CLDN6-induced apoptosis via regulating ASK1-p38/JNK signaling in breast cancer MCF-7 cells

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Abstract. Claudin 6 (CLDN6), a member of tight junction protein claudin (CLDN) family, inhibits proliferation and induces apoptosis of MCF-7 breast cancer cells. However, these molecular mechanisms of CLDN6-induced apoptosis remain largely elusive. We previously found that restoration of human CLDN6 gene expression was correlated with the expression level of apoptosis signal-regulating kinase 1 (ASK1) using cDNA array and bioinformatics analysis. ASK1, a mitogen-activated protein kinase kinase kinase, is involved in environmental stress-activation of the c-jun N-terminal kinase (JNK) and p38 pathways, which contribute to apoptosis-associated tumor cell death. In the present study, we show that the restoration of CLDN6 gene expression in MCF-7 cells marhedly decreased ASK1 phosphorylation at Ser967. Activated ASK1^{ser967} further induced the activation of downstream targets, JNK and p38 kinase. MCF-7/CLDN6 stable transfection cell clone treated with TRX1, an ASK1 inhibitor, showed suppressed JNK and p38 activation, and showed substantially increased survival and colony formation and reduced percent of apoptotic cells using TUNEL staining and DNA ladder. Furthermore, TRX1 treatment increased Bcl-2/Bax ratio and reduced caspase-3 cleavage in MCF-7/CLDN6 stable transfection cell clone. Therefore, these data show that CLDN6 mediates ASK1-p38/JNK apoptotic signaling in MCF-7 cells, and it is correlated with constitutive deregulation of the balance of Bcl-2 family proteins and activation of caspase-3.

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Introduction

Claudins (CLDNs) are scaffolding proteins of tight junction strands in epithelium and endothelium cells (1). Claudin 6 (CLDN6) is one of the 27 CLDN family members (2). Numerous studies have suggested that abnormal expression of CLDN6 could benefit the occurrence and development of tumor (3). We cloned and identified CLDN6 gene in Copenhagen rat mammary epithelial cells, which are almost completely resistant to mammary cancer, indicating that CLDN6 may be a breast cancer suppressor gene, we found that CLDN6 inhibits proliferation and induces apoptosis in MCF-7 breast cancer cells through p38 signaling (4-6). However, the precise molecular mechanisms by which CLDN6 promotes apoptosis remain largely elusive. We also previously found that CLDN6 expression levels were correlated with apoptosis signal-regulating kinase 1 (ASK1) expression levels using cDNA array and bioinformatics analysis (unpublished data). Another study showed that CLDN6 overexpression led to increased expression of ASK1 protein in breast cancer tissues (7). Together these data suggest a potential link between CLDN6 and ASK1 signaling.

ASK1 is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family that induces cells apoptosis, including in breast cancer cells, in response to various stresses (8). The phosphorylation state of serine/threonine kinase (serine/threonine kinase) as an essential component of the MAPK pathway plays an important role in the induction of cellular signaling. A previous study showed that phosphorylation of the Thr-838 site of ASK1 leads to ASK1 activation, while ASK1 is inactivated by Ser83, Ser1034 and Ser967 phosphorylation (9). Interactions between ASK1 and various binding proteins also regulate ASK1 active and inactive status. These mechanisms may play a major part in regulating the activation of ASK1 in cancer cells (10). ASK1 induces apoptosis via activation of downstream MAPKs, c-Jun N-terminal kinases (JNKs) and p38 MAPKs (11). The mechanisms of MAPK-induced apoptosis via Bcl-2 family proteins, and caspase family proteins in cancer cells have been documented (12-16).

Accumulating evidence suggests that CLDNs induce tumor apoptosis through activating MAPK signal pathway, such as CLDN6 induces tumor apoptosis through activating the ERK1/2 signaling pathway (17), and CLDN7 promotes apoptosis through the activation of p38 protein kinase in MKN28 gastric epithelial cells (18). The present study dissects the molecular mechanisms by which CLDN6 induced apoptosis in MCF-7 breast cancer cells. We hypothesize that CLDN6 induces MCF-7 cells apoptosis by early activation of ASK1 followed by the subsequent activation of p38/JNK pro-apoptotic signaling. We examined the potential relationship between CLDN6 and ASK1 signaling, and specifically through p-p38 and p-JNK downstream targets.

Materials and methods

Antibodies and chemicals. Antibodies agaist CLDN6, Bcl-2, Bax and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against p-ASK1^{ser976}, ASK1, p-JNK and JNK were from Bioworld (Dublin, OH, USA). Anti-human p-p38 and p38 antibodies were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibody against RIP1, caspase-3 and cleaved caspase-3 were purchased from BD Biosciences (San Diego, CA, USA). TRX1 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cells and culture conditions. MCF-7/pcDNA3.1(+) (MCF-7/vector) or MCF-7/pcDNA3.1(+)-CLDN6 (MCF-7/CLDN6) stable transfection cell clones were established and obtained as previously described (19). All cells were cultured in H-DMEM medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT USA) and 100 U/ml penicillin and 100 U/ml streptomycin in a 5% CO_2 humidified incubator at 37˚C. G418 (Sigma-Aldrich) was added to the medium of transfected cells at a concentration of 400 μg/ml.

TRX1 treatment. MCF-7/CLDN6 stably transfected cells were incubated with defined concentrations of Thioredoxin-1 (TRX1; Sigma-Aldrich) (a physiological inhibitor of ASK1) 0, 1, 10 and 100 ng/ml for 24 h and with 10 ng/ml of TRX1 for 0, 24, 48 and 72 h. p-ASK1^{ser967} and ASK1 expression levels were subsequently measured using western blot analysis (described below) to determine the optimal treatment concentration and time. Then these cells with TRX1 (10 ng/ml) treatment for 48 h were used to a follow-up study.

Western blot analysis. Cells were lysed with RIPA and PMSF (Roche Applied Science) buffer, and protein concentrations were quantitated based on the BCA (Protein assay kit; Beyotime Institute of Biotechnology, Haimen, China) method according to the manufacturer's protocol. Equal amounts of cell lysates were resolved on SDS-PAGE by electrophoresis, and proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA) and membranes were blocked with PBST buffered saline containing 5% (w/v) non-fat dried milk (20). The membranes were blotted with the appropriate primary antibodies and dilutions (Table I), followed by an HRP-conjugated secondary antibody. The detection of immunoreactive bands

Table I. Primary antibodies used by western blot analysis.

Antibody	Company	Dilution	Species
CLND6	Santa Cruz Biotechnology	1:1000	Goat
β-actin	Santa Cruz, Biotechnology	1:1000	Mouse
p-ASK1	Bioworld	1:500	Rabbit
ASK1	Bioworld	1:1000	Rabbit
p-JNK	Abcam	1:500	Rabbit
JNK	Abcam	1:500	Rabbit
p-p38	Abcam	1:500	Rabbit
p38	Abcam	1:500	Rabbit

was visualized by chemiluminiscence using ECL-plus reagent (Beyotime Institute of Biotechnology) via the western blot system (GeneSnap, Frederick, MD, USA).

Cell death assay. MCF-7/CLDN6 stably transfected cell death treated with TRX1 was measured by trypan blue (Beijing Donglinchangsheng Biotechnology, Co., Ltd., Beijing, China) exclusion assay (21). The cell relative viability percentage was recorded using the formula below: Survival ratio (%) = (number of viable cells/number of total cells) x 100%.

Colony formation experiment. MCF-7/CLDN6 stably transfected cells in the logarithmic growth phase were seeded in 6-well plate with the cell density of $3x10^2$ cells in each dish, and treated with TRX1. The visible clones appeared after 3 weeks, and were fixed with methanol for 20 min, stained with Giemsa solution for 5 min. Cell clones with diameter >0.5 mm were counted under an optical microscope and the cloned formation efficiency was calculated with the following formula: The cloning efficiency (CE) (%) = (number of clones formed/number of cells inoculated) x 100% (22).

Analysis of mitochondrial transmembrane potential using rhodamine 123 staining. The disruption of MCF-7/CLDN6 stably transfected cell mitochondrial membrane potential ΔΨm was analyzed by staining with rhodamine (Rho) 123 (Beijing Donglinchangsheng Biotechnology) according to manufacturer's protocol. Loss of ΔΨm resulting in strong yellow-green fluorescence was observed by fluorescence microscopy (Olympus, Tokyo, Japan) and measured by Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). Results are given in percentage of strong fluorescence cells with low ΔΨm compared to untreated control.

Evaluation of apoptotic cells using TUNEL staining. MCF-7/CLDN6 stably transfected cells were grown with TRX1 for 24 h and fixed in 1% paraformaldehyde in PBS, permeabilized in 70% ethanol. TUNEL staining procedure was carried out according to In Situ Cell Death Detection kit (Roche Products, Ltd., Basel, Switzerland), as per the manufacturer's protocol. The stained cells were analyzed and mounted under light microscope. The eosinophils count was done at x40 magnification and the apoptosis index was calculated by dividing the sum of eosinophils in apoptosis by the global sum of counted eosinophils (23).
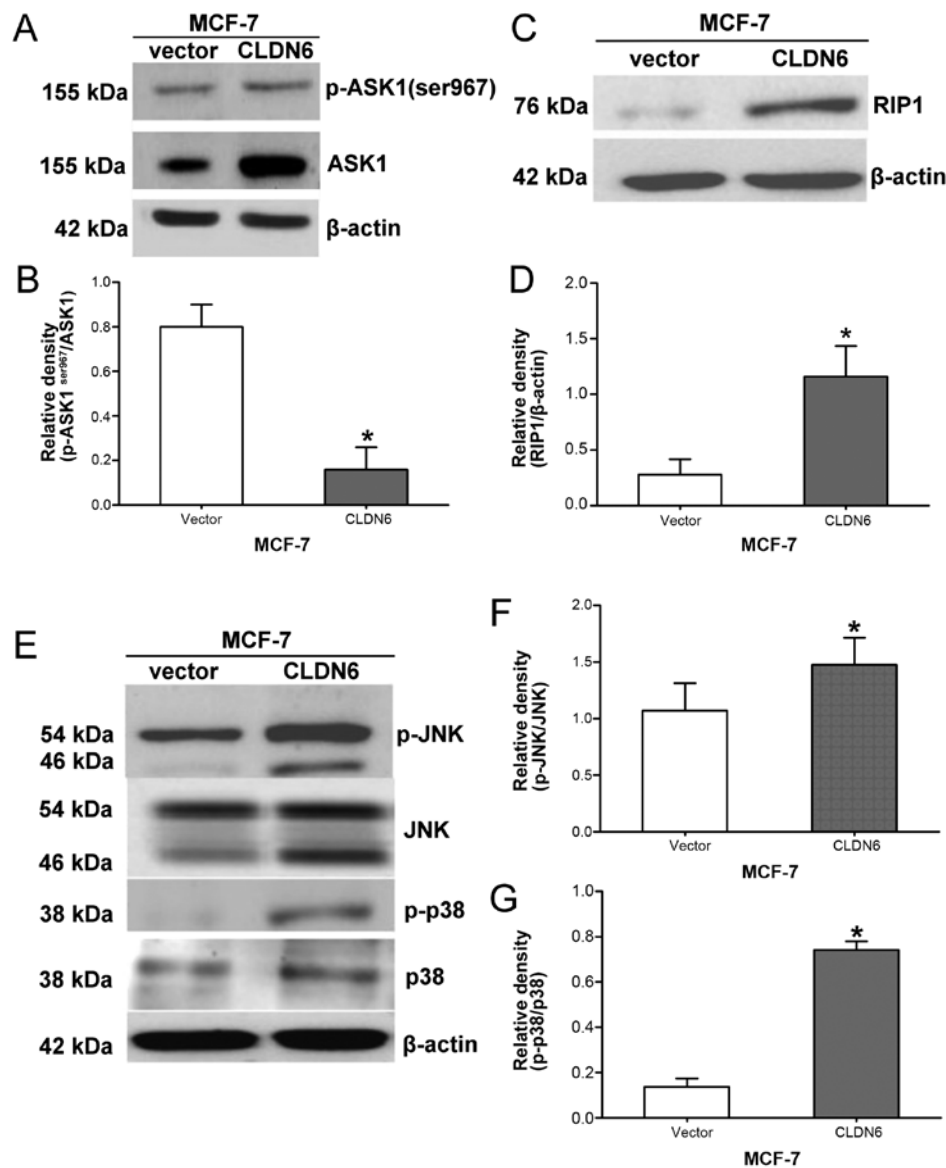
Figure 1. CLDN6 decreased the expression of ASK1 at Ser967 site, increased the expression of RIP1, and activated phosphorylation of p38, JNK proteins in human MCF-7 cells. (A and B) The expression level of p-ASK1^{ser967} and ASK1 proteins were detected by western blot analysis. Quantification of the protein levels are shown in the histograms, compared with the vector, *P=0.037<0.05; (C and D) RIP1 protein expression level was detected by western blot analysis. Compared with the vector, relative density of RIP1/β-actin was increased in MCF-7/CLDN6 stably transfected cells, *P=0.014<0.05. (E-G) p-JNK and p-p38 protein expression level were detected by western blot analysis. Compared with the vector, relative density of p-JNK/JNK and p-p38/p38 was increased in MCF-7/CLDN6 cells, *P=0.035<0.05, *P=0.026<0.05. Data are mean ± SD of vertical bars.

DNA fragmentation assay. MCF-7/CLDN6 stably transfected cells were harvested after 48-h incubation with 10 ng/ml TRX1 by centrifugation (300 x g for 5 min) and lysed in lysis buffer as previously described (24). The integrity of DNA was assessed by agarose gel electrophoresis.

Electron microscopy. MCF-7/vector and MCF-7/CLDN6 stably transfected cells were fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M, pH 7.4 phosphate buffer, followed by 1% OsO_4. After dehydration, thin sections were stained with uranyl acetate and lead citrate for observation under a FEI Tecnai Spirit electron microscope.

Statistical analysis. All data are presented as mean ± standard deviation (SD). The differences between groups were statistically analyzed using one-way analysis of variance (ANOVA)

followed by the Dunnett's test or LSD-t test for multiple comparisons. P<0.05 was considered as significant to evaluate the presence of a significant statistical difference.

Results

CLDN6 decreases p-ASK1^{ser967} levels and increases RIP1, p-p38 and p-JNK proteins in MCF-7 cells. To investigate the mechanism underlying CLDN6 induction of apoptosis, the levels of Ser967 phosphorylation of ASK1 in MCF-7 cells overexpressing CLDN6 and control cells were assessed by western blot analysis. The results showed that the relative expression level of p-ASK1^{ser967} was significantly decreased in MCF-7/CLDN6 stably transfected cells (Fig. 1A and B), suggesting that CLDN6-induced apoptosis may involve activation of ASK1. The expression levels of RIP1 (Fig. 1C and D),
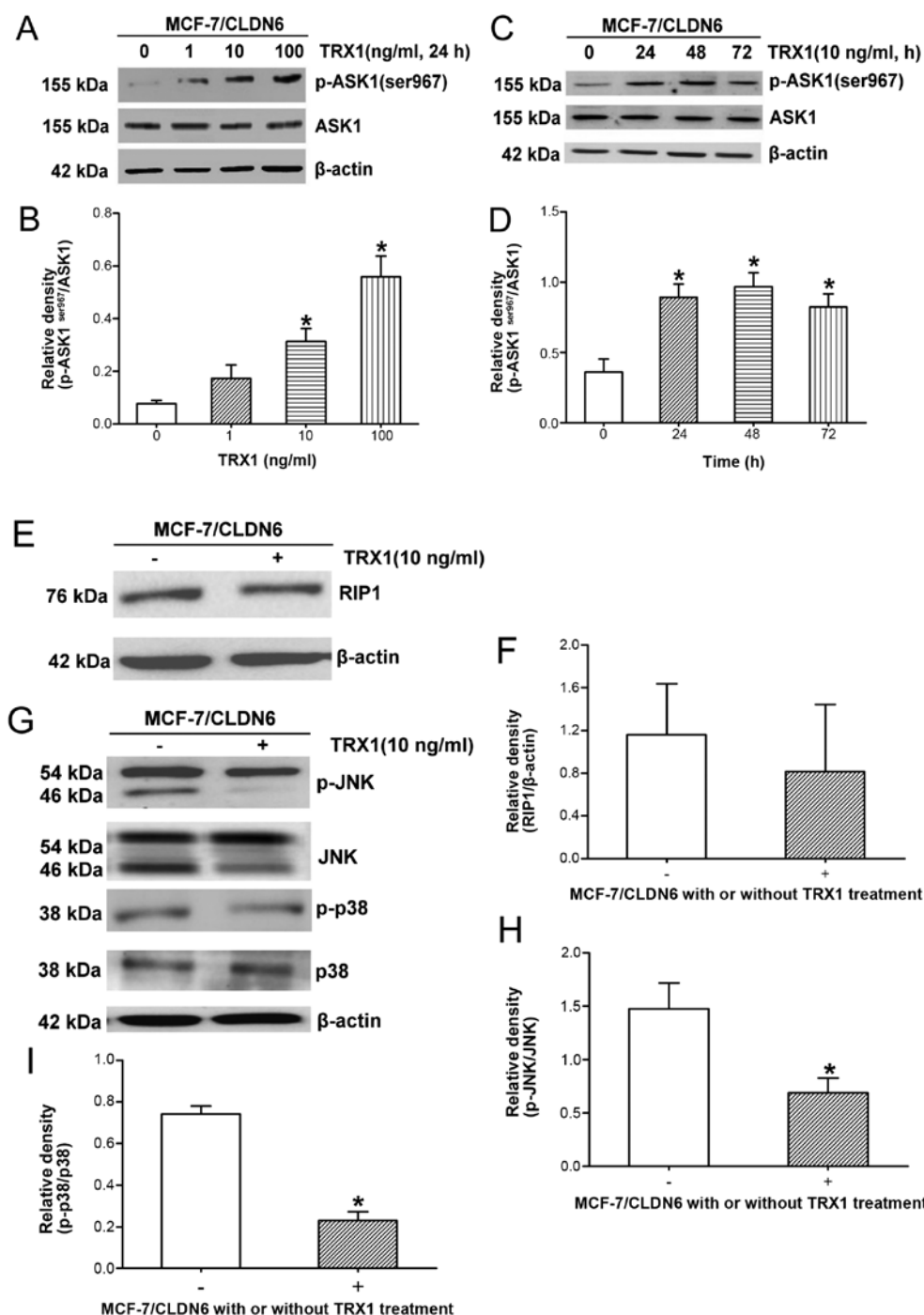
Figure 2. Effect of TRX1 on the molecular protein expression of ASK1 signal at different concentrations and various times after treatment in MCF-7/CLDN6 stably transfected cells. (A and C) Effect of TRX1 on the expression of ASK1 and p-ASK1[ser967] protein at different concentrations and various times after treatment of MCF-7/CLDN6 stably transfected cells. ASK1 and p-ASK1[ser967] protein expression levels time- and dose-dependent were increased in clone cells with TRX1 treatment for 24 h. (B and D) Histograms represent quantification of protein expression levels normalized to β-actin expression; *P=0.012; *P=0.017; *P=0.027; *P=0.024; *P=0.032; P<0.05. (E and G) Effect of TRX1 at 10 ng/ml for 48 h on the expression of ASK1 upstream of RIP1 and downstream of p-JNK, p-p38 protein levels were detected by western blot analysis in vector and clones cells before and after treatment. (F) Histograms represent quantification of protein expression levels normalized to β-actin expression; (H and I) Histograms represent quantification of p-JNK and p-p38 protein expression levels normalized to β-actin expression, *P=0.022 <0.05; *P=0.014 <0.05. *Compared with MCF-7/CLDN6 cells, P<0.05, mean ± SD of three independent experiments.

p-JNK and p-p38 (Fig. 1E-G) were significantly increased in MCF-7/CLDN6 stably transfected cells. We consider ASK1 is associated with upstream activators of p38 and JNK signaling in response to restoration of CLDN6 expression in MCF-7 cells. Taken together, these data suggest that CLDN6-induced apoptosis is associated with activation of ASK1 and upstream of RIP1 and downstream of JNK, and p38.

ASK1 plays a crucial role in CLDN6-activation of p38/JNK signaling molecules. We next assess the role of ASK1 in CLDN6-induced activation of p38/JNK signaling. MCF-7/CLDN6 stably transfected cells were treated with TRX1, a physiological inhibitor of ASK1 expression, for various exposure times and various doses (Fig. 2A-D). We confirmed increased p-ASK1[ser967] in response to TRX1 in a
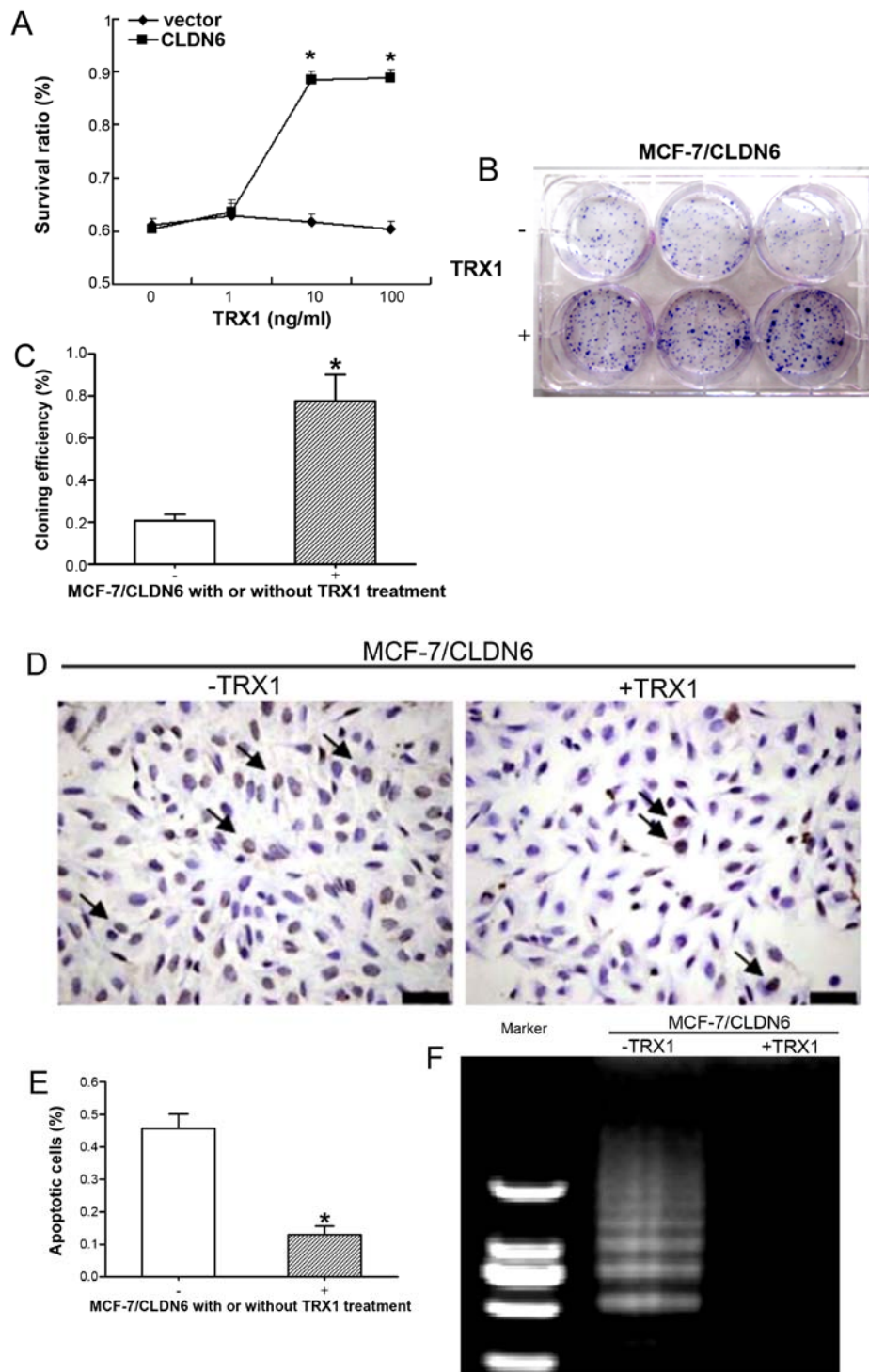
Figure 3. TRX1 inhibits cell survival rate, induces colony formation ability and suppresses apoptosis after CLDN6-overexpression. (A) Histograms represent cell survival rate (mean ± SD of three independent experiments, compared with 0 ng/ml TRX1 treatment for 24 h, **P=0.000941 <0.05 and **P=0.000806 <0.001, respectively). (B and C) Colony formation assay of MCF-7/CLDN6 stably transfected cells before and after TRX1 treatment. MCF-7/CLDN6 stably transfected cells were seeded at low density in individual wells of a standard 6-well plate and grown for 48 h. Colonies were visualized by crystal violet staining. In MCF-7/CLDN6 cells after treatment with TRX1, a small number of highly stained and tightly packed colonies were observed, representing clones within the overall cell population (*P=0.016 <0.05). (D and E) MCF-7/CLDN6 stably transfected cells with TRX1 treatment showed apoptotic cell morphological changes by TUNEL staining and brown counter stain (arrow represent apoptosis cells, magnification x400). Histograms represent apoptosis rate of positive cells with the overall cell population. *Compared with MCF-7/CLDN6 stably transfected cell group, *P=0.016 <0.05. (F) Effect of TRX1-suppressed apoptosis on CLDN6-overexpressed MCF-7 by DNA ladder analysis.

dose-dependent and time-dependent manner. Our results showed increased p-ASK1^{ser967} in response to TRX1 at 10 ng/ml for 48 h, suggesting that TRX1 effectively inhibited ASK1 activation under these conditions, therefore, we selected this concentration for further experiments. We next tested the impact of TRX1 on the phosphorylation of ASK1 downstream targets JNK and p38 in MCF-7/CLDN6 stably transfected cells. TRX1 significantly downregulated p-JNK and p-p38
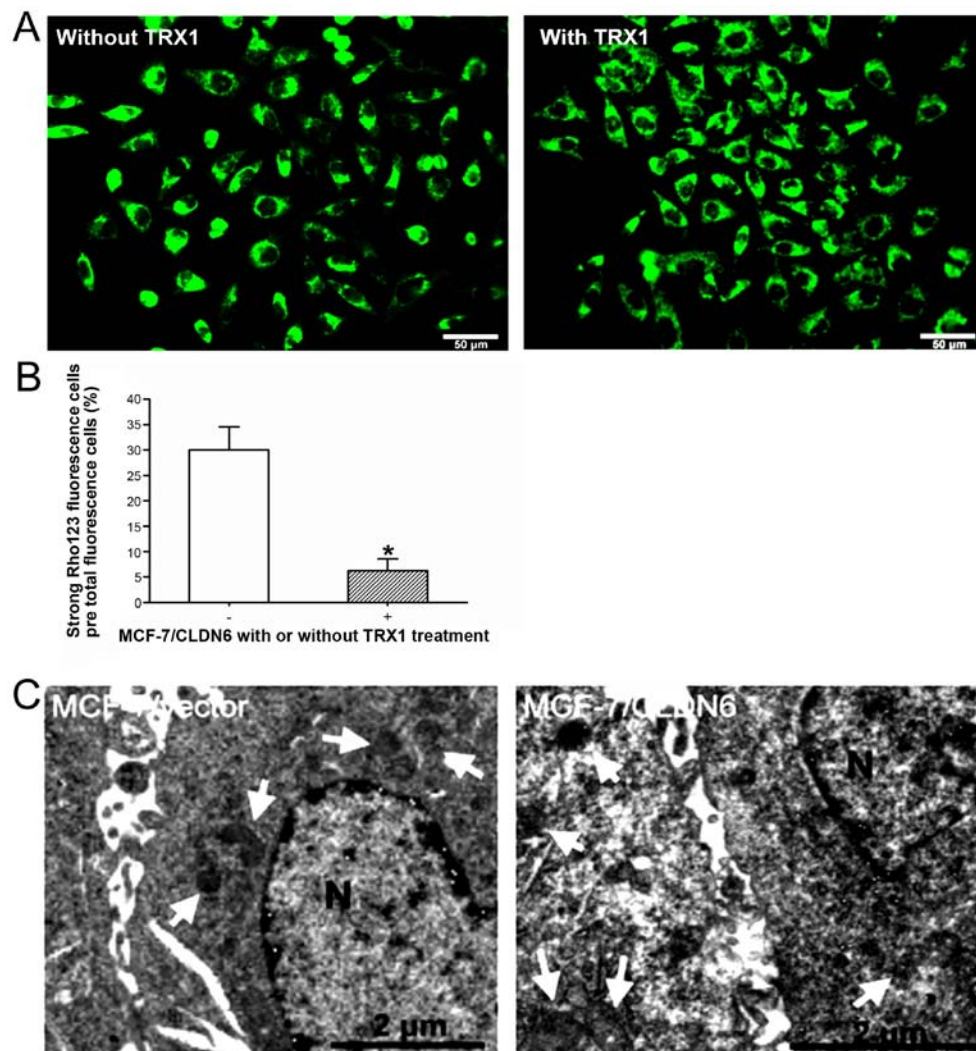
Figure 4. Disruption of the mitochondrial structure when MCF-7/CLDN6 stably transfected cells were treated with TRX1 for 48 h. (A) Rho 123 fluorescence was observed in MCF-7/CLDN6 stably transfected cells with or without TRX1 treatment (magnification, x400). (B) The loss of $\Delta\Psi$m was measured by software using the fluresescent dye of Rho 123. The percentage of cells with low $\Delta\Psi$m is indicated (P=0.016 <0.05). (C) Electron micrograph of MCF-7/CLDN6 stably transfected cells. Substantial disruption of the mitochondrial structure was observed.

levels (Fig. 2G-I). These results provide further evidence that ASK1 activation is required for CLDN6-promoted activation of JNK and p38.

TRX1 suppresses the apoptosis of MCF-7/CLDN6 stably transfected cells by inhibiting the cell viability and enhancing the clone formation ability. A previous study demonstrated that CLDN6 induced apoptosis of MCF-7 breast cancer cells (19). We analyzed cellular apoptosis using various assays including trypan blue staining, colony formation, TUNEL staining and DNA ladder analysis. MCF-7/CLDN6 stably transfected cells showed detectable levels of apoptosis in all assays (Fig. 3). Notably, treatment of MCF-7/CLDN6 stably transfected cells with TRX1 significantly decreased these apoptotic changes. Together these results suggest that ASK1 plays a major role in CLDN6-induced MCF-7 cell apoptosis.

TRX1 inhibits apoptosis by disruption of the mitochondrial structure in MCF-7/CLDN6 stably transfected cells. To analyze if ASK1 induces apoptosis via the mitochondrial pathway, we measured the disruption of the cell transmem-

brane mitochondrial potential ($\Delta\Psi$m) in MCF-7/CLDN6 stably transfected cells with TRX1 treatment. Using Rho-123 staining strong fluorescence was observed in MCF-7/CLDN6 stably transfected cells (Fig. 4A). TRX1 treatment of MCF-7/CLDN6 stably transfected cells induced a strong reduction of $\Delta\Psi$m after 48 h (Fig. 4A and B). Analysis of mitochondrial structure further confirmed disruption of mitochondrial structure in MCF-7/CLDN6 stably transfected cells and these changes were observed less frequently upon TRX1 treatment (Fig. 4C). These results indicate that CLDN6 induces the activation of mitochondrial apoptotic pathway in MCF-7 cells.

Changes of Bcl-2/Bax ratio and caspase-3 activation are involved in CLDN6-modulated pro-apoptotic effect. We continued investigating the molecular mechanisms of apoptosis induction by CLDN6 in MCF-7 cells. We evaluated expression levels of Bcl-2 and Bax proteins, which are involved in the mitochondrial apoptosis pathway, and the expression level of caspase-3 before and after TRX1 treatment. Bcl-2 levels were significantly lower, and Bax protein
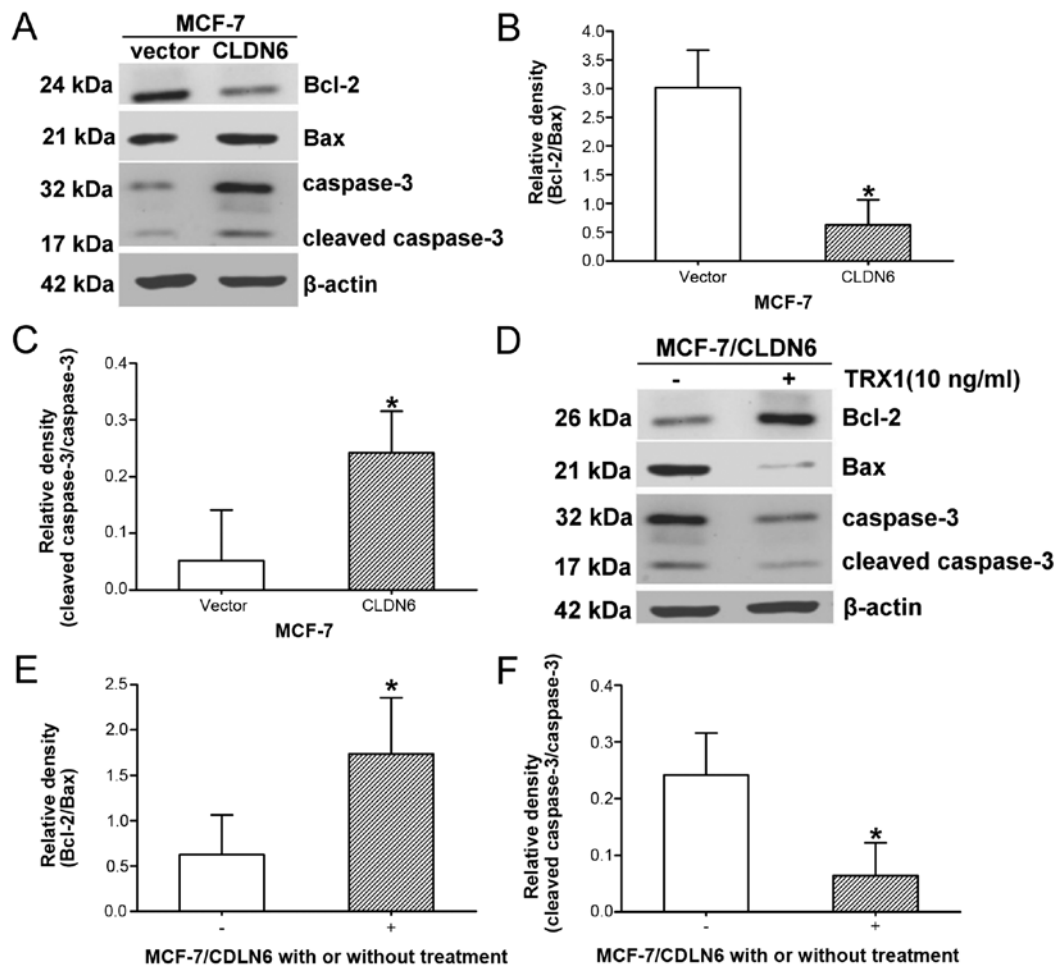
Figure 5. CLDN6 affects the expression levels of Bcl-2, Bax, cleaved caspase-3 proteins through ASK1 activation in MCF-7 breast cancer cells. (A and D) The protein expression levels of Bcl-2, Bax and cleaved caspase-3 was detected by western blot analysis in MCF-7/vector and MCF-7/CLDN6 stably transfected cells before and after TRX1 treatment. (B and C) Histograms represent relative quantitative Bcl-2/Bax (P=0.016) and cleaved caspase-3/caspase-3 (P=0.011) in vector and CLDN6 cell groups. *Compared with the vector cell group; P<0.05. (E and F) Histograms represent relative quantitative Bcl-2/Bax (P=0.012) and cleaved caspase-3/caspase-3 (P=0.042) in MCF-7/CLDN6 stably transfected cell group with TRX1 treatment. *Compared with MCF-7/CLDN6 cell group, P<0.05.

expression levels significantly increased in MCF-7/CLDN6 stably transfected cells compared with control cells, resulting in a decrease in Bcl-2/Bax ratio (Fig. 5A and B). However, TRX1 treatment caused an increase in Bcl-2 and decrease in Bax, resulting in significant increase in the Bcl-2/Bax ratio (Fig. 5D and E). Caspase-3 was significantly activated in MCF-7/CLDN6 stably transfected cells compared with control (Fig. 5C) and significantly inhibited in MCF-7/CLDN6 stably transfected cells after TRX1 treatment (Fig. 5F). Taken together, the constitutive deregulation of the balance of Bcl-2 family proteins and activation of caspase-3 are involved in the mitochondrial apoptotic pathways.

Discussion

Previous studies have demonstrated that CLDN6 functions as a cancer suppressor in MCF-7 breast cancer cells and CLDN6 could be attributed to inhibition of cell proliferation and induction of apoptosis (5). Immunohistochemical analysis in breast invasive ductal carcinomas tissues showed that ASK1 expression level is significantly related with CLDN6 (7). The purpose of the present study was to examine the involvement

of the ASK1 molecular signaling pathway in the anti-apoptotic effects of CLDN6 in MCF-7 cells.

ASK1 is a member of the mitogen-activated protein kinase kinase kinase family, in response to various stimuli such as oxidative stress, endoplasmic reticulum stress, infection and calcium influx, it is also activated by its upstream RIP1 and activates downstream JNK and p38 apoptotic signaling (25-28). Using cDNA microarray approaches, we previously analyed genes differentially expressed in the CLDN6 transfected cells (unpublished observations). The result showed ASK1 mRNA is increased, suggesting ASK1 may be a target of CLDN6. MCF-7/CLDN6 stable transfection cell clone treated with TRX1, an ASK1 inhibitor, showed p-ASK1[ser967] increased, suggesting that TRX1 effectively inhibited ASK1 activation. Some related genes of oxidative stress, endoplasmic reticulum stress, infection, and calcium influx were increased in transfection cells, they may play an important role in ASK1 activation and will be further investigated in our future work. Here ASK1 was activated, RIP1 was upregulated, p-p38 and p-JNK were increased by CLDN6 overexpression in MCF-7 cells. We considered that CLDN6 activated p-JNK and p-p38 through RIP1-ASK1 apoptotic signaling pathway.
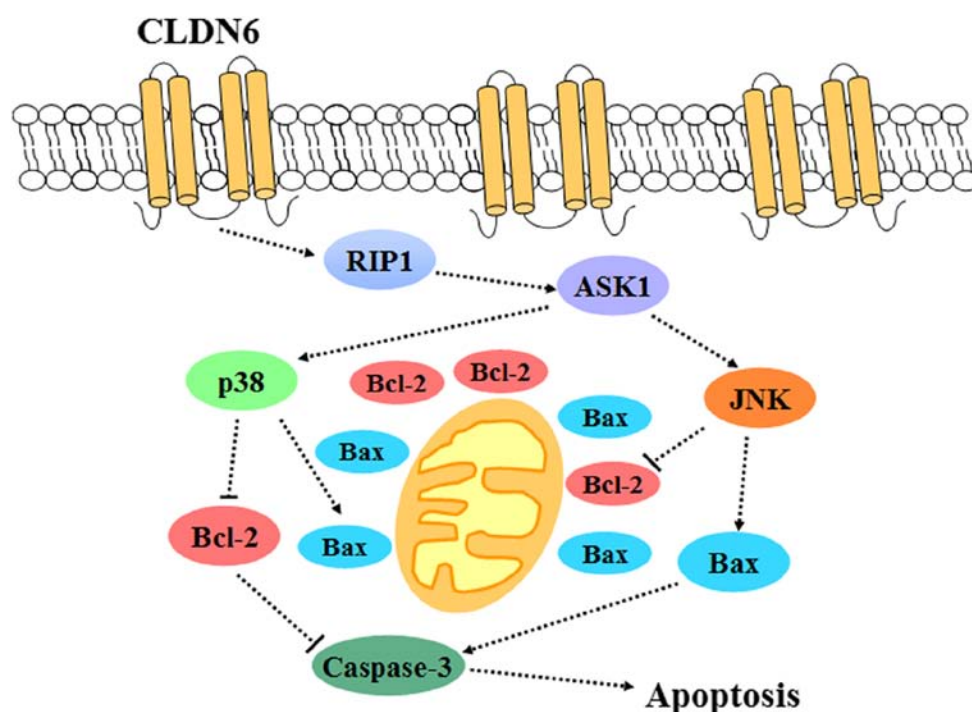
Figure 6. CLDN6 activates RIP1-ASK1-p38/JNK MAPK signaling pathways and their roles in regulating apoptosis. A number of representative up- and down-stream targets are shown. Both p38 and JNK MAPK can mediate apoptosis in response to restoration of CLDN6 gene expression through some downstream effectors such as Bcl-2, Bax, and caspase-3.

TRX1, a physiological inhibitor of ASK1, is a 12-kDa ubiquitous protein with a redox-active disulfide/dithiol within the conserved active site sequence (-Cys-Gly-Pro-Cys-) (29). Oxidized TRX1 with a disulfide on its active site is reduced by NADPH-dependent thioredoxin reductase1 (TR1) to restore its functions (30,31). TRX1 combined with ASK1 to form a TRX1-ASK1 complex which in return inactivated the ASK1 protein expression (27). To test the role of ASK1 during the effect of CLDN6 on JNK/p38 activation in MCF-7/CLDN6 stably transfected cells, these cells were incubated with 10 ng/ml of TRX1 for 48 h. The results showed that TRX1 treatment significantly downregulated p-JNK and p-p38 expression. These data further confirmed that ASK1 is required for CLDN6-promoted activation of JNK and p38. ASK1 serves as a general mediator of cell death such as apoptosis in cancer (32-34). Stress induced apoptosis worked through activation of the ASK1-p38 MAPK pathway (35-37). Several studied have shown that CLDNs induce apoptosis of cancer cells such as cervical carcinoma and breast cancer cells (17,38-40). Our data showed that CLDN6 significantly inhibited MCF-7 cell apoptosis with TRX1 treatment. Taken together, ASK1 by CLDN6 overexpression adjusted MAPK activity, increased JNK and p38 phosphorylation, and cell apoptosis reaction may be cascaded.

The dissociation between TRX1 and ASK1 induced by ROS leads to the activation of the ASK1/JNK signaling pathway and subsequent increase of apoptosis (41,42). The mechanism for the inhibition of apoptosis by ASK1 has been suggested such as Bcl-2 protein, caspase protein family members and mitochondria, whereby TRX1 could bind to and inhibit ASK1 further regulating the JNK/p38 signaling pathway in response to environmental stresses (26,27,43). Bcl-2 family proteins,

including Bax, Bak, Bik and Bid, promote apoptosis by translocating to and disrupting the mitochondrial membrane. The caspase protein family also plays a critical role in mediating apoptosis. Increasing studies have shown that caspase-1 and caspase-3 are associated with mitochondrial transmembrane potential and induce thymocyte apoptosis through the Fas/APO-1 (44). During apoptosis, mitochondria releasing caspase activation factors such as cytochrome *c*, undergo loss of electric transfer function and reduce generation of cellular energy.

We used Rho 123 staining on MCF-7 cells to evaluate the mitochondrial transmembrane potential. Changes in the cell membrane potential was detected in MCF-7/CLDN6 stably transfected cells, indicating a serious collapse of mitochondrial membrane potential and apoptosis rate. Our results further showed that TRX1 inhibited the ASK1 signaling pathway, reduced the degree of the mitochondrial transmembrane potential collapse and reduced the rate of apoptosis. These data suggest that CLDN6 regulates ASK1-JNK/p38 signal in apoptosis process and activates the mitochondrial apoptotic pathway. A previous study also found that JNK/p38-activated the inhibition of the growth of cancer cells through the mitochondrial apoptotic signaling pathway (45).

Among TJ proteins, multiple claudin isoforms are expressed in a homophilic and heterophilic manner and in varied patterns of expression that are tissue specific to regulate junctional permeability, and the selectivity and strength of the TJs are conferred by these proteins in most cell types (46). CLDN7 has a similar function and signaling transduction in the induction of tumor cell apoptosis. For example, CLDN7 expression shows a significant correlation with grading, locoregional and distant metastases, nodal involvement and cellular cohesion in

invasive carcinomas of the breast (47,48). Oshima *et al* (18) found that CLDN7 expression is increased in gastric cancer, and as a first report, showed that aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via CLDN7. To precisely define whether CLDN7 is involved in the same events as CLDN6, more studies are still needed.

In conclusion, the present study shows that CLDN6 is a tumor suppressor that inhibits MCF-7 cell survival and induces cell apoptosis. The breast cancer suppressive role of CLDN6 may be initiated by downregulation of ASK1 phosphorylation and subsequent decrease in JNK and p38 phosphorylation, along with decreasing the Bcl-2/Bax ratio and increasing caspase-3 activation (Fig. 6). This study highlighted the central role of CLDN6 in breast cancer cell survival and suggests that the CLDN6 gene is an ideal target for developing agents for breast cancer therapy.

Acknowledgements

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