Calcium promotes human gastric cancer via a novel coupling of calcium-sensing receptor and TRPV4 channel

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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 (GC) development. CaSR expression was enhanced in GC specimens, which positively correlated with serum calcium concentrations, tumor progression, poor survival, and male gender in GC patients. CaSR and transient receptor potential cation channel subfamily V member 4 (TRPV4) were co-localized in GC 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 GC cells through a Ca\(^{2+}\)/AKT/β-catenin relay which occurred only in GC cells or normal cells overexpressing CaSR. Tumor growth and metastasis of GC depended on CaSR in nude mice. Overall, our findings indicate that calcium may enhance expression and function of CaSR to potentially promote GC, and that targeting the novel CaSR/TRPV4/ Ca\(^{2+}\) pathway might serve as preventive or therapeutic strategies for GC.
INTRODUCTION

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide (1). Currently, GC 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 (CRC) and pancreatic cancer (PC), as a tumor suppressor therapy likely through activation of calcium-sensing receptor (CaSR) (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 since calcium supplement has been widely advocated in our daily life.

CaSR plays critical roles in systemic calcium homeostasis and diverse pathophysiological conditions (4, 5, 6, 7), including a pathogenic role in human pulmonary arterial hypertension recently identified by us (8). When stimulated by extracellular calcium ([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 ([Ca$^{2+}$]$_{cyt}$), 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 GC (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 GC.

In the present study, we examined the roles of calcium and CaSR in GC development and elucidated the underlying molecular mechanisms. Unexpectedly, we found that
calcium activates CaSR to promote GC growth and metastasis, instead of suppressing it as in the cases of CRC and PC. We further showed that CaSR activation triggers Ca^{2+} entry through TRPV4 channels and the downstream AKT/β-catenin pathways to facilitate GC growth. Our results indicate that calcium supplement may be problematic, especially for GC patients, and also suggest a novel strategy for GC 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 GC 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 histological 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 (St. Louis, MO). After dissolved in DMSO, they were diluted in cell culture media with final concentrations of below 0.1%. The rat intestinal epithelial cell 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, Shanghai, China. SGC-7901 and IEC-6 cells were STR-authenticated by Shanghai Biowing Applied Biotechnology Co. LTD, Shanghai, China. The cells lines were grown in RPMI-1640, or Iscove's DMEM medium (GIBCO-BRL, Gaithersburg, MA) supplemented with 10% fetal calf serum (GIBCO-BRL) in an incubator with 5% CO$_2$ at 37 °C. IEC-6 cells (2–4 × 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 GC tissues were incubated with an anti-CaSR monoclonal antibody (1:100 dilution, Abcam, Cambridge, MA). The primary antibodies were detected with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) secondary antibodies. Immunoreactivity was detected using a horseradish peroxidase (3’-, 3’-diaminobenzidine) kit (BioGenex, San Francisco, CA), 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 min, blocked for 30 min and incubated with primary antibodies against CaSR (1:100 dilution, thermo, Cambridge, MA) overnight at 4°C. Then the cells were incubated with FITC-IgG fluorescence secondary antibody (1:500 dilution; ZSGB-BIO, Beijing, China) at 37°C for 1 h.

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

The cells grown on coverslips or inserts were loaded with 5 μM fura-2 AM in physiological salt solution (PSS) at 22°C for 50 min and then washed in PSS for 30 min.
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, Downingtown, PA). PSS used in Ca\(^{2+}\) measurement contained the following (in mM): 140 Na\(^+\), 5 K\(^+\), 2 Ca\(^{2+}\), 147 Cl\(\text{--}\), 10 HEPES, and 10 glucose, pH 7.4. For the Ca\(^{2+}\)-free solution, Ca\(^{2+}\) was omitted and 0.5 mM EGTA was added.

Quantitative real-time PCR

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

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

Western blotting

For immunoprecipitation studies, lysates were incubated with a CaSR antibody for 1 h at 4°C. Pellets or cell lysates were resuspended in 2 x loading buffer, boiled for 5 min, and separated by SDS-PAGE (10%). Resolved proteins were transferred onto a PVDF membrane (Millipore Corporate, Billerica, MA). Membranes were blocked by 5% blocking buffer, followed by incubation with a monoclonal antibody CaSR, 1:500 (Abcam, Cambridge, MA), p-ERK and p-JNK, 1:1,000 (Bioworld), β-catenin, 1:1,000 (Cell Signaling Technology), and GAPDH, 1:5,000 (Ambion), TRPV4, 1:2000 (milibper),
respectively. After washing with TBST, secondary antibody was applied. The signals were visualized using enhanced chemiluminescence (ECL, Thermo, USA).

**Cell proliferation assay**

Cell viability was assessed by Cell Counting Kit- (CCK-) 8 (Tongren, Shanghai, China). CCK-8 reagent was added to each well at 0.5-2 hour 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 h and 24 h by using a microscope. Narrower widths indicate the migration distances.

**Transwell invasion assays**

A 24-well transwell chamber (8-μm pores; Corning, Corning, NY) was used for this assay. The upper surface of polycarbonate filters with 8-μm pores was coated with matrigel (Collaborative Biomedical, USA). The serum-free RPMI-1640/DMEM was used to suspend the cells (1×10⁶ cells/100 μl), then the cells were placed in the upper chambers and treated with different drugs. The lower chambers were filled with RPMI-1640 plus 10% FBS medium. After 24 h of incubation, the cells which had migrated onto the lower surface were stained with crystal violet, and then the number of cells was counted under a microscope (Olympus Corporation, Japan). The average number of three random selected fields was taken as the number of invasion and metastasis.

**Treatment of s.c. GC xenografts**

Approximate 1×10⁶ SGC-7901 cancer cells were injected into the armpits of male


nude mice (~4 weeks old). After tumor sizes reached around 1 mm$^3$, CaCl$_2$ (4 mM), NPS2143 (15 µM), or both (100 µl) 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 GC 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, USA). 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 mm$^3$ was calculated by the formula $V = \frac{1}{2} (\text{length} \times \text{width}^2)$. For the experiment in which CaCl$_2$ 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 mM). Afterwards, each mouse in treatment group received 1.38 mg CaCl$_2$ in 50 µl of 0.9% saline per day via an intravenous injection into tail veins to raise the final serum calcium concentrations to 6 mM, while each mouse in control group received 50 µl 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, i.e. Control group, Ca$^{2+}$ group and Ca$^{2+}$+NPS group. $1 \times 10^6$ MKN45 cells were injected into the abdominal cavity of nude mice, and then CaCl$_2$ (4 mM), NPS2143 (15 µM), or both (100 µl) 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 ± SEM, and analyzed by one-way ANOVA followed by the Student-Newman-Keul post hoc test or by Student’s t-tests for paired or unpaired samples with GraphPad Prism 5.0 (San Diego, CA). 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 IACUC 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 GC tissues
Since calcium stimulates plasma membrane CaSR to produce biological activities, we first compared the expression of CaSR in normal stomach and GC tissues at protein and mRNA levels. Immunohistological studies revealed little staining of CaSR in normal human gastric tissues but strong staining in primary GC tissues (Figure 1A). The expression of CaSR proteins and transcripts were significantly enhanced in GC tissues (Figure 1A and 1B). The human GC cell lines (SGC-7901 and MKN45) also exhibited greater expression of CaSR than the non-tumorigenic gastric epithelial cell line (GES-1), as determined by Western blot and qRT-PCR (Figure 1C), as well as immunofluorescence staining (Figure 1D). These results are consistent with the previous report showing that GC cells had copy number gain of the CaSR gene (14), suggesting that CaSR may be oncogenic in GC.

High serum calcium and CaSR expression correlate with severe GC progression and poor patient survival
Western blot analysis of a large cohort of 91 GC 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 (Figure 1E), indicating that a positive association of CaSR expression with GC progression. Since up to 30% of patients with cancers may develop hypercalcemia during their disease course (15, 16, 17), the correlation between serum calcium and the survival of GC patients was analyzed. We found the two parameters to be negatively correlated (Figure 1F), which is consistent with the notion that GC patients with hypercalcemia usually have poor prognosis (15, 16, 17). Since 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 GC patients and found the patients with high CaSR expression showed markedly lower survival than those with low CaSR expression (Figure 1G), suggesting that CaSR stimulation by high serum calcium may be involved in poor prognosis of GC. Furthermore, we compared serum calcium levels of the patients with the CaSR expression levels in their GC tissues and found the two parameters to be positively correlated (Figure 1H), which is consistent with the findings that \([\text{Ca}^{2+}]_o\) induces expression and function of CaSR. Therefore, unlike serving as suppressors in CRC, calcium and CaSR may serve as tumor promoters in GC.

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

To examine the roles of calcium and CaSR in GC, GES-1, SGC-7901 and MKN45 cells were cultured in varying concentrations of \(\text{CaCl}_2\) and the rates of proliferation were determined. Although \(\text{CaCl}_2\) did not alter proliferation of GES-1 cells, it dose-dependently enhanced proliferation of GC cells (Figure 2A). \(\text{CaCl}_2\) treatment also time-dependently increased protein expression of PCNA, a well-known biomarker of proliferation in GC cells (Supplementary Figure 1A). In MKN45 cells, the \(\text{CaCl}_2\)-enhanced proliferation of GC cells was attenuated by either a CaSR antagonist.
NPS 2143 or the knockdown of CaSR expression by its shRNA ([Figure 2B and Supplementary Figure 2A](#)). Moreover, CaCl$_2$-enhanced migration ([Figure 2C](#)) and invasion ([Figure 2D](#)) of GES-1 and MKN45 cells were also attenuated by either NPS 2143 or knockdown of CaSR ([Figure 2E and 2F](#)). However, overexpression of CaSR in GES-1 cell allowed CaCl$_2$ to increase proliferation ([Figure 2G](#)) and further accelerated migration of these cells ([Figure 2H](#)). Therefore, calcium promoted cell proliferation, migration and invasion of GC cells through activation of CaSR.

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

In subcutaneously xenografted GC model of nude mice, CaSR activation by daily administration of CaCl$_2$ increased tumor volumes and the effect was attenuated by co-injection with NPS 2143 ([Figure 3A and 3B](#)), which itself did not alter tumor volumes ([Supplementary Figure 2B](#)). The knockdown of CaSR from SGC-7901 cells with the use of CaSR-shRNA lentiviruses ([Supplementary Figure 2C and 2D](#)) also suppressed their ability to growth after implantation ([Figure 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 ([Figure 3D](#)). However, the tumors derived from the implants with calcium treatment had high PCNA staining than those without calcium treatment ([Supplementary Figure 2E](#)), 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 Figure 3A](#)). Therefore, an intravenous injection of CaCl$_2$ failed to induce a hypocalcaemia model ([Supplementary Figure 3B](#)).
We further explored whether metastasis of GC cell depends on CaSR in vivo. MKN45 cells-precocultured with either CaCl₂ alone or CaCl₂ plus NPS2143 were injected peritoneally into nude mice. In control group, the same amount of untreated GC cells was injected. Six weeks later, the numbers of metastatic tumors were counted in the mice. As shown in Figure 3E, the CaCl₂-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 GC growth and metastasis in vivo.

**CaSR activation mediates intracellular Ca²⁺ signals in GC cells**

We determined if CaSR mediates [Ca²⁺]_{cyt} increase in GC cells by Fura-2 Ca²⁺ imaging. While 5 mM CaCl₂ induced a slow and slight rise in [Ca²⁺]_{cyt} in normal GES-1 cells, it evoked a marked elevation of [Ca²⁺]_{cyt} in the two GC cell lines (Figure 4A), suggesting an enhanced function of CaSR in GC cells. The CaCl₂-induced [Ca²⁺]_{cyt} rise in MKN45 cells was abolished by NPS 2143 or shRNA knockdown of CaSR (Figure 4A), demonstrating that CaSR mediates the response. Like CaCl₂, spermine had similar effects on [Ca²⁺]_{cyt} changes in GES-1 and MKN45 cells (Supplementary Figure 4A)(18). Overexpression of CaSR in GES-1 cells also markedly increased [Ca²⁺]_{cyt} response to CaCl₂ (Figure 4B), further supporting a role of calcium in generating cell Ca²⁺ signal through activation of CaSR in gastric epithelial cells (Figure 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 intestinal epithelial cell (IEC-6) monolayer (the left panel in Figure 4D), because the gastric cell lines can not grow into monolayers. Spermine did not alter [Ca²⁺]_{cyt} when applied from the apical side, but induced a marked [Ca²⁺]_{cyt} increase when perfused onto to basolateral side (Figure 4E). As a control, ionomycin, a Ca²⁺ ionophore, evoked a Ca²⁺ transient when applied from each side of the monolayer and the response was
reduced by EGTA, a Ca\(^{2+}\) chelator (Figure 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 Figure 4D) and consistent with the GC patient data described above (Figure 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 GC 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 (Figure 4F and Supplementary Figure 4B). In contrast, re-addition of CaCl\(_2\) led to immediate elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) in the continued presence of spermine (Figure 4F and Supplementary Figure 4B). 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 (Figure 4G and Supplementary Figure 4C), 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 GC cells. Furthermore, the \([\text{Ca}^{2+}]_{\text{cyt}}\) rise induced by CaCl\(_2\) or spermine was abolished by U73122 (Figure 4C and Supplementary Figure 4D and 4E), a selective inhibitor of phospholipase C (PLC), suggesting its involvement in CaSR-stimulated Ca\(^{2+}\) entry in GC cells.

**TRPV4 plays a major role in CaSR-stimulated Ca\(^{2+}\) entry in GC cells**

Since SKF96365 did not alter CaCl\(_2\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) rise (Figure 5A), suggesting that TRPC channels are not involved, we therefore focused on TRPV channels. In MKN45 cells, whereas a selective TRPV1 agonist, capsaicin, even at very high concentration (100 µM) did not evoke Ca\(^{2+}\) signaling (Figure 5B), the selective TRPV4 agonist,
GSK1016790A, elicited [Ca\textsuperscript{2+}]\textsubscript{cyt} rise in the presence but not in the absence of extracellular Ca\textsuperscript{2+} (Figure 5C) and the response was abolished by RN1734, a specific TRPV4 antagonist (Figure 5C). More importantly, CaCl\textsubscript{2} and spermine-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} rise in MKN45 cells was also inhibited by RN1734 (Figure 5D), suggesting that CaSR activation is coupled to TRPV4 to mediate Ca\textsuperscript{2+} signal. We showed that TRPV4 was expressed in normal human gastric and GC tissues by immunohistochemistry (Figure 5E) and in cultured normal gastric and GC cells by western blotting (Figure 5F). Immunofluorescence labeling revealed a possible co-localization between CaSR and TRPV4 in the cultured cells (Figure 5G), but co-immunoprecipitation failed to detect physical association between the two proteins (data not shown). These data demonstrate that TRPV4 is expressed in the same GC cells as CaSR and serves to mediate Ca\textsuperscript{2+} entry upon stimulation of CaSR in these cells.

**Calcium activates TRPV4 currents via stimulation of CaSR in GC cells**

We further performed whole-cell patch-clamp experiments to characterize TRPV4 currents in GC cells. In MKN45 cells, bath application of GSK1016790A evoked outwardly rectifying currents that was abolished by RN1734 (60 µM) (Figure 5H, 5I and 5J), suggesting specific TRPV4 currents in GC cells. Similar currents were induced by bath application of CaCl\textsubscript{2}, which were also inhibited by RN1734 (Figure 5K, 5L and 5M). These data strongly indicate a functional coupling of CaSR and TRPV4 channels in GC cells.

**CaSR activation enhances Wnt/β-catenin signaling in GC cells but attenuates it in normal cells**

Since previous studies showed the important role of Wnt/β-catenin in CaSR-mediated suppression of CRC and pancreatic cancers (PC) (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 GC cells. Interestingly, p-β-catenin was decreased in normal cells but increased in cancer cells.
when CaSR was activated by CaCl₂ or spermine (*Figure 6A and Supplementary Figure 5A*). The CaCl₂-induced p-β-catenin in MKN45 cells was attenuated by knockdown of CaSR or XAV9396, a selective inhibitor of β-catenin signaling (*Figure 6B and Supplementary Figure 5B*). Conversely, over-expression of CaSR in GES-1 cells reversed the effect of CaCl₂ on p-β-catenin formation (*Figure 6C*). Therefore, CaSR activation enhances β-catenin signaling in GC cells but attenuates it in normal cells, suggesting a unique role of β-catenin in CaSR-mediated GC growth.

**PI3K/AKT are involved in CaSR-mediated activation of β-catenin in GC cells**

Since PI3K/AKT are upstream of β-catenin signaling, we thus compared AKT phosphorylation at Ser-473 in normal gastric and GC 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 (*Figure 6D and Supplementary Figure 5C*). The CaCl₂-induced p-AKT formation in MKN45 cells was attenuated by knockdown of CaSR (*Figure 6E*). The overexpression of CaSR in GES-1 cells also reversed the effect of CaCl₂ on AKT phosphorylation (*Figure 6F*). Therefore, AKT is likely a more immediate responder to CaSR activation, which in turn regulates β-catenin signaling in GC cells. By contrast, the CaSR enhances phosphorylation of ERK1/2 and JNK, similar in both normal and GC cells (*Supplementary Figure 6A and 6B, and Supplementary Figure 6C and 6D*), despite some differences in kinetics and degrees of the responses were at least partial dependence on CaSR expression (*Supplementary Figure 7A and Supplementary Figure 7B*). These pathways may thus play minor roles in CaSR-mediated GC growth.

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

To test the importance of Ca²⁺, AKT and Wnt/β-catenin signaling in GC, 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 (Figure 7A). All three drugs inhibited the effects of CaCl₂ on enhancing proliferation, migration and invasion of GC cells (Figure 7B, and Supplementary Figure 7C, 7D and 7E). Combining with the earlier results that CaSR enhanced Ca²⁺ signaling in GC cells, these data altogether suggest that CaSR stimulates proliferation, migration and invasion of GC cells through a Ca²⁺/AKT/β-catenin relay.

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

Furthermore, not only GSK1016790A induced AKT phosphorylation in GC cells (Figure 7C and Supplementary Figure 8A), but also RN1734 attenuated the phosphorylation of AKT and β-catenin stimulated by CaCl₂ (Figure 7D and Supplementary Figure 8B and 8C), indicating that activation of TRPV4 is both sufficient and necessary for the Ca²⁺/AKT/β-catenin relay. Supporting its role in GC growth, the CaCl₂-induced proliferation and migration of GC cells were near-completely suppressed by RN1743 (Figure 7E and Supplementary Figure 8D). These data strongly argue for an essential role of TRPV4 in CaSR-stimulated Ca²⁺/AKT/β-catenin relay in GC 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 GC growth and metastasis by a novel functional coupling to TRPV4. First, CaSR expression is enhanced in human primary GC tissues and GC cell lines. Second, CaSR overexpression positively correlates with serum calcium levels, tumor progression, poor survival and male of GC patients. Third, calcium and CaSR function are important for GC growth and metastasis both *in vitro* and *in vivo*. Fourth, CaSR stimulation in GC cells activates TRPV4, which in turn mediates Ca²⁺ entry to increase [Ca²⁺]_{cyt} and consequent
activation of AKT and β-catenin, a well-known oncogenic pathway (5, 21, 22, 23). Fifth, CaSR exhibits aberrant functions in GC cells as compared to non-tumorigenic gastric cells. Sixth, the non-tumorigenic gastric cells overexpressing CaSR resemble GC cells in a number of functional behaviors. Finally, pharmacological inhibition and genetic knockdown of CaSR in GC cells attenuated its downstream signaling and suppressed GC 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 GC cells was also evident in the AKT/β-catenin pathway, which was enhanced in GC cells but suppressed in normal cells. Thus, CaSR activation only stimulated proliferation, growth, migration and invasion of GC, but not normal cells. This is consistent with previous observation that in high [Ca$^{2+}$]$_{o}$, oesophago-gastric cancer cells proliferated while normal cells stayed quiescent (22). Collectively, our data suggest that calcium and CaSR are tumor promoters in GC, which contrasts their roles as tumor suppressors in colorectal cancer (CRC) and pancreatic cancer (PC) (3, 6, 7). Currently, it is unknown why the CaSR in GC has opposite roles from those in CRC and PC, but it is likely that: 1) the changes in CaSR expression are different in various types of tumors (enhanced expression in GC, but decreased expression in CRC and PC); 2) the different downstream signaling of CaSR activation (TRPV4-mediated Ca$^{2+}$ signaling in GC, but other signaling in CRC and PC); 3) even Ca$^{2+}$ signaling as shown in our study, low CaSR expression in normal gastric cells leads to low [Ca$^{2+}$]$_{c}$ concentration that inhibits AKT/β-catenin, but high CaSR expression in GC cells leads to high [Ca$^{2+}$]$_{c}$ concentration that activates AKT/β-catenin. Therefore, the differences in the amplitude of CaSR-evoked Ca$^{2+}$ 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 [Ca\(^{2+}\)]\(_{\text{cyt}}\) 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. Ca\(^{2+}\) 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 [Ca\(^{2+}\)]\(_{\text{cyt}}\) in normal gastric cells, it led to a marked increase in GC cells. This quantitative difference in [Ca\(^{2+}\)]\(_{\text{cyt}}\) may account for the tumorigenic effect of CaSR through the Ca\(^{2+}\)/AKT/β-catenin relay in GC. Supporting this notion, it was reported recently that [Ca\(^{2+}\)]\(_{\text{cyt}}\) regulates hepatic cancer growth through the CaM/CaMKK2/CaMKIV relay (27), underscoring the critical role of [Ca\(^{2+}\)]\(_{\text{cyt}}\) in tumorigenesis. It is well known that Ca\(^{2+}\) signaling plays essential roles in the activation of immune cells and inflammation and that GC may develop from chronic inflammation associated with Hp (28). Previously, Iimuro M et al. found that dietary calcium enhanced Hp-induced gastritis in Mongolian gerbils (29). In contrast, calcium channel blockers attenuated chemically induced gastritis and GC in rats (30, 31). Therefore, these available data not only provide the suggestion that [Ca\(^{2+}\)]\(_{\text{o}}\) entry could enhance gastritis and GC, but further support our notion that calcium supplement may promote GC 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 GC 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 GC cell proliferation and migration were attenuated by blocking [Ca\(^{2+}\)]\(_{\text{cyt}}\) rise, as well as inhibiting CaSR or TRPV4, demonstrating the importance of CaSR-TRPV4 coupling and the consequent Ca\(^{2+}\) entry in GC. TRPV4 channel has been shown to localize exclusively in basolateral membrane of IEC-6 cells (36). Consistently, we
demonstrated that $[\text{Ca}^{2+}]_{\text{cyt}}$ 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 GC patients, further arguing that $[\text{Ca}^{2+}]_{\text{cyt}}$ signal resulting from basolateral CaSR stimulation by serum calcium are likely involved in GC development and progression. Thus, persistent stimulation of basolateral CaSR by elevated serum calcium can induce $\text{Ca}^{2+}$ entry via activation of TRPV4, which leads to sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ increase and in turn promotes GC development. To prove this notion in vivo animal study, we tried to induce an acute hypocalcaemia 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, duo to the facts that the GC cells with aberrant expression and function of CaSR are hypersensitive to $[\text{Ca}^{2+}]_{o}$ as shown presently and that GC patients usually develop hypercalcemia during their disease course as reported previously (15, 16, 17), any magnitudes of serum calcium rise may aggravate GC progression.

Intracellular $\text{Ca}^{2+}$ can bind to calmodulin and stimulate PI3K/AKT (33), resulting in subsequent nuclear accumulation of $\beta$-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 $\beta$-catenin signaling (39), confirming negative effect of CaSR on normal gastrointestinal epithelial growth. We showed that CaSR activation decreased AKT and $\beta$-catenin phosphorylation in normal gastric epithelial cells, but increased them in GC cells with enhanced cell growth, migration and invasion. Notably, overexpression of CaSR in normal cells resulted in marked increases in their $[\text{Ca}^{2+}]_{\text{cyt}}$, AKT and $\beta$-catenin phosphorylation, and migration, all of which mimic the behaviors of GC cells. Together, our results indicate that aberrant
CaSR induces GC development and progression through TRPV4/Ca\textsuperscript{2+}/AKT/β-catenin relay (Figure 7F). The TRPV4-mediated [Ca\textsuperscript{2+}]\textsubscript{o} entry in GC cells contrasts from the previous report that Ca\textsuperscript{2+} release from internal stores of CRC cells enhanced nuclear export and degradation of β-catenin in the cytoplasm (40), suggesting that different Ca\textsuperscript{2+} sources might play different roles in upper and lower GI tumorigenesis.

It has been puzzling in the clinic why GC 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 GC patients are supplemented with the electrolytes, including high calcium. Therefore, high serum calcium could be problematic for GC patients as shown in the present 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\textsuperscript{2+}/β-catenin signaling could be potential novel preventive and therapeutic strategies for human GC.
REFERENCE


Dihydropyridine Ca(2+) channel blockers increase cytosolic [Ca(2+)] by activating Ca(2+)-sensing receptors in pulmonary arterial smooth muscle cells. *Circ Res* **2013**;112:640-650.
Figure Legends

Figure. 1. Enhanced CaSR expression and serum calcium are associated with human gastric cancer progression.

(A) Representative images (left panels) and summary data (right panels) of immunohistological staining in GC biopsy tissues (Can) normalized to their paraneoplastic normal tissues (Con) (***p<0.001, n=91 patients). Scale bars in a1- a4 are 100µm. (B) Transcript levels of CaSR determined by qRT-PCR in GC 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 are 25 µm. (E) Relative CaSR protein levels in GC 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 GC patients with low and high serum calcium concentration (*p<0.05, n=91). (G) Kaplan-Meier analysis of survival ratio of GC 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 GC 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 Ca\(^{2+}\) 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 CaCl\(_2\) (2.0 mM)-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 indicates negative control, (C) CaCl\(_2\) (1.2 mM) enhanced migration of GES-1 and MKN45 cells in scratch test. Control treatment had PBS. Scale bars are 100 µm. Summary of distance migrated in 24 h is shown to the right of the images (*p<0.05, ***p<0.001, n=3). (D) Ca\(^{2+}\)-enhanced invasion of GES-1 and MKN45 cells in the transwell study. Control and CaCl\(_2\) treatment had PBS and 1.2 mM CaCl\(_2\), respectively. Scale bars are 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 mM CaCl\(_2\) (**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 Ca\(^{2+}\) (**p<0.01, n=3). (G) Overexpression of CaSR proteins in GES-1 cells (G, left panel, ***p<0.001 vs. vector, n=3) enhanced CaCl\(_2\)-induced proliferation (G, righ panel, (*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) are 100 µm.
Figure. 3. CaSR enhances gastric tumor growth and gastric cancer cell metastasis in vivo.

(A) Ca\(^{2+}\) 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 mM CaCl\(_2\) in normal saline on the right and control PBS on the left side. 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 CaCl\(_2\) injection (*p<0.05, n=5 mice). (B) NPS 2143 inhibited CaCl\(_2\)-induced gastric tumor growth in the xenograft model. The xenografted nude mice were injected with either CaCl\(_2\) (4 mM) alone (left side) or CaCl\(_2\) plus NPS 2143 (15 µM, right side) 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 Ca\(^{2+}\)-induced gastric tumor growth in the xenograft model. Nude mice were xenografted with tumor cells pretreated with shRNA-CaSR (right side) or shRNA-NC (left side) and injected daily for 30 days with 4 mM CaCl\(_2\). Shown are fluorescence images of tumors taken with an in-vivo imaging system (left) and summary of tumor 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 are 100 µm. (***p<0.001, n=5 mice). (E) The images and summary data of nude mice injected peritoneally with MKN45 cells pre-cocultured with either 4 mM CaCl\(_2\) alone or CaCl\(_2\) plus 15 µM NPS 2143 (left panels), and the number of tumors in each group (right panel). The white arrows indicate metastatic tumors. **p<0.01, n=5 mice in each group.
Figure. 4. CaSR activation causes cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\)) increases in gastric epithelial and gastric cancer cells.

(A) Representative tracings showing CaCl\(_2\)-induced [Ca\(^{2+}\)\(_{\text{cyt}}\)] in GES-1(a1), SGC-7901(a2) and MKN45(a3) cells. CaSR knockdown (a4) and NPS 2143 (5 µM)(a5) on CaCl\(_2\)-induced [Ca\(^{2+}\)\(_{\text{cyt}}\)] 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\(^{2+}\)\(_{\text{cyt}}\)] in different cells with different treatments (\(@p<0.05\), or **,**,**,**,$$$, \&\&\&\&\&; p<0.001, n=20 cells). (D) Diagram of dual perfusion system (left panel) and confocal microscopy images of CaSR (green) with an xz-scan through polarized IEC-6 monolayer culture (right panels). Phalloidin (actin, red) was used as a control (AP: apical and BS: basolateral. Scale bar is 8 µm). (E) Representative tracings and summary of Ca\(^{2+}\) signaling using dual perfusion system to deliver drugs to each side of IEC-6 monolayer (*p<0.05, or ** *p<0.001, n=3). (F) Representative tracings and summary of spermine-induced [Ca\(^{2+}\)\(_{\text{cyt}}\)] signaling in MKN45 cells in the absence (0Ca) or presence of 2 mM extracellular Ca\(^{2+}\)(***p<0.001, n=20 cells). (G) Representative tracings and summary of CPA (10 µM) or UTP (10 µM)-induced [Ca\(^{2+}\)\(_{\text{cyt}}\)] signaling in Ca\(^{2+}\)-free (0Ca) solutions (**p<0.01, n=20 cells). The CaCl\(_2\) of 5 mM and spermine of 3 mM were used for all experiments.
Figure. 5. TRPV4 mediates currents and Ca\textsuperscript{2+} entry in response to CaSR activation human gastric epithelial and gastric cancer cells.

(A) CaCl\textsubscript{2}-induced [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in MKN45 cells was not affected by SKF96365 (30 µM, NS: no significance, n=20 cells). (B) Capsaicin (10 µM to 100 µM) did not elicit [Ca\textsuperscript{2+}]\textsubscript{cyt} increase in MKN45 cells. Spermine (3 mM) was used as a positive control (NS: no significance, n=20 cells). (C) GSK1016790A (1 µM) increased [Ca\textsuperscript{2+}]\textsubscript{cyt} in the presence, but not the absence of 2 mM extracellular Ca\textsuperscript{2+} (left, **p<0.01, n=20 cells) and the effect was blocked by 30 µM RN1734 (right, **p<0.01, n=20 cells). (D) RN1734 (30 µM) inhibited [Ca\textsuperscript{2+}]\textsubscript{cyt} increases induced by CaCl\textsubscript{2} (5 mM, left) and spermine (3 mM, right) in MKN45 cells (**p<0.01, n=20 cells). (E) Immunohistochemistry analysis of TRPV4 proteins in human normal gastric (upper) and GC (lower) biopsy tissues, representatives of 10 samples. Scale bars are 10 µm. (F) Western blot analysis of TRPV4 proteins in the indicated cell lines, representatives of 3 independent experiments. (G) Confocal immunofluorescence images showing co-localization of CaSR and TRPV4 in human gastric epithelial and GC cells, representatives of 3 independent experiments. Scale bars are 25 µm. (H) Whole-cell currents in MKN45 cells evoked by TRPV4 agonist GSK1016790A (50 nM,) and the inhibition by TRPV4 antagonist RN1734 (30 µM). (I) Current-voltage (I-V) relationships obtained from voltage ramps under basal, peak of GSK1016790A response and after inhibition by RN1734 (30 µM). (J) Summary of current densities of GSK1016790A and RN 1743 at +100 mV. (K) Whole-cell currents in MKN45 cells evoked by CaSR agonist CaCl\textsubscript{2} (5 mM) and the inhibition by RN1734 (60 µM). (L) Current-voltage (I-V) relationships obtained from voltage ramps under basal, peak of CaCl\textsubscript{2} response and after inhibition by RN1734 (30 µM). (M) Summary of current densities of CaCl\textsubscript{2} and RN 1743 at +100 mV. **p<0.01, n=5 cells for all experiments
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 mM) in GES-1, SGC-7901 and MKN45 cells. Shown are representative western blots (upper) and summary data normalized to total β-catenin (lower, *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).
Figure. 7. CaSR-mediated TRPV4/Ca\(^{2+}\)/AKT/β-catenin relay and its role in proliferation, migration and invasion of gastric cancer cells.

(A) BAPTA-AM (2 µM) abolished CaCl\(_2\) (2 mM)-induced AKT phosphorylation of AKT (left) and β-catenin (middle) in MKN45 cells. Shown are representative western blots (upper) and summary data normalized to total AKT or β-catenin (lower, *p<0.05, or **p<0.01, n=3). LY294002 (5 µM) inhibited Ca\(^{2+}\)-induced β-catenin phosphorylation in MKN45 cells (right) (*p<0.05, n=3). (B) Chelating cytosolic Ca\(^{2+}\) with BAPTA-AM or inhibiting PI3K/AKT with LY294002 or β-catenin with XAV939 (1 µM) abolished CaCl\(_2\)-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 µM) in MKN45 cells (*p<0.05, n=3). (D) RN1734 (30 µM) suppressed CaCl\(_2\)-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 Ca\(^{2+}\)-enhanced proliferation (left) and migration (right) of MKN45 cells (**p<0.01, n=3). (F) Signaling cascade for the aberrant CaSR activation in gastric tumonogenesis.
Figure 3

A

B

C

D

E

Con

CaCl₂

CaCl₂+NPS

Number of tumors

0

5

10

15

20

25

***

***

***

CaSR protein level

0.0

0.5

1.0

1.5

***

***

***

shRNA

NC

CaCl₂

NC

shRNA

CaCl₂

NPS

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A

a1 GES
Ca
F(340/380)
0 100 200 300 400(s)

a2 7901
Ca
F(340/380)
0 100 200 300 400(s)

a3 MKN
Ca
F(340/380)
0 100 200 300 400(s)

a4 MKN
Ca+NPS
F(340/380)
0 100 200 300 400(s)

a5 MKN
Ca+shRNA
F(340/380)
0 100 200 300 400(s)

B

GES(Vec)
Ca
F(340/380)
0 100 200 300 400(s)

GES(Over)
Ca
F(340/380)
0 100 200 300 400(s)

C

CaSR
Phalloidin
Merge

D

IEC-6 monolayer model

Microvilli
Tight junction
Semipermeable membrane
Apical side
Medium
Basolateral side

E

Basolateral
Ion EGTA
Sp

Apical
Ion EGTA
Sp

G

MKN
CPA+0Ca
UTP+0Ca
0Ca 2Ca

F

MKN
Spermine

ΔF(340/380)

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