 hour at 4°C . Pellets or cell lysates were resuspended in 2x loading buffer, boiled for 5 minutes, and separated by SDS-PAGE (10%). Resolved proteins were transferred onto a PVDF membrane (Millipore Corporate). Membranes were blocked by 5% blocking buffer, followed by incubation with a monoclonal antibody CaSR, 1:500 (Abcam), p-ERK and p-JNK, 1:1,000 (Bioworld), β -catenin, 1:1,000 (Cell Signaling Technology), and GAPDH, 1:5,000 (Ambion), TRPV4, 1:2,000 (Miliper), respectively. After washing with TBST, secondary antibody was applied. The signals were visualized using enhanced chemiluminescence (ECL; Thermo).

Cell proliferation assay

Cell viability was assessed by Cell Counting Kit- (CCK-) 8 (Tongren). CCK-8 reagent was added to each well at 0.5 to 2 hours before the endpoint of incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader. Experiments were repeated at least three times each time in triplicate.

Cell scratch test

The monolayer was gently scratched across the center of the well with a 10 μL pipette tip. After scratching, the well was gently washed with medium to remove the detached cells. After different treatments, images were obtained at 0 and 24 hours by using a microscope. Narrower widths indicate the migration distances.

Transwell invasion assays

A 24-well transwell chamber (8- μ m pores; Corning) was used for this assay. The upper surface of polycarbonate filters with 8- μ m pores was coated with matrigel (Collaborative Biomedical). The serum-free RPMI1640/DMEM was used to suspend the cells (1×10^4 cells/100 μ L), then the cells were placed in the upper chambers and treated with different drugs. The lower chambers were filled with RPMI1640 plus 10% FBS medium. After 24 hours of incubation, the cells that had migrated onto the lower surface were stained with Crystal violet, and then the number of cells was counted under a microscope (Olympus Corporation). The average number of three random selected fields was taken as the number of invasion and metastasis.

Treatment of subcutaneous gastric cancer xenografts

Approximate 1×10^6 SGC-7901 cancer cells were injected into the armpits of male nude mice (~4 weeks old). After tumor sizes reached around 1 mm³, CaCl₂ (4 mmol/L), NPS2143 (15 μ mol/L), or both (100 μ L) were injected into the tumors in one side of their armpits once a day, and DMSO (0.1%) into the tumors in another side as controls. The volumes of gastric cancer xenografts were assessed each week. Similarly, the SGC-7901 infected with CaSR-shRNA or CaSR-NC was separately injected into each side of their armpits. After 4 weeks, the tumors were visualized with an *in vivo* imaging system (Cambridge Research & Instrumentation). On day 30 after implantation of SGC-7901 cells, the mice were killed and then the xenografted tumor volumes were measured using a digital caliper. The tumor volume in mm³ was calculated by the formula $1/2 (\text{length} \times \text{width}^2)$. For the experiment in which CaCl₂ was injected intravenously in mouse tail vein with serum calcium measurement, male nude mice with xenografts were randomly assigned to control group and treatment group, between them there was no significantly difference in their basal values of serum calcium (ranging from 2.20 to 2.65 mmol/L). Afterward, each mouse in treatment group received 1.38 mg CaCl₂ in 50 μ L of 0.9% saline per day via an intravenous injection into tail veins to raise the final serum calcium concentrations to 6 mmol/L, whereas each mouse in control group received 50 μ L of 0.9% saline. Three mice in each group were randomly assigned to be killed every other day for a total of 4 weeks to measure serum calcium concentrations.

In vivo metastasis assay

For the peritoneal dissemination assay, a total of 15 mice were divided into three groups, that is, Control group, Ca²⁺ group, and Ca²⁺+NPS group. A total of 1×10^6 MKN45 cells were injected into the abdominal cavity of nude mice, and then CaCl₂ (4 mmol/L), NPS2143 (15 μ mol/L), or both (100 μ L) were injected into abdominal cavity of nude mice once a day, and DMSO (0.1%) as controls. Six weeks later, the mice were killed, and the nodules were observed and counted. The mice were euthanized, and tumors were counted.

Statistical analysis

All data were expressed as the means for a series of *n* experiments \pm SEM, and analyzed by one-way ANOVA followed by the Student–Newman–Keul *post hoc* test or by Student *t* tests for paired or unpaired samples with GraphPad Prism 5.0. $P < 0.05$ was considered statistically significant.

Study approval

All experimental procedures involving animals and humans in this study were reviewed and approved by the Institutional Animal Care and Use Committee and the Clinical Research Ethics Committee of the Third Military Medical University, Chongqing, China. Written informed consent was received from participants prior to inclusion in the study. Participants were identified by number.

Results

CaSR expression is enhanced in human primary gastric cancer tissues

Because calcium stimulates plasma membrane CaSR to produce biological activities, we first compared the expression of CaSR in normal stomach and gastric cancer tissues at protein and mRNA levels. Immunohistologic studies revealed little staining of CaSR in normal human gastric tissues but strong staining in primary gastric cancer tissues (Fig. 1A). The expression of CaSR proteins and transcripts were significantly enhanced in gastric cancer tissues (Fig. 1A and B). The human gastric cancer cell lines (SGC-7901 and MKN45) also exhibited greater expression of CaSR than the nontumorigenic gastric epithelial cell line (GES-1), as determined by Western blot analysis and qRT-PCR (Fig. 1C), as well as immunofluorescence staining (Fig. 1D). These results are consistent with the previous report showing that gastric cancer cells had copy number gain of the CaSR gene (14), suggesting that CaSR may be oncogenic in gastric cancer.

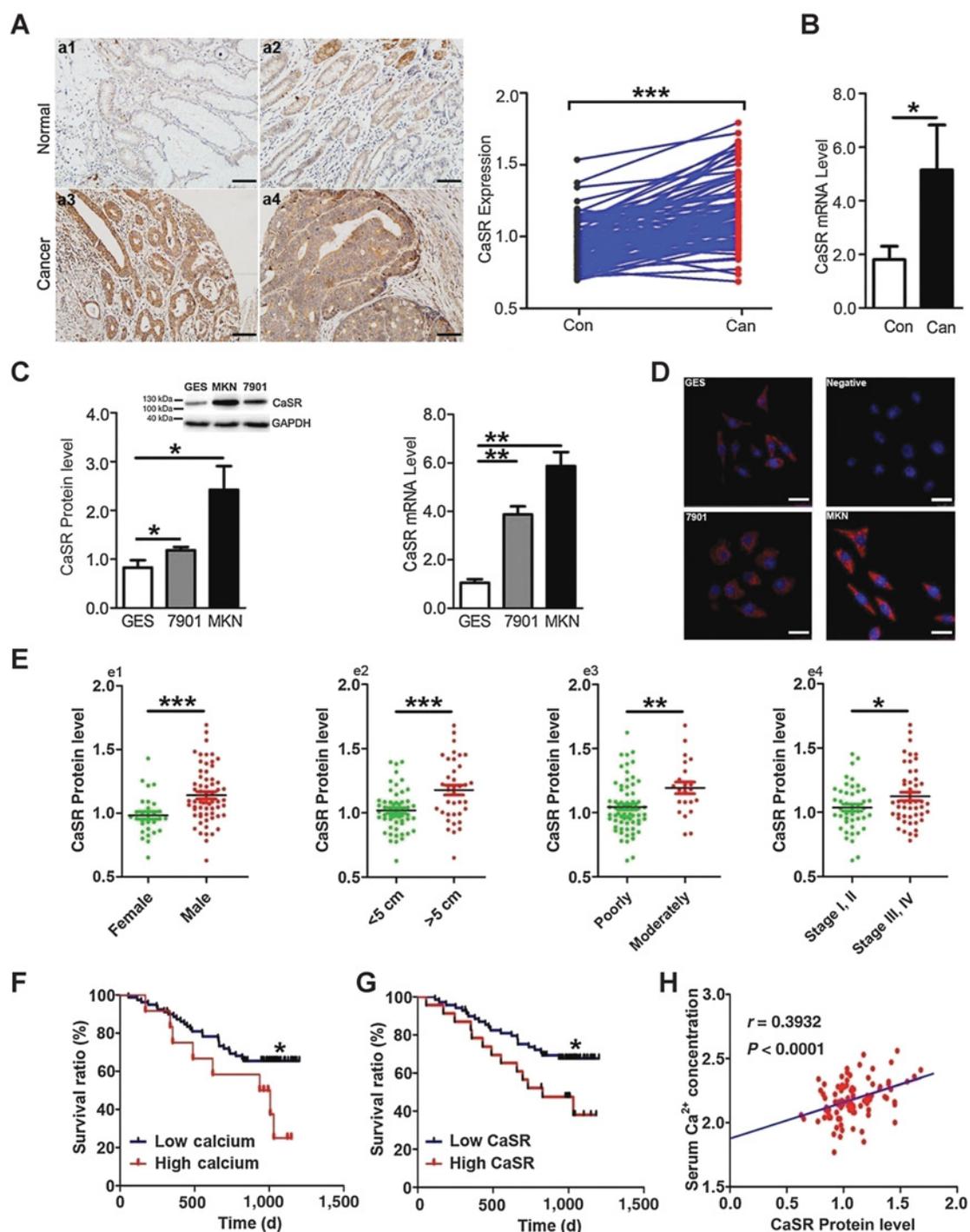
High serum calcium and CaSR expression correlate with severe gastric cancer progression and poor patient survival

Western blot analysis of a large cohort of 91 gastric cancer patients with different sexes, ages, and different extents of tumor progression revealed significantly increased CaSR expression in male patients, bigger tumors, moderate differentiation, and late stage (Fig. 1E), indicating that a positive association of CaSR expression with gastric cancer progression. Because up to 30% of patients with cancers may develop hypercalcemia during their disease course (15–17), the correlation between serum calcium and the survival of gastric cancer patients was analyzed. We found the two parameters to be negatively correlated (Fig. 1F), which is consistent with the notion that gastric cancer patients with hypercalcemia usually have poor prognosis (15–17). Because CaSR on the serosal side of gastric epithelial cells can be activated by serum calcium, we also analyzed the correlation between CaSR expression and survival of gastric cancer patients and found the patients with high CaSR expression showed markedly lower survival than those with low CaSR expression (Fig. 1G), suggesting that CaSR stimulation by high serum calcium may be involved in poor prognosis of gastric cancer. Furthermore, we compared serum calcium levels of the patients with the CaSR expression levels in their gastric cancer tissues and found the two parameters to be positively correlated (Fig. 1H), which is consistent with the findings that $[\text{Ca}^{2+}]_o$ induces expression and function of CaSR. Therefore, unlike serving as suppressors in colorectal cancer, calcium and CaSR may serve as tumor promoters in gastric cancer.

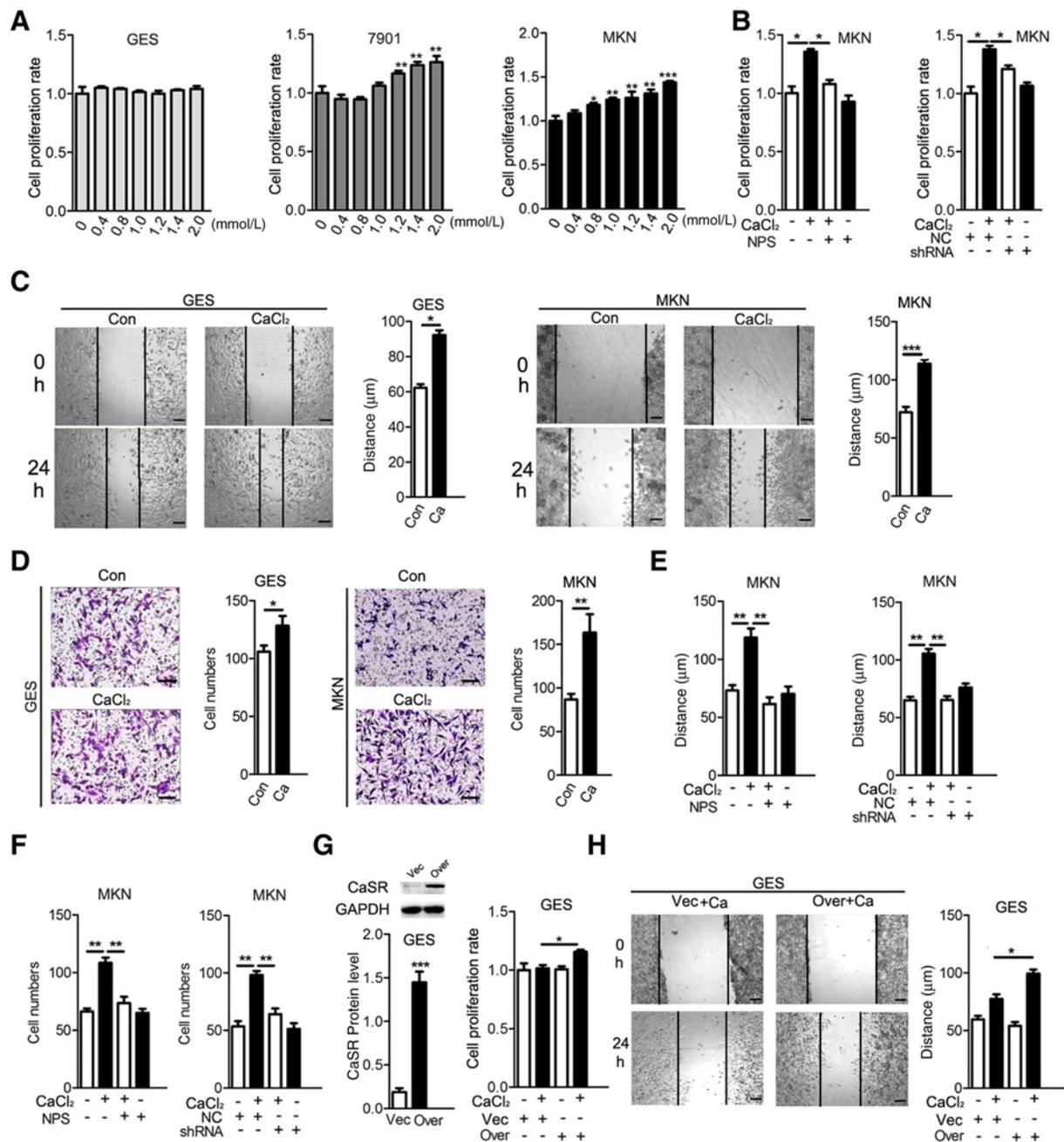
Calcium enhances gastric cancer cell proliferation, migration, and invasion through activation of CaSR *in vitro*

To examine the roles of calcium and CaSR in gastric cancer, GES-1, SGC-7901, and MKN45 cells were cultured in varying

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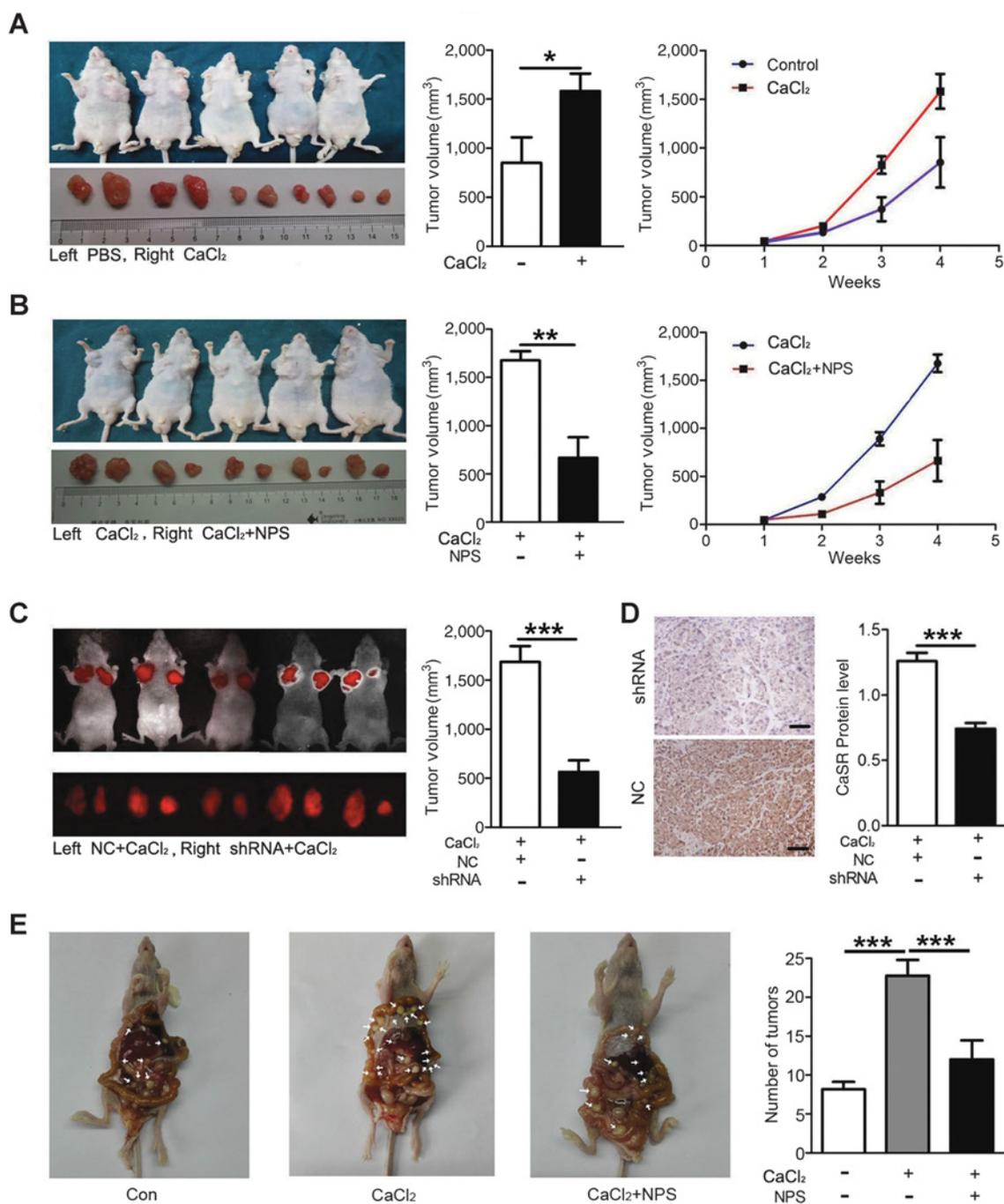
**Figure 1.**

Enhanced CaSR expression and serum calcium are associated with human gastric cancer progression. **A**, Representative images (left) and summary data (right) of immunohistologic staining in gastric cancer biopsy tissues (Can) normalized to their paraneoplastic normal tissues (Con; ***, $P < 0.001$, $n = 91$ patients). Scale bars in a1–a4, 100 μm . **B**, Transcript levels of CaSR determined by qRT-PCR in gastric cancer biopsy tissues (Can) normalized to their paraneoplastic normal tissues (Con; *, $P < 0.05$, $n = 22$ patients). **C**, Western blot and qRT-PCR analyses of CaSR protein and mRNA in a human normal gastric epithelial cell line (GES-1) and gastric cancer cell lines (SGC-7901 and MKN45; *, $P < 0.05$; **, $P < 0.01$, $n = 3$). Inset shows representative Western blots. **D**, Representative images of immunofluorescence labeling of CaSR proteins with primary antibody in GES-1, SGC-7901, and MKN45 cells and a negative control without the antibody. Scale bars, 25 μm . **E**, Relative CaSR protein levels in gastric cancer samples from the patients with different sexes (e1), tumor sizes (e2), differentiation (e3), and stages (e4; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, $n = 91$ patients). **F**, Kaplan–Meier analysis of survival ratio of gastric cancer patients with low and high serum calcium concentration (*, $P < 0.05$, $n = 91$). **G**, Kaplan–Meier analysis of survival ratio of gastric cancer patients with low and high CaSR expression levels (*, $P < 0.05$, $n = 91$). **H**, Correlation analysis of CaSR protein levels and serum calcium concentrations in gastric cancer patients ($r = 0.3932$, $P < 0.0001$, $n = 91$).

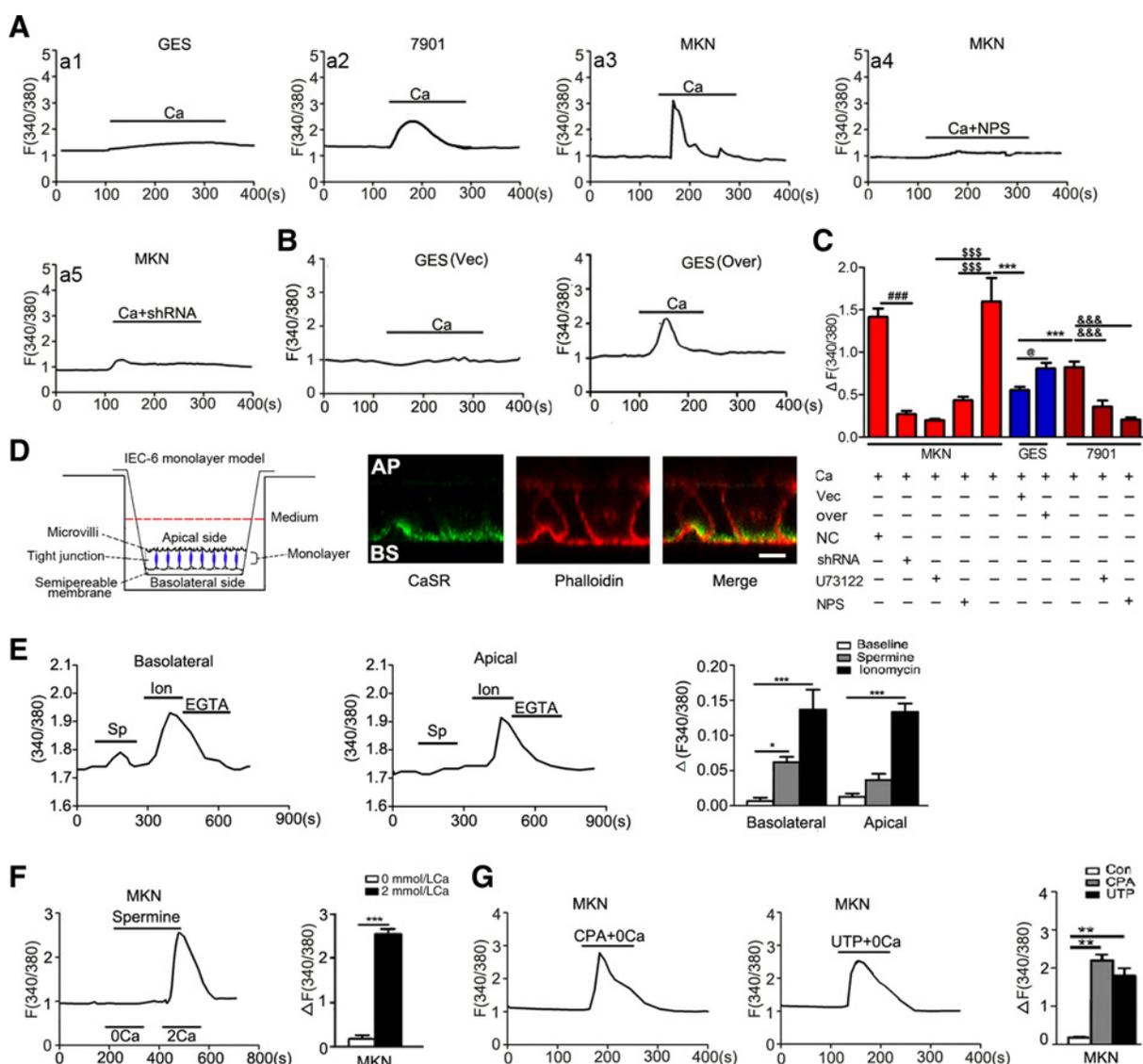
**Figure 2.**

CaSR promotes proliferation, migration, and invasion of gastric cancer cells. **A**, Concentration dependence of cell proliferation on extracellular Ca²⁺ of human normal gastric epithelial cell line (GES-1) and gastric cancer cell lines (SGC-7901 and MKN45; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001, *n* = 3). **B**, Effects of NPS 2143 (NPS) and CaSR knockdown by shRNA on CaCl₂ (2.0 mmol/L)-induced proliferation of MKN45 cells, and the efficiency of shRNA knockdown on the expression of CaSR protein in MKN45 cells (**, *P* < 0.01, *n* = 3; *, *P* < 0.05, *n* = 3). NC, negative control. **C**, CaCl₂ (1.2 mmol/L) enhanced migration of GES-1 and MKN45 cells in scratch test. Control treatment had PBS. Scale bars, 100 μm. Summary of distance migrated in 24 hours is shown to the right of the images (*, *P* < 0.05; ***, *P* < 0.001, *n* = 3). **D**, Ca²⁺-enhanced invasion of GES-1 and MKN45 cells in the transwell study. Control and CaCl₂ treatment had PBS and 1.2 mmol/L CaCl₂, respectively. Scale bars, 50 μm. Summary of relative cell numbers migrated normalized to control is shown to the right of the images (*, *P* < 0.05; **, *P* < 0.01, *n* = 3). **E**, Effects of NPS 2143 and CaSR knockdown on migration of MKN45 cells in the presence and absence of 1.2 mmol/L CaCl₂ (*, *P* < 0.05, *n* = 3). **F**, Effects of NPS 2143 and CaSR knockdown on the invasion of MKN45 cells in the presence and absence of Ca²⁺ (*, *P* < 0.01, *n* = 3). **G** and **H**, Overexpression of CaSR proteins in GES-1 cells (**G**, left; ***, *P* < 0.001 vs. vector, *n* = 3) enhanced CaCl₂-induced proliferation (**G**, right; *, *P* < 0.05 vs. vector, *n* = 3) and migration (**H**, left, representative images; right, summary; *, *P* < 0.05 vs. vector, *n* = 3) of GES-1 cells. Scale bars in **H**, 100 μm.

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**Figure 3.**

CaSR enhances gastric tumor growth and gastric cancer cell metastasis *in vivo*. **A**, Ca²⁺ enhanced the growth of xenografted gastric tumors. Nude mice were subcutaneously xenografted in both armpits. The implanted areas were injected daily for 30 days with 4 mmol/L CaCl₂ in normal saline on the right and control PBS on the left. Shown are images of the mice and their tumors (left), summary of tumor volumes (middle), and the time courses of volume changes (right) without or with CaCl₂ injection (*, $P < 0.05$, $n = 5$ mice). **B**, NPS 2143 inhibited CaCl₂-induced gastric tumor growth in the xenograft model. The xenografted nude mice were injected with either CaCl₂ (4 mmol/L) alone (left) or CaCl₂ plus NPS 2143 (15 μ mol/L, right) daily for 30 days. Shown are images of the mice and their tumors (left), summary of tumor volumes (middle), and the time courses of volume changes (right) without or with NPS 2143 (**, $P < 0.01$, $n = 5$ mice). **C**, CaSR knockdown inhibited Ca²⁺-induced gastric tumor growth in the xenograft model. Nude mice were xenografted with tumor cells pretreated with shRNA-CaSR (right) or shRNA-NC (left) and injected daily for 30 days with 4 mmol/L CaCl₂. Shown are fluorescence images of tumors taken with an *in vivo* imaging system (left) and summary of CaCl₂ volumes (right, ***, $P < 0.001$, $n = 5$ mice). **D**, Comparisons of CaSR proteins with or without shRNA knockdown by immunohistochemistry analysis of gastric cancer tissues. Scale bars, 100 μ m (***, $P < 0.001$, $n = 5$ mice). **E**, The images and summary data of nude mice injected peritoneally with MKN45 cells pre-cultured with either 4 mmol/L CaCl₂ alone or CaCl₂ plus 15 μ mol/L NPS 2143 (left), and the number of tumors in each group (right). White arrows, metastatic tumors. **, $P < 0.01$, $n = 5$ mice in each group.

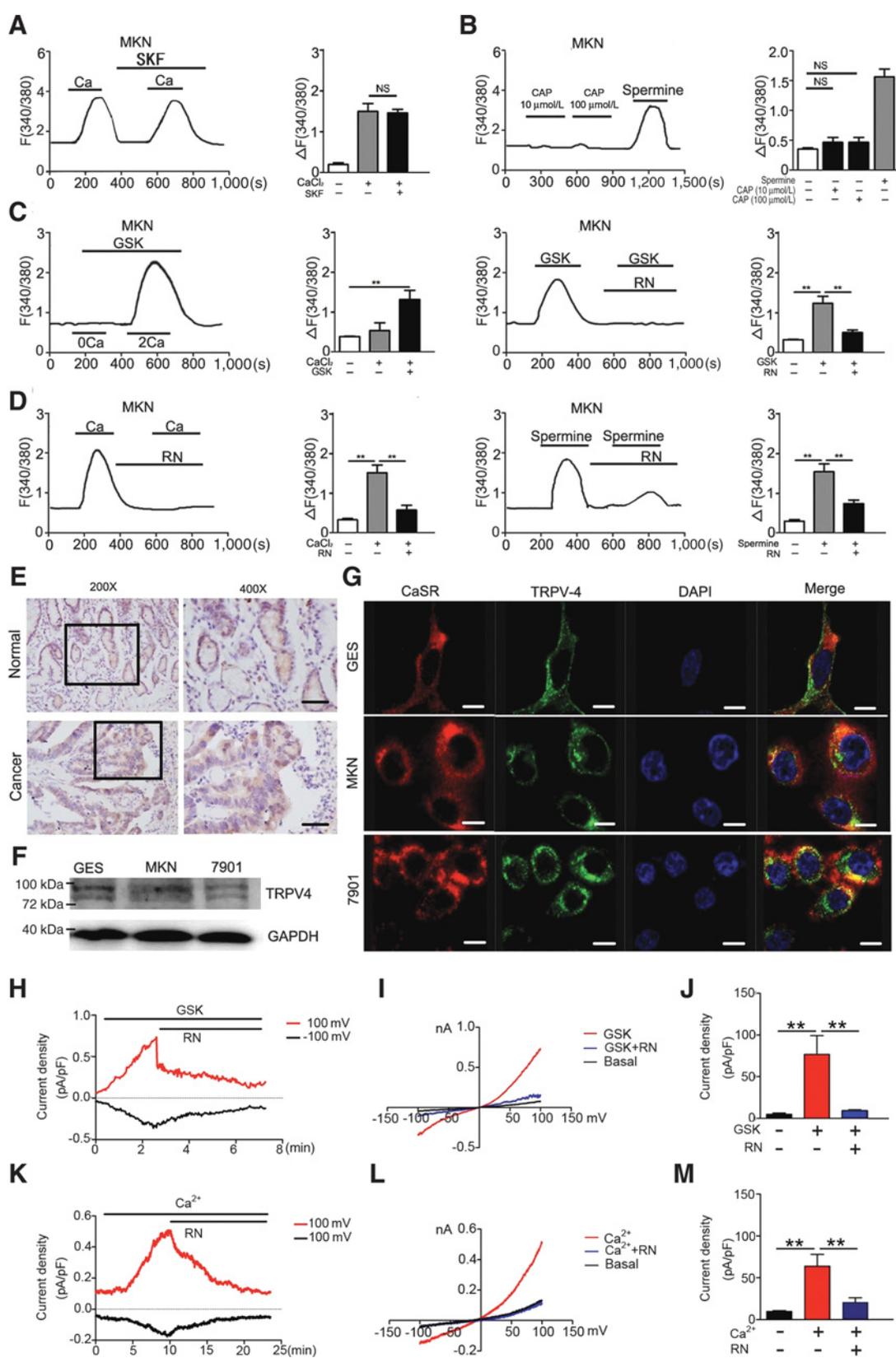
**Figure 4.**

CaSR activation causes cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increases in gastric epithelial and gastric cancer cells. **A**, Representative tracings showing $CaCl_2$ -induced $[Ca^{2+}]_{cyt}$ in GES-1 (**a1**), SGC-7901 (**a2**), and MKN45 (**a3**) cells. CaSR knockdown (**a4**) and NPS 2143 (5 μ mol/L; **a5**) on $CaCl_2$ -induced $[Ca^{2+}]_{cyt}$ in MKN45 cells. **B**, Representative tracings of $CaCl_2$ -induced Ca^{2+} signaling in GES-1 cells with (Over) or without (Vec) CaSR overexpression. **C**, Summary of $CaCl_2$ -induced $[Ca^{2+}]_{cyt}$ in different cells with different treatments ($^{\circ}$, $P < 0.05$ or *** , $^{###}$, $^{&&&}$, $^{&&&}$, $^{&&&}$, $^{&&&}$, $^{&&&}$, $^{&&&}$, $P < 0.001$, $n = 20$ cells). **D**, Diagram of dual perfusion system (left) and confocal microscopy images of CaSR (green) with an xz-scan through polarized IEC-6 monolayer culture (right). Phalloidin (actin, red) was used as a control (AP, apical and BS, basolateral; scale bar, 8 μ m). **E**, Representative tracings and summary of Ca^{2+} signaling using dual perfusion system to deliver drugs to each side of IEC-6 monolayer ($^{\circ}$, $P < 0.05$ or *** , $P < 0.001$, $n = 3$). **F**, Representative tracings and summary of spermine-induced $[Ca^{2+}]_{cyt}$ signaling in MKN45 cells in the absence (0Ca) or presence of 2 mmol/L extracellular Ca^{2+} (*** , $P < 0.001$, $n = 20$ cells). **G**, Representative tracings and summary of CPA (10 μ mol/L) or UTP (10 μ mol/L)-induced $[Ca^{2+}]_{cyt}$ signaling in Ca^{2+} -free (0Ca) solutions (** , $P < 0.01$, $n = 20$ cells). The $CaCl_2$ of 5 mmol/L and spermine of 3 mmol/L were used for all experiments.

concentrations of $CaCl_2$ and the rates of proliferation were determined. Although $CaCl_2$ did not alter proliferation of GES-1 cells, it dose-dependently enhanced proliferation of gastric cancer cells (Fig. 2A). $CaCl_2$ treatment also time-dependently increased protein expression of PCNA, a well-known biomarker of proliferation in gastric cancer cells (Supplementary Fig. S1A). In MKN45 cells, the $CaCl_2$ -enhanced proliferation of gastric cancer cells was attenuated by either a CaSR antagonist NPS 2143 or the knockdown of CaSR expression by its shRNA

(Fig. 2B and Supplementary Fig. S2A). Moreover, $CaCl_2$ -enhanced migration (Fig. 2C) and invasion (Fig. 2D) of GES-1 and MKN45 cells were also attenuated by either NPS 2143 or knockdown of CaSR (Fig. 2E and F). However, overexpression of CaSR in GES-1 cell allowed $CaCl_2$ to increase proliferation (Fig. 2G) and further accelerated migration of these cells (Fig. 2H). Therefore, calcium promoted cell proliferation, migration, and invasion of gastric cancer cells through activation of CaSR.

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Calcium enhances the growth and metastasis of GC through activation of CaSR in nude mice *in vivo*

In subcutaneously xenografted gastric cancer model of nude mice, CaSR activation by daily administration of CaCl_2 increased tumor volumes and the effect was attenuated by coinjection with NPS 2143 (Fig. 3A and B), which itself did not alter tumor volumes (Supplementary Fig. S2B). The knockdown of CaSR from SGC-7901 cells with the use of CaSR-shRNA lentiviruses (Supplementary Fig. S2C and S2D) also suppressed their ability to grow after implantation (Fig. 3C). Immunohistochemical analysis showed that tumors derived from the implants treated with CaSR shRNA indeed had lower CaSR expression than those treated with control shRNA (Fig. 3D). However, the tumors derived from the implants with calcium treatment had high PCNA staining than those without calcium treatment (Supplementary Fig. S2E), backing up our earlier studies done on cell culture *in vitro*. Moreover, calcium was also injected intravenously into the tail veins per day, and then serum calcium concentrations were measured every other day for a total of 28 days. Our data showed that serum calcium concentrations in the treatment group injected with calcium were not changed compared to those in the control group injected with 0.9% saline (2.48 ± 0.02 vs. 2.46 ± 0.02 , $P > 0.05$, $n = 42$ for each group; Supplementary Fig. S3A). Therefore, an intravenous injection of CaCl_2 failed to induce a hypocalcemia model (Supplementary Fig. S3B).

We further explored whether metastasis of gastric cancer cell depends on CaSR *in vivo*. MKN45 cells precultured with either CaCl_2 alone or CaCl_2 plus NPS2143 were injected peritoneally into nude mice. In control group, the same amount of untreated gastric cancer cells was injected. Six weeks later, the numbers of metastatic tumors were counted in the mice. As shown in Fig. 3E, the CaCl_2 -treated group was found to carry much more metastatic tumors, which was significantly inhibited by NPS2143. Collectively, these results strongly suggest that calcium and CaSR are promoters of gastric cancer growth and metastasis *in vivo*.

CaSR activation mediates intracellular Ca^{2+} signals in gastric cancer cells

We determined if CaSR mediates $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in gastric cancer cells by Fura-2 Ca^{2+} imaging. Although 5 mmol/L CaCl_2 induced a slow and slight rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in normal GES-1 cells, it evoked a marked elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in the two gastric cancer cell lines (Fig. 4A), suggesting an enhanced function of CaSR in gastric cancer cells. The CaCl_2 -induced $[\text{Ca}^{2+}]_{\text{cyt}}$ rise in MKN45

cells was abolished by NPS 2143 or shRNA knockdown of CaSR (Fig. 4A), demonstrating that CaSR mediates the response. Like CaCl_2 , spermine had similar effects on $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in GES-1 and MKN45 cells (Supplementary Fig. S4A; ref. 18). Overexpression of CaSR in GES-1 cells also markedly increased $[\text{Ca}^{2+}]_{\text{cyt}}$ response to CaCl_2 (Fig. 4B), further supporting a role of calcium in generating cell Ca^{2+} signal through activation of CaSR in gastric epithelial cells (Fig. 4C).

CaSR in basolateral membrane plays a functional role

To examine on which side of the epithelial cells CaSR functions, we delivered spermine using a dual perfusion system to either side of the small IEC (IEC-6) monolayer (the left panel in Fig. 4D), because the gastric cell lines cannot grow into monolayers. Spermine did not alter $[\text{Ca}^{2+}]_{\text{cyt}}$ when applied from the apical side, but induced a marked $[\text{Ca}^{2+}]_{\text{cyt}}$ increase when perfused onto to basolateral side (Fig. 4E). As a control, ionomycin, a Ca^{2+} ionophore, evoked a Ca^{2+} transient when applied from each side of the monolayer and the response was reduced by EGTA, a Ca^{2+} chelator (Fig. 4E). The specific response by CaSR at the basolateral side is in agreement with immunofluorescence labeling of CaSR at the basolateral membrane of IEC-6 monolayer (the right panels in Fig. 4D) and consistent with the gastric cancer patient data described above (Fig. 1H), showing that CaSR expression is correlated with Ca^{2+} levels in the serum, to which only the serosal side of gastric epithelial cells is exposed.

CaSR stimulates extracellular Ca^{2+} entry rather than internal store Ca^{2+} release

To distinguish whether Ca^{2+} arose from external or internal sources in response to CaSR activation in gastric cancer cells, spermine was first applied in a Ca^{2+} -free solution. This did not elicit $[\text{Ca}^{2+}]_{\text{cyt}}$ change in either MKN45 or SGC-7901 cells (Fig. 4F and Supplementary Fig. S4B). In contrast, re-addition of CaCl_2 led to immediate elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in the continued presence of spermine (Fig. 4F and Supplementary Fig. S4B). However, re-addition of CaCl_2 to Ca^{2+} -free solution did not induce $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the absence of spermine. Both CPA, a SERCA inhibitor, and UTP, a selective agonist of P2Y receptors, were able to induce sizeable Ca^{2+} transients in the Ca^{2+} -free solution (Fig. 4G and Supplementary Fig. S4C), indicating the functional integrity of internal Ca^{2+} pools under these conditions. Therefore, Ca^{2+} entry represents the major route of CaSR-stimulated Ca^{2+} signal in gastric cancer cells. Furthermore, the $[\text{Ca}^{2+}]_{\text{cyt}}$ rise induced by

Figure 5.

TRPV4 mediates currents and Ca^{2+} entry in response to CaSR activation human gastric epithelial and gastric cancer cells. **A**, CaCl_2 -induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in MKN45 cells was not affected by SKF96365 (30 $\mu\text{mol/L}$; NS, no significance; $n = 20$ cells). **B**, Capsaicin (10–100 $\mu\text{mol/L}$) did not elicit $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in MKN45 cells. Spermine (3 mmol/L) was used as a positive control (NS, no significance; $n = 20$ cells). **C**, GSK1016790A (1 $\mu\text{mol/L}$) increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in the presence, but not the absence of 2 mmol/L extracellular Ca^{2+} (left; **, $P < 0.01$, $n = 20$ cells), and the effect was blocked by 30 $\mu\text{mol/L}$ RN1734 (right; **, $P < 0.01$, $n = 20$ cells). **D**, RN1734 (30 $\mu\text{mol/L}$) inhibited $[\text{Ca}^{2+}]_{\text{cyt}}$ increases induced by CaCl_2 (5 mmol/L; left) and spermine (3 mmol/L; right) in MKN45 cells (**, $P < 0.01$, $n = 20$ cells). **E**, Immunohistochemistry analysis of TRPV4 proteins in human normal gastric (top) and gastric cancer (bottom) biopsy tissues, representatives of 10 samples. Scale bars, 10 μm . **F**, Western blot analysis of TRPV4 proteins in the indicated cell lines, representatives of three independent experiments. **G**, Confocal immunofluorescence images showing colocalization of CaSR and TRPV4 in human gastric epithelial and gastric cancer cells, representatives of three independent experiments. Scale bars, 25 μm . **H**, Whole-cell currents in MKN45 cells evoked by TRPV4 agonist GSK1016790A (50 nmol/L) and the inhibition by TRPV4 antagonist RN1734 (30 $\mu\text{mol/L}$). **I**, Current-voltage (I - V) relationships obtained from voltage ramps under basal, peak of GSK1016790A response, and after inhibition by RN1734 (30 $\mu\text{mol/L}$). **J**, Summary of current densities of GSK1016790A and RN1734 at +100 mV. **K**, Whole-cell currents in MKN45 cells evoked by CaSR agonist CaCl_2 (5 mmol/L) and the inhibition by RN1734 (60 $\mu\text{mol/L}$). **L**, Current-voltage (I - V) relationships obtained from voltage ramps under basal, peak of CaCl_2 response, and after inhibition by RN1734 (30 $\mu\text{mol/L}$). **M**, Summary of current densities of CaCl_2 and RN1734 at +100 mV. **, $P < 0.01$, $n = 5$ cells for all experiments.

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CaCl₂ or spermine was abolished by U73122 (Fig. 4C and Supplementary Fig. S4D and S4E), a selective inhibitor of phospholipase C (PLC), suggesting its involvement in CaSR-stimulated Ca²⁺ entry in gastric cancer cells.

TRPV4 plays a major role in CaSR-stimulated Ca²⁺ entry in gastric cancer cells

Because SKF96365 did not alter CaCl₂-induced [Ca²⁺]_{cyt} rise (Fig. 5A), suggesting that TRPC channels are not involved, we therefore focused on TRPV channels. In MKN45 cells, although a selective TRPV1 agonist, capsaicin, even at very high concentration (100 μmol/L) did not evoke Ca²⁺ signaling (Fig. 5B), the selective TRPV4 agonist, GSK1016790A, elicited [Ca²⁺]_{cyt} rise in the presence but not in the absence of extracellular Ca²⁺ (Fig. 5C) and the response was abolished by RN1734, a specific TRPV4 antagonist (Fig. 5C). More importantly, CaCl₂⁻ and spermine-induced [Ca²⁺]_{cyt} rise in MKN45 cells was also inhibited by RN1734 (Fig. 5D), suggesting that CaSR activation is coupled to TRPV4 to mediate Ca²⁺ signal. We showed that TRPV4 was expressed in normal human gastric and gastric cancer tissues by immunohistochemistry (Fig. 5E) and in cultured normal gastric and gastric cancer cells by Western blotting (Fig. 5F). Immunofluorescence labeling revealed a possible colocalization between CaSR and TRPV4 in the cultured cells (Fig. 5G), but coimmunoprecipitation failed to detect physical association between the two proteins (data not shown). These data demonstrate that TRPV4 is expressed in the same gastric cancer cells as CaSR and serves to mediate Ca²⁺ entry upon stimulation of CaSR in these cells.

Calcium activates TRPV4 currents via stimulation of CaSR in gastric cancer cells

We further performed whole-cell patch-clamp experiments to characterize TRPV4 currents in gastric cancer cells. In MKN45 cells, bath application of GSK1016790A evoked outwardly rectifying currents that was abolished by RN1734 (60 μmol/L; Fig. 5H–J), suggesting specific TRPV4 currents in gastric cancer cells. Similar currents were induced by bath application of CaCl₂, which were also inhibited by RN1734 (Fig. 5K–M). These data strongly indicate a functional coupling of CaSR and TRPV4 channels in gastric cancer cells.

CaSR activation enhances Wnt/β-catenin signaling in gastric cancer cells but attenuates it in normal cells

Because previous studies showed the important role of Wnt/β-catenin in CaSR-mediated suppression of colorectal cancer and pancreatic cancers (19, 20), we therefore examined the effect of CaSR stimulation on β-catenin phosphorylation at Ser-675 (21), which indicates β-catenin's transcriptional activity in normal gastric and gastric cancer cells. Interestingly, p-β-catenin was decreased in normal cells but increased in cancer cells when CaSR was activated by CaCl₂ or spermine (Fig. 6A and Supplementary Fig. S5A). The CaCl₂-induced p-β-catenin in MKN45 cells was attenuated by knockdown of CaSR or XAV9396, a selective inhibitor of β-catenin signaling (Fig. 6B and Supplementary Fig. S5B). Conversely, over-expression of CaSR in GES-1 cells reversed the effect of CaCl₂ on p-β-catenin formation (Fig. 6C). Therefore, CaSR activation enhances β-catenin signaling in gastric cancer cells but attenuates it in normal cells, suggesting a unique role of β-catenin in CaSR-mediated gastric cancer growth.

PI3K/AKT are involved in CaSR-mediated activation of β-catenin in gastric cancer cells

Because PI3K/AKT are upstream of β-catenin signaling, we thus compared AKT phosphorylation at Ser-473 in normal gastric and gastric cancer cells before and after stimulation by CaCl₂ or spermine. In a similar pattern to p-β-catenin, CaCl₂ and spermine decreased p-AKT in normal cells, but increased its levels in the cancer cells (Fig. 6D and Supplementary Fig. S5C). The CaCl₂-induced p-AKT formation in MKN45 cells was attenuated by knockdown of CaSR (Fig. 6E). The overexpression of CaSR in GES-1 cells also reversed the effect of CaCl₂ on AKT phosphorylation (Fig. 6F). Therefore, AKT is likely a more immediate responder to CaSR activation, which in turn regulates β-catenin signaling in gastric cancer cells. By contrast, the CaSR enhances phosphorylation of ERK1/2 and JNK, similar in both normal and gastric cancer cells (Supplementary Fig. S6A and S6B, and Supplementary Fig. S6C and S6D), despite some differences in kinetics and degrees of the responses were at a least partial dependence on CaSR expression (Supplementary Fig. S7A and S7B). These pathways may thus play minor roles in CaSR-mediated gastric cancer growth.

The Ca²⁺/AKT/β-catenin relay is involved in CaSR-stimulated gastric cancer cell proliferation, migration, and invasion

To test the importance of Ca²⁺, AKT, and Wnt/β-catenin signaling in gastric cancer, we applied BAPTA-AM to load the cells with the fast Ca²⁺ chelator to prevent [Ca²⁺]_{cyt} rise, or selective inhibitors to suppress PI3K/AKT (LY294002). BAPTA-AM and LY294002 attenuated CaCl₂-induced phosphorylation of AKT and β-catenin in MKN45 cells (Fig. 7A). All three drugs inhibited the effects of CaCl₂ on enhancing proliferation, migration, and invasion of gastric cancer cells (Fig. 7B and Supplementary Fig. S7C–S7E). Combining with the earlier results that CaSR enhanced Ca²⁺ signaling in gastric cancer cells, these data altogether suggest that CaSR stimulates proliferation, migration, and invasion of gastric cancer cells through a Ca²⁺/AKT/β-catenin relay.

TRPV4 plays an essential role in CaSR-stimulated Ca²⁺/AKT/β-catenin relay in gastric cancer cells

Furthermore, not only GSK1016790A induced AKT phosphorylation in gastric cancer cells (Fig. 7C and Supplementary Fig. S8A), but also RN1734 attenuated the phosphorylation of AKT and β-catenin stimulated by CaCl₂ (Fig. 7D and Supplementary Fig. S8B and S8C), indicating that activation of TRPV4 is both sufficient and necessary for the Ca²⁺/AKT/β-catenin relay. Supporting its role in gastric cancer growth, the CaCl₂-induced proliferation and migration of gastric cancer cells were near-completely suppressed by RN1734 (Fig. 7E and Supplementary Fig. S8D). These data strongly argue for an essential role of TRPV4 in CaSR-stimulated Ca²⁺/AKT/β-catenin relay in gastric cancer cells.

Discussion

In this study, we demonstrate for the first time that calcium exerts an oncogenic action in the stomach through activation of CaSR and TRPV4 channels. Several lines of evidence suggest that CaSR promotes human gastric cancer growth and metastasis by a novel functional coupling to TRPV4. First, CaSR expression is enhanced in human primary gastric cancer tissues and gastric

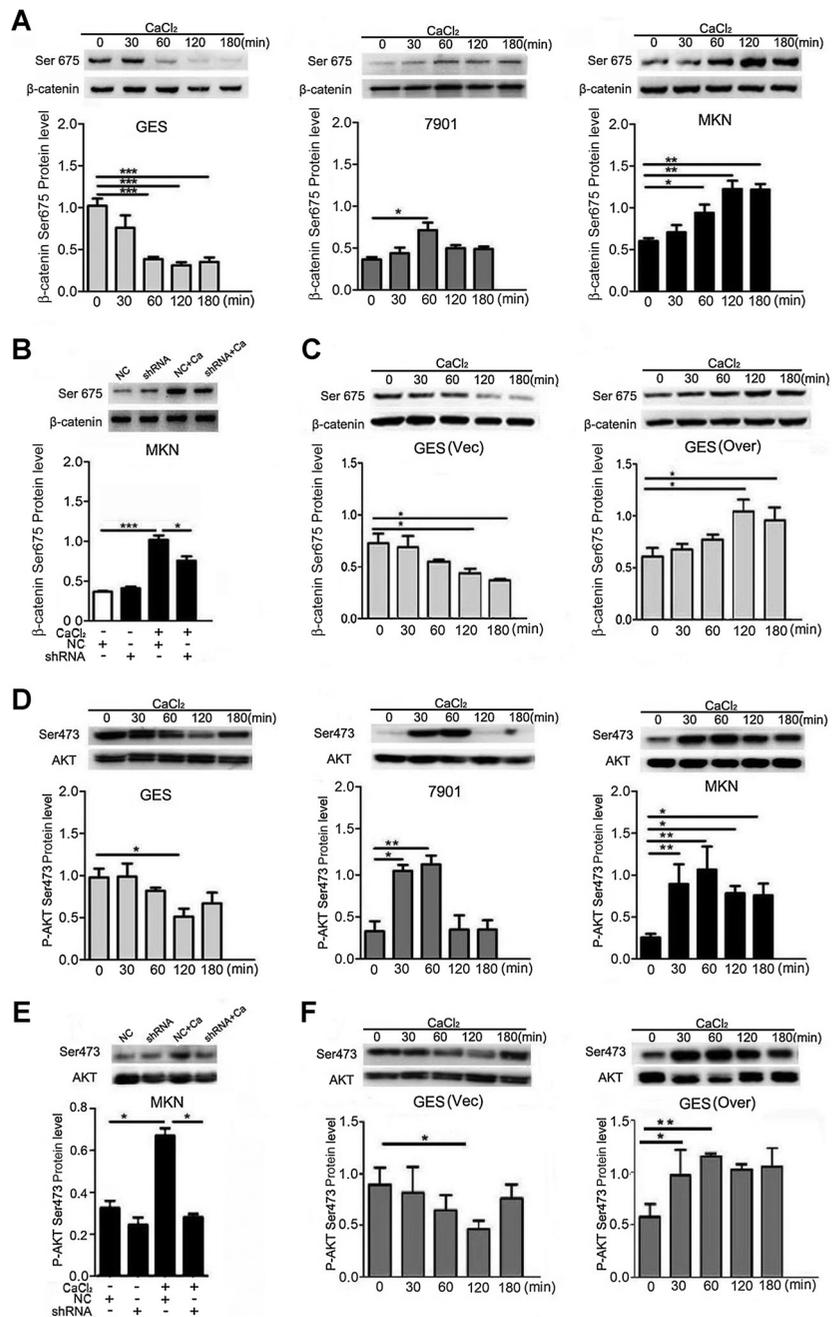


Figure 6.

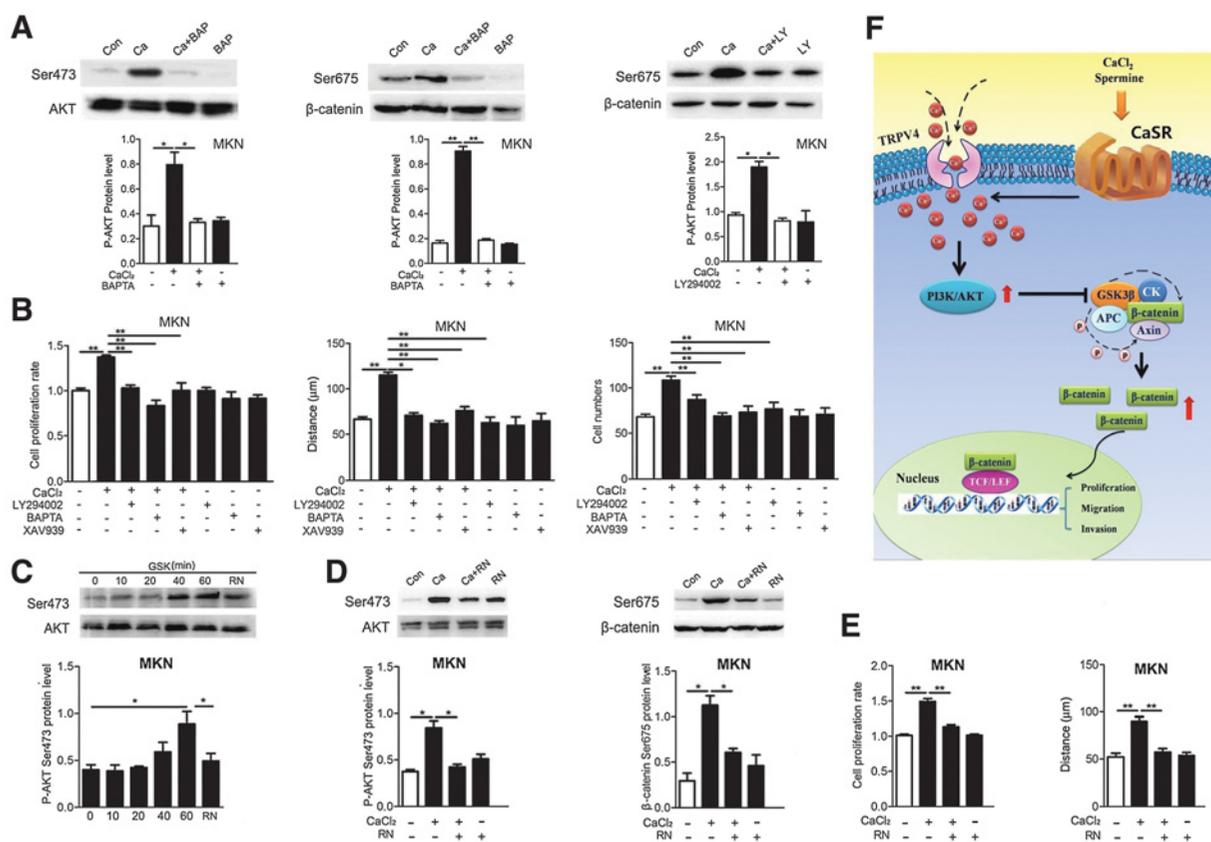
Extracellular Ca^{2+} induces phosphorylation of β -catenin and AKT in human gastric epithelial and gastric cancer cells. **A**, Time-dependent phosphorylation of β -catenin (at Ser675) induced by extracellular CaCl_2 (2 mmol/L) in GES-1, SGC-7901, and MKN45 cells. Shown are representative Western blots (top) and summary data normalized to total β -catenin (bottom; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, $n = 3$). **B**, CaSR knockdown suppressed Ca^{2+} -induced β -catenin phosphorylation in MKN45 cells (*, $P < 0.05$; ***, $P < 0.001$, $n = 3$). **C**, Time-dependent phosphorylation of β -catenin induced by CaCl_2 in GES-1 cells with or without CaSR overexpression (*, $P < 0.05$, $n = 3$). **D**, Time-dependent phosphorylation of AKT (at Ser473) induced by CaCl_2 in GES-1, SGC-7901, and MKN45 cells (*, $P < 0.05$; **, $P < 0.01$, $n = 3$). **E**, CaSR knockdown abolished CaCl_2 -induced AKT phosphorylation in MKN45 cells (*, $P < 0.05$, $n = 3$). **F**, Time-dependent AKT phosphorylation induced by CaCl_2 in GES-1 cells with or without CaSR overexpression (*, $P < 0.05$; **, $P < 0.01$, $n = 3$).

cancer cell lines. Second, CaSR overexpression positively correlates with serum calcium levels, tumor progression, poor survival, and male of gastric cancer patients. Third, calcium and CaSR function are important for gastric cancer growth and metastasis both *in vitro* and *in vivo*. Fourth, CaSR stimulation in gastric cancer cells activates TRPV4, which in turn mediates Ca^{2+} entry to increase $[\text{Ca}^{2+}]_{\text{cyt}}$ and consequent activation of AKT and β -catenin, a well-known oncogenic pathway (5, 21–23). Fifth, CaSR exhibits aberrant functions in gastric cancer cells as compared to nontumorigenic gastric cells. Sixth, the nontumorigenic gastric cells overexpressing CaSR resemble gastric cancer cells in a number of functional behaviors. Finally, pharmacologic inhibition and genetic knockdown of CaSR in gastric cancer cells attenuated

its downstream signaling and suppressed gastric cancer growth and metastasis.

Calcium and CaSR have been reported to either suppress or promote cancer depending on tumor types (7, 24, 25), and the aberrance in CaSR function in gastric cancer cells was also evident in the AKT/ β -catenin pathway, which was enhanced in gastric cancer cells but suppressed in normal cells. Thus, CaSR activation only stimulated proliferation, growth, migration, and invasion of gastric cancer, but not normal cells. This is consistent with previous observation that in high $[\text{Ca}^{2+}]_{\text{o}}$, esophago-gastric cancer cells proliferated whereas normal cells stayed quiescent (22). Collectively, our data suggest that calcium and CaSR are tumor promoters in gastric cancer, which contrasts their roles as tumor

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**Figure 7.**

CaSR-mediated TRPV4/Ca²⁺/AKT/β-catenin relay and its role in proliferation, migration, and invasion of gastric cancer cells. **A**, BAPTA-AM (2 μmol/L) abolished CaCl₂ (2 mmol/L)-induced AKT phosphorylation of AKT (left) and β-catenin (middle) in MKN45 cells. Shown are representative Western blots (top) and summary data normalized to total AKT or β-catenin (bottom; *, *P* < 0.05 or **, *P* < 0.01, *n* = 3). LY294002 (5 μmol/L) inhibited Ca²⁺-induced β-catenin phosphorylation in MKN45 cells (right; *, *P* < 0.05, *n* = 3). **B**, Chelating cytosolic Ca²⁺ with BAPTA-AM or inhibiting PI3K/AKT with LY294002 or β-catenin with XAV939 (1 μmol/L) abolished CaCl₂-enhanced proliferation (*, *P* < 0.01, *n* = 3), migration (*, *P* < 0.05; **, *P* < 0.01, *n* = 3), and invasion (**, *P* < 0.01, *n* = 3) of MKN45 cells. **C**, Time-dependent phosphorylation of AKT induced by GSK1016790A (0.1 μmol/L) in MKN45 cells (*, *P* < 0.05, *n* = 3). **D**, RN1734 (30 μmol/L) suppressed CaCl₂-induced phosphorylation of AKT (left; *, *P* < 0.05, *n* = 3) and β-catenin (right; *, *P* < 0.05, *n* = 3) in MKN45 cells. **E**, RN1734 inhibited Ca²⁺-enhanced proliferation (left) and migration (right) of MKN45 cells (**, *P* < 0.01, *n* = 3). **F**, Signaling cascade for the aberrant CaSR activation in gastric tumorigenesis.

suppressors in colorectal cancer and pancreatic cancer (3, 6, 7). Currently, it is unknown why the CaSR in gastric cancer has opposite roles from those in colorectal cancer and pancreatic cancer, but it is likely that: (i) the changes in CaSR expression are different in various types of tumors (enhanced expression in gastric cancer, but decreased expression in colorectal cancer and pancreatic cancer); (ii) the different downstream signaling of CaSR activation (TRPV4-mediated Ca²⁺ signaling in gastric cancer, but other signaling in colorectal cancer and pancreatic cancer); (iii) even Ca²⁺ signaling as shown in our study, low CaSR expression in normal gastric cells leads to low [Ca²⁺]_{cyt} concentration that inhibits AKT/β-catenin, but high CaSR expression in gastric cancer cells leads to high [Ca²⁺]_{cyt} concentration that activates AKT/β-catenin. Therefore, the differences in the amplitude of CaSR-evoked Ca²⁺ signaling may account for the different roles of CaSR in normal cells and in various types of tumor cells as well. Deciphering the molecular mechanisms underlying different roles of CaSR in tumors could lead to the development of new drugs that may selectively target CaSR and have therapeutic potential for a special cancer.

CaSR activation stimulates [Ca²⁺]_{cyt} rise, as well as PI3K/AKT, β-catenin, and MAPK signaling (5, 21, 23, 24), all of which are involved in malignant progression of cancers. Ca²⁺ signaling is well recognized to regulate proliferation, migration, epithelial mesenchymal transition (EMT), and malignant transformation (22, 26). Although CaSR activation only induced a slight increase in [Ca²⁺]_{cyt} in normal gastric cells, it led to a marked increase in gastric cancer cells. This quantitative difference in [Ca²⁺]_{cyt} may account for the tumorigenic effect of CaSR through the Ca²⁺/AKT/β-catenin relay in gastric cancer. Supporting this notion, it was reported recently that [Ca²⁺]_{cyt} regulates hepatic cancer growth through the CaM/CaMKK2/CaMKIV relay (27), underscoring the critical role of [Ca²⁺]_{cyt} in tumorigenesis. It is well known that Ca²⁺ signaling plays essential roles in the activation of immune cells and inflammation and that gastric cancer may develop from chronic inflammation associated with Hp (28). Previously, Iimuro and colleagues found that dietary calcium enhanced Hp-induced gastritis in Mongolian gerbils (29). In contrast, calcium channel blockers attenuated chemically induced gastritis and gastric cancer in rats (30, 31). Therefore, these available data

not only provide the suggestion that $[Ca^{2+}]_o$ entry could enhance gastritis and gastric cancer, but further support our notion that calcium supplement may promote gastric cancer development and progression.

We and others have shown that CaSR activation triggers Ca^{2+} entry (32, 33); however, Ca^{2+} entry channels that mediate this effect have not been defined. We show here that TRPV4 is responsible for CaSR-induced Ca^{2+} entry in gastric cancer cells using calcium imaging and patch clamp techniques. Although how CaSR activates TRPV4 channels is not known, PLC activity that has been shown to regulate TRPV4 (34, 35) appears to be required in this process. Furthermore, CaSR-induced gastric cancer cell proliferation and migration were attenuated by blocking $[Ca^{2+}]_{cyt}$ rise, as well as inhibiting CaSR or TRPV4, demonstrating the importance of CaSR-TRPV4 coupling and the consequent Ca^{2+} entry in gastric cancer. TRPV4 channel has been shown to localize exclusively in basolateral membrane of IEC-6 cells (36). Consistently, we demonstrated that $[Ca^{2+}]_{cyt}$ rise occurred only when CaSR was stimulated from the basolateral side of these cells. Confocal fluorescence imaging further confirmed the basolateral localization of CaSR. The basolateral side of action of CaSR echoes the positive correlation found between CaSR overexpression and serum calcium levels in gastric cancer patients, further arguing that $[Ca^{2+}]_{cyt}$ signal resulting from basolateral CaSR stimulation by serum calcium is likely involved in gastric cancer development and progression. Thus, persistent stimulation of basolateral CaSR by elevated serum calcium can induce Ca^{2+} entry via activation of TRPV4, which leads to sustained $[Ca^{2+}]_{cyt}$ increase and in turn promotes gastric cancer development. To prove this notion *in vivo* animal study, we tried to induce an acute hypocalcemia model via an intravenous injection of calcium, but failed finally. This is not surprise due to the existence of accurate regulatory system that maintains a stable serum calcium homeostasis in the body. Therefore, we did not further compare the tumor growth in the mouse xenograft models with or without an intravenous injection of calcium. However, due to the facts that the gastric cancer cells with aberrant expression and function of CaSR are hypersensitive to $[Ca^{2+}]_o$ as shown presently and that gastric cancer patients usually develop hypercalcemia during their disease course as reported previously (15–17), any magnitudes of serum calcium rise may aggravate gastric cancer progression.

Intracellular Ca^{2+} can bind to calmodulin and stimulate PI3K/AKT (33), resulting in subsequent nuclear accumulation of β -catenin, a process that plays a significant role in tumorigenesis (37, 38). Targeted deletion of CaSR in intestinal epithelium of mice resulted in hyperplasia and enhanced β -catenin signaling (39), confirming negative effect of CaSR on normal gastrointestinal epithelial growth. We showed that CaSR activation decreased AKT and β -catenin phosphorylation in normal gastric epithelial cells, but increased them in gastric cancer cells with enhanced cell growth, migration, and invasion. Notably, overexpression of

CaSR in normal cells resulted in marked increases in their $[Ca^{2+}]_{cyt}$, AKT, and β -catenin phosphorylation, and migration, all of which mimic the behaviors of gastric cancer cells. Together, our results indicate that aberrant CaSR induces gastric cancer development and progression through TRPV4/ Ca^{2+} /AKT/ β -catenin relay (Fig. 7F). The TRPV4-mediated $[Ca^{2+}]_o$ entry in gastric cancer cells contrasts from the previous report that Ca^{2+} release from internal stores of colorectal cancer cells enhanced nuclear export and degradation of β -catenin in the cytoplasm (40), suggesting that different Ca^{2+} sources might play different roles in upper and lower GI tumorigenesis.

It has been puzzling in the clinic why gastric cancer patients with hypercalcemia usually have a poor prognosis. Our study may provide a plausible explanation. More important is that due to the difficulty in dietary intake, most gastric cancer patients are supplemented with the electrolytes, including high calcium. Therefore, high serum calcium could be problematic for gastric cancer patients as shown in this study and other diseases, such as pulmonary hypertension as shown in our previous study revealing that CaSR activated by calcium may exacerbate pulmonary hypertension (41). Moreover, modulation of CaSR and its TRPV4/ Ca^{2+} / β -catenin signaling could be potential novel preventive and therapeutic strategies for human gastric cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Dong, S. Yang

Development of methodology: J. Liu, S. Wang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Xie, J. Xu, Y. Xiao, B. Tang, J. Liu, Y. Fan, Y. Wu, T.X. Dong

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Xie, J. Xu, Y. Xiao, H. Wan, B. Tang, J. Liu, Y. Fan, T.X. Dong

Writing, review, and/or revision of the manuscript: M.X. Zhu, J.M. Carethers, H. Dong

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Wu, H. Wan, S. Wang, Y. Wu

Study supervision: H. Dong, S. Yang

Grant Support

These studies were supported by research grants from the National Natural Science Foundation of China (no. 81301731 to R. Xie) and the National Key Research and Development Program of China (no. 2016YFC1302200 to H. Dong).

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Received February 7, 2017; revised June 19, 2017; accepted September 21, 2017; published OnlineFirst September 26, 2017.

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Cancer Res 2017;77:6499-6512. Published OnlineFirst September 26, 2017.

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