# Anti-apoptotic role of peroxiredoxin III in cervical cancer cells☆

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ABSTRACT

As a member of peroxiredoxin (Prx) family, PrxIII is predominantly located in mitochondria and plays an important role as a scavenger of reactive oxygen species (ROS). Since previous reports demonstrated over-expression of PrxIII in cervical cancer, we conducted the present study to investigate the significance of PrxIII in cervical cancer development and/or progression.

Cervical cancer cells were cultured from tissues derived from cervical cancer patients. After successful knockdown of PrxIII expression by small interfering RNA, we evaluated ROS level, viable cell number, and apoptosis of cervical cancer cells along with the culture time.

The production of ROS was increased in cervical cancer cells as compared with normal cervical epithelia. Knockdown of PrxIII expression induced up-regulation of other Prx members including PrxI, PrxII, and PrxV. ROS level was higher in down-regulated cervical cancer cells than in controls and the difference was increasing with culture time. We also observed increased apoptosis and decreased viable cell number in down-regulated cervical cancer cells.

Our results suggest that PrxIII is an indispensable ROS scavenger, which protects tumor cells against oxidative damage and subsequent apoptosis.

## 1. Introduction

As an energy-provider for cellular activities, mitochondrion is a major source of reactive oxygen species (ROS) production. Since excessive ROS may cause oxidative damage on DNA, lipids, and proteins, organisms have developed multiple antioxidant systems to control the ROS at baseline level that serves as cellular second messengers. Under pathophysiological conditions or outer stimuli, ROS generation is exaggerated to an extent that overwhelms cellular antioxidant defenses and a state called "oxidative stress" occurs. As a member of peroxiredoxin (Prx) family, PrxIII is mainly located in mitochondria. By reducing $H_2O_2$ with its two active cysteines, PrxIII acts as a mitochondrial scavenger of ROS, which protects mitochondria against oxidative damage and influences diverse cellular processes including growth, differentiation, apoptosis, and carcinogenesis [1–3].

Up to now, accumulating evidence demonstrated that PrxIII was up-regulated in various types of carcinomas [4–7]. According to the report by Kim et al., PrxIII expression was increased with the severity of cervical lesions [8]. We recently demonstrated that PrxIII was over-expressed in cervical cancer and the immunostaining pattern was consistent with that of the proliferation marker Ki67 [9], suggesting an active response of PrxIII to cell expansion-induced oxidative stress in cervical cancer. Nevertheless, the precise function of PrxIII in cervical cancer development and/or progression remains to be obscure. The present study was conducted to investigate the significance of PrxIII in cervical cancer cells.

## 2. Material and methods

This study was approved by the Institutional Review Boards of Tsinghua University Medical School. All samples were obtained after informed consent from patients at Tsinghua University Second Hospital. Cervical cancer tissues from squamo-columnar zone were obtained from 10 patients who underwent radical surgery. The mean age of the cancer patients was $50.2 \pm 2.6$. All the patients were diagnosed as squamous cervical cancer stage IA according to the International Federation of Gynaecology and Obstetrics criteria for cervical carcinoma.

### 2.1. Cell culture

Cancerous tissues and the adjacent normal epithelial tissues were cut and cultured separately at the same time. Primary culture was

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**Table 1**
Primer sequences for qRT-PCR analysis.

| Genes | Sequences | Size (bp) |
|---|---|---|
| PrxI | Forward 5′-TATGCCAGATGGTCAGTTT-3′ | |
| | Reverse 5′-CCCAGTCCTCCTTGTTTC-3′ | 166 |
| PrxII | Forward 5′-TGTCGGACTACAAAGGGAA-3′ | |
| | Reverse 5′-GACGCCCAGCACTTCACA-3′ | 203 |
| PrxIII | Forward 5′-TGCCTGGATAAATACACC-3′ | |
| | Reverse 5′-AGTCTCGGGAAATCTGCT-3′ | 198 |
| PrxIV | Forward 5′-GCTGGGAGACAGAGGAGA-3′ | |
| | Reverse 5′-AAATGTGAAATCAAGTGGG-3′ | 156 |
| PrxV | Forward 5′-ATTCGCTGGTGTCCATCTTT-3′ | |
| | Reverse 5′-GTGCCATCTGGTTCCACATTCA-3′ | 211 |
| PrxVI | Forward 5′-ATTCTCAGGGTAGTCATCTC-3′ | |
| | Reverse 5′-TTTGGCTTCTTCTTCAGG-3′ | 179 |
| β-actin | Forward 5′-ACGTTGACATCCGAAAGACC-3′ | |
| | Reverse 5′- CCACCGATCCACACAGAGTA-3′ | 154 |



**Fig. 1.** Expression of PrxIII and other 2-Cys Prx genes in cervical cancer cells analyzed by Western blot. (A) The PrxIII expression was significantly inhibited by siRNA. There was no significant difference of PrxIII expression among cells without transfection (Con), cells transfected with siRNA containing the 5′-UUCUCCGAACGUGUCACGUTT-3′ sequence (SiNc), and cells transfected with Lipo-fectamine only (SiLipo). (B) The expression of *other* 2-Cys Prx genes was up-regulated after knockdown of PrxIII.



**Fig. 2.** Expression analysis of Prx genes by qRT-PCR. After knockdown of PrxIII expression (SiPrxIII vs. SiNc: **$P < 0.01$), other Prx genes including PrxI, PrxII, and PrxV were significantly up-regulated (SiPrxIII vs. SiNc: *$P < 0.05$).

performed in keratinocyte serum-free medium (Invitrogen Corporation) supplemented with human recombinant EGF, bovine pituitary extract, and antibiotics. All cells were maintained at 37 °C in a humidified incubator with 5% $CO_2$ and passaged at a 1:4 split ratio.

## 2.2. Knockdown of PrxIII expression

Cells at passage 3 were seeded in six-well plates with a density of $1 \times 10^6$ cells/ml. After culture for 24 h, down-regulation of PrxIII expression was achieved by small interfering RNA (siRNA) that targeted the 5′-GCCAAGUCCAGCUGCUUCCTT-3′ sequence of human PrxIII mRNA. The interfering effectiveness was confirmed by Western blot analysis using mouse monoclonal antibody against human PrxIII (1:1000 dilution, Abcam Biochemicals).

## 2.3. Changes of PrxIII and other Prx genes

Expression changes of PrxIII and other Prx genes in PrxIII-knockdown cells were analyzed by Western blot and quantitative real-time PCR (qRT-PCR) as described previously [10]. The primer sequences for qRT-PCR were summarized in Table 1. We used mouse monoclonal anti-2 Cys Prx antibody to recognize 2-cys Prxs (1:1000 dilution, Abcam Biochemicals).

## 2.4. Detection of ROS level

Detection of ROS level was performed as previously described [11]. Briefly, harvested cells at passage 3 were re-cultured overnight in six-well plates ($1 \times 10^6$ cells/ml) with 4 ml of serum- and phenol red-free medium per well. After incubation for 20 min with 2 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes), the ROS levels in cancer cells and the adjacent normal epithelia were respectively estimated at indicated time points through the oxidation of DCFH-DA. After siRNA experiment, we detected ROS level in six PrxIII-knockdown cancer cell lines and six control cancer cell lines respectively.

## 2.5. Evaluation of cell apoptosis and viable cell number

After confirmation of PrxIII down-regulation, cancer cells were harvested and sub-cultured in 96-well plates at a density of $1 \times 10^4$ cells/ml for 48 h. Cell apoptosis was detected by flow cytometry using Annexin V-FITC Kit (Invitrogen). In addition, we used Cell Counting Kit-8 (CCK8, Dojindo Laboratories, Japan) to evaluate the number of viable cells. This assay was based on the conversion of tetrazolium salt WST-8 to formazan. The amount of yellow-colored formazan generated by the activity of mito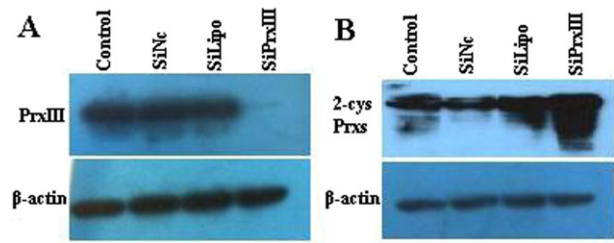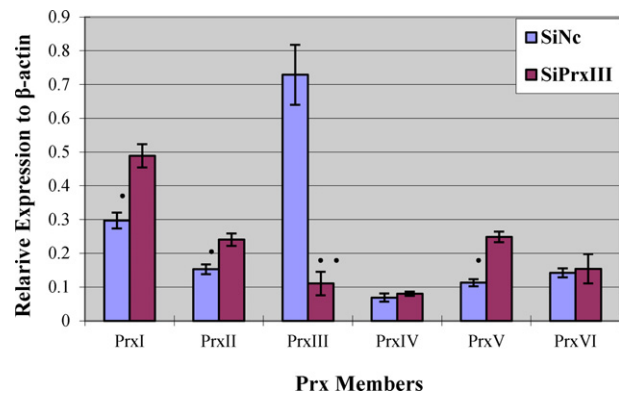chondrial dehydrogenases in cells was directly proportional to the number of living cells. OD450nm was assayed at indicated time points.

## 2.6. Statistical analysis

Data was presented as mean ± SD of three separate experiments. Cell apoptosis, viable cell number, and ROS level between PrxIII down-regulated cells and controls were compared by analysis of variance. It was considered a significant difference when $P < 0.05$.

## 3. Results

### 3.1. Knockdown of PrxIII expression induced up-regulation of other Prx genes

Among the 10 patients with stage IA cervical cancer, six cases were successfully cultured for both cancerous cells and adjacent normal epithelia. We used these cells (tentatively called "cell lines") to perform siRNA experiment. As shown in Fig. 1(A), PrxIII expression in cancer cells was significantly down-regulated after siRNA. Interestingly, other Prx members were significantly up-regulated after knockdown of PrxIII expression (Fig. 1(B)). qRT-PCR analysis recognized that the up-regulated Prx members included PrxI, PrxII, and PrxV (Fig. 2). We achieved the similar effectiveness of PrxIII down-regulation and observed the similar compensation of other Prx members in adjacent normal epithelia (data not shown).

### 3.2. ROS level was increased in PrxIII down-regulated cancer cells

We compared the ROS level in the six cancer cell lines to that in the six epithelial cell lines. As shown in Fig. 3(A), the ROS production
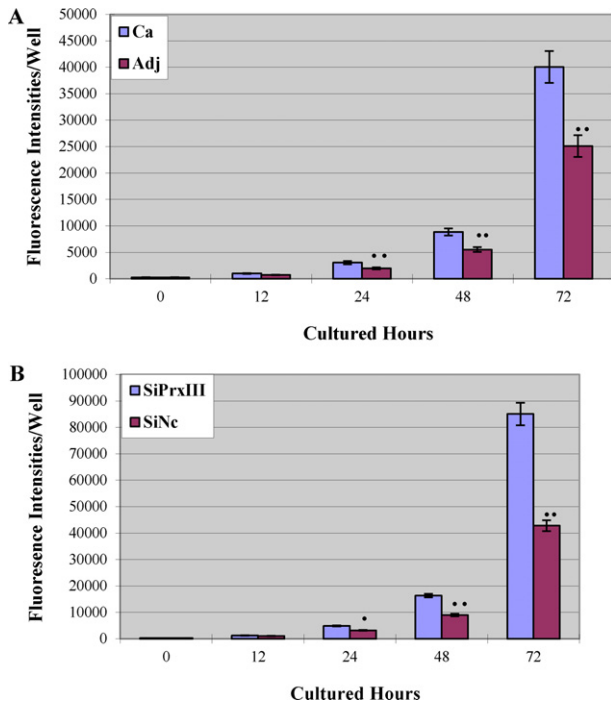
**Fig. 4.** The number of viable cervical cancer cells along with culture time analyzed by Cell Counting Kit-8 (CCK8). We detected significant decrease of viable cell number after PrxIII down-regulation (SiPrxIII vs. SiNc: *$P < 0.05$ at 48 h and 72 h points respectively).

**Fig. 3.** Detection of ROS level in cervical cells by DCF fluorescence. (A) The ROS production in cervical cancer cells (Ca) was higher than that in the adjacent normal epithelia (Adj) and the difference was increasing with culture time (Ca vs. Adj: **$P < 0.01$ from 24 h point). (B) After down-regulation of PrxIII in cervical cancer cells, the ROS level was increased and reached a significant difference from 24 h point as compared with controls (SiPrxIII vs. SiNc: *$P < 0.05$ at 24 h point, **$P < 0.01$ at 48 h and 72 h points respectively).

was higher in cervical cancer cells than in adjacent normal epithelia and the difference was increasing with culture time (Ca vs. Adj: $P < 0.01$ from 24 h point). To examine the significance of PrxIII in removing ROS, we compared the ROS level in cancerous cells before and after knockdown of PrxIII. As indicated in Fig. 3(B), the ROS level was significantly increased in PrxIII down-regulated cancer cells as compared with control cancer cells (SiPrxIII vs. SiNc: $P < 0.05$ at 24 h-point; $P < 0.01$ at 48 h and 72 h-points respectively).

### 3.3. Cell apoptosis was enhanced after knockdown of PrxIII expression

To investigate the significance of PrxIII in apoptotic regulation, we compared the apoptotic percentage in six cancer cell lines with PrxIII-knockdown to that in six cancer cell lines without PrxIII-knockdown. After transfection of SiPrxIII, the apoptotic percentage was significantly higher (5.64 ± 1.76%) as compared with controls (0.67 ± 0.53%) (SiPrxIII vs. SiNc: $P < 0.01$). At the same time, we observed significant decrease of viable cell number after PrxIII down-regulation (Fig. 4, SiPrxIII vs. SiNc: $P < 0.05$).

### 4. Discussion

In the present study, we provided direct evidence that the ROS production was increased in cervical cancer cells as compared with normal epithelia. After down-regulation of PrxIII expression, we noticed further increase of the ROS level and subsequent apoptosis in cancer cells, although other Prx members provided compensation to some extents. Our results suggest that PrxIII is an indispensable ROS scavenger in cervical cancer cells.

Because of active and indefinite growth of cancer cells, ROS production from mitochondria is accordingly increased [12]. It has been reported that excessive ROS may result in disruption of mitochondrial
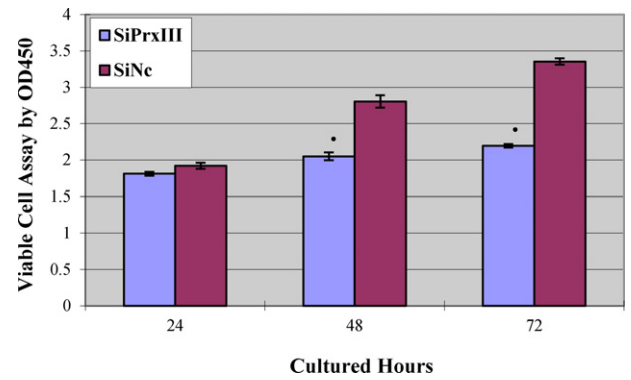
membrane permeabilization and alteration of mitochondrial membrane transition pores, which leads to the release of apoptotic effectors [13–15]. Since mitochondrion is the main apoptotic mediator, it is imaginable that the control of ROS level by PrxIII is involved in apoptotic inhibition. In fact, accumulating studies have suggested that PrxIII plays an active inhibitory role in chemical-induced oxidation and subsequent apoptosis [16–18]. According to the report by Cox et al., PrxIII provided the first-line response to oxidative stress and acted as a "trigger" in the apoptosis-signaling pathway of cancerous cells [19].

By using cervical cancer cell lines, Shih et al. and Chang et al. demonstrated that PrxIII was responsive to oxidation-inducing chemicals and protected against oxidative apoptosis [20,21]. To the best of our knowledge, there have been few reports concerning the role of PrxIII in cervical cancer cells at "natural state". In the present study, we successfully cultured primary cervical cancer cells and showed that PrxIII silence in cervical cancer cells resulted in increased apoptosis and decreased proliferation. Our results suggest that PrxIII plays an important role in apoptotic inhibition of cervical cancer cells by controlling ROS level. This study can explain at least partially the underlying mechanism for the over-expression of PrxIII in diverse type carcinomas including cervical cancer. Fast growing cancer cells produce large amount of ROS that disturb the redox balance. As an active responder to oxidative stress, PrxIII is accordingly up-regulated to remove cellular ROS and inhibit apoptosis, which is beneficial to cancerous growth and invasion. Since most of chemotherapy or radiotherapy for cancers is through ROS increase and apoptotic induction, our results have provided a clue that PrxIII may be involved in the chemotherapeutic resistance of cervical cancer.

We previously reported that PrxI provided a compensatory function in the absence of PrxIII in mouse erythrocytes [10]. In the present study, we showed that PrxIII silence in cervical cancer cells resulted in the up-regulation of PrxI, PrxII, and PrxV. Our result implies that the members of Prx family respond cooperatively to oxidative stress in cervical cancer cells.

### 5. Conclusion

Combining the present study with previous reports of PrxIII over-expression in cervical cancer [8,9], we conclude that PrxIII is an important regulator of intracellular ROS, which provides a favorable microenvironment for tumor growth and protects against oxidation-induced apoptosis. Our results have provided a new clue to the understanding of the mechanism for tumor progression or recurrence.

## Acknowledgement and funding

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