

RESEARCH ARTICLE

H₂O₂ Inhibits Proliferation and Mediates Suppression of Migration via DLC1/RhoA Signaling in Cancer Cells

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Abstract

Background: RhoGTPase-activating proteins (RhoGAPs) regulate RhoGTPases in cells, but whether individual reactive oxygen species (ROS) regulate RhoGAPs is unknown. Our previous published papers have shown that deleted in liver cancer 1 (DLC1) inhibits cancer cell migration by its RhoGAP activity. The present study was designed to explore the role of H₂O₂ in regulation of DLC1. **Materials and Methods:** We treated cells with H₂O₂ for 24h and phenotypic changes were analyzed by MTT, RT-PCR, Western blotting, immunofluorescence staining and wound healing assays. **Results:** H₂O₂ downregulated cyclin D1 and cyclin E to inhibit proliferation, and upregulated BAX to induce apoptosis in MCF-7 cells. Compared with non-tumorigenic cells, H₂O₂ increased expression of DLC1 and reduced activity of RhoA in cancer cells. Stress fiber production and migration were also suppressed by H₂O₂ in MDA-MB-231 cells. **Conclusions:** Our study suggests that H₂O₂ inhibits proliferation through modulation of cell cycle and apoptosis-related genes, and inhibits migration by decreasing stress fibers via DLC1/RhoA signaling.

Keywords: H₂O₂ - proliferation - deleted in liver cancer 1 - RhoA - migration

Asian Pac J Cancer Prev, 16 (4), 1637-1642

Introduction

ROS, such as H₂O₂, O₂^{•-} and •OH, play an important role in cell physiological and pathophysiological responses. Under normal physiological conditions, cells keep ROS levels in balance between their generation and elimination by a redox reaction system (Winterbourn, 2008). However, under pathophysiological conditions, the disorder of ROS gives rise to fatal lesions in cells, which causes a lot of human diseases, including cancers (Gupta et al., 2012). The association of ROS with cancers is difficult to understand because each kind of ROS has specific effect on cancer cells (Veal et al., 2007). H₂O₂, as an individual ROS, can diffuse freely into cells and is relatively more stable compared with O₂^{•-} and •OH (Davies, 1999). It becomes a signaling molecule in regulation of cancer cell proliferation and migration.

H₂O₂ inhibits proliferation in various cancer cells, but the mechanism is unclear. The proliferation of breast cancer cells is inhibited by H₂O₂ with cell cycle arrest and apoptosis (Chua et al., 2009). H₂O₂-mediated cell cycle arrest is correlated with downregulation of cyclin D1 and cyclin E in lung carcinoma cells (Upadhyay et al., 2007). And H₂O₂ induces apoptosis with upregulation of BAX in colon cancer cells as well (Nie et al., 2008).

Migration and invasion result in spreading of cancers. RhoGTPases function as critical regulators for rearrangements in actin cytoskeleton during cancer cell spreading and migration. RhoA is one of the best characterized members in RhoGTPase family. The activity of RhoA is determined by its guanine nucleotide-bound state. RhoGTPase-activating protein (RhoGAP) can catalyze the conversion of RhoA from its GTP-bound active state to GDP-bound inactive state. Only few reports about the role of ROS on GAP are documented. Nimmual et al. found that Rac-induced ROS downregulates RhoA-GTP via elevation of p190RhoGAP activity in HeLa cell line (Nimmual et al., 2003), but it is unclear which kind of ROS regulates RhoGAP. Li et al. demonstrated that H₂O₂ regulates another GAP family member Cdc42GAP in canine tracheal smooth muscle cells (Li et al., 2009). As a new member of GAP family, deleted in liver cancer 1 (DLC1) has been proved to possess RhoGAP activity (Yuan et al., 1998; Wong et al., 2003). Our previous studies show that DLC1 suppresses cancer cell migration by its RhoGAP activity (Qian et al., 2007; Li et al., 2011). In this paper, we investigated effects of H₂O₂ on proliferation by checking cell cycle and apoptosis-related genes and explored H₂O₂-mediated suppression of migration via DLC1/RhoA signaling pathway.

Materials and Methods

Chemical

30% H₂O₂ solution (Sinopharm chemical reagent, China) was used to prepare different concentrations of H₂O₂.

Cell lines and culture

Human breast cancer cell lines MCF-7, MDA-MB-231, non-tumorigenic human breast epithelial cell line MCF 10A and human lung cancer cell line H1299 (ATCC, USA) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin, and maintained at 37°C in humidified atmosphere with 5% CO₂.

MTT assay

MCF-7 cells were plated in 96-well microtiter plates respectively at a density of 10 000 cells/well. Next day, the cells were treated with 0-200 μM H₂O₂ for 24h and subsequently incubated with MTT for 4h. The amount of formazan product was measured at 492 nm absorbance to evaluate cell proliferation.

Hoechst/PI staining

Cell apoptosis was examined by Hoechst/PI staining. MCF-7 cells were exposed to 0, 10, 50, 100 and 200 μM H₂O₂ for 24h. Then the cells were incubated with Hoechst 33342 and PI (Beyotime biotechnology, China) for 30min. The apoptotic cells were observed using a fluorescence microscopy with 365/480 nm excitation/emission.

Semi-quantitative RT-PCR

MCF-7 cells were used to detect expressions of cyclin D1, cyclin E, BAX and DLC1. The expression of DLC1 was also assayed in MCF 10A cells. The cells were treated with or without 100 μM H₂O₂ for 24h. Total RNA was extracted with RNAsimple Total RNA Kit (TIAN GEN, China) as described by the manufacturer and rapidly transcribed to cDNA. PCR amplifications were performed with each pair of primers.

Western blot analyses

Because DLC1 is frequently deleted or downregulated in many cancer cell lines and H1299 expresses endogenous DLC1. We used H1299 cells to examine the protein expression of DLC1. Cells were treated with or without 100 μM H₂O₂ for 24h. The protein was electrophoresed on a SDS-PAGE and then electro-transferred to PVDF membranes. The membranes were probed with anti-DLC1 antibody (1: 350, BD Biosciences, USA), followed by incubation with anti-mouse IgG (ZSGB-BIO, China). Protein expression was detected with the ECL system (Beyotime biotechnology, China).

Rhotekin affinity precipitation/immunoblot

Measurement of RhoA activity was assessed by Rhotekin affinity precipitation/immunoblot, using a RhoA activation assay kit (Merck &Millipore, USA). H1299 cells were treated with or without 100 μM H₂O₂ for 24h. And 2.5 mg protein was incubated with 19 μl

GST-Rhotekin agarose beads on a rocker platform at 4°C for 45min. After incubation, the beads were washed and GTP-bound protein was eluted with Laemmlis's reducing sample buffer boiling for 5min. The GTP-bound protein and total protein were performed on western blot analysis using anti-Rho antibody (1: 330, Merck &Millipore, USA).

Immunofluorescence staining

Stress fibers were detected by immunofluorescence staining. MDA-MB-231 cells were plated onto coverslips in 24-well plates and treated with 0, 50, 100 μM H₂O₂ for 24h. After fixing/permeabilizing, cells were stained with FITC-phalloidin (Sigma, USA). Immunofluorescence images were taken with a laser scanning confocal microscope. FITC-positive cells were scored for the presence of stress fibers in more than 300 cells with Image Pro Plus 6.0 software. FITC-positive cells/total cells in each condition was plotted as an "induction of stress fiber index" (Aghajanian et al., 2009).

Wound healing assay

MDA-MB-231 cells were cultured in 24-well plates at a density of 500 000 cells/well. Cells were wounded with a 20 μl tip and treated with 0, 10, 50, 100, 150 and 200 μM H₂O₂ for 24h. Then, we used crystal violet staining solution to stain the cells. Photographs were taken at 0 and 24h. The relative migration by the leading edge from 0 to 24h was assessed using Image Pro Plus 6.0 software.

Statistical analyses

All of the assays were performed three times independently at least. Data represented mean±standard deviation (SD). Statistical analysis was determined by two-tailed Student's *t* test or two-way ANOVA. *P*-value<0.05 was considered statistically significant.

Results

H₂O₂ inhibits proliferation of MCF-7 cells with the downregulation of cyclin D1 and cyclin E

Breast cancer cells MCF-7 were treated with 0-200 μM H₂O₂ for 24h. We used MTT assay to measure the effect of H₂O₂ on MCF-7 cells. The result showed that 5-15 μM H₂O₂ increased proliferation, and higher concentrations at 30-200 μM H₂O₂ inhibited proliferation (Figure 1A). This displayed dual effects of H₂O₂ on proliferation, and that H₂O₂ inhibited the proliferation in a dose-dependent manner. To explore the suppressed proliferation in MCF-7 cells, we investigated the expressions of cell cycle-related genes.

Cell cycle progression is essential for cell proliferation. Cyclin D1 and cyclin E control the transition of cell cycle from G1 to S phase. Here, our study detected mRNA expressions of cyclin D1 and cyclin E to investigate their roles in proliferation of MCF-7 cells. We found that cyclin D1 and cyclin E were downregulated when MCF-7 cells were exposed to 100 μM H₂O₂ (Figure 1B and C). It implied that the downregulation of cyclin D1 and cyclin E by H₂O₂ might cause cell cycle arrest to inhibit the proliferation of MCF-7 cells.

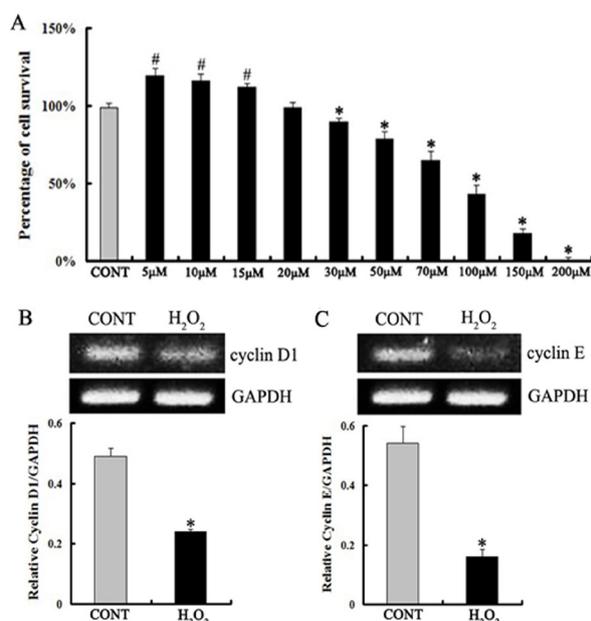


Figure 1. H₂O₂ Regulates Proliferation with the Modulation of Cyclin D1 and Cyclin E in MCF-7 Cells.

A) 5-15 μM H₂O₂ promoted proliferation and 30-200 μM H₂O₂ inhibited proliferation as determined by MTT assay. **B/C)** Cell cycle-related genes cyclin D1 and cyclin E were significantly downregulated by 100 μM H₂O₂. [#]*p*<0.05, ^{*}*p*<0.05

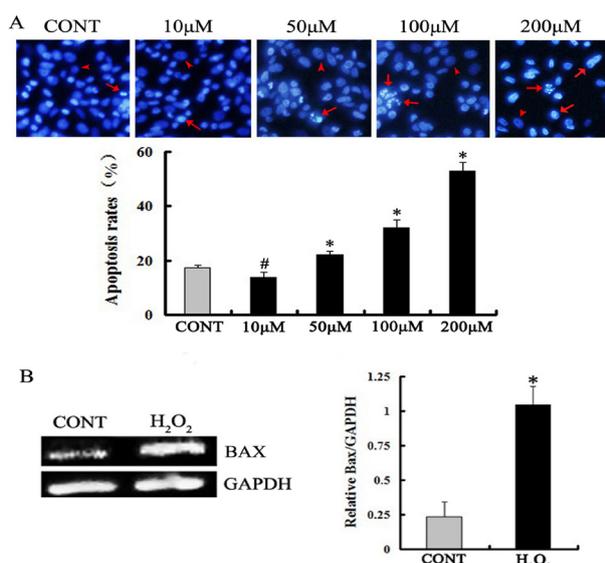


Figure 2. H₂O₂ Induces Apoptosis with the Upregulation of BAX in MCF-7 Cells. **A)** Hoechst/PI staining discriminated apoptotic cells (arrows) and non-apoptotic cells (arrowheads). The apoptosis rates were analysed by Image Pro Plus 6.0 software. 10 μM H₂O₂ reduced apoptosis rate and 50-200 μM H₂O₂ promoted cell apoptosis. **B)** Pro-apoptosis gene BAX was significantly upregulated by 100 μM H₂O₂. [#]*p*<0.05, ^{*}*p*<0.05

H₂O₂ induces apoptosis with the upregulation of pro-apoptosis gene BAX in MCF-7 cells

It is known that proliferation is affected by cell apoptosis. 0-200 μM H₂O₂ were used to induce apoptosis of breast cancer cells MCF-7. The result showed that apoptotic cells decreased at 10 μM H₂O₂, and higher concentrations of 50-200 μM H₂O₂ promoted cell apoptosis (Figure 2A). It appeared a similar trend with the proliferation inhibited by 50-200 μM H₂O₂ in MCF-7

cells. This suggested that cell apoptosis contributed to the inhibition of proliferation by H₂O₂. We further explored the expression of pro-apoptosis gene BAX, as it participated in release of apoptosis factors. The result showed that BAX was significantly upregulated by 100 μM H₂O₂ (Figure 2B). It implied H₂O₂ increased the expression of BAX to promote apoptosis of MCF-7 cells.

H₂O₂ increases the mRNA and protein expressions of DLC1 and reduces the activity of RhoA

Tumor suppressor gene DLC1 is frequently downregulated in cancer cells. In order to examine the role of H₂O₂ in regulation of DLC1, we used 100 μM H₂O₂ to treat cancer cells MCF-7, H1299 and non-tumorigenic cells MCF 10A, and detected the expression of DLC1. The result showed that H₂O₂ increased mRNA and protein expression of DLC1 (Figure 3A and B), whereas H₂O₂ did not change mRNA expression of DLC1 in non-tumorigenic MCF 10A cells (Figure 3C). It indicated that the expression of DLC1 was increased by H₂O₂ only in cancer cells.

DLC1 has a RhoGAP domain, which can hydrolyze RhoA-GTP to RhoA-GDP. To further explore whether H₂O₂ regulated the activity of RhoA, we treated H1299 cells with 100 μM H₂O₂ and examined the level of RhoA-GTP by Rhotekin affinity precipitation/immunoblot. The result indicated that cells treated with H₂O₂ had decreased the level of RhoA-GTP compared to that in control group (Figure 3D). This showed that the activity of RhoA was significantly reduced by H₂O₂ in H1299 cells. It suggested that H₂O₂ regulated DLC1/RhoA signaling pathway by upregulating DLC1 and subsequently reducing RhoA activity.

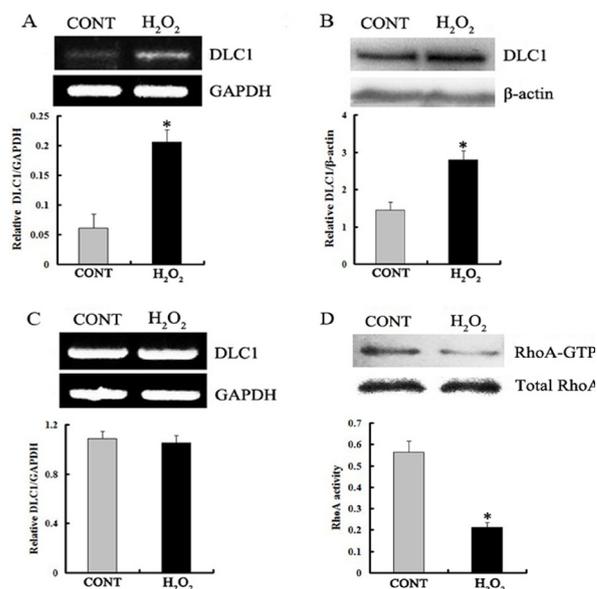


Figure 3. H₂O₂ Upregulates Expression and Reduces RhoA Activity.

A) The mRNA expression of DLC1 was upregulated by 100 μM H₂O₂ in MCF-7 cells. **B)** DLC1 protein production was increased by 100 μM H₂O₂ in H1299 cells. **C)** H₂O₂ did not affect DLC1 mRNA expression in non-tumorigenic MCF 10A cells. **D)** The activity of RhoA was detected by Rhotekin affinity precipitation/immunoblot. RhoA-GTP was downregulated by 100 μM H₂O₂ in H1299 cells. Total RhoA serves as a loading control. ^{*}*p*<0.05

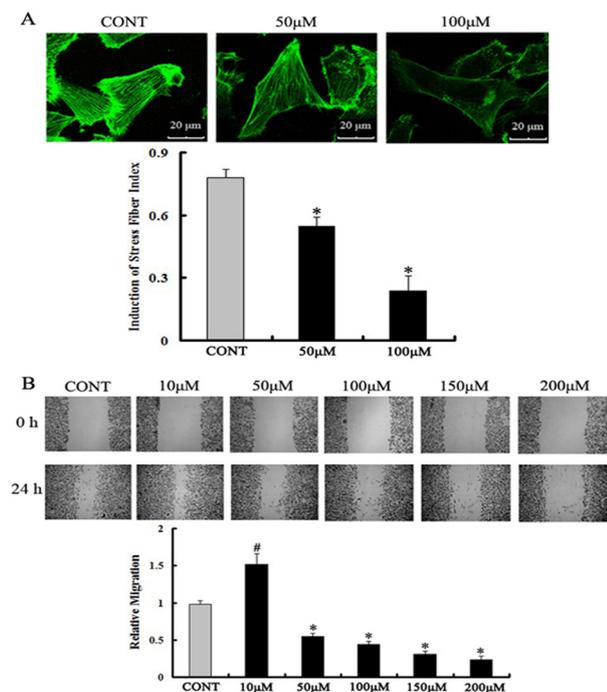


Figure 4. H₂O₂ Inhibits Formation of Stress Fibers and Migration in MDA-MB-231 Cells. A) Stress fibers were detected with FITC labeled phalloidin. Induction of stress fiber index represented the formation of stress fibers. 50 μ M and 100 μ M H₂O₂ significantly decreased stress fibers in MDA-MB-231 cells. Scale bar = 20 μ m. B) Cell migration was measured by wound healing assay. 10 μ M H₂O₂ promoted migration and 50-200 μ M H₂O₂ inhibited migration in MDA-MB-231 cells. #*p*<0.05, **p*<0.05

H₂O₂ inhibits migration by decreasing stress fibers in MDA-MB-231 cells

RhoA-mediated signaling pathways involve in formation of stress fibers and cell migration. This study used H₂O₂ to treat breast cancer cells MDA-MB-231 and measured the formation of stress fibers. We found that stress fiber organization was reduced after 50 μ M H₂O₂ treatment, and the concentration at 100 μ M H₂O₂ aggravated the decrease of stress fibers compared to the control group (Figure 4A). It turned out that H₂O₂ reduced the formation of stress fibers in MDA-MB-231 cells.

Stress fibers generate contraction to improve cell mobility and are essential for migration of cancer cells. We investigated the regulatory effect of H₂O₂ on the migration of MDA-MB-231 cells. The results of wound healing assay indicated that 10 μ M H₂O₂ promoted cell migration and 50, 100, 150 and 200 μ M H₂O₂ suppressed cell migration compared to the control group (Figure 4B). It is noted that the suppression of migration by H₂O₂ was dose-dependent in MDA-MB-231 cells.

Together with our results mentioned above, we speculated that H₂O₂ inhibited proliferation by downregulating cyclin D1, cyclin E and upregulating BAX, and suppressed migration by reducing stress fibers via DLC1/RhoA signaling pathway in cancer cells.

Discussion

Research during last decades suggests that the disorder of ROS is critically associated with cancer

cell development. The individual ROS, H₂O₂ plays an important role in cell proliferation and migration. Increasing evidence shows that H₂O₂ has dual effects on cancer cell proliferation which depends on its concentration. For instance, H₂O₂ at concentrations from 50 μ M to 200 μ M inhibits the proliferation of human breast cancer cells MCF-7 (Chua et al., 2009), while 1-10 μ M increases hepatoma 7721 cell proliferation (Liu et al., 2002). The cell proliferation is improved at 10 μ M H₂O₂, whereas 1000 μ M H₂O₂ induces apoptosis in colon cancer cells HT-29 (Park et al., 2006). Our results showed that the proliferation of breast cancer cells MCF-7 was promoted by 5-15 μ M H₂O₂ and inhibited by 30-200 μ M H₂O₂. This is not only consistent with the above-mentioned studies, but also indicates that the inhibitory effect of H₂O₂ on MCF-7 cells is dose-dependent. So, we suggest that administrating H₂O₂ in an appropriate concentration may suppress the proliferation of breast cancer cells effectively.

H₂O₂-mediated suppression of proliferation is correlated with cell cycle arrest in breast cancer cells MCF-7 (Chua et al., 2009). Cell cycle-related genes, cyclin D1 and cyclin E are responsible for regulating the progression of cell cycle through G1 to S phase transition (Kang et al., 2002; Keyomarsi et al., 2002; Liu et al., 2013). It has been reported that H₂O₂ can induce a transient multiphase cell cycle arrest with reduction in cyclin D1-CDK4 and cyclin E-CDK2 activities in mouse fibroblasts (Barnouin, 2002). Subsequent study shows that H₂O₂ causes downregulation of cyclin D1 and cyclin E in lung carcinoma cells A549 (Upadhyay et al., 2007). A recent published paper also proves that reduced expression of cyclin D1 is associated with H₂O₂-mediated cell cycle arrest in HeLa cells (Pyo et al., 2013). Our study showed that H₂O₂ downregulated the expressions of cyclin D1 and cyclin E in breast cancer cells MCF-7, which is in agreement with previous reports. We think that the downregulation of cyclin D1 and cyclin E by H₂O₂ may block cell cycle progression and further result in the suppression of breast cancer cell proliferation.

Apoptosis is recognized as a process of cell death that affects cell proliferation (Fattahi et al., 2013). It has been demonstrated that H₂O₂ induces apoptosis in several cancer cell lines. 150 μ M H₂O₂ significantly increases the number of apoptotic neuroblastoma cells SH-SY5Y (Zhang et al., 2007). And the apoptosis is induced by 2 mM H₂O₂ in glioma cells U251 with reduction of cell viability (Tanaka et al., 2004). Nevertheless, there is no detectable apoptosis in 10 μ M H₂O₂-treated colon cancer cells HT-29 (Park et al., 2006). In concert with these studies, our result showed that 50-200 μ M H₂O₂ increased levels of apoptosis, while lower concentration at 10 μ M decreased apoptosis rate in breast cancer cells MCF-7. Obviously, the trend of apoptosis consists with the decrease of cell proliferation we measured. It suggests that the apoptosis induced by H₂O₂ contributes to the suppression of proliferation in MCF-7 cells. Pro-apoptosis gene BAX is essential for release of cytochrome c from mitochondria, which is a central process in apoptosis (Friberg and Wieloch, 2002; Rengarajan et al., 2014). BAX is increased with treatment of H₂O₂ in endometrial Ishikawa cancer cells (Jung et al., 2011). Our result showed that 100 μ M H₂O₂ upregulated the expression of BAX in MCF-7 cells. It implies that the

increased expression of BAX by H₂O₂ promotes breast cancer cell apoptosis. Therefore, we speculate that H₂O₂ inhibits breast cancer cell proliferation by downregulating the expressions of cyclin D1 and cyclin E to make cell cycle arrest, and upregulating BAX expression to induce cell apoptosis.

The role of ROS in regulation of cell migration has been reported, but the role of H₂O₂ and its regulatory mechanism have not been well investigated. Ho et al. found that 0.1-5 μM H₂O₂ increased the number of invading colorectal cancer cells SW620 (Ho et al., 2011). But the migration is inhibited by 100 μM H₂O₂ in lung cancer cells H460 (Luanpitpong et al., 2010). In this study, we showed that 10 μM H₂O₂ promoted migration, whereas higher concentrations from 50 μM to 200 μM suppressed migration of breast cancer cells MDA-MB-231 in a dose-dependent manner. It is in agreement with the works mentioned above. As we known, the migration of cancer cells is generated by consecutive contractions of stress fibers which create a tension and drive cell movement (Kaverina et al., 2002). Previous report indicates that 1 μM H₂O₂ increases stress fibers in rat fibroblasts REF-52 (Aghajanian et al., 2009). We measured the formation of stress fibers in response to higher concentrations of H₂O₂. The results showed that 50 μM and 100 μM H₂O₂ reduced the formation of stress fibers in breast cancer cells MDA-MB-231. It reflects that the suppression of breast cancer cell migration results from the decrease of stress fibers.

The formation of stress fibers is regulated by RhoA (Chrzanowska-Wodnicka and Burridge, 1996). The activated-RhoA interacts with its downstream effectors Rho-kinase, Citron kinase and M-Dia1, and tightly controls cell motility (Kimura et al., 1996; Nayak et al., 2013). Our previous studies have demonstrated that DLC1 exerts RhoGAP activity to reduce RhoA-GTP and inhibits cancer cell migration (Qian et al., 2007; Li et al., 2011). So we explored the role of H₂O₂ in regulation of DLC1/RhoA signaling pathway. Nimnual et al. indicates that Rac-derived ROS can lead to activation of p190RhoGAP and subsequent reduction of RhoA activity in HeLa cells (Nimnual et al., 2003). Our results showed that H₂O₂ upregulated the expression of DLC1 and downregulated the level of RhoA-GTP to reduce RhoA activity. These results are similar to the effect of Rac-derived ROS on p190RhoGAP and RhoA. Although DLC1 expression was elevated in cancer cells MCF-7 and H1299, H₂O₂ did not affect the expression of DLC1 in non-tumorigenic cells MCF 10A. Therefore, we think that H₂O₂ upregulates tumor suppressor gene DLC1 expression only in cancer cells. Collectively, we suggest that H₂O₂ inhibits cancer cell migration with the reduction of stress fibers through DLC1/RhoA signaling pathway.

In conclusion, we demonstrated that H₂O₂ inhibits proliferation and induces apoptosis with downregulation of cyclin D1 and cyclin E, and upregulation of BAX in breast cancer cells MCF-7. H₂O₂ upregulates DLC1 expression and reduces the activity of RhoA in H1299 cells. The formation of stress fibers and migration are also inhibited by H₂O₂ in breast cancer cells MDA-MB-231. These suggest that the proliferation of breast cancer cells is inhibited by H₂O₂ through the modulation of cell cycle

and apoptosis-related genes to make cell cycle arrest and induce cell apoptosis. And we speculate that H₂O₂ inhibits cancer cell migration by decreasing stress fibers via DLC1/RhoA signaling pathway. H₂O₂-mediated DLC1/RhoA signal transduction pathway provides a new sight for anti-cancer studies.

Acknowledgements

This work was supported by the Special Funds of the National Natural Science Foundation of China (Grant No. 31340035), the Key Program of Shandong Provincial Natural Science Foundation of China (ZR2013CZ002), the Research Fund for the Doctoral Program of Higher Education of China (20113704110004), Science and Technology Development Program of Shandong Province (2012CSF12112), Science and Technology Program of Jinan (201202033).

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