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Original Article

Ring box protein-1 mediates the disassembly of cilia in esophageal squamous cell carcinoma cells

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Abstract: Cilia are structurally and evolutionarily conserved organelles in many cells with an origin similar to that of basal bodies. Recent studies suggest that nearly all types of human cells have cilia. Cilia are predominantly lost in cancer cells. Our previous study reported that the ubiquitin-proteosome was probably associated with ciliary disassembly. As part of the ubiquitin-proteasome complex, RBX1 may respond to ciliary disassembly in esophageal squamous cell carcinoma (ESCC) cells. Compared with Het-1A, which represents the non-cancerous esophageal epithelial cells, cilia are lost in ESCC cells. Therefore, we explored the function of RBX1 in the ciliary disassembly of ESCC cells. Our results showed that RBX1 was overexpressed in ESCC cells of Eca109, EC1 and EC9706. Furthermore, the suppression of RBX1 by the shRNA lentivirus in ESCC cells reversed the phenotype of lost cilia. The expressions of the tumor suppressors LKB1 and AMPK were increased, while the oncogene Aurora A was inhibited in shRBX1 ESCC cells. These findings initially demonstrated that RBX1 mediated the disassembly of human cilia and suggested that the abnormal expression of the cilia-related gene RBX1 influenced cilia formation and the initiation of cancer.

Keywords: RBX1, esophageal squamous cell carcinoma, ciliary disassembly

Introduction

Cilia are structurally and evolutionarily conserved organelles in many cells. Cilia were once thought to be vestigial structures in vertebrates but are now confirmed as important sensory organelles [1, 2]. Recent studies have revealed that nearly all types of human cells possess cilia [3]. The intraflagellar transport (IFT) machinery was initially identified in *Chlamydomonas reinhardtii*, and later in the cilia of higher organelles [4, 5]. Primary cilia play a central role in different signaling pathways, such as PDGFαα, hedgehog, and mTOR [6-9]. In addition, cilia are predominantly lost in prostate cancer, glioblastoma, and breast and renal cell cancers [10-13]. The hypothesis that dysfunctional cilia result in tumorigenesis has not been well proven.

Previous studies of *C. reinhardtii* and *Dunaliella salina* found that the *C. reinhardtii* Aurora-like kinase is a crucial factor for flagellar disassembly [14]. Further investigations also established that LKB1 and its target AMPK are localized to the cilium or the basal body of epithelial cells [15] and inhibit the HEF1-AuroraA-HDAC6 signaling pathway [16, 17], which contributes to ciliary disassembly in hTERT-RPE1 cells [18]. Furthermore, recent studies have also indicated that the expression of LKB1, AMPK and Aurora A regulate ciliary disassembly in ESCC cells [19].

The SCF E3 ubiquitin ligases, consisting of Skp1, cullins, F-box proteins, and the RING domain containing Ring box protein-1 (RBX1), represent the largest family of E3 ubiquitin ligases and are critical regulators of cellular processes under pathological and physiological conditions [20, 21]. As a crucial subunit of SCF E3 ubiquitin ligases, RBX1 binds to ubiquitin-loaded E2 at its C terminus and cullins at its N terminus and catalyzes the transfer of ubiquitin from E2 ubiquitin ligases to specific substrates for protein degradation [22, 23]. RBX1 is essen-

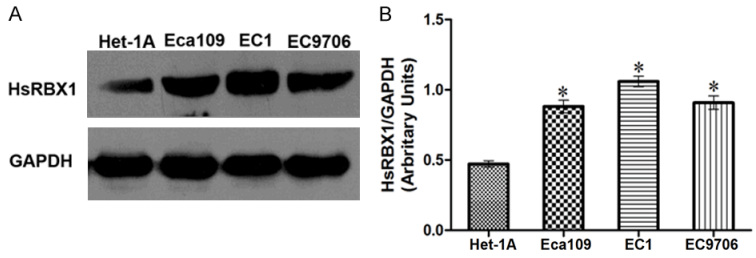

Figure 1. RBX1 expression in ESCC cells. A. The expression of RBX1 protein in Eca109, EC1, EC 9706 and Het-1A cells was analyzed by Western blot. B. Semi-quantitative values of Western blot were statistically analyzed. $P < 0.05$, compared with that of shCon cells.

tial for embryonic development of *Caenorhabditis elegans*, *Drosophila*, mice and humans by regulating cell proliferation and differentiation [21, 24, 25]. Our previous study reported that the ubiquitin-proteosome was probably associated with ciliary disassembly [26]. DsRBX1 responds to the flagellar disassembly of *Dunaliella salina* [27]. As part of the ubiquitin-proteosome system, RBX1 may respond to ciliary disassembly in esophageal squamous cell carcinoma (ESCC) cells. Recent studies suggested that RBX1 is overexpressed and plays a central role in a large number of human cancers, including liver, gastric, and bladder cancers [28-30]. Cilia are lost in ESCC cells compared with the noncancerous esophageal epithelial cells (Het-1A) [19]. Our results showed that RBX1 is overexpressed in ESCC cells, including Eca109, EC1, and EC9706, compared with Het-1A cells. The ciliogenesis and expression of LKB1 and AMPK are increased, while Aurora A was inhibited in shRBX1 cells compared with shCon cells. These findings suggest that RBX1 plays a significant role in the disassembly of cilia. This study established a link between ciliary disassembly mediated by cilia-related genes including RBX1 and carcinogenesis. The results provide important insights into the molecular mechanisms of carcinogenesis and potential therapeutic targets.

Materials and methods

Cell lines and cell cultures

ESCC cell lines Eca109, EC1 and EC9706 were provided by the Chinese Academy of Medical Sciences. The noncancerous esophageal epithelial cell line Het-1A was provided by Professor Zhenyu Ji (Henan Academy of Medical and Pharmaceutical Sciences, Henan, P. R. China). The cell lines were cultured in the RPMI 1640 medium (Gibco-BR2, USA) with 10% fetal bovine serum (HyClone Laboratories, Logan, USA) at 37°C in the presence of 5% CO_2, and the protein expression was detected using a Chemiluminescence Kit (Santa Cruz, USA).

Lentiviral production and transduction

The shRNA targeting RBX1 (GenBank accession number: NM_014248.3) sequence (TTCCACTGCATCTCTCGCT) and a scramble sequence (TTCTCCGAACGTGTCACGT) were cloned into GV-115-Lentivirus vectors with *Age* I/*Eco*R I sites, respectively. All the recombinant lentiviral particles were prepared as described previously [31]. The ESCC cells were incubated for 12 h at a final concentration of 5 μg/mL polybrene and 10 μL of packaged lentiviral particles, and the fresh growth medium was added to the cells, which were collected for experiments at 72 h after transfection.

Detection of cilia by immunofluorescence in ESCC cells

The cells were grown to 80% confluence in 24-well plates, and the fresh medium without serum was added to culture for 24 h to induce visible cilia. Detection of cilia was performed as previously described. The cells were incubated with a monoclonal antibody against acetyl-α-tubulin (1:300, Cell Signaling Technology) for 2 h, and incubated with a secondary antibody (1:1000, Sangon Biotech, China). Nuclei were stained with Hoechst 33342 for 30 min in the dark. Stained cells were viewed under an immunofluorescence microscope. Quantification was performed using at least three independent experiments.

Western blot

The protein concentrations were measured using a Bradford protein assay kit (Sangon Biotech, China) as a standard. Typically, 15-20 μg of proteins were run in each lane. The protein extracts were separated on 10% SDS-PAGE for RBX1 (Cell Signaling Technology, USA), LKB1 (Cell Signaling Technology, USA), AMPKα (Cell

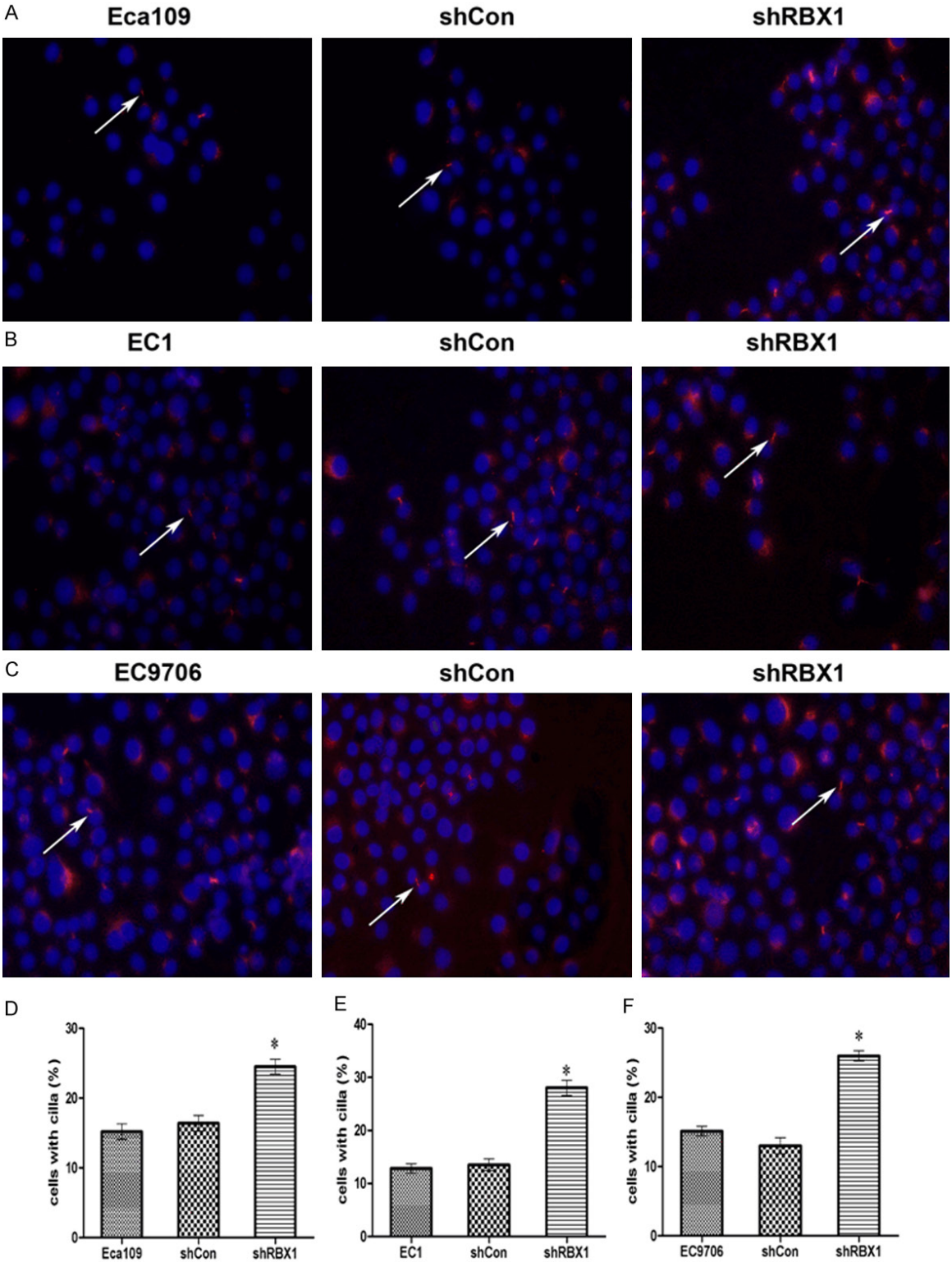

Figure 2. Ciliogenesis was restored in shRBX1 ESCC cells. A-C. Cilia were visualized in shRBX1 Eca109, EC1 and EC 9706 cells and their control cells using specific antibodies targeting acetyl-α-tubulin (red) and indicated with white arrows. Nuclei were stained by Hoechst33342 (blue). D-F. Quantitative analysis of ciliated cells in shRBX1 Eca109, EC1 and EC 9706 cells. $P < 0.05$, compared with that of shCon cells.

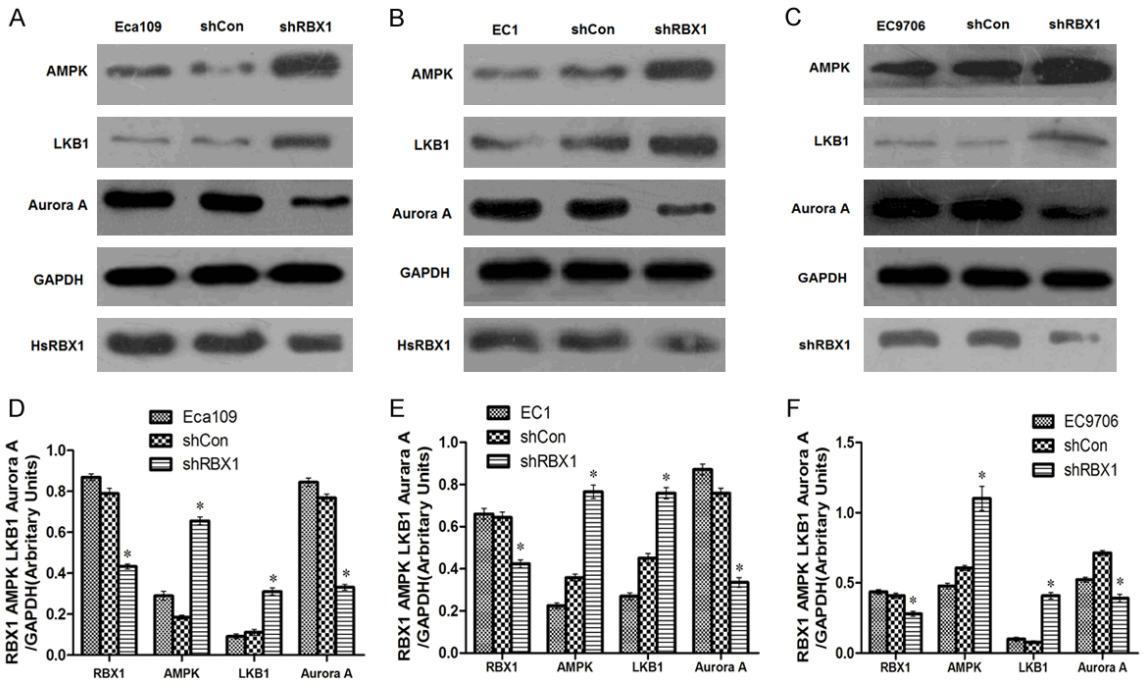

Figure 3. LKB1/AMPK-Aurora A pathway was suppressed in shRBX1 ESCC cells. A-C. The expressions of RBX1, LKB1, AMPK and Aurora A proteins were detected by Western blot in the shRBX1 Eca109, EC1 and EC 9706 cells, respectively. D-F. Semi-quantitative values of Western blot were statistically analyzed, respectively. $P < 0.05$, compared with that of shCon cells.

Signaling Technology, USA), Aurora A (Cell Signaling Technology, USA) and GAPDH (Cell Signaling Technology, USA). Finally, the specific protein bands on the PVDF membranes were detected using N Enhanced Chemiluminescence Kit (Santa Cruz, USA).

Statistical analysis

All the results were derived from at least three independently repeated experiments. The data were analyzed using SPSS version 17.0 (SPSS, Chicago, USA). The summary statistics were expressed as the means ± standard deviations. A *P* value < 0.05 was considered statistically significant.

Results

RBX1 is overexpressed in ESCC cell lines

We investigated the role of RBX1 in human cilia. The expression of RBX1 was initially examined using Western blot in esophageal cells (**Figure 1A**). The results demonstrated an increase in RBX1 by 1.87-fold ($P < 0.05$), 2.24-fold ($P < 0.05$) and 1.92-fold ($P < 0.05$) in Eca109, EC1 and EC 9706 cells, respectively compared with of the increase in Het-1A cells (**Figure 1B**). Based on the results, we inferred a possible relationship between RBX1 overexpression and ciliary loss.

Ciliogenesis is restored in shRBX1 ESCC cells

To examine the relationship between RBX1 and cilia, we observed the ciliary changes in RBX1-suppressed cells of Eca109, EC1, and EC 9706. Western blot results demonstrated that the levels of RBX1 were decreased to 0.55, 0.66 and 0.68 in shRBX1 cells of Eca109, EC1 and EC 9706 compared with shCon cells, respectively (**Figure 3D-F**). Consequently, 24.51% (n = 247) of the Eca109 cells were ciliated after transfection with the shRBX1 lentivirus. By contrast, only 16.41% (n = 182) of shCon cells were ciliated ($P < 0.05$) (**Figure 2A** and **2D**). Similarly, 28.05% (n = 165) of the EC1 cells were ciliated after RBX1 inhibition compared with 13.51% (n = 297) of the ciliated shCon cells ($P < 0.05$) (**Figure 2B** and **2E**), 25.98% (n = 323) of ciliated EC9706 cells, and 13.00% (n = 262) of ciliated shCon cells ($P < 0.05$) (**Figure 2C** and **2F**). These results suggest that ciliogenesis was restored in shRBX1 ESCC cells.

Activity of LKB1/AMPK and Aurora A was regulated by shRBX1

To examine whether RBX1 inhibited ciliogenesis by regulating the LKB1-Aurora A pathway, the expressions of LKB1, AMPK, and Aurora A were determined through a Western blot of the shRBX1 ESCC cells (**Figure 3A-C**). The protein levels of LKB1 were increased 2.79-fold, 1.69-fold and 5.22-fold in the RBX1-suppressing cells of Eca109, EC1 and EC 9706 compared with the shCon cells ($P < 0.05$), respectively (**Figure 3D-F**). The protein levels of AMPK were increased 3.54-fold, 2.14-fold, and 1.82-fold in the RBX1-suppressing cells of Eca109, EC1, and EC 9706 compared with the shCon cells ($P < 0.05$), respectively (**Figure 3D-F**). Furthermore, the expression of Aurora A was decreased to 0.43, 0.44, and 0.55 in RBX1-suppressing cells of Eca109, EC1, and EC 9706 compared with shCon cells ($P < 0.05$), respectively (**Figure 3D-F**). These results suggest that ciliary disassembly occurred via the LKB1-Aurora A signaling pathway and was regulated by shRBX1, and further establishes the role of RBX1 in ciliary disassembly.

Discussion

Cilia are evolutionarily conserved microtubule-based organelles that emanate from the apical membranes of cells [19]. Cilia are predominantly lost in a number of cancers compared with normal cells. Signaling transduction pathways, such as hedgehog, Wnt, and mTOR, are associated with ciliary expression in carcinogenesis. The relationship between ciliary loss and carcinogenesis attracted our attention. We predicted that RBX1 was overexpressed in ESCC cells without the cilia, and ciliogenesis was restored in shRBX1 ESCC cells. The results indicated that RBX1 may mediate ciliary loss in esophageal cancer.

Previous studies showed that LKB1 regulated ciliary disassembly by controlling the HEF1-Aurora A-HDAC6 signaling pathway. Several lines of evidence established that primary cilia regulated the mTOR pathway and cell size via the LKB1/AMPK signaling pathway [15, 16]. In this study, the results showed that the expression of LKB1 and AMPK was increased and Aurora A was decreased in the shRBX1 ESCC cells, indicating that RBX1 regulated ciliogenesis via LKB1/AMPK-Aurora A signaling.

In this study, the tumor suppressor expression of LKB1 was increased, and the expression of Aurora A oncogene was decreased in shRBX1 ESCC cells, indicating that RBX1 inhibition may contribute to cancer therapy. Previous studies showed that RBX1 downregulation inhibited cell proliferation and invasion and induced cell apoptosis [20-23]. Furthermore, recent reports demonstrated that the inhibition or depletion of HDAC6, HEF1, Aurora A and Prdx1 restored ciliogenesis and suppressed cancer cell proliferation [32, 33]. Thus, the link between ciliary disassembly mediated by cilia-related genes including RBX1 and carcinogenesis further provides a promising therapeutic strategy against cancers.

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Disclosure of conflict of interest

None.

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