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Induced Expression of STIM1 Sensitizes Intestinal Epithelial Cells to Apoptosis by Modulating Store-Operated Ca^{2+} Influx

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Abstract

Apoptosis plays a critical role in the maintenance of gut mucosal epithelial homeostasis and is tightly regulated by numerous factors including intracellular Ca^{2+}. Canonical transient receptor potential channel-1 (TRPC1) is expressed in intestinal epithelial cells (IECs) and functions as a store-operated Ca^{2+} channel. We have recently demonstrated that increased TRPC1 activity sensitizes IECs to apoptosis, but the upstream signaling initiating TRPC1 activation remains elusive. The novel protein, Stromal interaction molecule 1 (STIM1), is shown to act as a store Ca^{2+} sensor and it can rapidly translocate to the plasma membrane where it directly interact with TRPC1. The current study determined whether STIM1 plays an important role in the regulation of IEC apoptosis by activating TRPC1 channel activity.

Methods—Studies were conducted in IEC-6 cells (derived from rat intestinal crypts) and stable TRPC1-transfected IECs (IEC-TRPC1). Apoptosis was induced by tumor necrosis factor-α (TNF-α)/cycloheximide (CHX), and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was measured by fluorescence digital imaging analysis. Functions of STIM1 were investigated by specific siRNA (siSTIM1) and ectopic overexpression of the constitutively active STIM1 EF-hand mutants.

Results—Stable STIM1-transfected IEC-6 cells (IEC-STIM1) showed increased STIM1 protein expression (~5 fold) and displayed a sustained increase in Ca^{2+} influx after Ca^{2+} store depletion (~2 fold). Susceptibility of IEC-STIM1 cells to TNF-α/CHX-induced apoptosis increased significantly as measured by changes in morphological features, DNA fragmentation, and caspase-3 activity. Apoptotic cells were increased from ~20% in parental IEC-6 cells to ~40% in stable IEC-STIM1 cells 4 h after exposure to TNF-α/CHX ($p<0.05$). In addition, stable IEC-TRPC1 cells also exhibited an increase sensitivity to TNF-α/CHX-induced apoptosis, which was prevented by STIM1 silencing through siSTIM1 transfection. STIM1 silencing by siSTIM1 also decreased Ca^{2+} influx after store depletion in cells overexpressing TRPC1. Levels of Ca^{2+} influx due to store depletion were decreased by ~70% in STIM1-silenced populations. Similarly, exposure of IEC-STIM1 cells to Ca^{2+} free medium also blocked increased sensitivity to apoptosis.

Conclusions—These results indicate that 1) STIM1 plays an important role in the regulation of IEC apoptosis by altering TRPC1 activity and, 2) ectopic STIM1 expression sensitizes IECs to apoptosis through induction in TRPC1-mediated Ca^{2+} influx.

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Keywords

STIM1; TRPC1; apoptosis; calcium influx; intestinal epithelial cell

Introduction

Maintaining homeostasis within the gastrointestinal mucosa requires a balance of cell restitution, proliferation, and apoptosis. The intestinal epithelial cells line the entire gastrointestinal mucosal layer and are integral to gastrointestinal homeostasis. Undifferentiated cells migrate up from the crypts as they mature and differentiate to replace injured and senescent cells that are shed, thus preserving villus integrity and homeostasis.(1–2) Preserving a balance of intracellular and extracellular calcium is vital for numerous biologic processes within the human body. Defects in calcium homeostasis lead to a variety of pathologies, from cardiac and gastrointestinal disturbances to immune dysfunction and cancer. Calcium signaling has been shown to be an integral part of cell proliferation, differentiation, motility, and apoptosis.(3–4) Both influx of extracellular calcium through calcium channels in the cell membrane as well as release from intracellular stores (e.g. sarcoplasmic and endoplasmic reticulum) serve to maintain adequate cytoplasmic calcium levels.(5)

Our previous studies have demonstrated that the canonical transient receptor potential channel-1 (TRPC1) is highly expressed in intestinal epithelial cells (IECs) and functions as a store-operated Ca^{2+} channel to restore intracellular calcium levels after store depletion although the mechanism by which TRPC1 channel activity is regulated remains unknown.(6) Recently, stromal interaction molecule-1 (STIM-1) is shown to act as a calcium sensor protein that exists within the membrane of the endoplasmic reticulum that is known to aggregate as well as translocate to the plasma membrane upon depletion of intracellular calcium stores and interact with various calcium channels, (7–9) although the full details of its scope and function are still being elucidated. In this study we wanted to test the hypothesis that STIM1 plays an integral role in calcium homeostasis in intestinal epithelial cells and furthermore that it interacts with TRPC1 to regulate TRPC1 induced apoptosis in intestinal epithelial cells.

Materials and Methods

Chemicals and Supplies

Disposable culture ware was purchased from Corning Glass Works (Corning, NY) and Sarstedt Inc. (Newton, NC). Tissue culture media, LipofectAMINE and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Cycloheximide and other biochemicals were obtained from Sigma. Recombinant mouse tumor necrosis factor alpha (TNF-α) was purchased from R&D Systems, Inc. (Minneapolis, MN). The monoclonal antibodies against Caspase-3 were purchased from Cell Signaling Technology (Danvers, MA). Stromal interaction molecule 1 (STIM1) monoclonal antibodies were purchased from BD Transduction Laboratories (San Jose, CA) and Cell Signaling Technology (Danvers, MA). Secondary antibodies, anti-mouse IgG conjugated to horseradish peroxidase, and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

The IEC-6 cell line was purchased from the American Type Culture Collection (ATCC) at passage 13. This cell line is a nontumorigenic cell line derived from normal rat intestinal crypt cells as judged by morphological and immunologic criteria and was developed and

characterized by Quaroni et al.(10) Stock cells were maintained in T-150 flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with FBS, insulin and gentamicin (Invitrogen).

Plasmid Construction and Infection

The full-length human STIM1 cDNA was inserted into the *HindIII* and *XhoI* sites of the pcDNA3.1 (+) expression vector (Invitrogen) containing the human cytomegalovirus promoter (pcDNA-STIM1). IEC-6 cells were transfected with pcDNA-STIM1 or pcDNA3.1 (+) vectors containing no STIM1 cDNA using a LipofectAMINE kit per manufacturer's instructions (Invitrogen). Cells were incubated for 5 hours at 37°C in the transfection medium. The transfection medium was then replaced with normal growth medium containing 5% FBS and cells grown for 2 days prior to exposure to selection medium containing 0.6mg/mL Geneticin (G418). Clones resistant to the selection medium were isolated, cultured and screened for STIM1 expression by STIM1 antibody specific Western blot analysis.

RNA Interference

The small interfering RNA (siRNA) specifically targeting and cleaving STIM1 mRNA was purchased from Dharmacon RNAi Technologies (Lafayette, CO). The GenBank database was used to screen siRNA nucleotides with no matches found to other target genes. Control siRNA (C-siRNA), with no sequence homology to any known genes, was used as a control. Briefly, 80 μL of the siSTIM1 stock or 20μL of control siRNA was mixed with 2 mL of Opti-MEM medium (Invitrogen) and incubated at room temperature for 5 minutes. An equal volume (2 mL) of Lipofectamine/Opti-MEM medium was then added to the 15 mL tubes containing siRNA and incubated at room temperature for 20 minutes. One milliliter of the Opti-MEM/siRNA/Lipofectamine medium was added to each plate as well as an additional 3 mL of regular medium for a final concentration of 150 pmol/mL siSTIM1 and 100 pmol/mL control siRNA per plate. The plates were then incubated for 24–48 hrs at 37°C and the medium subsequently changed to regular medium after this incubation period. The cells were allowed to grow an additional 24 hrs prior to being treated and harvested for various assays.

Western Blot Analysis

Total cell lysates were obtained by scraping plates and centrifuging medium and scraped cells at 1500 – 4000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet rinsed with 3–5 mL cold PBS and then recentrifuged at 1500–4000rpm × 5 minutes ad 4°C. An appropriate amount of RIPA lysis buffer was added and the cells were sonicated briefly, incubated on ice for 40 minutes and subsequently centrifuged at 12,000 rpm for 12 minutes at 4°C. Protein concentrations were calculated from the supernatant using BCA protein assay (Pierce Chemical Co.). Supernatants were boiled for 5 minutes and then subjected to electrophoresis on 10 or 12% SDS-PAGE gels (Bio-Rad). The protein was transferred to nitrocellulose filters and the filters then incubated at room temperature (~24°C) for 1 hour in 5% non-fat dry milk in 1x tris buffered saline + 0.5% vol/vol Tween 20 (TBS-T). Immunological evaluation was conducted either for 1 hour at room temperature or overnight in 4°C in 5% milk/TBS-T or plain PBS containing 1–2 μg/mL of the appropriate antibody (STIM1 or Caspase-3) according to the suppliers' instructions. The filters were then washed with 1x TBS-T and incubated for 1 hour with the horseradish peroxidase conjugated secondary antibody. After multiple washings with 1x TBS-T, the filters were developed by enhanced chemiluminescence.

Measurement of [Ca^{2+}]

Cells from both the control IEC-6 and two different stable IEC-STIM1 lines were plated onto 25 mm cover slips and were incubated in culture medium containing 3.3 μM fura 2-AM (fura 2 acetoxymethyl ester) for 30–40 min at room temperature under an atmosphere of 10% CO_2. Fura 2-AM-loaded cells were then superfused with standard bath solution for 20–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura 2-AM into active fura-2. Images of fura 2 fluorescence in the cells and background fluorescence were viewed using a Nikon Diaphot microscope equipped for epifluorescence. A microchannel plate image intensifier (Amperex XX1381; Opelco, Washington DC) coupled by fiber optics to a Pulnix charge coupled device (Stanford Photonics, Stanford, CA) was used to obtain the fluorescent images. The Metamorph Imaging System (Universal Imaging) was used to perform image analysis and acquisition and $[Ca^{2+}]_{cyt}$ was calculated from fura 2 fluorescent emission spectra, excited at 380 and 340 nm using the ratio method.

Measurement of Caspase-3 Activation

Caspase-3 activation was measured using the Caspase-3 Colorimetric Assay kit (R&D Systems, Inc., Minneapolis, MN) and performed according to the manufacturer's protocol. In brief, cells were grown in culture and treated with TNF-α/CHX for the appropriate length of time (2 and 4 hrs). Plates were scraped and the media and cells were collected into conical tubes and spun at 4500 rpm for 10 minutes at 4°C. The pellet was rinsed once with ~3 mL of cold PBS and then respun at 4500 rpm for 10 minutes at 4°C. The PBS supernatant was removed and an appropriate amount of lysis buffer (based on estimated cell number) was added to the pellet in two parts (the initial portion was used to remove the majority of the pellet and the remainder used to rinse the tube) and transferred to a 1.7 mL microcentrifuge tube. The tubes were vortexed for ~10 seconds and then incubated on ice for 10 minutes. Samples were spun at 14,000 rpm × 1 minute and the supernatant transferred to a new tube. Protein concentrations were determined using BCA Protein Assay (Pierce Chemical Co.). The reaction was carried out using a 96-well flat bottom plate on which 200 μg of sample was loaded (a volume of 50 μL/well) in duplicate. Subsequently 50 μL of 2X Reaction Buffer + DTT was added and finally 5 mL of the DEVD-pNA, the Caspase-3 colorimetric substrate. Control wells were also added to the plate containing either no substrate or no cell lysate. The plate was incubated for 1 to 2 hrs at 37°C and then read at 405 nm on a microplate reader.

Determination of the Amount of Apoptosis and DNA Fragmentation

Cells were photographed at time zero and after the various treatments using a Nikon Eclipse TE200 inverted microscope. Images were printed off, blinded and live and dead cell counts obtained. Percentage apoptosis was then calculated.

Determination of the amount of DNA fragmentation was conducted using (R&D Systems, Inc., Minneapolis, MN) per the manufacturer's instructions. Briefly, cells were grown in culture and treated with TNF-α/CHX for the appropriate length of time (2 and 4 hrs). The plates were then scraped and the media and cells pooled into 15-mL conical tubes. Each plate was rinsed with a small amount of cold PBS to remove any remaining cells and this was then added to the tube. Tubes were spun at ~4000 rpm × 5 minutes and then the supernatant was removed. Samples were resuspended in sample buffer and incubated at room temperature for 10 minutes. Lysis buffer was then added and samples gently mixed. They were subsequently transferred to 2 mL microcentrifuge tubes and the appropriate volumes of Extraction Buffers added per manufacturer protocol. The samples were vortexed for 10 seconds and spun at 14,000 rpm for 5 minutes. The upper aqueous layer was removed and transferred to a new tube, and 0.1 volume of Sodium Acetate was added and mixed.

This was followed by the addition of an equal total volume of isopropyl alcohol. After mixing, the samples were spun again at 14, 000 rpm x10 minutes. The supernatant was removed and the pellet washed with 1 mL of 70% ethanol and then respun at 14,000 rpm for 5 minutes. After carefully decanting the ethanol the samples were briefly placed in an autodryer to remove any remaining liquid and then resuspended in 100 μL H_20. One microgram of DNA per well was loaded onto a 1.5% TreviGel (powder supplied by the manufacturer) that had been incubated in 1x TAE + ethidium bromide and run at 100V for ~2hrs. Photographs were taken using a BioRad VersaDOC imaging system.

Statistics

Experiments were carried out in triplicate and statistical analyses conducted using GraphPad Prism 5 and Microsoft Excel.

Results

STIM1 overexpression is associated with an increase in intestinal epithelial cell growth

In order to evaluate the effect of STIM1 expression on epithelial cell apoptosis, IEC-6 cells were transfected with a STIM1 construct (Fig. 1) in order to constitutively express STIM1 protein. Multiple clones were created and characterized within our laboratory and these were used to conduct the experiments in this paper.(11) A couple of stable transfected clones expressing high amounts of STIM1 protein were selected to conduct experiments. Growth studies were conducted using one STIM1 cell clone and parental IEC-6 cells in which cells were plated in triplicate on 12-well plates and subsequently trypsinized and counted. IEC-STIM1 cells were noted to replicate approximately twice as fast as parent IEC-6 cells grown under the same conditions (Fig. 2). Although not formally quantified, wild type STIM1 overexpression cells (IEC-WT-STIM1) were also noted to grow faster than the parental IEC-6 cells, although not as fast as STIM1 overexpression cells.

IEC-STIM1 Cells Exhibit Sustained Increase in Ca^{2+} Influx after Ca^{2+} Store Depletion

Calcium influx was measured in two different STIM1 clones and compared to that of parental undifferentiated intestinal epithelial cells. STIM1 cells exhibited an approximately 75% increase in calcium influx over IEC-6 cells upon depletion of intracellular calcium stores with cyclopiazonic acid (P value is <0.05, data not shown).(11)

TNFα/Cycloheximide (TNFα/CHX) induced apoptosis in IEC-STIM1 cells results in an increased susceptibility when compared to parent IEC-6 cells

We hypothesized that STIM1 plays a key role in intestinal epithelial cell (IEC) apoptosis through interaction with TRPC1. In order to examine the effect of STIM1 gene overexpression on apoptosis in intestinal epithelial cells, STIM1 overexpression and IEC-6 parent cells were plated and then exposed to TNFα and cycloheximide for 2 and 4 hours. STIM1 cells were noted to have a significant increase in apoptosis after 2 and 4 hours of TNFα/CHX exposure compared to IEC-6 cells (Fig. 3). After 2 hours of exposure approximately 1% of IEC-6 cells were dead compared to 10% in the STIM1 cells ($p<0.05$). Four hours of TNFα/CHX exposure yielded ~40% of STIM1 cells dead compared to ~20% in the control group ($p<0.05$). As a confirmatory test for the increase in apoptosis observed morphologically between IEC-6 and STIM1 cells, these cells were again exposed to TNFα/CHX for 2 and 4 hours, and caspase-3 activation was evaluated by Western Blot analysis. Caspase-3 activation was seen to increase ~20–30% (per western blot densitometry) in STIM1 overexpression cells when compared to parental IEC-6 cells after 4 hours of exposure to the apoptotic agent (Fig. 4). DNA fragmentation, a sign of apoptosis, was also

evaluated in IEC-6 versus STIM1 cells. Increased DNA fragmentation, and therefore apoptosis, was seen in STIM1 cells over IEC-6 cells upon exposure to TNFα/CHX (Fig. 5).

Effect of siSTIM1 on Caspase-3 Activation in IEC-TRPC1 Cells

Our previously published data has shown that constitutive expression of TRPC1 makes IEC cells more sensitive to apoptosis.(6) TRPC1 has also been shown to bind to and interact with STIM1 in human embryonic kidney (HEK) cells. (12) In light of this known interaction we sought to determine whether silencing STIM1 using small interfering RNA in TRPC1 overexpression cells would affect their sensitization to apoptosis. TRPC1 cells were transfected with control siRNA as well as STIM1 siRNA and the effects on caspase-3 activation after induction of apoptosis with TNFα/CHX at 2 and 4 hours were observed. Western immunoblotting was conducted to confirm silencing of the STIM1 gene in the TRPC1 cells (Fig. 6a). Western blotting analysis after induction of apoptosis revealed that by 4 hours of exposure caspase-3 activation decreased ~40% in TRPC1 cells with inhibition of STIM1 expression (Fig. 6b).

Effect of Ca^{2+} Free Medium on TNFα/CHX Induced Apoptosis in IEC-STIM1

In light of the effect of STIM1 on calcium influx and the known role of calcium in apoptosis, we sought to determine whether depleting calcium levels in STIM1 overexpression cells would reverse the observed increase in apoptosis over control cells under normal growth conditions. IEC-6 and IEC-STIM1 cells were grown to confluence and then calcium containing medium was removed and the cells washed to remove any remaining calcium. The cells were then subjected to TNFα/CHX in calcium free conditions for 0, 2, and 4 hrs. Western blot analysis revealed that after TNFα/CHX exposure the amount of caspase-3 activation was significantly decreased (~75% by Western immunoblotting densitometry) after 4 hours of TNFα/CHX exposure when compared to that of control cells (Fig. 7a). This decrease was confirmed by colorimetric assay with separate sets of experiments in cells grown under the same treatment conditions with a return of caspase-3 levels to approximately that of cells untreated with calcium free medium (Fig. 7b).

Discussion

Stromal interaction molecule 1 (STIM1) is a recently described protein shown to act as a store Ca^{2+} sensor through facilitating calcium entry at the plasma membrane with TPRC1 interaction. In the present study, we demonstrated that STIM1 plays a critical role in intestinal epithelial cells through the regulation of calcium concentration and susceptibility to apoptosis. Cells with increased STIM1 expression showed increased growth, increased cytoplasmic Ca^{2+} concentrations, and greater susceptibility to apoptosis. This effect was abolished by inhibition of STIM1, and even in cells overexpressing TRPC1 this effect was abolished if STIM1 inhibition occurred concomitantly.

Calcium is required for many cellular functions including growth and proliferation. Abundant evidence indicates that calcium helps to regulate the integrity of the gastrointestinal mucosal layer in physiological and pathophysiological states. Calcium helps to regulate cell growth, apoptosis, and myriad of other functions; its levels require precise regulation, and cells must be able to rapidly increase cytosolic levels to survive insults. In non-excitable cells such as intestinal epithelial cells, much of the calcium regulation occurs through calcium permeable channels. TRPC1 has been proposed by our group as one candidate for regulation of cytosolic calcium levels. TRPC1 expression results in formation of calcium permeable channels and our previous studies have shown that TRPC1 is expressed in IECs, and that TRPC1 overexpression leads to increased cytosolic calcium levels, increased cellular migration, and increased mucosal restitution.(6, 13–14) The

mechanism by which TRPC1 enacts this has been unknown, and the recent discovery of STIM1 has allowed speculation that it may be involved in TRPC1 regulation of calcium influx in intestinal epithelial cells.

Stromal interaction molecule-1 has recently been identified as an intracellular protein found on the endoplasmic reticulum (ER) that senses intracellular calcium store levels and is required for calcium release activating channel (CRAC) mediated store-operated Ca^{2+} entry in non-excitable cells.(8, 15–16) By residing on the ER, STIM1 is able to sense decreases in ER intraluminal calcium concentrations via calcium binding to its EF-hand.(8) In our experiments this EF-hand was altered to make STIM1 constitutively active in sensing calcium levels in order to examine STIM1's potential role in intestinal epithelial cell apoptosis. STIM1 has been shown to aggregate within the ER and translocate to the plasma membrane (PM) interacting with PM bound calcium channels to stimulate store-operated calcium entry under depletion conditions.(8–9, 17) Prior studies by Chiu et al (18) revealed that STIM1 is upregulated upon inducing apoptosis in cervical epithelial cells thus upregulating its interaction with store-operated Ca^{2+} channels. In the present study we demonstrated that overexpression of STIM1 protein indeed leads to increased calcium influx under calcium store depletion conditions. This data, coupled with that of other experiments in our lab as well as others showing an interaction between STIM1 and the calcium channel TRPC1, was used to further investigate the nature of their interaction and its potential effect on apoptosis.(12)

In our experiments IEC-STIM1 cells were noted to grow at a faster rate than parent IEC-6 cells. This is most likely due at least in part to their ability to stimulate calcium influx into intestinal epithelial cells. Similar effects were seen in rat vascular smooth muscle cells where inhibition of STIM1 expression using small interfering RNA lead to an inhibition of cell proliferation and cell cycle arrest which was reversed by co-transfection of rat siSTIM1 with human STIM1.(19)

Our previous studies have shown that TRPC1 overexpression sensitizes IECs to apoptosis.(6) The results of the present study, as shown in figures 3 through 5, further indicate that upon overexpression of the STIM1 gene, these cells become more sensitized to induction of apoptosis. The results of the current study parallel those seen in IECs that overexpress the TRPC1 gene. The current study demonstrates that the apoptotic effects of TNFα/CHX are reversed, at least in part, with the removal of calcium, leading us to believe that STIM1's ability to increase calcium influx under calcium free conditions is crucial to its ability to sensitize cells to apoptosis. In addition, we were able to see a reversal in TRPC1 induced sensitization to apoptosis through silencing the STIM1 gene in TRPC1 overexpression cells, leading us to believe that the interaction between STIM1 and TRPC1 is key to the regulation of apoptosis in intestinal epithelial cells.

Aberrancies in calcium regulation, such as those seen with derangements of Orai1 and TRPC channels, have been linked to disorders of immune system modulation as well as platelet function and atherosclerosis.(20–24) A recent case report by Picard et al.(21) has shown that a homozygous nonsense mutation in the STIM1 gene with resultant undetectable STIM1 expression lead to defects in store-operated calcium entry and subsequent "immunodeficiency, autoimmune hemolytic anemia, thrombocytopenia, muscular hypotonia, and disturbed enamel dentition."(21) STIM1 in recent studies has been shown to be crucial to calcium regulation in multiple cell types. This case report is just one example of how further elucidation of the role of STIM1 and defining methods to regulate it and the calcium channels with which it interacts can potentially lead to treatment modalities for a wide range of immune, vascular and neoplastic diseases.

In conclusion, our data indicate that STIM1 plays an important role in the regulation of IEC apoptosis through interaction with TRPC1 and subsequent induction of TRPC1-mediated store operated calcium entry (SOCE).

Acknowledgments

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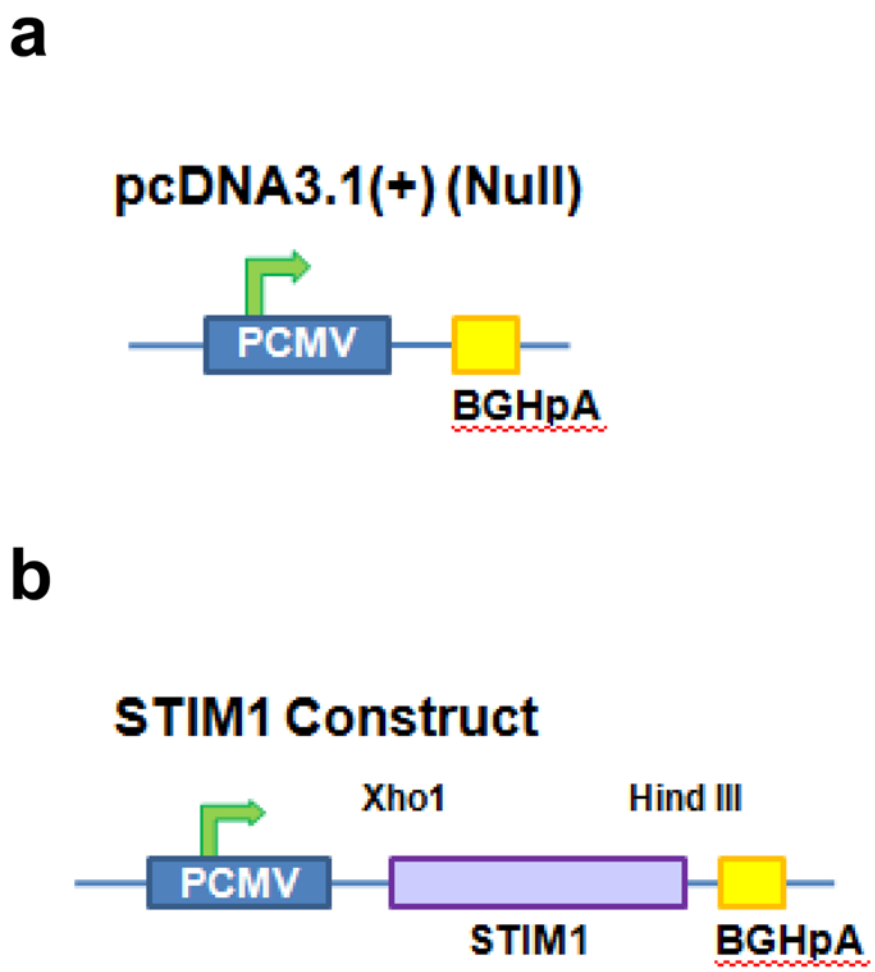

Fig. 1.
Schematic of expression vector constructs. A: null vector. B: STIM1 expression vector.

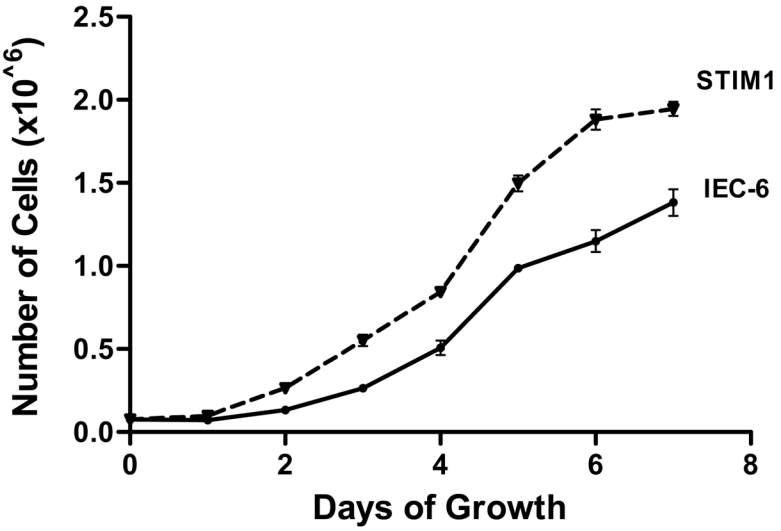

Fig. 2.

Growth rate differences between IEC-6 cells and IEC-STIM1 overexpression cells. The rate of growth in STIM1 cells was approximately twice that of IEC-6 cells.

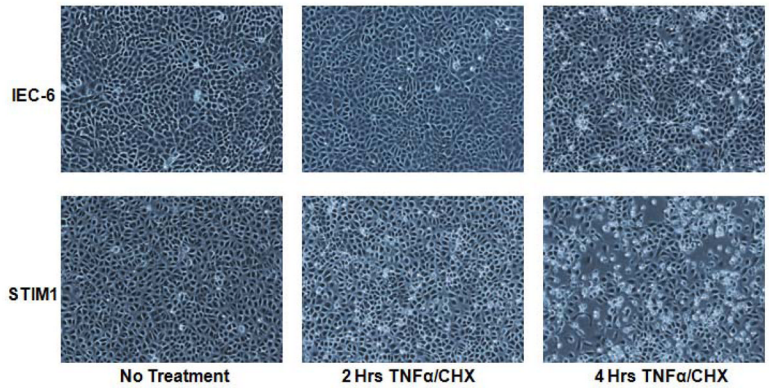

Fig. 3.

Effect of TNFα/CHX induced apoptosis on IEC-6 and IEC-STIM1 cells. Cells were grown in standard DMEM medium until confluence and then treated with 20 ng/mL TNFα + 25 μg/mL cycloheximide for 2 and 4 hours and morphologic effects on apoptosis observed.

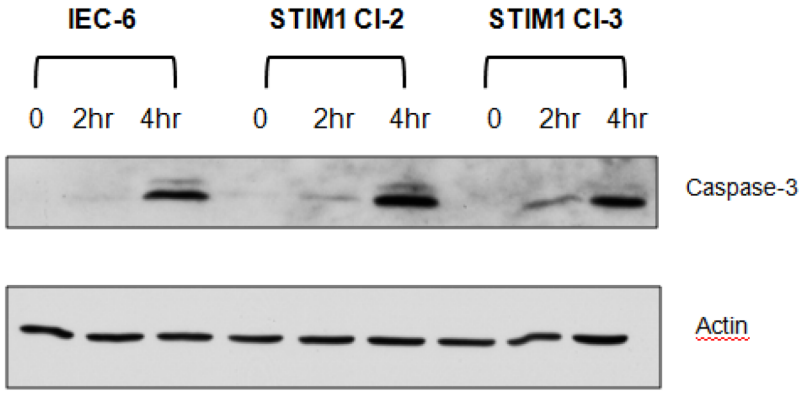

Fig. 4.

Changes in caspase-3 activation after induction of apoptosis in IEC-6 cells and two different IEC-STIM1 cell clones. Cells were grown to confluence in standard DMEM and subsequently treated with 20 ng/mL TNFα + 25 μg/mL cycloheximide for 2 and 4 hours. Total protein was extracted from all cells (floating and attached) and caspase-3 protein levels measured by Western blotting technique. Actin levels were detected as a loading control.

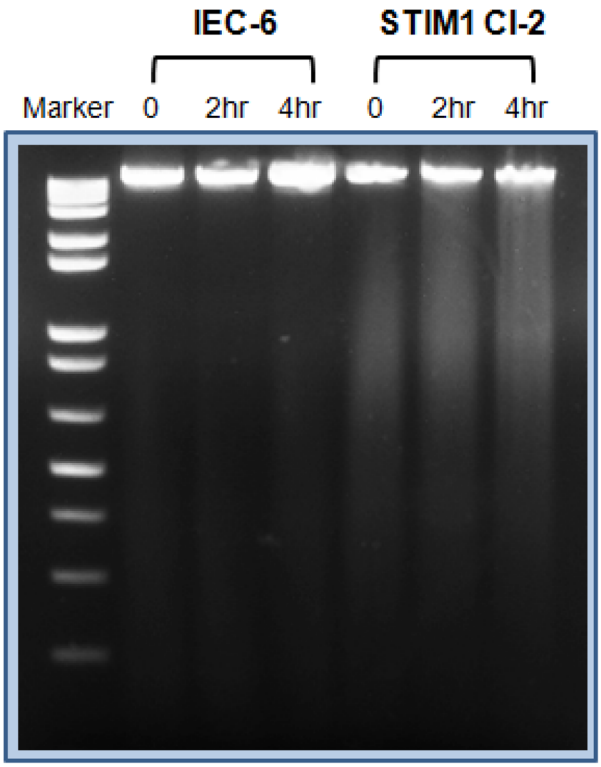

Fig. 5.
Change in DNA Fragmentation in control cells (IEC-6) and STIM1 overexpression cells after 0, 2 and 4 hours of TNFα/CHX exposure.

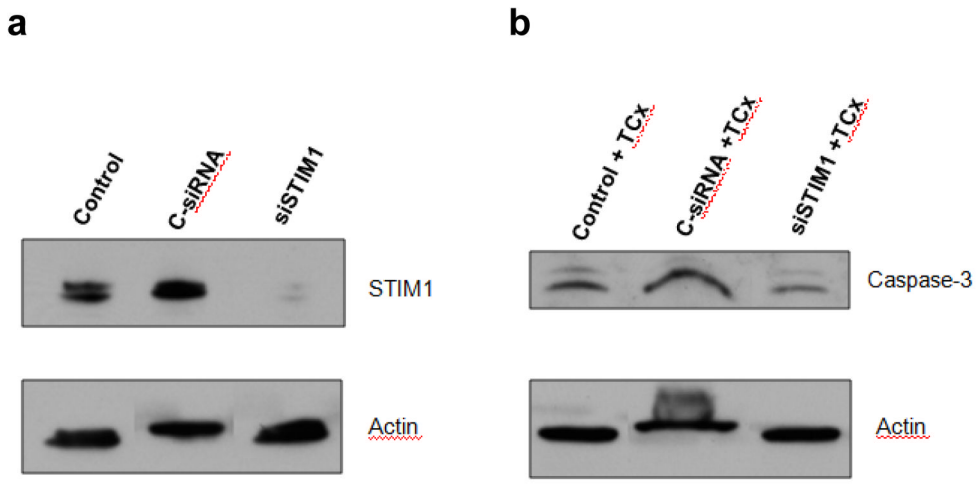

Fig. 6.

Effect of STIM1 silencing on TRPC1 induced apoptosis. A: Stable IEC-TRPC1 cells were transfected with either control siRNA (C-siRNA) or siSTIM1 and the level of STIM1 protein was measured by Western immunoblotting. B: Apoptosis was induced with TNFα/CHX (TCx) in stable IEC-TRPC1 cells transfected with either C-siRNA or siSTIM1 and caspase-3 levels were measured by Western immunoblotting. Both blots were stripped and actin immunoblotting was conducted as an internal control.

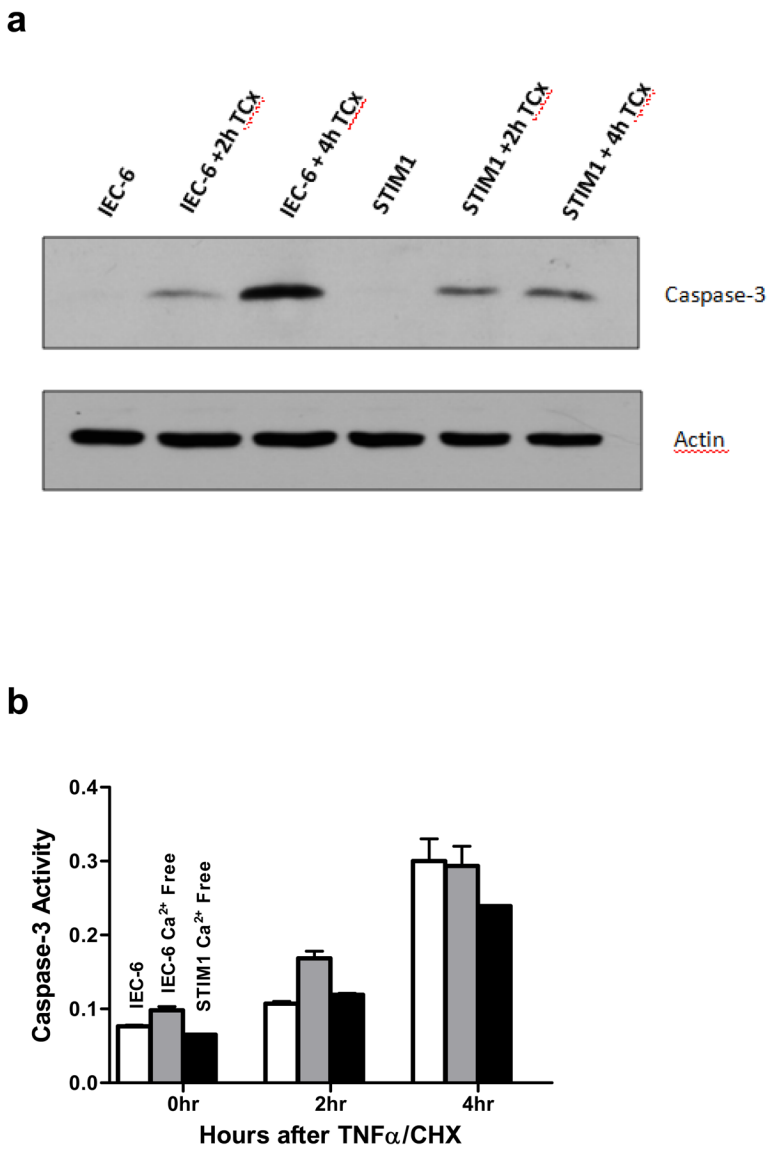

Fig. 7.

Effect of calcium depletion on TNFα/CHX (TCx) induced apoptosis in IEC-STIM1 cells. Control cells (IEC-6) and STIM1 overexpression cells were grown to confluence. Cells were washed with calcium free medium + EGTA and then subjected to apoptosis by TNFα/CHX in a calcium free medium environment for 0, 2 and 4 hrs. A: Caspase-3 levels were measured by Western immunoblotting and actin was used as an internal control after stripping the blot. B: Changes in caspase-3 activation was confirmed using a colorimetric ELISA protein assay.