T-type voltage-activated calcium channel $Ca_v3.1$, but not $Ca_v3.2$, is involved in the inhibition of proliferation and apoptosis in MCF-7 human breast cancer cells

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Abstract. T-type voltage-gated Ca^{2+} channels have unique electrophysiological properties, suitable for generating Ca^{2+} oscillations and waves and thus controlling the proliferation of various tumor cells. In the present study, we investigated the role of $Ca_v3.1$, a candidate tumor suppressor gene, in neoplastic processes, and compared the differences between $Ca_v3.1$ with $Ca_v3.2$ channels. While the overexpression of a full-length $Ca_v3.1$ clone suppressed cell proliferation, the knockdown of the $Ca_v3.1$ gene by siRNA, or treatment with ProTx-I, a relatively selective inhibitor for $Ca_v3.1$, promoted the cell proliferation of MCF-7 cells (a human breast adenocarcinoma cell line). Although $Ca_v3.1$ and $Ca_v3.2$ channels possess comparable biophysical properties and are often co-expressed in various tissues, gene knockdown or the overexpression of $Ca_v3.2$ channels exhibited no effect on cell proliferation. Using immunocytochemical co-staining, the $Ca_v3.1$ channels were specifically visualized in the plasma membranes of apoptotic cells, identified by Annexin V and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assays and nuclear condensation. On the contrary, $Ca_v3.2$ channels were expressed at the membrane of large portions of cells, with no likely relation to $Ca_v3.1$ expression or apoptosis. An apoptosis assay revealed that the overexpression of the $Ca_v3.1$ clone caused an increase in the number of apoptotic cells. Furthermore, $Ca_v3.1$ knockdown blocked cyclophosphamide-induced apoptosis. These results suggest that $Ca_v3.1$ channels may contribute to the repression of tumor proliferation and the promotion of apoptosis mediated via $Ca_v3.1$-specific Ca^{2+} influx.

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Introduction

There is increasing evidence that intracellular Ca^{2+} signaling regulates cellular processes, such as proliferation and apoptosis, in both normal and tumor cells. To organize such functions, the Ca^{2+} signal must be precisely regulated. Ca^{2+} signaling is generated by ions acting in various contexts of space, time and amplitude, and a unique combination of these factors influencing the Ca^{2+} signal is formulated depending upon cellular events, such as the cell cycle and cell death, or depending upon the physiological cell type (1). As one such component for producing the Ca^{2+} signal, T-type voltage-gated calcium channels have unique electrophysiological characteristics. T-type calcium channels open after a small depolarization of the membrane, and exhibit a unique low voltage-dependent activation/inactivation and slow deactivation; these channels are known to mediate a phenomenon known as 'window current' at near resting membrane potentials (2). These properties may be particularly important in controlling intracellular Ca^{2+} levels (3), especially for non-excitable tumor cells. At present, three isoforms of T-type Ca^{2+} channel α1 subunits are known: $Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H) and $Ca_v3.3$ (α1I) (4-8). Among them, the $Ca_v3.1$ and $Ca_v3.2$ channels resemble each other electrophysiologically.

The expression of T-type Ca^{2+} channels has been reported in numerous types of tumor cells (9). However, there is a divergence in views among reports regarding the effects of T-type channels on neoplastic processes, and information on this topic has remained insufficient. One of the T-type channel-isoform genes, the $Ca_v3.1$ (CACNA1G) gene, has been reported to be a target of aberrant methylation and silencing in human tumors, such as colorectal and lung cancers (10,11). Since the hypermethylation of CpG islands located in the promoter region is thought to be a major epigenetic modification that represses the transcription of tumor suppressor genes, the $Ca_v3.1$ (CACNA1G) gene has been regarded as a candidate tumor suppressor gene (10,11). Nowadays, the CACNA1G gene is regarded as a marker of the CpG island methylation phenotype (CIMP) for identifying specific clinicopathological features and determining the prognosis of patients with cancer. Furthermore, in prostate carcinoma cells, an increase in T-type $Ca_v3.1$ channel expression has been found to correlate with the inhibition of proliferation and the promotion of apoptosis (12). Alternatively,

a number of reports have speculated on the progressive roles of T-type channels in tumor growth (13-16). In addition to such conflicts, almost all these reports have not distinguished among T-type isoforms, since the currently available blockers that have been used have poor specificities for each isoform. This lack of specificity not only raises doubts as to the exact role of T-type channels, but also may complicate each function of the isoforms in tumor development. The purpose of this study was to investigate whether T-type channels are involved in the neoplastic process and, if so, whether they suppress or promote tumor growth. Using the MCF-7 breast cancer cell line, which has been reported to express two isoforms of T-type Ca^{2+} channels, $Ca_v3.1$ and $Ca_v3.2$ (9), and T-type currents (17), we show that differences exist between the two isoforms and that $Ca_v3.1$ channels may have an antitumorigenic function.

Materials and methods

Cell culture. MCF-7 cells (human breast adenocarcinoma cell line) and MCF-10F cells (non-tumorigenic human breast epithelial cell line) were obtained from the Health Science Research Resources Bank and the American Type Culture Collection, respectively. These cells were maintained in MEM (Eagle) with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 μg/ml insulin, and 10% fetal bovine serum at 37˚C in a CO_2 incubator.

Gene knockdown, gene overexpression and channel inhibition. siRNAs (Silencer® Select Pre-designed siRNA; Applied Biosystems/Ambion, Foster City, CA, USA) for human $Ca_v3.1$ (CACNA1G: s17051) and human $Ca_v3.2$ (CACNA1H: s17048) channels were used. The effects of these siRNAs on gene knockdown were estimated in advance using real-time PCR (see 'Microarray analysis of $Ca_v3.1$ and $Ca_v3.2$ gene knockdown' in Materials and methods). For non-silencing siRNA, control siRNA (Negative Control siRNA no. 1; Applied Biosystems/Ambion) was used. We assessed in advance that almost no difference in the cell number was observed between untransfected cells and cells transfected with Negative Control siRNA no. 1. Twenty-four hours after inoculation, the cells were transfected with each siRNA using the transfection reagent (siPORT™ NeoFX™; Applied Biosystems/Ambion) indicated according to the manufacturer's instructions. For the gene overexpression experiment, human $Ca_v3.1$ clone ($Ca_v3.1$/pcDNA3.1) or human $Ca_v3.2$ clone ($Ca_v3.2$/pcDNA3.1/V5-His) was transfected into MCF-7 cells using the TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA) on the same schedule as the gene knockdown experiment. The cDNA clone of the human $Ca_v3.1$ α1 subunit (GenBank: NM018896) was a generous gift from Dr T. Kobayashi (Mitsubishi Tanabe Pharma, Osaka, Japan). The human $Ca_v3.2$ isoform (GenBank: AF073931) was cloned using a PCR cloning method, as described previously in detail (18). ProTx-I (Peptide Institute Inc., Osaka, Japan) was used as a $Ca_v3.1$ channel blocker (19).

Cell proliferation assay. Cells at a cell density of $8x10^3$ cells/well were seeded in eight-sets per treatment in a 96-well culture plate. Twenty-four hours after inoculation, the cells were treated with siRNAs, channel clones, or channel blocker. Cell proliferation was determined using the Premix WST-1 (tetrazolium salt) assay system (Takara Bio Inc., Otsu, Japan) at 24 h after the treatments by measuring the absorbance at 450 nm using a multi-well plate reader.

Immunocytochemistry. Briefly, after washing the cells with PBS, the cells were fixed in 4% PFA-PBS for 20 min, and permeabilized with 0.2% Triton-X100-PBS. After blocking with 10% goat serum-PBS, the cells were incubated with the primary antibodies for 1 h, followed by incubation with the secondary antibodies for 1 h. The primary antibodies used were as follows: rabbit anti-$Ca_v3.1$ channel polyclonal antibody (dilution, 1:100) (C5488; Sigma-Aldrich, St. Louis, MO, USA), mouse anti-$Ca_v3.2$ channel monoclonal antibody (dilution, 1:100) (ab84815; Abcam, Cambridge, UK), and mouse anti-Annexin V monoclonal antibody (dilution, 1:50) (ab84815; Abcam). Alexa Fluor 488- or 568-conjugated anti-rabbit for $Ca_v3.1$, Alexa Fluor 488- or 568-conjugated anti-mouse for $Ca_v3.2$, and Alexa Fluor 568-conjugated anti-mouse for Annexin V (dilution, 1:1000 each) (Molecular Probes, Eugene, OR, USA) were used as the secondary antibodies. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed using the *In situ* Apoptosis Detection kit (Takara Bio Inc., Shiga, Japan), and FITC-dUTP labeling was performed together with the secondary antibody reaction. The cells were also counterstained with the nuclear stain, DAPI. The co-localization of apoptotic markers was detected using multiple label immunofluorescence and confocal microscopy (model LSM710; Carl Zeiss MicroImaging Inc., Jena, Germany). Images were processed using LSM software ZEN 2008 (Carl Zeiss MicroImaging Inc.).

Apoptosis assay. The APOPercentage™ Apoptosis assay kit (Biocolor Ltd., Newton Abbey, UK) was used. Briefly, the cells were incubated on a 24-well culture plate for 24 h; then, the siRNAs or channel clones were transfected as mentioned above. Forty-eight hours later, the cells were incubated with the APOPercentage dye (to allow dye transport into apoptotic cells) for 30 min at 37˚C in a CO_2 incubator. Cyclophosphamide (Sigma-Aldrich) was added 24 h after the siRNA treatment. After washing gently with PBS to remove the unbound dye, the red-stained cells were counted in a field of view containing 2,500-3,000 cells in total. Three fields per well were sampled and averaged. The averaged values from four wells per experimental group were collected, and the average and standard error (SEM) was calculated for each experimental group.

Microarray analysis of $Ca_v3.1$ and $Ca_v3.2$ gene knockdown. For each sample, 650 ng of total RNA isolated from $Ca_v3.1$-siRNA-treated cells or $Ca_v3.2$-siRNA-treated cells (collected at 24 h post-transfection) were used in a microarray analysis to evaluate the changes in gene expression in comparison with the untreated MCF-7 cells (Agilent Technologies Inc., Santa Clara, CA, USA). The T-type channel siRNAs caused a -55% gene knockdown for $Ca_v3.1$ and a -63% gene knockdown for $Ca_v3.2$, as measured using real-time PCR. To analyze the sets of genes identified as being the most significantly differentially expressed by the knockdown experiments, that is, as estimated by the z-score of the fold change (z-score was under -2 or over +2), Gene Ontology (GO) clustering was performed using GOstat, an analysis program (http://gostat.wehi.edu.au/)

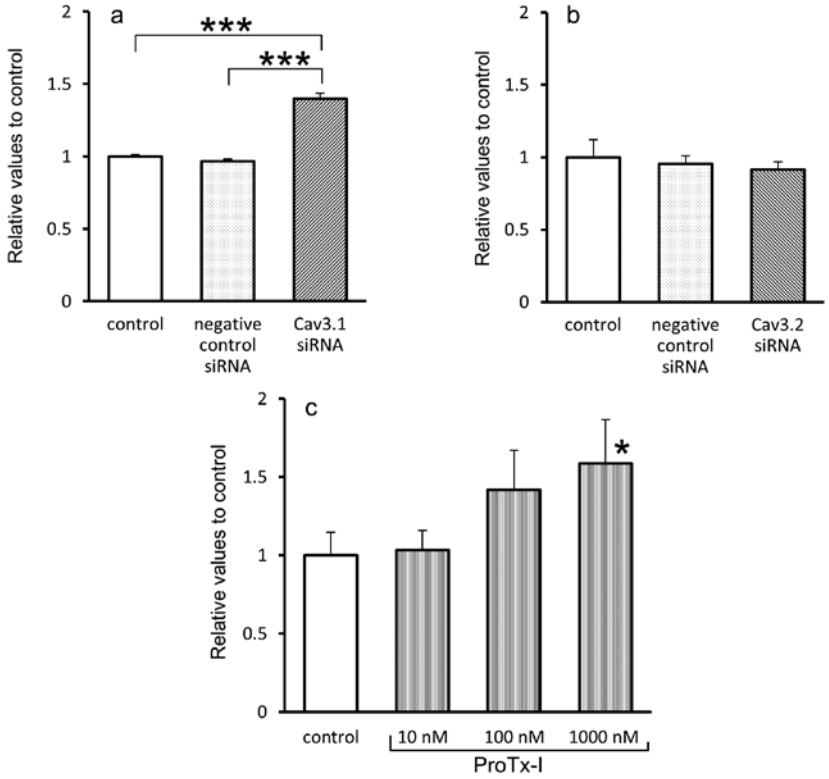

Figure 1. Effects of siRNAs and channel blockers for $Ca_v3.1$ and $Ca_v3.2$ channels on the proliferation of MCF-7 cells. (a) Knockdown of the $Ca_v3.1$ gene. (b) Knockdown of the $Ca_v3.2$ gene. According to the manufacturer's instructions, a 5-nM final concentration of siRNA was used. The control group was treated with only the transfection reagent. Data were statistically analyzed within the groups. (c) ProTx-I treatment: 1, 10 and 1,000 nM final concentrations of the blocker were used. Data were statistically analyzed compared with the control group. The data represent the means ± SEM of seven to eight observations. $^{*}P<0.05$, $^{***}P<0.001$.

(20) with the 'goa_human' database and a P-value cut-off of 0.01 indicating statistically significant differences.

Statistical analysis. Different values among the experimental groups were analyzed using one-way ANOVA and Scheffe's t-test, with a value of $P<0.05$ indicating statistically significant differences. The results are presented as the means ± SEM.

Results

Influence of T-type channels on cell proliferation. As the first step in investigating whether voltage-gated T-type channels are related to tumor suppression or promotion, the influences of the channels on cell growth were examined by modulating the expressions of the $Ca_v3.1$ and $Ca_v3.2$ isoforms using siRNA for gene targeting, or isoform clones for overexpression, in addition to modulating the Ca^{2+} influx using a channel blocker. The gene knockdown experiments using siRNA for the $Ca_v3.1$ channel gene showed that silencing the $Ca_v3.1$ gene significantly enhanced cell proliferation, compared with that in the untreated control (only the transfection reagent) and the negative control (Fig. 1a). As for isoform $Ca_v3.2$, gene knockdown did not result in any change in cell growth (Fig. 1b). Similar to the $Ca_v3.1$ knockdown data, the T-type channel blocker tarantula toxin, ProTx-I (10-1,000 nM), a relatively selective blocker for $Ca_v3.1$ channels, stimulated the cell proliferation dose-dependently and significantly (1,000 nM; $P<0.05$) (Fig. 1c).

As shown by the results of the gene silencing experiments, the overexpression of $Ca_v3.1$ channels caused a significant decrease in cell proliferation, compared with that in the untreated control or empty vector control (Fig. 2a). Similarly, as expected by the ineffective data in the $Ca_v3.2$ knockdown experiment, the $Ca_v3.2$ clone produced no change in cell proliferation (Fig. 2b). To confirm whether the regulation of proliferation mediated by $Ca_v3.1$ is distinctive in cancer cells or not, gene knockdown and channel blocking experiments were performed using MCF-10F cells, a non-tumorigenic human breast epithelial cell line. Using real-time PCR, the mRNA expression of the $Ca_v3.1$ channels was verified in advance (data not shown). In the MCF-10F cells, neither $Ca_v3.1$ gene knockdown using siRNA nor ProTx-I (100-4,000 nM) enhanced the cell proliferation (Fig. 3a and b).

Immunocytochemical expression of T-type channels in MCF-7 cells. When the localization of $Ca_v3.1$ channels in MCF-7 cells was examined, the $Ca_v3.1$ channel protein was clearly located at the membrane of cells exhibiting cell shrinkage, surface blebbing and chromatic agglutination identified by excessive blue-fluorescence from DAPI staining; in the other remaining cells, localization was rarely observed at the cell membrane but was observed in the cytosol (Fig. 4a-c). A correlation between apoptosis and $Ca_v3.1$ localization at the cell membrane was speculated, and additional immunocytochemical double staining using an apoptotic marker supported this possibility. $Ca_v3.1$ channels were identified specifically at the membrane of

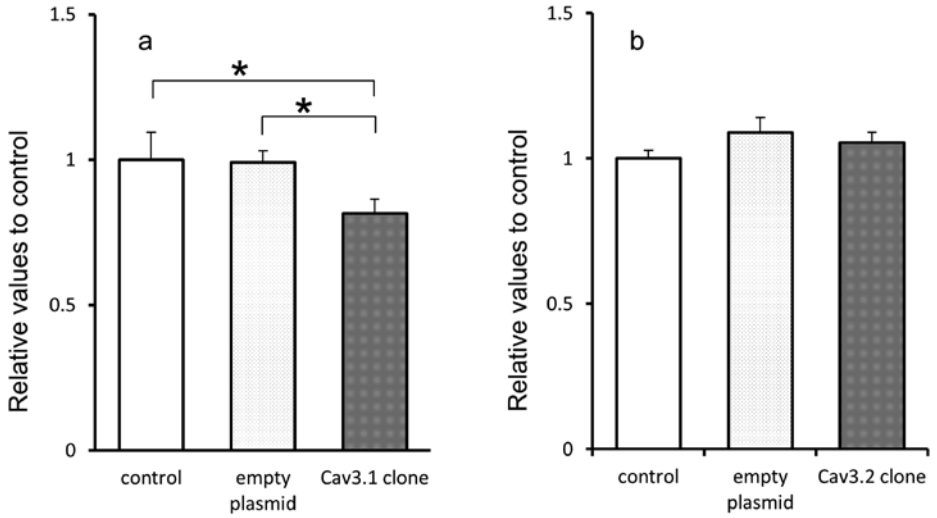

Figure 2. Effect of the overexpression of (a) $Ca_v3.1$ and (b) $Ca_v3.2$ channels on the proliferation of MCF-7 cells. According to the manufacturer's instructions, 0.1 μg of plasmid DNA per each well was used in a 96-well plate. The control group was treated with only the transfection reagent. Data were statistically analyzed within the groups. Data represent the means ± SEM of seven to eight observations. *P<0.05.

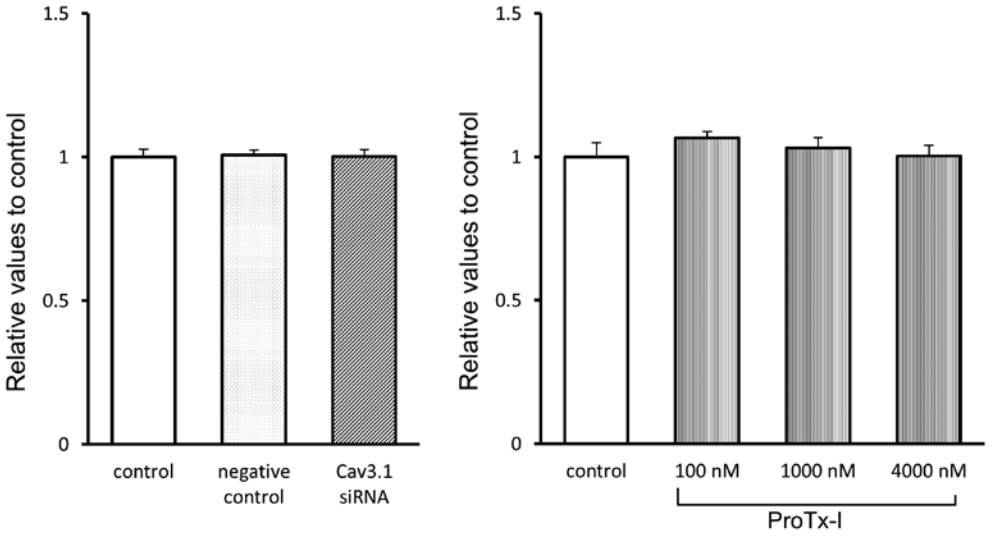

Figure 3. Effects of siRNAs and the channel blocker ProTx-I for $Ca_v3.1$ channels on the proliferation of MCF-10F cells (see Fig. 1 for details). Data represent the means ± SEM of seven to eight observations.

apoptotic/Annexin V-positive cells (Fig. 4c-e). $Ca_v3.1$ channels were also expressed specifically at the membrane of apoptotic/TUNEL-positive cells (Fig. 4f-j). Furthermore, in proportion to the intensity of Annexin V red fluorescence, $Ca_v3.1$ green fluorescence was stronger in the cytosol (Fig. 4c and d, arrow). By contrast, $Ca_v3.2$ channels were abundantly found at the plasma membrane in a large portion of the MCF-7 cells (Fig. 5a and b), with no characteristics of apoptosis, such as cell shrinkage and chromatic agglutination. A few cells were co-localized with the $Ca_v3.1$ protein at the cell membrane (Fig. 5c); however, the expressions of $Ca_v3.1$ and $Ca_v3.2$ differed in the majority of cells (Fig. 5d).

Apoptosis induced by $Ca_v3.1$ channels. Since an association between $Ca_v3.1$ channels and apoptosis was expected from the immunostaining data, we then assessed whether $Ca_v3.1$ channels are involved in the apoptotic process. The change in the number of apoptotic cells was measured using an APOPercentage apoptosis assay kit and MCF-7 cells. Overexpression of the $Ca_v3.1$ clone significantly increased the number of apoptotic cells labelled with red dye by 2.3-fold, compared with that in the empty vector control (Fig. 6a). Treatment with cyclophosphamide (CPA) (1.8 mM), an alkylating anti-cancer drug, known to induce apoptosis, increased the number of apoptotic MCF-7 cells; however, transfection with siRNA for $Ca_v3.1$ 24 h prior to the CPA treatment significantly blocked the CPA-induced apoptosis (Fig. 6b). Immunocytochemical staining revealed that CPA treatment increased the number of $Ca_v3.1$-positive cells, compared with that in the vehicle-treated control cells (Fig. 6c and d).

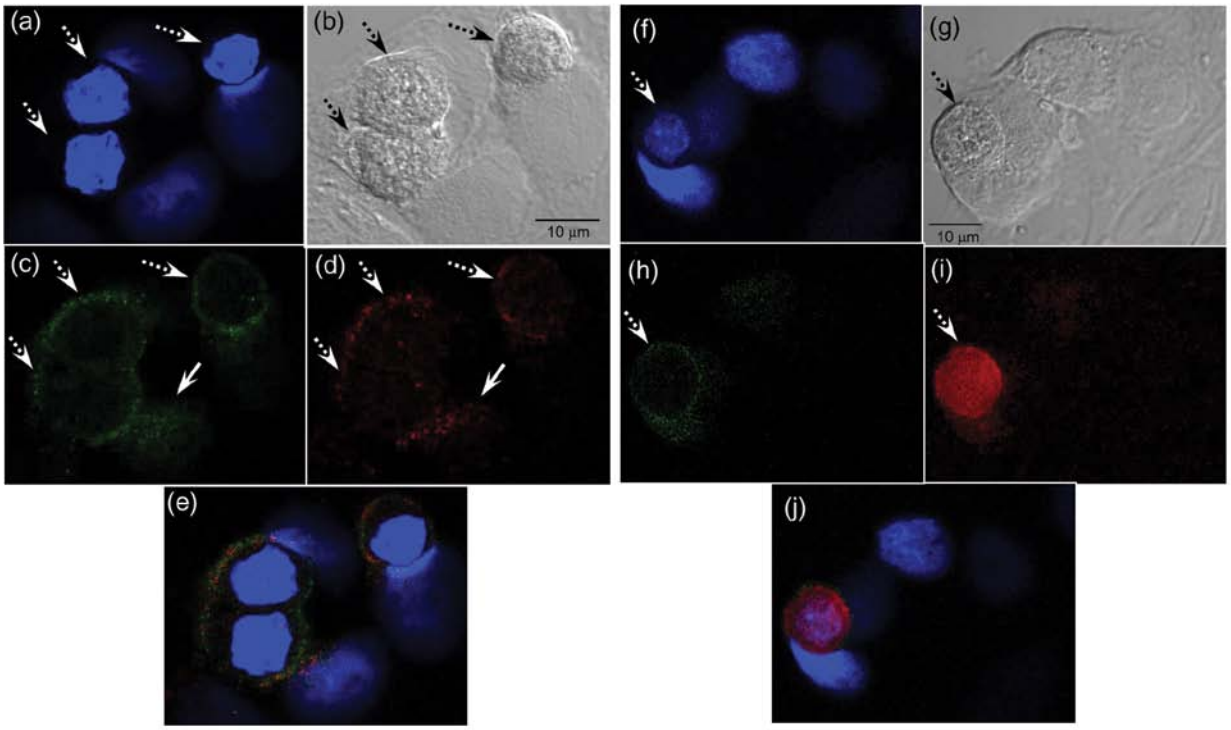

Figure 4. Immunocytochemical studies for evaluating the expression of $Ca_v3.1$ channels and apoptotic markers in MCF-7 cells. Images were acquired using a Plan-Apochromat 63x/1.4 DIC oil immersion objective. (a-e) Triple staining was performed for the nucleus, $Ca_v3.1$ channels, and Annexin V. (f-j) Triple staining was performed for the nucleus, $Ca_v3.1$ channels, and terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay. (a and f) Nucleus staining (blue): DAPI, blue diode laser, 405 nm excitation. (b and g) Phase contrast image. (c) $Ca_v3.1$-channel staining (green): Alexa 488, Argon, 488 nm excitation. (d) Annexin V staining (red): Alexa 568, He/Ne Laser, 563 nm excitation. (e) Merged image of nucleus, $Ca_v3.1$ and Annexin V. (h) $Ca_v3.1$-channel staining (green): the red color excited with the He/Ne Laser at 563 nm was changed to green after acquisition to enable a unified comparison with (c), though it was actually stained with Alexa 568. (i) TUNEL assay (red): the green fluorescence color excited with Argon at 488 nm was changed to red after acquisition to enable a unified comparison with (d), though it was actually labeled with green fluorescein-dUTP. (j) Merged image of nucleus, $Ca_v3.1$ and TUNEL labeling.

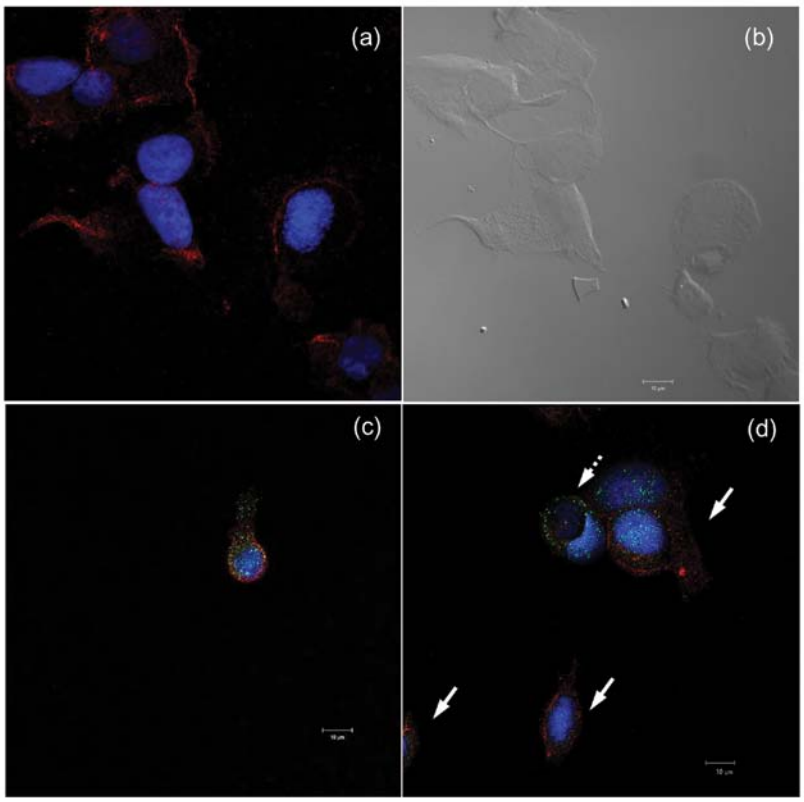

Figure 5. Immunocytochemical analysis for evaluating the expression of $Ca_v3.2$ channels and a comparison with $Ca_v3.1$ expression (see Fig. 4 for details). (a) Merged image of nucleus (DAPI, blue) and $Ca_v3.2$ (Alexa 568, red). The largest area contains $Ca_v3.2$-positive cells. (b) Phase contrast image. (c and d) Merged image of $Ca_v3.1$ (Alexa 488, green) and $Ca_v3.2$ (Alexa 568, red). The arrow in (d) indicates $Ca_v3.2$ expression that is independent of $Ca_v3.1$ expression and the dotted arrow shows the co-expression of $Ca_v3.1$ and $Ca_v3.2$.

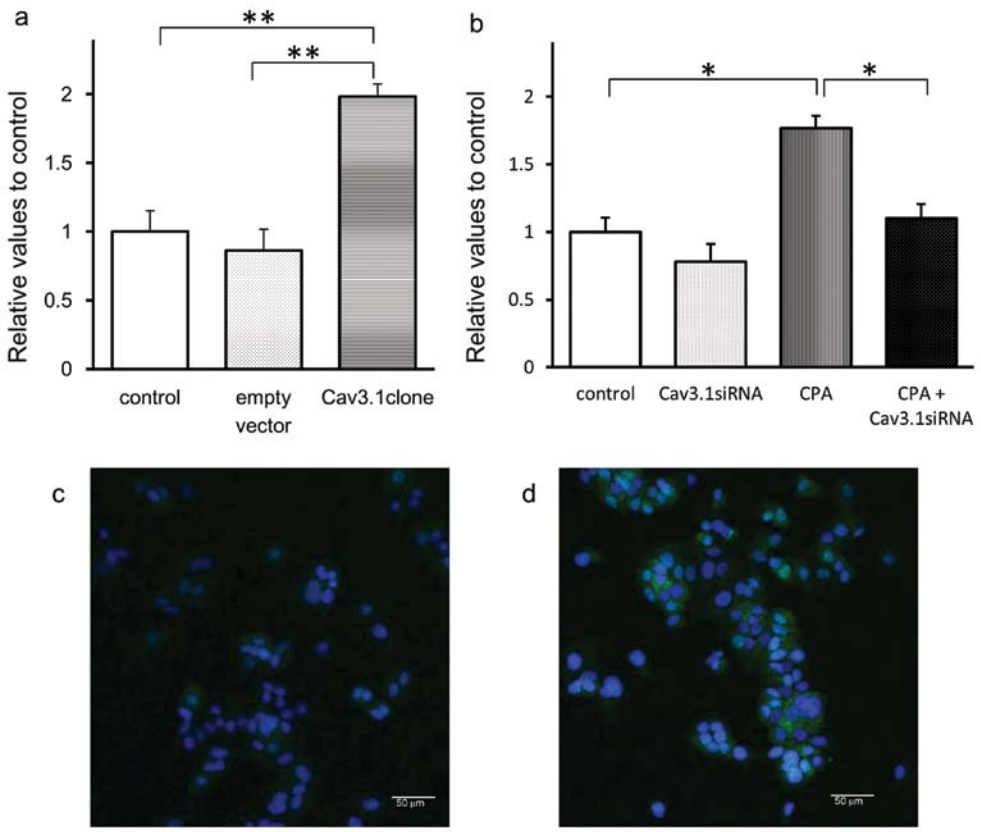

Figure 6. (a) Apoptosis induced by the overexpression of $Ca_v3.1$ channels and (b) the inhibition of cyclophosphamide (CPA)-induced apoptosis by $Ca_v3.1$ siRNA. Data were statistically analyzed within the groups. Data represent the means ± SEM of four wells (average of three fields/well). $^{**}P<0.05$, $^{**}P<0.01$. (c and d) Immunocytochemical staining revealed that CPA treatment increased the number of $Ca_v3.1$-positive cells, compared with the vehicle treatment.

Changes in gene expression by knockdown of T-type channels. A microarray analysis was performed to identify alterations in the gene expression profiles involved in the regulation of proliferation/apoptosis mediated by $Ca_v3.1$. For this purpose, knockdown experiments using siRNA for the $Ca_v3.1$ gene were performed in MCF-7 cells, and the gene expression profiles were compared with those of the $Ca_v3.2$ knockdown experiments. To analyze the microarray data, a list of genes (365 genes) that exhibited significant changes in expression (z-score <-2 or z-score >+2) as a result of the $Ca_v3.1$ knockdown was submitted to the program, GOstat, which identifies statistically over-represented Gene Ontologies. The top function term listed by the analysis with a P-value cut-off of 0.01 was 'protein binding (GO:0005515)', one of the annotations belonging to molecular function (one of three sub-ontologies) in the GO hierarchy, and 75 of the 365 genes were categorized in this GO term. On the contrary, this term was not found in the GOstat analysis data using the same P-value cut-off and the $Ca_v3.2$-microarray data. Among the 75 genes belonging to the term, GO:0005515, we focused on the GO term, GO:0007049 (cell cycle), located in the lower tree in the GO hierarchy for 'protein binding'. Eight genes that were classified as belonging to this term were found. The expressions of three of these genes were decreased: ERCC2 [annotated as GO:0000075 (induction of apoptosis)], and seven in absentia homolog 1 (SIAH1) and growth arrest and DNA damage-inducible protein (GADD34) [annotated as GO:0006915 (apoptosis)]. The expression of STRADB [annotated as GO:0006916 (anti-apoptosis)] was increased. The expressions of MIS12 [GO:0007067 (mitosis)], synaptonemal complex protein 1 (SYCP1) and XRN1 [GO:0007126 (meiosis)], and cyclin D2 (CCND2) [GO:0050679 (positive regulation of epithelial cell proliferation)] were also increased (Table I).

Discussion

As we have already mentioned above, divergent observations have been reported regarding the function of T-type channels in the tumor process. We investigated the differences between two isoforms of T-type channels, $Ca_v3.1$ and $Ca_v3.2$. These two isoforms possess comparable biophysical properties and are often co-expressed in various tissues. The two T-type channels open near the resting membrane potential, inactivate rapidly, exhibit slow deactivation, and have small unitary conductances (4,5,8). However, both isoforms reportedly demonstrate distinct kinetics of Ca^{2+} entry in response to different action potential frequencies and duration (21). Furthermore, while these subtypes show high sequence identity across their transmembrane-spanning regions, the amino and carboxy termini and intracellular loops exhibit a considerable divergence, making these regions possible targets for differential regulation (4,5). These findings suggest that these two isoforms may serve different functional roles in various tissues. At first, our interests were focused on whether T-type channels are involved in the regulation of tumor development as either an inhibitor or a facilitator, and whether there were

Table I. Cell cycle-related genes changed by $Ca_v3.1$ knockdown.

Gene symbol	Description	Z-score for $Ca_v3.1$	Z-score for $Ca_v3.2$
ERCC2	Xeroderma pigmentosum group D (XPD)	-3.4980806	-0.1718786
SIAH1	Seven in absentia homolog 1	-6.0084969	0.3178764
GADD34	Growth arrest and DNA damage-inducible protein	-3.6228875	-0.4750755
STRADB	E20-related kinase adaptor β	3.9179519	1.5596587
MIS12	MIND kinetochore complex component	4.6366151	-0.5762074
SYCP1	Synaptonemal complex protein 1	3.1491646	0.5514925
CCND2	Cyclin D2	3.5136368	-0.046792
XRN1	5'-3' exoribonuclease 1	2.7172767	-0.246792

any differences in the roles of $Ca_v3.1$ and $Ca_v3.2$ in the regulation of tumor formation.

The knockdown of the $Ca_v3.1$ gene with siRNA significantly promoted cell proliferation in MCF-7 tumor cells. The T-type channel blocker, ProTx-I, also resulted in an increase in the number of MCF-7 cells. These results are well-matched with each other. We previously showed that ProTx-I is a more selective blocker for cloned $Ca_v3.1$ channels (IC_{50}, 0.2 μM) than for cloned $Ca_v3.2$ (IC_{50}, 17.8 μM), with the ratio of potency for blocking the effects of each channel being approximately 160-fold (19). The concentration range used in the present study (at 1 μM, significantly potentiated) hardly blocked the $Ca_v3.2$ channels. Although ProTx-I is also known as a potent blocker for voltage-gated Na^+ channels, tetrodotoxin (TTX; 1-1,000 nM) did not have any effects on cell proliferation in MCF-7 cells (data not shown). The existence of a ProTx-I-sensitive and TTX-resistant isoform (sensory neuron-specific Nav1.8) has not been reported in MCF-7 cells. Thus, it is conceivable that the suppressive effect of ProTx-I on cell proliferation is due to the blocking action of $Ca_v3.1$ channels; in other words, Ca^{2+} influx via $Ca_v3.1$ channels may be important for the regulation of cell proliferation.

In the immunocytochemical experiment, the expression of $Ca_v3.1$ channels was specifically condensed on the plasma membrane of apoptotic/Annexin V-positive and /TUNEL-positive cells. These observations revealed that $Ca_v3.1$ channels may also be related to apoptotic events. This assumption was further supported by the results that the overexpression of $Ca_v3.1$ channels increased the number of apoptotic MCF-7 cells, and siRNA for $Ca_v3.1$ opposed the CPA-induced apoptosis. Although the mechanism responsible for the link between the CPA-induced apoptosis and $Ca_v3.1$ expression is unknown, CPA elicited the increase in $Ca_v3.1$-positive cells, suggesting a pathway mediated via $Ca_v3.1$ channels from the activity of the genotoxic drug to apoptosis. A recent study consistent with our results indicated that $Ca_v3.1$ channels are involved in apoptosis in tumor cells (12). They showed that ghrelin, a multifunctional peptide hormone, significantly decreased proliferation and induced apoptosis in PC-3 human prostate carcinoma cells; these processes were prevented by T-type channel antagonists. Consistent with a role in apoptosis, an increase in intracellular free Ca^{2+} levels was observed in the ghrelin-treated cells, and this increase was accompanied by the up-regulated expression of $Ca_v3.1$. Additionally, taking into consideration the epigenetic silencing of the $Ca_v3.1$ gene reported in many tumor tissues (10,11), our results suggest that $Ca_v3.1$ promoted apoptosis but also prevented tumor proliferation, indicating the possibility that $Ca_v3.1$ acts as a tumor suppressor.

As for the $Ca_v3.2$ channels, the results were quite distinct from those for $Ca_v3.1$. The $Ca_v3.2$ gene has been suggested as a candidate oncogenic gene, possibly regulated by hypomethylation (22). Despite the resemblance in electrophysiological characteristics, previous studies have indicated opposing functions for the two isoforms of T-type channels in cancer, with $Ca_v3.1$ acting as a tumor suppressor gene (10) and $Ca_v3.2$ acting as an oncogene and with the two genes regulated by hypermethylation and hypomethylation, respectively. In this study, however, the positive involvement of $Ca_v3.2$ in cell proliferation was not detected using siRNA or the overexpression of the $Ca_v3.2$ clone in MCF-7 cells. Furthermore, immunostaining data presented no positive correlation between $Ca_v3.2$ expression and apoptotic events in MCF-7 cells. The stable overexpression of a $Ca_v3.2$ clone reportedly resulted in an increase in the proliferation of glioma and neuroblastoma cell lines (14). Alternatively, Mariot *et al* showed that $Ca_v3.2$ channels are responsible for Ca^{2+} entry into cells, facilitating the neurite lengthening of LNCaP prostate cancer cells (23). The neurite lengthening of LNCaP is thought to be related to a decrease in malignancy (24); therefore, $Ca_v3.2$ may not promote tumor development. In view of these results, whether the $Ca_v3.2$ channel has tumorigenic or anti-tumorigenic functions remains uncertain, and further study is required.

The gene knockdown experiment for $Ca_v3.1$ and channel blocking by ProTx-I had no effect on MCF-10F non-tumorigenic breast epithelial cells. Ca^{2+} signaling in tumor cells reportedly uses an altered pathway during cell cycling. Whitfield has shown that during carcinogenesis, colon carcinomas (which have lost their tumor-suppressing genes), have a noticeably changed Ca^{2+} signaling mechanism (25). In a previous study, the overexpression of T-type channels, such as the $Ca_v3.1$ and $Ca_v3.2$ isoforms, reportedly did not affect the proliferation rate of HEK-293 cells (26). Thus, the demand for Ca^{2+} ions during proliferation may differ between tumor and non-tumor cells, possibly explaining the difference between MCF-7 and MCF-10F cells. Further study is required to clarify the difference.

In previous studies, T-type channel blockers such as mibefradil and pimozide, which cannot distinguish between $Ca_v3.1$

and $Ca_v3.2$ channels, were used to investigate the regulation of cancer cell proliferation (13-17). Since there is a possibility that these isoforms exhibit different functions in tumors, if both isoforms are inhibited by such drugs to the same extent, the results should be interpreted with caution. Furthermore, we should always consider other cytotoxic actions arising from these drugs. The anti-proliferative and cytotoxic actions of pimozide may be mediated via other complex mechanisms and may not involve T-type channel blockade (17). Mibefradil has also been reported to inhibit cell proliferation through an association with cell swelling and the inhibition of volume-sensitive Cl^- channels (27,28) or several other ion channels (29-31), in addition to a mechanism via the inhibition of T-type channels. Flunarizine, which has been reported to be a T-type antagonist (32), also produces its anti-apoptotic action via the direct inhibition of lipid peroxidation and mitochondrial permeability transition (33). Thus, these findings suggest that the cytotoxic effects of non-specific blockers should be carefully considered in studies reporting inhibitory effects on cell proliferation or apoptosis.

The location, degree and temporal aspects of changes in cytosolic free Ca^{2+} from the resting state (~100 nM) regulate many important pathways in tumorigenesis, such as motility, proliferation, cell cycle and apoptosis (34). As for apoptosis, the accumulation of excessive Ca^{2+} by the mitochondria and the activation of mitochondrial membrane permeabilization are often linked to apoptosis as well as necrosis (35,36). The overexpression of $Ca_v3.1$ channels in MCF-7 tumor cells can increase Ca^{2+} influx into the cell, thereby promoting cellular Ca^{2+} overload, which may initiate apoptotic pathways. However, it is also possible that $Ca_v3.1$ channels may produce appropriate changes in cellular Ca^{2+} for the regulation of gene expression required for the induction of apoptosis. Microarray and GO analysis showed that the expressions of three genes, ERCC2, SIAH1 and GADD34, related to the induction or progression of apoptosis (37-39), were significantly decreased in a $Ca_v3.1$ gene knockdown experiment, while the expression of STRADB, which has been reported to have an anti-apoptotic function (40), was increased. These changes are compatible with their functions, and apoptotic events by $Ca_v3.1$ channels could possibly be explained. The ubiquitin ligase SIAH1, which was decreased by $Ca_v3.1$ knockdown, has been reported to be expressed during apoptosis and tumor suppression during physiological apoptosis occurring in the intestinal epithelium (38). In addition, the stable transfection of SIAH-1 in MCF-7 cells blocked cell growth by altering the mitotic process and inducing the expression of $p21^{Waf\text{-}1/Cip\text{-}1}$ (blocks cell-cycle progression) (41). Furthermore, in our study, $Ca_v3.1$ knockdown resulted in increases in the expressions of MIS12, SYCP1 and CCND2 (which are thought to be involved in cell cycle progression) (42-44). Consequently, $Ca_v3.1$ may also suppress the cell cycle, thus inhibiting proliferation. Further study is required to clarify the complex correlation between $Ca_v3.1$ channels and these events. However, these alterations in gene expression are at least due to $Ca_v3.1$-specific changes in Ca^{2+} influx, since $Ca_v3.2$ knockdown did not cause any changes in the expressions of these genes related to apoptosis and the cell cycle.

If only one set of Ca^{2+} responses, such as apoptosis, can be modified without changing the others, such activity could be a positive strategy for promoting cell death in tumors. $Ca_v3.1$ may act as a growth regulator and may be a useful target for controlling cancer, if, for example, the expression of $Ca_v3.1$ channels could be induced. Anti-cancer agents or drugs/substances with the ability to activate the expression of $Ca_v3.1$ channels, such as ghrelin (12), may already exist, and a pharmacological search for such reagents may be a useful future direction for anti-cancer strategies.

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