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Peroxiredoxin 1 is involved in disassembly of flagella and cilia

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ABSTRACT

Cilia/flagella are evolutionarily conserved cellular organelles. In this study, we demonstrated that *Dunaliella salina* Peroxiredoxin 1 (DsPrdx1) localized to the flagella and basal bodies, and was involved in flagellar disassembly. The link between DsPrdx1 and flagella of *Dunaliella salina* (*D. salina*) encouraged us to explore the function of its human homologue, *Homo sapiens* Peroxiredoxin 1 (HsPrdx1) in development and physiology. Our results showed that HsPrdx1 was overexpressed, and cilia were lost in esophageal squamous cell carcinoma (ESCC) cells compared with the non-cancerous esophageal epithelial cells Het-1A. Furthermore, when HsPrdx1 was knocked down by short hairpin RNA (shRNA) lentivirus in ESCC cells, the phenotype of cilia lost can be reversed, and the expression levels of tumor suppressor genes LKB1 and p-AMPK were increased, and the activity of the oncogene Aurora A was inhibited compared with those in cells transfected with scrambe-shRNA lentivirus. These findings firstly showed that Prdx1 is involved in disassembly of flagella and cilia, and suggested that the abnormal expression of the cilia-related gene including Prdx1 may affect both ciliogenesis and cancernogenesis.

1. Introduction

Cilia (also known as flagella) are microtubule-based signaling platforms that protrude as filiform organelles from the surface of cells, and are highly conserved in organisms ranging from *Chlamydomonas reinhardtii* (*C. reinhardtii*), *Drosophila melanogaster*, *Caenorhabditis elegans* and to all vertebrates so far examined [1]. Formerly, the cilia have been viewed as an evolutionary vestige in vertebrates. But, recent studies revealed that the cilia are sensory organelles, which act as machinery and chemical receptors [2–3]. Additional evidences showed that the cilia play important roles in several crucial signaling pathways of cancernogenesis, tissue development and cell homeostasis, such as, PDGFαα, Hedgehog, Wnt and mTOR [4–7]. Furthermore, it has been indicated that cilia are predominantly lost or disassembly in cancers including renal cell, breast, glioblastoma cells and pancreatic cancers in comparison with their normal cellular counterparts [8–11]. Until now, evidence has not been reported on whether the cilia are lost in esophageal squamous cell carcinoma (ESCC) cells.

Previous studies on *Chlamydomonas* and *Dunaliella* have begun to dissect the process of flagellar disassembly or assembly [12–15], i.e., identified that *C. reinhardtii* Aurora-like kinase (CALK) was an essential factor for flagellar disassembly [16], End-binding protein 1 (EB1) localized to flagellar tip and basal bodies of *C. reinhardtii* [17] and was required for cilia assembly in fibroblasts [18], and S-adenosylhomocysteine hydrolase (SAHH) accelerated flagellar regeneration of *D. salina* [19]. Additional investigations also showed that Liver kinase B 1 (LKB1) and its target AMP-activated protein kinase (AMPK) localize to the cilium or basal body of epithelial cells [20], and it negatively regulates HEF1-Aurora A-HDAC6 signaling pathway [21,22], which contributes to ciliary disassembly in hTERT-RPE1 cells [23].

Peroxiredoxin 1 (Prdx1) is an ubiquitously expressed protein for scavenging reactive oxygen species (ROS) in cells, and overexpressed in many cancers [24–28], but its biology functions in cancers remain enigmatic. In this study, we found that *D. salina* Peroxiredoxin 1 (DsPrdx1) localized to the flagella and basal bodies, and was involved in flagellar disassembly in *D. salina*. The link between DsPrdx1 and flagella of *D. salina* encouraged us to explore the function of *Homo sapiens* Peroxiredoxin 1 (HsPrdx1) in development and physiology. The results showed that HsPrdx1 was overexpressed and the cilia were lost in ESCC cells compared with the non-cancerous esophageal epithelial cells Het-1A cells. The ciliogenesis and the expression levels of LKB1 and p-AMPK were increased, while the activity of Aurora A was inhibited in HsPrdx1-knockdown ESCC cells. These findings suggest that Prdx1 may play important roles in disassembly of cilia, and this study establishes a link between ciliary disassembly mediated by cilia-related genes

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and cancernogenesis, which is likely to provide not only new sights into the molecular mechanism of cancernogenesis but also potential therapeutic or diagnostic targets for cancer patients.

2. Materials and methods

2.1. Cell line and cell cultures

ESCC cell lines Eca109 and EC9706 were purchased from the State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Sciences. The control cell was non-cancerous esophageal epithelial cell Het-1A, which immortalized by transfection with plasmid pRSV-T containing the RSV-LTR promotor and SV40 T-antigen [29] was kindly provided by Professor Zhenyu Ji (Henan Academy of Medical and Pharmaceutical Sciences, Zhengzhou University, Henan, PR China). Each of the cell lines was cultured in RPMI 1640 medium (Gibco-BR2, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, USA), at 37 °C in the presence of 5% CO_2.

2.2. Antibody preparation and Western blotting of D. salina

The DsPrdx1 cDNA fragment (GenBank accession number KC999111) was subcloned into the prokaryotic protein expression vector pET28a (+), and expressed in competent cells of *Escherichia coli* (*E. coli*) BL21 to produce fusion DsPrdx1 proteins. Transformants were cultured in Luria–Bertani medium for 3 h, and then isopropyl β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was added to induce the expression of the fusion proteins. The inclusion bodies were purified using Ni-IDA Sefinose Kit (BBI, Canada), and the anti-DsPrdx1 polyclonal antibodies were prepared in rabbit using purified recombinant fusion proteins. In Western blotting, protein samples were subjected to 12% SDS–PAGE gels and transferred to PVDF membranes (Millipore, USA), which were blocked in 5% skimmed milk in TBST buffer, and then incubated with the anti-DsPrdx1 polyclonal antibody (1:2000), and the horseradish-peroxidase labeled secondary antibodies (1:3000, Sangon Biotech, China). Finally, the bands of specific proteins on the membranes were detected by Enhanced Chemiluminescence Kit (Santa Cruz, USA).

2.3. Protein extraction and treatment of D. salina cells

The *D. salina* cells were treated with 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, USA) at a final concentration of 1 mM to induce flagellar disassembly [30,31]. Flagella were detached from *D. salina* cell bodies by pH shock method, and purified by sucrose density gradient centrifugation as described previously [13]. Total proteins were extracted from cells of *D. salina* at particular times during IBMX treated, and the DsPrdx1 protein in cell bodies (CP) and flagella (FL) was examined by Western blotting.

2.4. Examination of DsPrdx1 localization

The *D. salina* cells were collected and fixed by 2% glutaraldehyde in the centrifuge tube, and then they were washed with PBS at room temperature (RT). The cells were smeared on the slides and air-dried, and the slides were treated with 0.5% Nonidet P 40 (NP-40) for 5 min and washed by water and ice-Acetone three times with every time for 5 min. Subsequently, the slides were incubated with anti-DsPrdx1 antibody of 1:200 diluted with antibody diluent at 37 °C for 2 h. The slides were rinsed with PBS and incubated with FITC-conjugated goat anti-rabbit IgG (1:100, Sangon Biotech, China) at 37 °C for 1 h. Stained cells were viewed under confocal laser scanning biological microscope (LEICA, TCS-SP2).

2.5. Lentiviral production and transduction

Small interfering RNA (siRNA) targeting HsPrdx1 (GenBank accession number: NM_181696) sequence (TGTCTGACTACAAAGGAAA) and a scrambe sequence (TTCTCCGAACGTGTCACGT) were transformed into short hairpin RNA (shRNA) (stem-loop-stem structure) and were cloned into GV112-Lentivirus vectors with *Age* I/*EcoR* I sites, respectively. All the recombinant lentiviral particle and transduction were prepared as described previously [32]. A final concentration of 5 μg/mL Polybrene and 10 μL of concentrated packaged lentiviral particle were added to the cells, allowed to incubate for 12 h, and then the medium was changed for fresh growth medium and the cultures were collected for experiments at 72 h after transfection.

2.6. Detection of cilia by immunofluorescence in ESCC cells

To induce visible cilia, the cells were grown to 80% confluence in 24-well plate, and then replaced for fresh medium without serum for 24 h. The cells were fixed with 4% paraformaldehyde for 30 min, and incubated with 200 μL glycine (2 mg/mL) for 5 min. The cells were permeabilized with 0.5% Triton-X 100 in PBS, and then blocked in blocking solution (5% goat serum and 0.5% Triton-X 100 in PBS) for 1 h. The cells were incubated with the monoclonal antibody Acetyl-α-Tubulin (1:500, Cell Signaling Technology) for 2 h. After cells were washed with PBS three times, followed incubated with secondary antibody Goat Anti-Rabbit IgG/FITC (1:500, Sangon Biotech, China). Nuclei were stained by Hoechst 33342 at RT for 30 min in the dark. Stained cells were viewed under inverted immunofluorescence microscope (OLYMPUS I, X71). Quantitation was performed on at least three independent experiments, counting about 100–200 cells per sample each time.

2.7. Western blotting of ESCC cells

Protein concentrations were determined using Bradford protein assay kit (Sangon Biotech, China) as a standard. Typically, 10–15 μg of proteins were subjected to each lane. Each of the antibodies sera against HsPrdx1 (Cell Signaling Technology, USA), LKB1 (Cell Signaling Technology, USA), phospho-AMPKα^{Thr172} (Cell Signaling Technology, USA), phospho-Aurora A^{Thr288} (Cell Signaling Technology, USA) and GAPDH (Cell Signaling Technology, USA) were used at 1:1000, and the horseradish-peroxidase labeled secondary antibodies (Sangon Biotech, China) at 1:3000. Finally, the bands of specific proteins on the PVDF membranes were detected by Enhanced Chemiluminescence Kit (Santa Cruz, USA).

2.8. Statistical analysis

All experiments were repeated at least three separate experiments, and the data were performed using SPSS version 17.0 (SPSS, Chicago, USA). Results were expressed as means ± standard deviations. In all statistical analyses, a P value <0.05 was considered statistically significant and was two-sided.

3. Results

3.1. Function and localization of DsPrdx1

Polyclonal antibodies of DsPrdx1 were prepared against the purified prokaryotic expressed protein and used in Western blotting and immunolocalization experiments. As shown in Fig. 1B, DsPrdx1 antibodies specifically recognized a single band of the predicted molecular weight of DsPrdx1 at ~22 kDa, and the Western blotting result also showed that DsPrdx1 existed in the

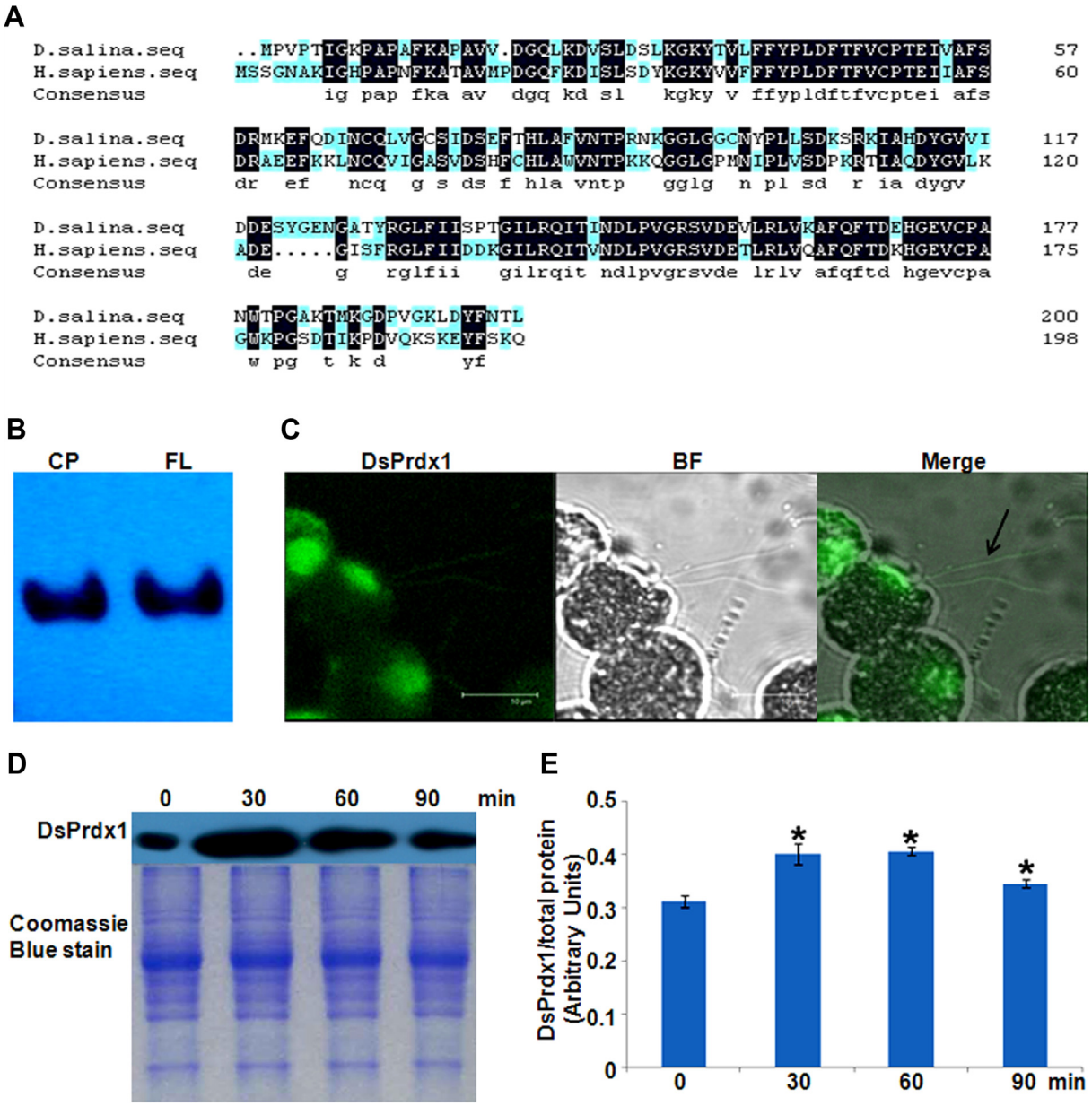

Fig. 1. Alignment analysis, localization and the function of DsPrdx1. (A) Amino acid sequence alignment was made by DNAMAN between the deduced DsPrdx1 and *Homo sapiens* Prdx1. Identical and similar amino acid residues were dark shaded and shown in the line of consensus. (B) The existence of DsPrdx1 in the flagella of *D. salina*. Cell proteins (CP) and flagella proteins (FL) were extracted from *D. salina* cells of the same batch, subjected to SDS–PAGE gels and detected with anti-DsPrdx1 antibody, respectively. (C) Cellular localization of DsPrdx1 by indirect immunofluorescence. The bright-field micrograph (BF) indicated the position of flagella and the merged image showed that the DsPrdx1 was localized to flagella and basal bodies of *D. salina* cell. (D) Western blotting of DsPrdx1 during flagellar disassembly. The amount of proteins in each assay was indicated by the coomassie brilliant blue-stained SDS–PAGE gels for loading control. (E) Semi-quantitated values of Western blotting experiments were statistically analyzed. $^{*}P < 0.05$, compared to those of *D. salina* cells which treated with IBMX 0 min.

isolated *D. salina* flagella. Furthermore, DsPrdx1 was observed to be localized in the flagella and basal bodies of *D. salina* (Fig. 1C) by immunolocalization, suggesting that it may be related to flagellar assembly or disassembly. In the present study, the expression levels of DsPrdx1 during flagellar disassembly were respectively examined by Western blotting (Fig. 1D), and the results showed that the expression level of DsPrdx1 was increased obviously at 30 min (Fig. 1E) after flagella disassembly which further demonstrated that DsPrdx1 may be involved in flagellar disassembly.

3.2. Cilia are lost in ESCC cells

Homologous analysis revealed that the putative protein DsPrdx1 shared high identities with the Prdx1 from *H. sapiens* (65%) (Fig. 1A). Thus we want to know whether the protein of HsPrdx1 plays a similar function in human cilia as DsPrdx1 does in *D. salina* flagellar disassembly. The protein expression levels of HsPrdx1 were firstly examined by Western blotting in esophageal cells (Fig. 2A), and the results demonstrated that HsPrdx1 was increased to ~2.12-fold ($P < 0.001$) and ~1.72-fold ($P < 0.001$) in EC9706 and Eca109 cells compared with that in Het-1A cells, respectively (Fig. 2B).

After serum starved for 24 h, the primary cilia of esophageal cells were examined using immunofluorescence with antibodies against acetylated α-tubulin (red), a cilia/flagellar marker [33], and counterstained with Hoechst 33342 (blue) (Fig. 2c–e). As shown in Fig. 2f, primary cilia were observed at a lower frequency in Eca109 (14.27%, $n = 496$) and EC9706 (12.52%, $n = 576$) cells

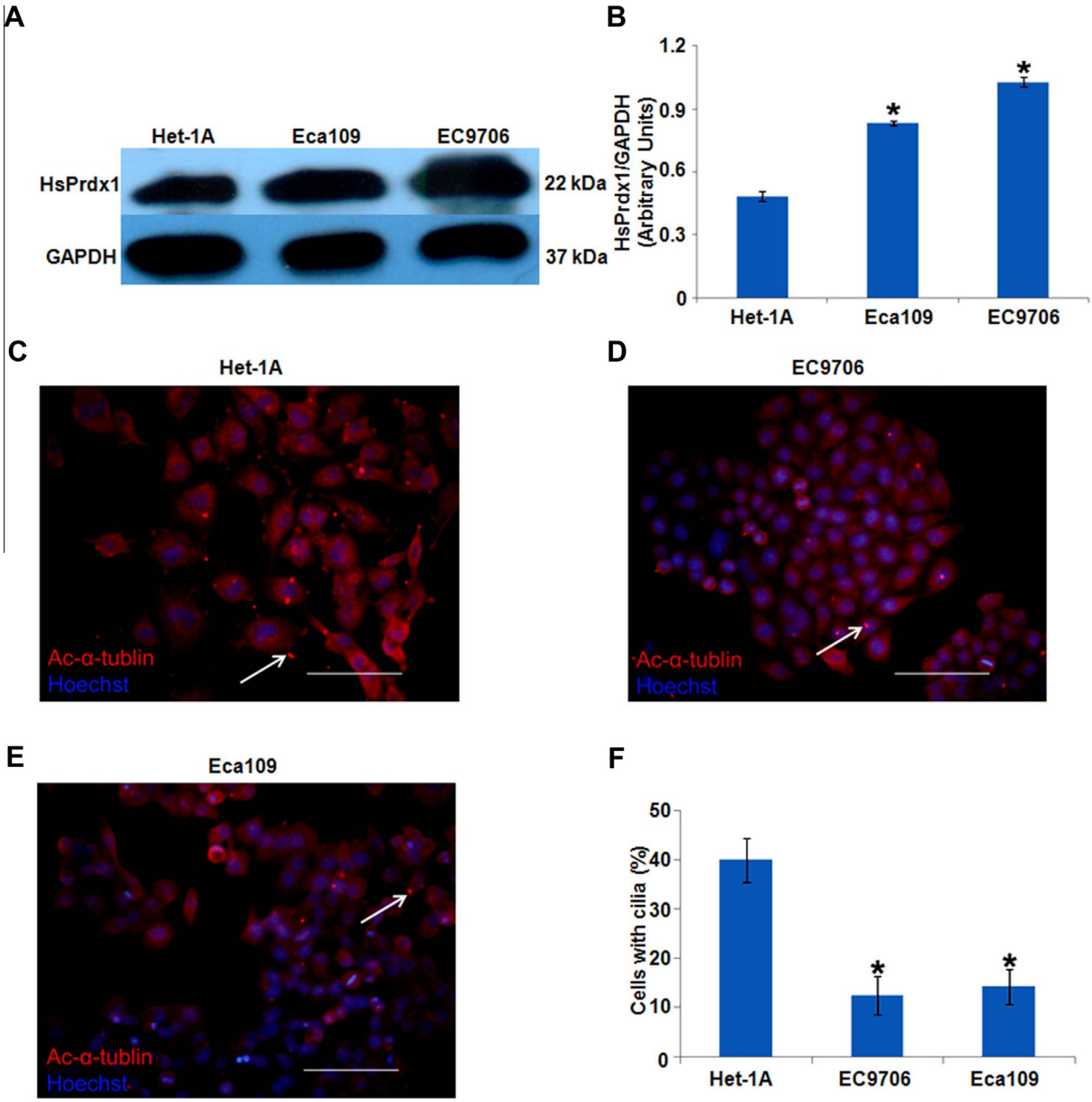

Fig. 2. HsPrdx1 expression and quantitative analysis of ciliated cells of ESCC cells. (A) The expression level of HsPrdx1 protein in EC9706, Eca109 and Het-1A cells was examined by Western blotting. (B) Semi-quantitated values of Western blotting experiments were statistically analyzed. $^{*}P < 0.05$, compared to those of Het-1A cells. (C–E) Cilia were visualized in Het-1A, EC9706 and Eca109 cells with antibodies specific for acetylated α-tubulin (red) and indicated with white arrows. Nuclei were stained by Hoechst 33342 (blue). Scale Bars: 100 μm. (F) Quantitative analysis of ciliated cells of ESCC cells and Het-1A cell. $^{*}P < 0.05$, compared to those of Het-1A cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compared with that in Het-1A cells (39.95%, $n = 431$) ($P < 0.05$). Taken together with the discoveries above that HsPrdx1 overexpressed in ESCC cells while primary cilia lost in ESCC cells, we conjecture that there may be a relationship between HsPrdx1 overexpression and ciliary loss.

3.3. Ciliogenesis is restored in HsPrdx1-suppressing ESCC cells

To test for a direct relationship between HsPrdx1 and cilia, we observed the cilia changes in ESCC cells when the expression of HsPrdx1 was inhibited by transfecting HsPrdx1-shRNA lentivirus. The Western blotting results showed that the protein levels of HsPrdx1 were reduced to ~2.52-fold and ~1.43-fold in HsPrdx1-suppressing cells of EC9706 and Eca109 compared with scramble-shRNA cells, respectively (Fig. 4b and d). Consequently, 24.73% ($n = 454$) of the EC9706 cells were ciliated after transfecting with HsPrdx1-shRNA lentivirus, but only 11.11% ($n = 580$) of the control cells were ciliated ($P < 0.05$), similarly, 25.67% ($n = 447$) of the Eca109 cells were ciliated after HsPrdx1 inhibition, but only 11.32% ($n = 578$) of the control cells were ciliated ($P < 0.05$) (Fig. 3e). These results suggest that ciliogenesis is able to be restored in HsPrdx1-suppressing ESCC cells, and these results provide the evidence that the lost of primary cilia in ESCC cells is directly or indirectly mediated by the overexpression of HsPrdx1 in ESCC cells.

3.4. The activity of LKB/AMPK and Aurora A were regulated by inhibition of HsPrdx1 in ESCC cells

To test whether HsPrdx1 inhibited the ciliogenesis by regulating LKB1-Aurora A pathway, we examined the expression changes of LKB1, p-AMPK and p-Aurora A using Western blotting in

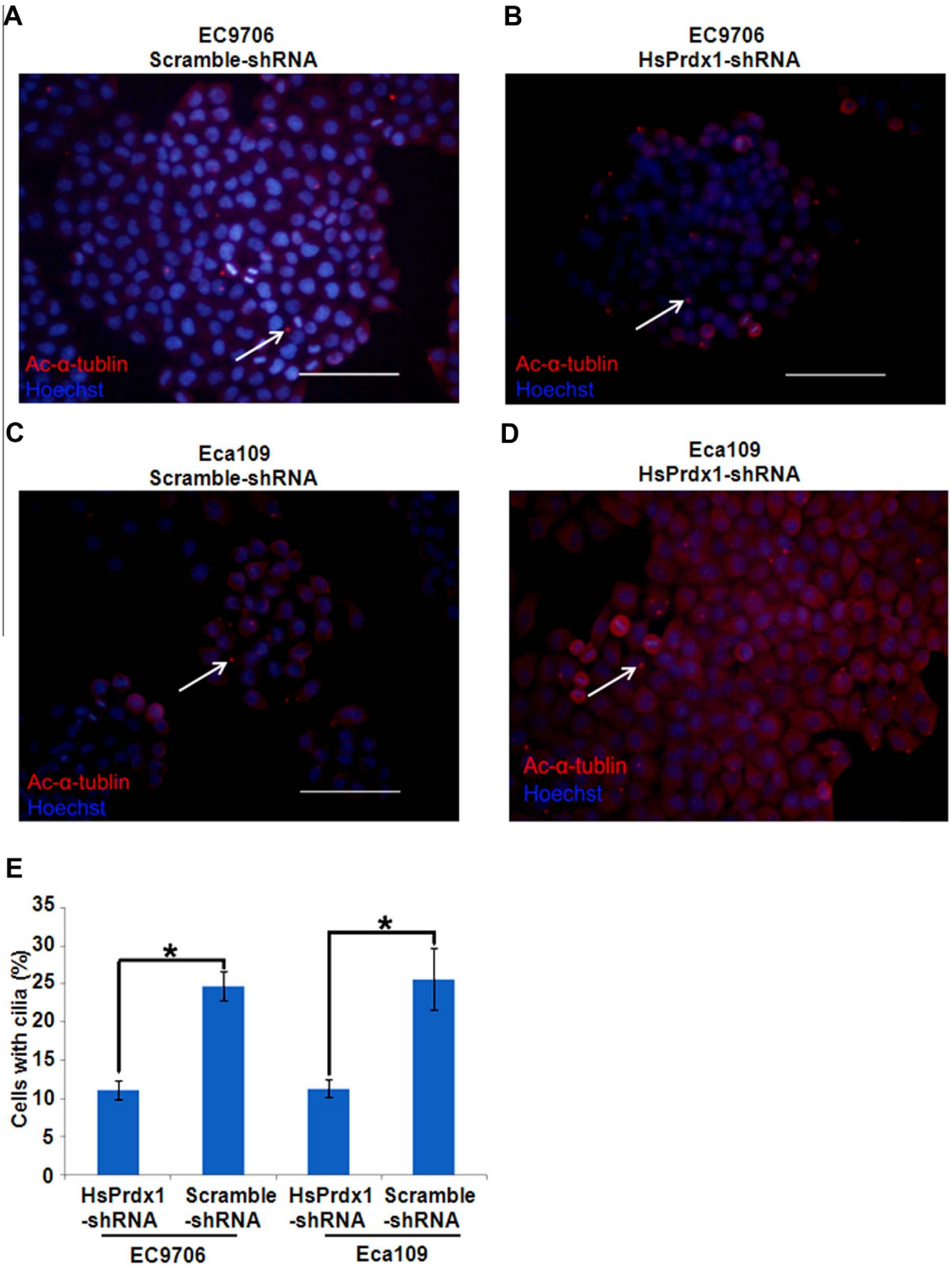

Fig. 3. Ciliogenesis was restored in HsPrdx1-suppressing ESCC cells. (A–D) Cilia were visualized in HsPrdx1-suppressing EC9706 and Eca109 cells and their control cells with antibodies specific for acetylated α-tubulin (red) and indicated with white arrows. Nuclei were stained by Hoechst 33342 (blue). Scale Bars: 100 μm. (E) Quantitative analysis of ciliated cells of HsPrdx1-suppressing EC9706 and Eca109 cells. $^{*}P < 0.05$, compared to those of control cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HsPrdx1-suppressing ESCC cells (Fig. 4a and c). The protein levels of LKB1 were increased to ~1.12-fold and 2.01-fold in EC9706 HsPrdx1-suppressing cells and Eca109 HsPrdx1-shRNA cells compared with their control cells ($P < 0.05$), respectively (Fig. 4b and d). And the protein levels of p-AMPK were increased to ~1.13-fold and ~4.22-fold in EC9706 and Eca109 HsPrdx1-shRNA cells compared with their control cells ($P < 0.05$), respectively (Fig. 4b and d). Furthermore, the expression level of p-Aurora A were decreased to ~1.79-fold and ~1.36-fold in EC9706 and Eca109 HsPrdx1-shRNA cells compared with their control cells ($P < 0.05$), respectively (Fig. 4b and d). These results indicate that ciliary disassembly related signaling pathway LKB1-Aurora A can be regulated by

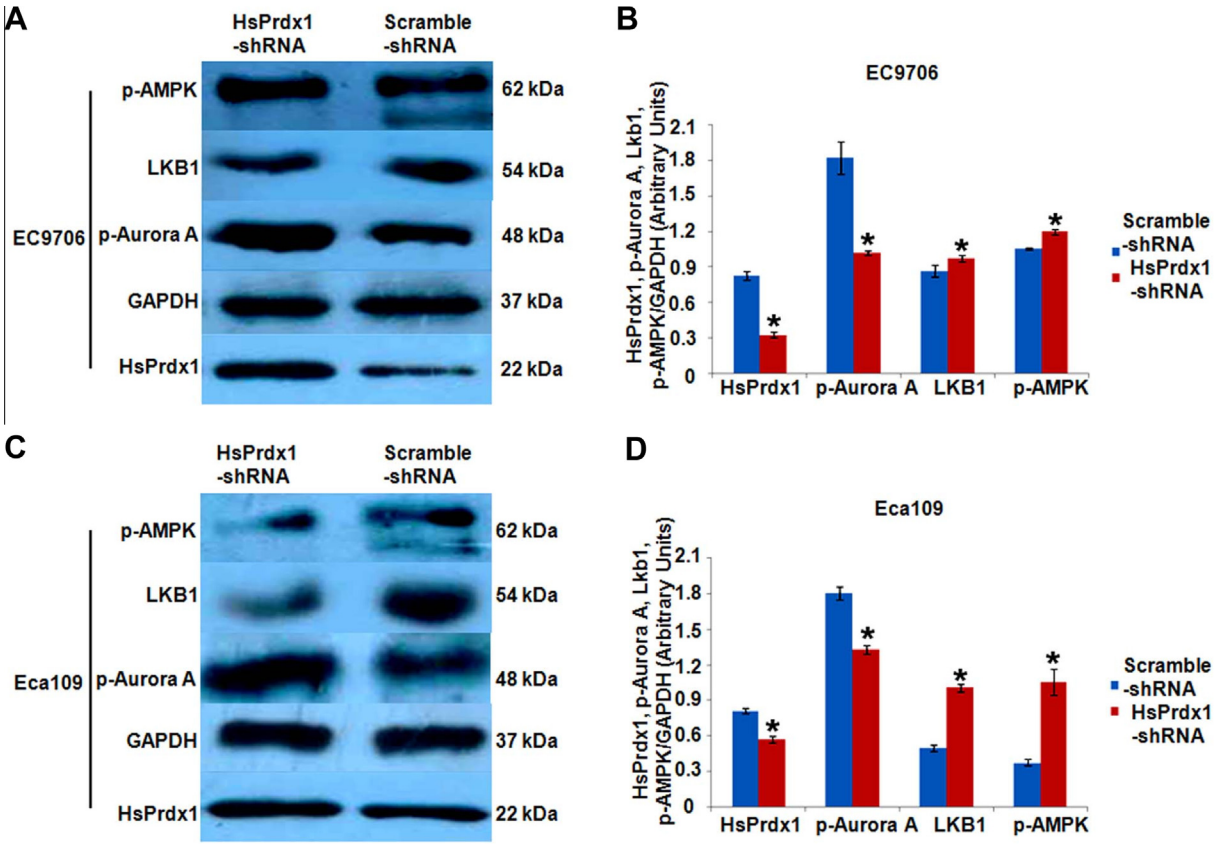

Fig. 4. LKB1-AMPK-Aurora A pathway was inhibited in HsPrdx1-suppressing ESCC cells. (A and C) The expression levels of HsPrdx1, LKB1, p-AMPK and p-Aurora A proteins were examined by Western blotting in HsPrdx1-suppressing EC9706 and Eca109 cells, respectively. (B and D) Semi-quantitated values of Western blotting experiments (A and C) were statistically analyzed, respectively. $^{*}P < 0.05$, compared to control cells.

HsPrdx1 inhibition, and further confirmed that HsPrdx1 is involved in ciliary disassembly.

4. Discussion

Cilia/flagella are evolutionarily conserved microtubule-based organelles and emanate from the apical membrane of cells. Over the past decade, the relationship between primary cilia and human diseases has been revealed gradually. Several cancernogenesis crucial signaling pathways for hedgehog, Wnt and mTOR associate with the cilia, thus we are attracted by the relationship between ciliary loss or disassembly and cancernogenesis. Loss of cilia is frequently observed in human disorders including cancers and polycystic kidney disease. In this study, firstly, we demonstrates that DsPrdx1 existed in *D. salina* flagella by both Western blotting and immunofluorescence results, and the protein level of DsPrdx1 increased obviously during the flagella disassembly and reached to the maximum at 30 min, suggesting that DsPrdx1 is involved in flagellar disassembly. As a connection, the DsPrdx1 protein shared high identities with the HsPrdx1, thus it is reasonable to examine the relationship between the expression level of human HsPrdx1 in ESCC cells and cilia disassembly. As we predicted that HsPrdx1 is overexpressed, and the cilia are lost in ESCC cells compared with the control Het-1A cells, and ciliogenesis is restored in HsPrdx1-suppressing ESCC cells, indicating that HsPrdx1 may be implicated in cilia loss in esophageal cancer.

Recent studies revealed that LKB1 may be an regulator of ciliary disassembly through control of HEF1-Aurora A-HDAC6 signaling pathway [22], and additional evidences further demonstrated that primary cilia control mTOR pathway and cell size through LKB1/AMPK [20]. Here, our results showed that expression level of LKB1 and p-AMPK was increased, and p-Aurora was decreased in HsPrdx1-suppressing ESCC cells, suggesting that HsPrdx1 mediated ciliogenesis may be through LKB1/AMPK-Aurora A.

As we known, LKB1 is a tumor suppressor, and Aurora A is an oncogene in cancers. In the present study, the expression level of LKB1 is increased, and the activity of Aurora A is decreased in HsPrdx1-suppressing cells, suggesting that inhibition of HsPrdx1 may be contributed to cancer therapy. Indeed, previous study confirmed that inhibition of HsPrdx1 induced cell apoptosis in cancers [34]. In addition, recent reports indicated that inhibition or depletion of CCRK, HEF1, Aurora A or HDAC6 could restore ciliogenesis, and decrease the cell proliferation of cancer cells [35–37]. Thus, restoration of ciliogenesis by inhibiting of ciliary disassembly related genes in cancer cells may become a novel, promising therapeutic strategy for cancers in the future.

Acknowledgments

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