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Cytoprotective Roles of Senescence Marker Protein 30 against Intracellular Calcium Elevation and Oxidative Stress

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Senescence marker protein 30 (SMP30) is identified as an important aging marker molecule and known to play multifunctional roles as an intracellular calcium regulatory protein in the signaling process. To elucidate the functional significance of SMP30, we established the stably transfected P19 cell line with SMP30 expression vector. Overexpression of SMP30 slightly suppressed the proliferation of P19 cells. However, SMP30 overexpression was cytoprotective against calcium-mediated stress such as calcium ionophore (A23187), and thapsigargin. We found that SMP30 overexpression reduced the elevated intracellular calcium levels induced by A23187, but not by thapsigargin. In addition, SMP30 transfected P19 cells were more protective to *tert*-butylhydroperoxide induced cytotoxicity, indicating the antioxidative properties of SMP30. Taken together, our results suggest that external calcium regulation and antioxidant properties are involved in the cytoprotective mechanism of SMP30.

Key words: Aging, Calcium homeostasis, Endoplasmic reticulum, Oxidative stress, Plasma membrane, Senescence marker protein 30

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl-tetrazolium bromide; PM, the plasma membrane; SER, the sarco/endoplasmic reticulum; PMCA, plasma membrane calcium ATPase; SMP30, Senescence marker protein 30; tBHP, *tert*-butylhydroperoxide; Tg, thapsigargin

INTRODUCTION

Calcium is one of the most important intracellular messengers involved in many biological processes. However, overflow of external calcium induced by biological stimulation can lead to cell death. In addition to the excessive calcium influx from the outside of cells, calcium release from intracellular stores such as endoplasmic reticulum (ER) and mitochondria can elevate intracellular calcium load up to the level that exceed the cellular calcium-regulatory capacity (James and Murphy, 2002, Takadera *et al*., 2006). Thapsigargin, the sarco/endoplasmic reticular Ca^{2+}-ATPase (SERCA) pump inhibitor, is known to induce apoptosis in many cell types because it suppresses calcium ion re-uptake from cytosol to ER leading to elevated intracellular calcium concentration (Nguyen *et al.*, 2002, Yoshida *et al*., 2006).

Oxidative stress causes cellular damages, including DNA, lipid membranes and proteins (Yin *et al.*, 1999). A growing number of studies have described that many different reactive species are major causes of endogenous and exogenous cellular damages (Rahman *et al.*, 2006, Yin *et al.*, 1999, Zhu *et al.*, 2004). *tert*-Butylhydroperoxide (tBHP), an analog of lipid hydroperoxides can generate peroxy radicals and induce apoptosis (Zhu *et al.*, 2004). Therefore, regulation of intracellular calcium homeostasis and redox balance plays important roles in protecting cells against calcium and oxidative stress-mediated cytotoxicity.

Senescence marker protein 30 (SMP30) has been known as an aging marker because its expression level decreases with aging (Fujita *et al.*, 1996). SMP30 is later recognized as a novel class of Ca^{2+} binding protein and named regucalcin (Fujita, 1999). However, it has been lately reported that purified SMP30 has no Ca^{2+} binding activity, indicating that SMP30 is not a Ca^{2+} binding protein at all (Kondo *et al.*, 2004). Nonetheless, evidence has

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suggested that SMP30 might participate in Ca^{2+} efflux by activating the calmodulin-dependent Ca^{2+} pump in the plasma membrane (PM) (Fujita *et al.*, 1998). In this report, Ca^{2+} efflux across the PM was enhanced in HepG2 cells overexpressing SMP30, indicating that SMP30 might be involved in the activity of the PM Ca^{2+} ATPase (PMCA). Another known function of SMP30 is an antioxidative property. There are many evidences providing the antioxidative property of SMP30 in kidney, liver, and brain (Jung *et al.*, 2004, Son *et al.*, 2006). SMP30 also participates in controlling the antioxidant enzyme activity and suppresses oxidative stress in liver (Ishigami *et al.*, 2001).

In the present study, we demonstrated that SMP30 has the cytoprotective roles of SMP30 against Ca^{2+}-related stress and oxidative stress by using the stably transfected P19 cell lines with vector or SMP30.

MATERIALS AND METHODS

Cell culture and establishment of stably transfected cell lines

A multipotent mouse embryonic carcinoma, P19 cells (purchased from ATCC, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM) with supplements 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin (all components from Sigma, U.S.A.), 10% fetal bovine serum (Gibco, U.S.A.), and adjusted pH 7.4 by sodium bicarbonate (Sigma, U.S.A.). To generate the stably transfected cell lines, the cells were transfected with pcDNA3/SMP30, or as a control with pcDNA3 empty vector by using Lipofectamine 2000 (Invitrogen, U.S.A.). Human SMP30 cDNA (pHSMP6) and empty vector (pcDNA3) were kindly provided by Dr. Akihito Ishigami at Tokyo Metropolitan Institute of Aging (TMIG), Japan. Transfected cell lines were selected and maintained at the medium containing G418 antibiotics at the concentration of 400 μg/mL for selection, and 100 μg/mL for maintenance (Sigma, U.S.A.).

Western blot analysis

Cells transfected with empty vector (VT) or SMP30 (ST) were cultured and scraped into a lysis buffer. Cell lysates were sonicated for 10 sec to reduce sample viscosity. After removing unbroken cells by centrifugation at 10,000 *g* for 15 min, the supernatant was used for the following experiments. The expression of SMP30 in the established cells was analyzed by Western blot analysis using a rabbit anti-rat SMP30 antibody and horseradish peroxidase conjugated anti-rabbit antibody (Rabbit anti-rat SMP30 antibody was kindly provided by Dr. Akihito Ishigami at TMIG). Horseradish peroxidase conjugated secondary antibody was detected by ECL Western blot detection reagents (Amersham Biosciences, U.S.A.).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from VT or ST cells using the RNase kit from Gibco and was quantified spectrophotometrically. One microgram RNA was subjected to reverse transcription to make cDNA. To carry out the PCR, cDNA amplification was performed in a PCR master mix containing 1×PCR buffer (Perkin Elmer, U.S.A.), 0.2 mM dNTP, 0.25 U of Taq polymerase (Perkin Elmer, U.S.A.), and 50 ng of sense and anti-sense primers. For SMP30, sense was 5'-AGT GTC CAA CTC TCT GCT CT-3' and anti-sense was 5'-GTT GGA GAT CTG TCC TGT CT-3'. Reaction conditions were consisted of 94°C for 30 sec denaturation, 54°C for 30 sec annealing, and 72°C for 1 min extension. Electrophoresis was performed in 1% agarose gel. After staining in ethidium bromide solution, the band intensity of the gel was observed by UV transilluminator. GAPDH primers were used as control for the efficiency of cDNA synthesis in each sample. For GAPDH, sense was 5'-GGG TGA TGC TGG TGC TGA GTA TGT-3', and anti-sense was 5'-AAG AAT GGG AGT TGC TGT TGA AGTC-3'.

Cell proliferation assay

Cells (5×10^3/well) were seeded in 96-well culture plates and maintained in DMEM supplemented 10% FBS. After the indicated time, cell proliferation was measured by the treatment of 100 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, U.S.A.) at 500 μg/mL to each well and incubated for 4 h at 37°C. One hundred microliters of solubilization solution (ethyl alcohol to dimethyl sulfoxide = 1 to 1 ratio) was replaced to each well and read the absorbance at 560 nm and 650 nm by an ELISA reader, respectively. The OD was represented as the difference between two wavelengths. All samples were assayed in triplicate and each experiment was repeated at least three times.

Cell viability assay

Cells were seeded at approximately 2×10^4 cells in 96-well plate and incubated for the cell attachment. Medium was changed to serum-free DMEM for 2 h until cells were stabilized. Cells were then treated with 500 nM of A23187, calcium ionophore, or 1 μL of Tg, SER-associated membrane calcium ATPase inhibitor for 24 h. Cell viability was assessed by MTT assay. After treatment, the cells were incubated with MTT solution for 4 h at 37°C and the medium was replaced with 100 μL of DMSO solubilization solution. The amount of solubilized MTT formazan was measured by the absorbance at 560 nm using a spectrophotometer. All samples were assayed in triplicate and each experiment was repeated at least three times.

Measurement of intracellular calcium concentration

Ca^{2+}-containing medium (pH 7.4) had: NaCl 140 mM; KCl 5 mM; $MgCl_2$ 1 mM; $CaCl_2$ 2 mM; Hepes 10 mM; glucose 5 mM. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that $CaCl_2$ was substituted with 0.1 mM EGTA. Agents were dissolved in water, ethanol or dimethyl sulfoxide as stock solutions. Fura-2 (1 μM) was loaded into cells for 40 min at 37°C. The cells were washed and re-suspended in Ca^{2+}-containing medium (in Ca^{2+}-free medium for measuring source of calcium). Fura-2 fluorescence measurements were performed in a water–jacketed cuvette with continuous stirring; the cuvette contained 1 ml of medium and 1 million cells. Calcium influx to cytosol was induced by adding 500 nM A23187 or 1 μM Tg. Fluorescence was monitored with Hitachi F4500 fluorophotometer (Japan) by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1 sec intervals.

Statistical analysis

The statistical significance of the differences between vector and SMP30 overexpressed P19 cells were determined by ANOVA with PLSD. Values of $p<0.05$ were considered statistically significant. Analyses were performed using Statview software®.

RESULTS

SMP30 was stably transfected in P19 cells

To examine the effects of SMP30 overexpression in cells, we established the stable cell lines transfected with pcDNA3 vector (VT) or pcDNA3-SMP30 (ST) constructs. Transfected cell lines were selected and maintained at the medium containing G418 antibiotics. One clone from VT, and 2 clones from ST were chosen and used for the following experiments. We confirmed that expressions of SMP30 proteins and RNA transcripts were significantly elevated in ST cells, compared to VT cells (Fig. 1).

Proliferation of P19 cells was suppressed by overexpressing SMP30

It is important to evaluate whether SMP30 overexpression affects normal physiological changes such as cell proliferation prior to cell viability experiments. To verify the effect of SMP30 on the cell proliferation, P19 cell proliferation was measured by MTT assay at 0, 12, 24, 48 h time points (Fig. 2). Growth rate of ST cells was significantly decreased at 24 h and 48 h time points, compared to VT cells.

SMP30 protects cells from Ca^{2+}-mediated stress

SMP30 participates in regulation of $[Ca^{2+}]_i$ (Fujita *et al.*, 1998), suggesting protective role of SMP30 against Ca^{2+}-related stress. To clarify the cytoprotective actions of SMP30 against Ca^{2+} overloading induced by Ca^{2+} ionophore (A23187) and thapsigargin (Tg), a highly specific inhibitor of ER-associated calcium ATPase pump, cytoprotective studies were carried out. SMP30 overexpression efficiently prevented cell death at the concentration of 500 nM of A23187 in ST cells (Fig. 3A), compared to VT cells showing approximately 35% cell death at the same doses. Tg also

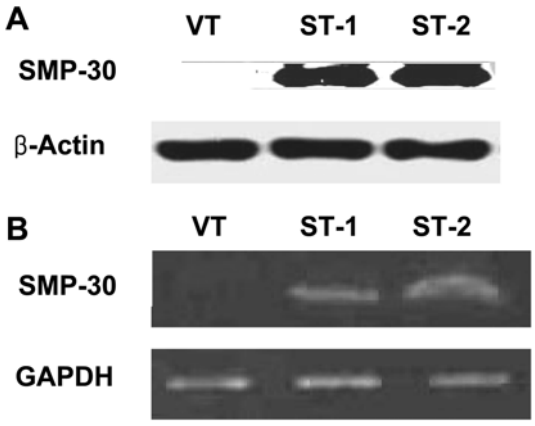

Fig. 1. Expression of SMP30 in P19 cells transfected with empty vector and SMP30. (A) Expression of SMP30 protein. Total proteins were isolated from stable cell lines transfected with pcDNA3 (VT) or pcDNA3-SMP30 (ST) constructs. Western blot was performed using a rabbit anti-SMP30. (B) Expression of SMP30 mRNA. Total RNA was extracted from stable cell lines and analyzed by RT-PCR using the primer described in the method. Lane 1: P19 cells transfected with pcDNA3-vector (VT), Lane 2: P19 cells transfected with pcDNA3-SMP30 clone 1 (ST1), and Lane 3: P19 cells transfected with pcDNA3-SMP30 clone2 (ST2).

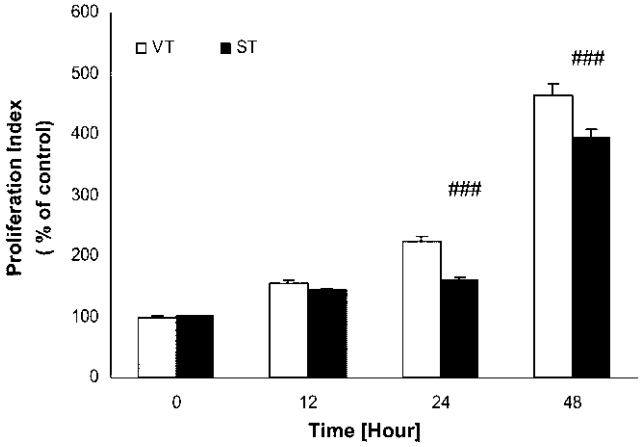

Fig. 2. Effect of SMP30 on the proliferation of P19 cells. The proliferation of VT and ST cells was determined by the MTT assay. Proliferation of ST cells was suppressed, compared to VT cells. Data are expressed as the mean ± SE (n = 6). ### $p<0.001$ compared to VT. VT: P19 cells transfected with empty vector, ST cells: P19 cells transfected with SMP30.

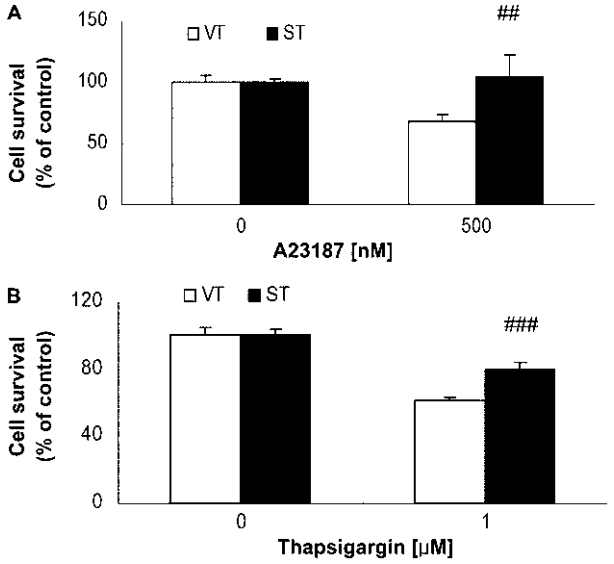

Fig. 3. SMP30 enhances the cell viability against A23187 or thapsigargin-induced calcium stress. VT and ST cells were cultured in 96-well plates and treated 500nM A23187 (A) or 1 μM Tg (B) to measure cell viability. A significant difference in cell viability was observed between VT and ST cells in response to calcium stress. Each value is the mean ± SE (n = 6). Results of one factor ANOVA: ## p<0.01, ###p< 0.001 compared to VT cells. VT: P19 cells transfected with empty vector, ST cells: P19 cells transfected with SMP30.

showed the similar pattern of cell survival in a dose dependent manner (Fig. 3B).

Increased $[Ca^{2+}]_i$ induced by external calcium stress was modulated by SMP30

Furthermore, we investigated whether there is the direct link between Ca^{2+} regulatory role of SMP30 and cell protection. Cytoprotective effects of SMP30 were shown in cell viability experiments using VT and ST cells treated with A23187 or Tg, $[Ca^{2+}]_i$ in the presence or absence of Ca^{2+} in media. Addition of A23187 to the cells significantly elevated $[Ca^{2+}]_i$ in P19 cells, and A23187-mediated $[Ca^{2+}]_i$ levels were significantly modulated by SMP30 overexpression (Fig. 4A). However, SMP30 overexpression couldn't affect the Ca^{2+} responses mediated by Tg in Ca^{2+} free media (Fig. 4B).

Oxidative stress-induced cell death was attenuated by SMP30 overexpression

Many reports indicate that SMP30 expression is associated with oxidative stress (Jung *et al.*, 2004, Son *et al.*, 2006). To examine the antioxidative effect of SMP30, we carried out the following experiment. Cells were exposed to tBHP for the oxidative stress and the cell

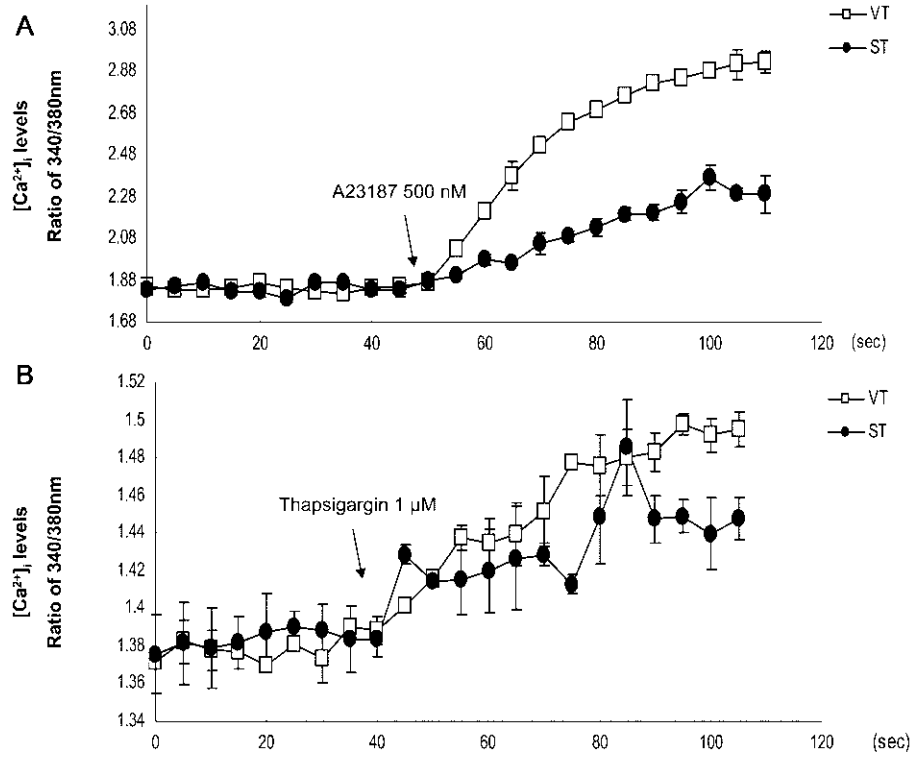

Fig. 4. SMP30 stabilizes intracellular calcium in response to calcium stress. Cells were loaded with 1 μM Fura-2/AM for 40 min at 37°C, and then added 500 nM A23187 in Ca^{2+} media or 1 μM thapsigargin in Ca^{2+}-free media. (A) Representative recording showed levels of $[Ca^{2+}]_i$ before and after addition of 500 nM A23187 (arrow indicates time of addition) to VT and ST cells. (B) Representative recording showed levels of $[Ca^{2+}]_i$ prior to and after exposure of VT and ST cells to 1 μM thapsigargin (arrow indicates time of addition). Values are mean ± SE from three independent experiments. ### p<0.001 compared to VT (ANOVA with PLSD test).

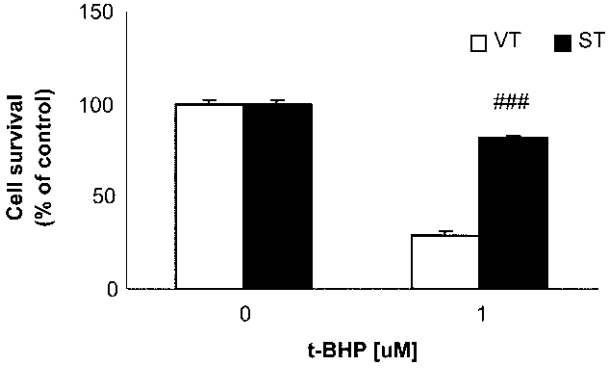

Fig. 5. Cytoprotective effect of SMP30 against tBHP-induced oxidative stress. Cells were exposed to 1 μM of tBHP for 24 h, and cell viability was measured by MTT assay. Results are expressed as percentage of the control (without tBHP), and are mean ± SE (n = 6). Results of one factor ANOVA: ###p<0.001 compared to VT cells. VT: P19 cells transfected with empty vector, ST cells: P19 cells transfected with SMP30.

viability was measured by MTT assay. Cell viability was decreased in VT cells as treatment of 1 μM tBHP for 24 h. However, ST cells exposed to tBHP stress showed the protective effect on cell death (Fig. 5).

DISCUSSION

SMP30, also known as a regucalcin, is a calcium regulatory protein but dose not contain EF-hand motifs, which is a typical character of calcium binding proteins (Fujita *et al.*, 1996). Therefore, SMP30 is regarded as a novel calcium binding protein. However, there are opposite reports that SMP30 is not a calcium binding protein (Kondo *et al.*, 2004), but is still believed to contribute the regulation of $[Ca^{2+}]_i$. In present study, P19 cells stably overexpressing SMP30 were established to examine the functional roles of SMP30. Regulation of $[Ca^{2+}]_i$ and cell protective effect of SMP30 against Ca^{2+} stress by A23187 and Tg were determined. SMP30 showed suppressive effects on the proliferation in ST cells, consistent with previously reported study with rat hepatoma H4-II-E cells (Yamaguchi and Daimon, 2005). In fact, SMP30 enhances p21 mRNA expression and suppresses IGF-1 mRNA expression in the hepatoma cells (Yamaguchi and Daimon, 2005). Moreover, the suppressive effect of SMP30 on proliferation was also confirmed by using neural progenitor cells (NPCs) overexpressing SMP30 (Data not shown). These results suggest that SMP30 may be associated with cell cycling in suppressing cell proliferation.

SMP30 has a cytoprotective effect in both A23187 and Tg treated cells. It is not clear to explain the phenomenon, but it is presumed due to antioxidative property of SMP30 (Feng *et al.*, 2004, Son *et al.*, 2006). Oxidative stress affects intracellular redox regulators, cell survival, and cell proliferation (Rahman *et al.*, 2006). For instance, oxidative stress can increase p21 expression as cell proliferation is decreased (O'Reilly *et al.*, 2001). In addition, overexpression of p21 increases cellular susceptibility to oxidative stress (Yin *et al.*, 1999).

The cytoprotective effects of SMP30 could be suggested its functional roles of calcium regulator or antioxidative property (Feng *et al.*, 2004, Fujita *et al.*, 1998, Son *et al.*, 2006). For instance, as extracellular ATP stimulates to increase transient $[Ca^{2+}]_i$, Ca^{2+} efflux is significantly increased in SMP30 transfectants compared with vehicle transfectants. Our results showed that overexpression of SMP30 protected cells against $[Ca^{2+}]_i$ overloading derived from external Ca^{2+} influx and internal Ca^{2+} pool. Furthermore, A23187 elevates $[Ca^{2+}]_i$ by increasing the permeability of the cell membrane (Abramov and Duchen, 2003). In contrast, Tg acts as an ER specific Ca^{2+} ATPase inhibitor, which is widely used to deplete Ca^{2+} stores in ER and thus to $[Ca^{2+}]_i$ (Yoshida *et al.*, 2006). Therefore, cell death induced by A23187 and Tg is mediated by elevation of $[Ca^{2+}]_i$. In the present study, when treated with A23187, the sustained elevation of cytosolic calcium was prevented by SMP30 overexpression. However, Tg induced increased $[Ca^{2+}]_i$ was not affected by SMP30 overexpression. These results support that Ca^{2+} efflux across the PM might be enhanced only in SMP30 transfected cells (Fujita *et al.*, 1998). It is also plausible that SMP30 enhance SERCA pump activity, causing sequestration of calcium into internal calcium store. However, SMP30 might not be involved in SERCA regulation in this study since Tg-induced $[Ca^{2+}]_i$ was not modulated in SMP30-overexpressed ST cells. Thus, the effects of SMP30 on SERCA remain to be further studied. At present, it was not known yet why SMP30 reduces Tg-induced cell death, we presumed that multi-functional roles of SMP30 such as anti-oxidative property might be involved in cell protection (Fukaya and Yamaguchi, 2004, Ichikawa and Yamaguchi, 2004, Son *et al.*, 2006). Overexpression of SMP30 enhanced cell viability in response to oxidative insults, tBHP (Fig. 5). Many signals other than increased $[Ca^{2+}]_i$ have been implicated in the mechanisms of cell death, including reactive oxygen species (Rosenstock *et al.*, 2004), the production of ceramide and the activation of adenylate cyclase or various cytoplasmic enzymes (Gibson and Huang, 2004). Therefore, Tg-evoked Ca^{2+} independent cytotoxicity suggests that other signal transduction pathways might be involved in the cytoprotective actions of SMP30.

In conclusion, our results demonstrated that suppression of cell proliferation is a phenotypic phenomenon when SMP30 is overexpressed but SMP30 has a protective role against cell death, particularly induced by Ca^{2+}-mediated stress. The cytoprotective effect of SMP30 on external

Ca^{2+} stress might be regulated by $[Ca^{2+}]_i$ via PMCA pump. Moreover, antioxidative property of SMP30 protects cells against Tg-mediated internal calcium stress and oxidative stress.

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