

MicroRNA-340 Inhibits Epithelial-Mesenchymal Transition by Impairing ROCK-1-Dependent Wnt/ β -Catenin Signaling Pathway in Epithelial Cells from Human Benign Prostatic Hyperplasia

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To the Editor: Benign prostatic hyperplasia (BPH) is a prevalent chronic disease that predominately affects males aged over 40 years, characterized by benign unregulated hyperplasia that arises from stromal and epithelial prostate cells.^[1,2] Although the association between altered microRNA (miRNA) expressions and the incidence of prostate cancer has been widely investigated, there still remains a poor understanding of the mechanism underlying miRNA expressions in BPH of the prostatic stroma.^[3] More recently, emerging evidence has highlighted the role of antitumor miRNA-340 (miR-340) in the event of prostate cancer progression and metastasis.^[4] However, there remains an inadequate understanding regarding the role of miR-340 in BPH. In this study, we collected prostatic tissues from 75 BPH patients and normal prostatic tissues from 67 non-BPH patients who were admitted into Beijing Friendship Hospital, between June 2012 and December 2013. In an attempt to examine the expression patterns of miR-340 in BPH *in vivo*, we quantified a decreased expression level of miR-340 among obtained prostatic tissues from patients with BPH in contrast to normal prostatic tissues [Figure 1a], which suggested that downregulated miR-340 was associated with the occurrence of BPH. The study was conducted with the approval of the Institutional Ethics Committee of Beijing Friendship Hospital, Capital Medical University, with all participating patients giving informed consent in accordance with the requirements of the *Declaration of Helsinki*.

The Rho-kinase (ROCK) pathway is involved in the process of proliferation in the human prostate, with reports indicating its suitability as an additional target for a more effective treatment strategy for BPH, whereby contractility and proliferation are targeted accordingly.^[5] ROCK is a downstream effector of the Rho family GTPases, with two highly homologous isoforms, ROCK1 and ROCK2.^[6] However, the molecular mechanism by which ROCK influences BPH still requires further elucidation. In order to identify the role of ROCK-1 in BPH, an RNA quantification assay [Figure 1a and Table 1], Western blot analysis [Figure 1b], as well as immunohistochemistry [Figure 2]

methods were conducted to determine the expression patterns of ROCK-1 among the collected prostatic tissues from patients with BPH as well as normal prostatic tissues. During Western blot analysis, rabbit monoclonal antibodies specific to human were employed as primary antibodies including ROCK-1 (ab97592, Abcam, Cambridge, MA, USA) (1:1000), β -catenin (ab32572, Abcam) (1:5000), cyclin D1 (ab134175, Abcam) (1:10,000), E-cadherin (ab1416, Abcam) (1:50), N-cadherin (ab18203, Abcam) (1:1000), and vimentin (ab92547, Abcam) (1:1000). Murine monoclonal antibody GAPDH (ab8245, Abcam) (1:1000) was prepared for GAPDH detection. The obtained results indicated that ROCK-1 was robustly induced in the prostatic tissues from patients with BPH, which further highlighted the reciprocal relationship between miR-340 and ROCK-1 in BPH.

Epithelial-mesenchymal transition (EMT) has been implicated in the pathogenesis of a variety of fibrotic disorders.^[7] Recent studies have highlighted that an association between the development of BPH development is associated with accumulation of mesenchymal-like cells derived from the prostatic epithelium.^[8,9] During the present study, the role of miR-340 and ROCK-1 in BPH was investigated, with particular emphasis placed on the process of EMT in BPH, aiming to elucidate the pathogenesis of BPH. RNA quantification assay [Figure 3a] and Western blot analysis [Figure 3b] methods revealed there to be reduced E-cadherin and increased N-cadherin and vimentin in the collected prostatic tissues from patients with BPH and normal prostatic tissues, suggesting the involvement of EMT in the pathogenesis of BPH.

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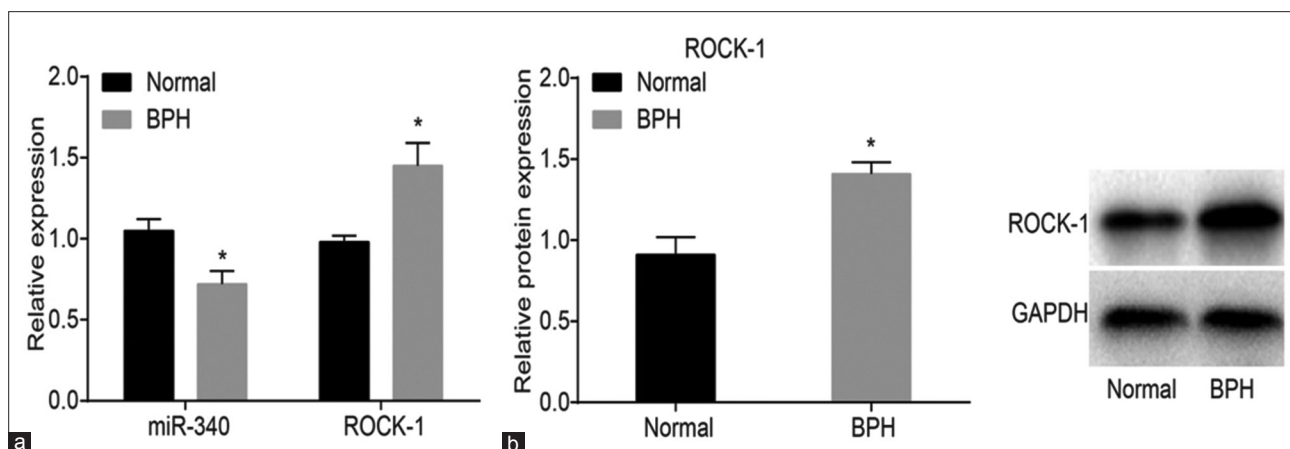


Figure 1: Detection of decreased miR-340 while ROCK-1 is reciprocal in the collected prostatic tissues from patients with BPH when compared to the normal prostatic tissues. (a) miR-340 expression and ROCK-1 mRNA expression determined by RNA quantification assay; (b) ROCK-1 protein levels determined by Western blot analysis; * $P < 0.05$ versus normal prostatic tissues. ROCK-1: Rho-kinase 1; BPH: Benign prostatic hyperplasia.

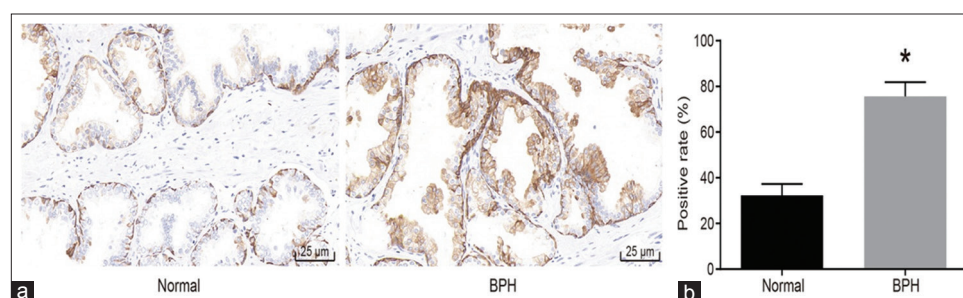


Figure 2: High positive rates of ROCK-1 protein detected in the collected prostatic tissues among patients with BPH as opposed to normal prostatic tissues. (a) ROCK-1-positive cells detected by immunohistochemistry ($\times 400$); (b) the positive rate of ROCK-1 protein; * $P < 0.05$ versus normal prostate epithelial tissues. ROCK-1: Rho-kinase 1; BPH: Benign prostatic hyperplasia.

Table 1: Primer sequences for RNA quantification assay

Genes	Upstream sequence 5'-3'	Downstream sequence 5'-3'
miR-340	GCGGTTATAAAGCAATGAGA	GTGCGTGTCTGGAGTCG
ROCK-1	ACGCCGAGCTCAGCAAGAT	TCCACGTACGGCCTCTTCTG
β -catenin	AAAGTAGAGTGATGAAAGTTGTT	CACCATGTCTCTTGTCTATTC
Cyclin D1	AACTACCTGGACCGCTTCTCT	CCACTTGAGCTTGTTACCA
E-cadherin	AACATGGTTCAGATCAAATC	AAGCTTGAAGATCGGAGGATTATCG
N-cadherin	CAACTTGCCAGAAACTCCAGG	GCGTTCCTGTTCACCTCATAGGAGG
Vimentin	CGCCAGATGCGTGAAATGG	ACCAGAGGGAGTGAATCCAGA
U6	CTCGTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	ATGGAGAAGGCTGGGGCTC	AAGTTGTCATGGATGACCTTG

miR-340: MicroRNA-340; GAPDH: Glyceraldehyde phosphate dehydrogenase; ROCK-1: Rho kinase-1.

To ascertain as to whether miR-340 alternation is responsible for ROCK-1 overexpression in BPH, isolated epithelial cells from prostatic tissues of patients with BPH were treated with a series of miR-340 mimics, miR-340 inhibitors, or anti-ROCK-1 small interfering RNA (siRNA). Initially, the results of the RNA quantification assay and Western blot analysis [Figure 4a and 4b] demonstrated that miR-340 mimics or anti-ROCK-1 siRNA could increase E-cadherin and decrease ROCK-1, N-cadherin, and vimentin. MiR-340 inhibitors produced a reciprocal effect. The subsequent experiments, comprised a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry analysis of annexin

V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining (annexin V-FITC kits from CA1020-20T, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China; 400 μ l PI from Shanghai Lianshuo Biotechnology Co., Ltd., Shanghai, China) [Figure 5a–5e], revealed that miR-340 mimics or anti-ROCK-1 siRNA reduced human BPH epithelial cell proliferation, while inducing apoptosis. Hence, we subsequently asserted that miR-340 acts to negatively regulate ROCK-1, while inhibiting EMT and proliferation in human BPH epithelial cells. The ROCK is a downstream effector of Rho GTPases usually activated in the process of EMT, with various reports suggesting that ROCK could induce EMT in human ovarian cancer cells.^[10]

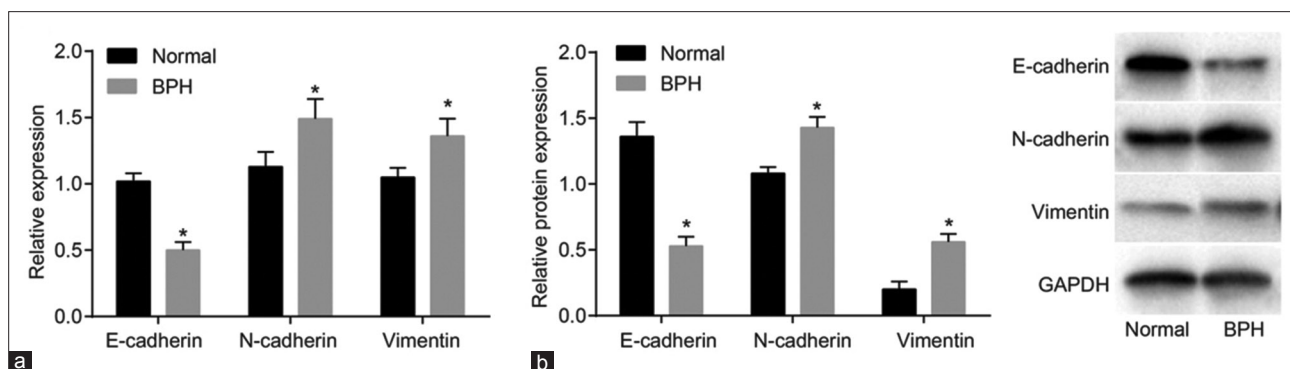


Figure 3: The incidence of EMT is implicated with the development of BPH. Reduced E-cadherin and increased N-cadherin and vimentin detected among the collected prostatic tissues from patients with BPH and normal prostatic tissues; (a) E-cadherin, N-cadherin, and vimentin mRNA expression levels quantified by RNA quantification assay; (b) E-cadherin, N-cadherin, and vimentin protein levels determined by Western blot analysis; * $P < 0.05$ versus normal prostatic tissues. EMT: Epithelial-mesenchymal transition; BPH: Benign prostatic hyperplasia.

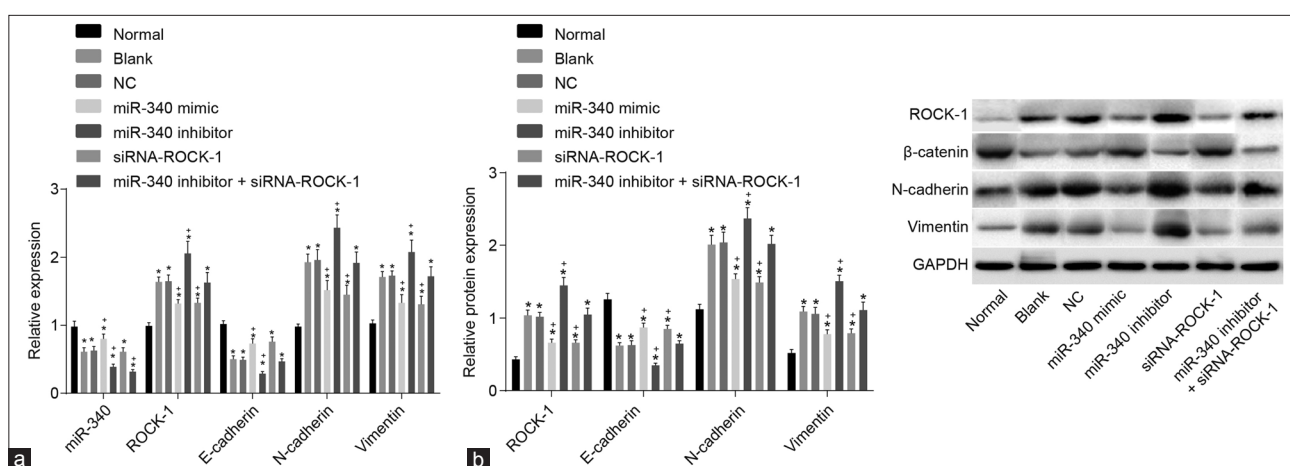


Figure 4: Reversal of EMT achieved through the inhibition of miR-340-dependent ROCK-1 among human BPH epithelial cells *in vitro*. MiR-340 mimics or anti-ROCK-1 siRNA could increase E-cadherin and decrease ROCK-1, N-cadherin, and vimentin, and anti-ROCK-1 siRNA could rescue the phenomena caused by miR-340 inhibitors. (a) miR-340 expression, ROCK-1, E-cadherin, N-cadherin, and vimentin mRNA expression levels quantified by RNA quantification assay; (b) ROCK-1, E-cadherin, N-cadherin, and vimentin protein levels determined by Western blot analysis; * $P < 0.05$ versus normal prostatic epithelial cells; † $P < 0.05$ versus untreated BPH epithelial cells or BPH epithelial cells treated with scramble siRNA. EMT: Epithelial-mesenchymal transition; ROCK-1: Rho-kinase 1; siRNA: Small interfering RNA.

Similarly, Li *et al.*^[11] demonstrated that the knockdown of ROCK1 reversed EMT in non-small cell lung cancer. In addition, in an attempt to verify whether miR-340 governs ROCK-1 in human BPH epithelial cells *in vitro*, human BPH epithelial cells were treated with a combination of miR-340 inhibitors and anti-ROCK-1 siRNA. The results revealed that anti-ROCK-1 siRNA could rescue the phenomena caused by miR-340 inhibitors, which ultimately suggested that miR-340 could negatively regulate ROCK-1. It is suggested that miR-340-dependent ROCK-1 inhibition could lead to both a reversal of EMT as well as enhanced proliferation levels in human BPH epithelial cells.

At a molecular level, the issue of a more efficient route form iR-340-dependent ROCK-1 inhibition and its influence on human BPH epithelial cells is yet to be identified. Intriguingly, miR-340 has previously been observed to inhibit β -catenin, which is essential for the modulation of the Wnt/ β -catenin signaling pathway in bone marrow-derived mesenchymal stem cells.^[12] Kim *et al.*^[13] demonstrated the cross-talk between canonical and noncanonical signaling pathways of Wnt3A, which increases glycogen synthase kinase-3 β phosphorylation and β -catenin accumulation through the activation of RhoA and ROCK.

Wnt/ β -catenin signaling activation by Wnt3A treatment has been reported to induce resistance to gemcitabine in pancreatic cancer cells.^[14] Hence, the Wnt/ β -catenin signaling pathway, as an efficient route form miR-340-dependent ROCK-1 inhibition, is suggested to influence human BPH epithelial cells. During the current study, we quantified the downstream effectors of the Wnt/ β -catenin signaling pathway among the collected prostatic tissues from patients with BPH and human BPH epithelial cells. β -Catenin and cyclin D1 were found to have increased in the collected prostatic tissues from patients with BPH [Figure 6a and 6b], indicating that Wnt/ β -catenin signaling activation was associated with BPH development. Mechanistically, we found that miR-340 mimics or anti-ROCK-1 siRNA reduced the expressions of β -catenin and cyclin D1, while miR-340 inhibitors were observed to have elicited a reciprocal effect [Figure 6c and 6d].

Taken together, the results obtained indicated that miR-340 inhibits EMT by impairing ROCK-1-dependent Wnt/ β -catenin signaling pathway in epithelial cells from human BPH, which supports the conceptual perspective that downregulated miR-340 may be associated with the process of EMT in BPH, thus providing a novel target for the molecular treatment of BPH. However, further

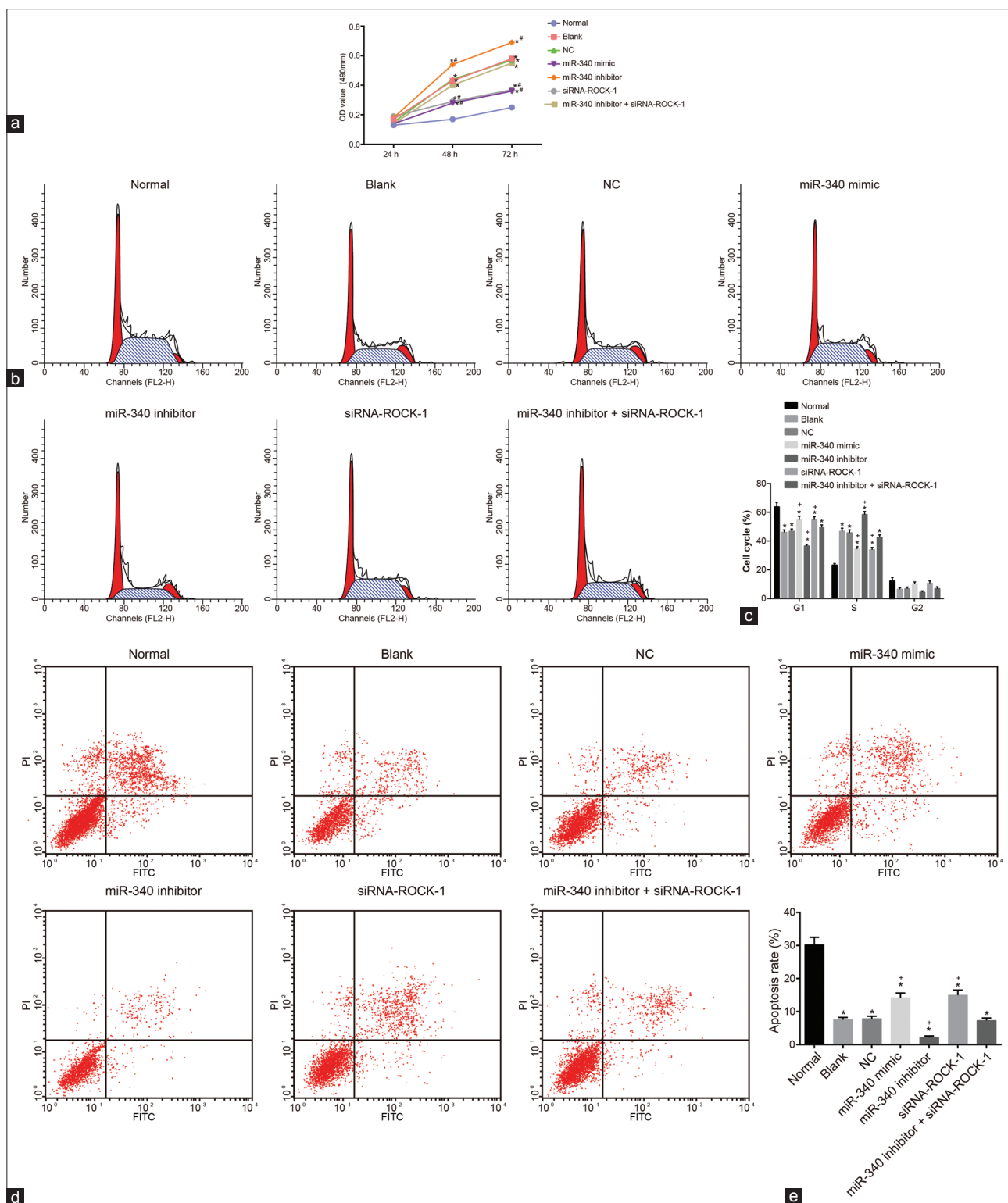


Figure 5: Results suggesting that miR-340 mimics or anti-ROCK-1 siRNA reduces human BPH epithelial cell proliferation and induces apoptosis. (a) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (optical density values detected at a wavelength of 490 nm), indicating that BPH epithelial cells treated with miR-340 mimics or anti-ROCK-1 siRNA results in decreased cell viability; (b and c) flow cytometry analysis of PI staining reflects that reduced BPH epithelial cells treated with miR-340 mimics or anti-ROCK-1 siRNA are arrested in the S stage; (d and e) flow cytometry analysis of annexin V-FITC/PI staining demonstrates that apoptosis is induced in BPH epithelial cells treated with miR-340 mimics or anti-ROCK-1 siRNA. * $P < 0.05$ versus normal prostatic epithelial cells; † $P < 0.05$ versus untreated BPH epithelial cells or BPH epithelial cells treated with scramble siRNA. BPH: Benign prostatic hyperplasia; ROCK-1: Rho-kinase 1; siRNA: Small interfering RNA.

investigation is required to validate the growth-inhibitory role of miR-340 in human BPH epithelial cells *in vivo* and to also identify

the additional regulatory mechanism involved with miR-340 in BPH.

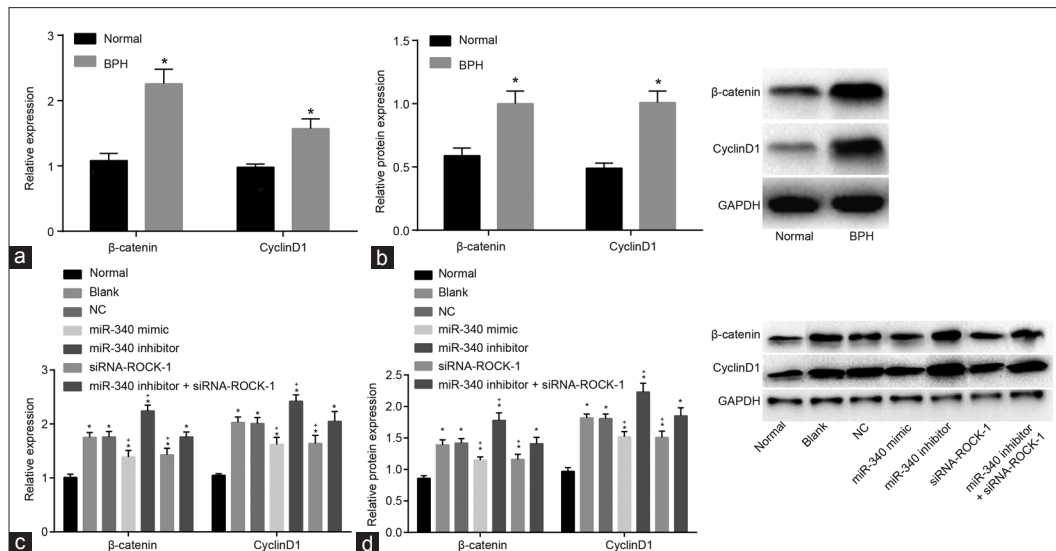


Figure 6: miR-340-dependent ROCK-1 inhibition affects Wnt/ β -catenin signaling pathway during BPH. (a and b) RNA quantification assay and Western blot analysis *in vivo* reflect that β -catenin and cyclin D1, downstream effectors of Wnt/ β -catenin signaling pathway, were determined to have increased in the collected prostatic tissues from patients with BPH; (c and d) RNA quantification assay and Western blot analysis *in vitro* demonstrating that miR-340 mimics or anti-ROCK-1 siRNA reduces the expressions of β -catenin and cyclin D1 in human BPH epithelial cells. ROCK-1: Rho-kinase 1; BPH: Benign prostatic hyperplasia; siRNA: Small interfering RNA.

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Conflicts of interest

There are no conflicts of interest.

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