Am J Cancer Res 2022;12(1):108-122
www.ajcr.us /ISSN:2156-6976/ajcr0139125

Original Article

δ-Catenin promotes cell migration and invasion via Bcl-2-regulated suppression of autophagy in prostate cancer cells

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Received September 14, 2021; Accepted November 8, 2021; Epub January 15, 2022; Published January 30, 2022

Abstract: As a member of the catenin family, δ-catenin is overexpressed in many cancers, including prostate cancer, and the role of δ-catenin in prostate tumor growth has been reported. However, the involvement of δ-catenin in the migration and invasion of prostate cancer has rarely been studied. In this study, we innovatively proposed that δ-catenin would enhance the migration and invasion ability of prostate cancer cells. It is worth noting that the molecular mechanism underlying the effect involved the downregulation of autophagy. We demonstrated that δ-catenin could suppress autophagy by Bcl-2-regulated disruption of the Beclin1-Vps34 autophagosome complex. Furthermore, the effect of δ-catenin on promoting cell migration and invasion was dependent upon β-catenin-mediated Bcl-2 transcription. Finally, using rapamycin and bafilomycin, we largely confirmed that the degradation of Snails by autolysosomes may be related to δ-catenin regulated migration and invasion. Overall, our results indicated that δ-catenin promoted cell migration and invasion of prostate cancer cells via Bcl-2-regulated autophagy suppression.

Keywords: δ-catenin, prostate cancer, autophagy, Bcl-2, migration, invasion

Introduction

δ-Catenin belongs to the p120ctn subfamily of armadillo proteins and was identified for the first time due to its interaction with presenilin-1 [1]. Initially, it was thought to play an important regulatory role in brain and neuronal functions [2]. However, many recent studies show that it was overexpressed in a variety of cancers, including colorectal cancer [3], prostate cancer [4], lung cancer [5], and ovarian cancer [6]. It is noteworthy that many studies have shown that δ-catenin is overexpressed in prostate tumors. Strong evidence has indicated that δ-catenin transcription and protein are both overexpressed in prostate cancer [4, 7]. Moreover, δ-catenin mRNA was overexpressed in prostate cancer compared to benign prostatic hyperplasia [7]. The overexpression of δ-catenin promoted the growth and progression of prostate cancer cells by inducing cell cycle and survival gene profiles [8]. An analysis of human prostate cancer tissues and normal prostate tissues found that 85% of the prostate cancer tissues showed δ-catenin immunoreactivity, and the expression of δ-catenin was associated with an increase in Gleason score [4]. Our previous studies showed that δ-catenin could increase β-catenin, thereby transcriptionally regulating the expression of its target genes, and promoting the growth and proliferation of prostate cancer cells [9]. In recent years, there have been many studies on the role of δ-catenin in cancer growth and progression [10], but there are few reports on the relationship between δ-catenin and tumor invasion, and the mechanisms involved are still unclear.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose polarization and homotypic cell adhesion, and develop slender fibroblast-like morphology [11, 12]. A

process similar to EMT has also been observed in metastatic tumors, indicating that EMT plays an important role in cancer metastasis [13]. EMT exerts a vital role in the initiation of cancer metastasis. The characteristic loss of epithelial properties leads to the acquisition of mesenchymal properties, thereby increasing cell fluidity and anti-apoptotic ability [14-16]. E-cadherin is considered to inhibit metastasis during tumor development, and one of the characteristics of EMT is the loss of E-cadherin function [17]. Snail is an important transcriptional inhibitor of E-cadherin in EMT and plays a key role in tumor progression [18]. New evidence showed that Snail conferred cancer stem cell-like characteristics to cancer cells and promoted cancer metastasis. In a variety of cancers, the expression of Snail was closely related to tumor metastasis [19-21]. For instance, a previous study confirmed that Snail was necessary for lymph node metastasis in breast cancer [22]. According to another study, the expression of Snail was increased in metastatic ovarian cancer [23]. In addition, Snail knockout significantly suppressed tumor growth and metastasis by increasing tumor-infiltrating lymphocytes and systemic immune responses [24]. Therefore, these findings indicate that Snail can be used as an effective target to prevent tumor metastasis, and the inactivation of Snail protein become a potential target for cancer treatment.

Autophagy is one of the major intracellular degradation systems, transporting organelles and proteins into lysosomes for degradation [25]. Autophagy can be a spontaneous process, or it can be activated under different external stimuli. In addition to maintaining cellular homeostasis, autophagy is involved in the regulation of many physiological and pathological conditions, such as cancer, apoptosis, and senescence [26-28]. In recent years, autophagy regulation has become a new strategy for treating neurodegenerative diseases and cancer [29, 30]. However, the role of autophagy in tumorigenesis and tumor progression is still controversial. Autophagy was shown to limit the instability of the genome and prevent the accumulation of damaged organelles and proteins, which had an inhibitory effect on tumor formation [31]. In contrast, developed tumor cells can promote their survival in adverse microenvironments such as hypoxia and stress through autophagy, thereby evading the immune system under unfavorable environmental conditions [30, 32, 33]. That means that autophagy exerts a double-edged sword effect in cancers. Furthermore, the tumor suppressor function of autophagy is partly due to its ability to induce cell death [34]. The role of autophagy is complex and diverse and varies in different types of tumors. Autophagy inhibits tumor initiation and the progression of certain cancers, but also promotes tumor survival and the development of other cancers, which makes it a potential therapeutic target for cancer treatment [35]. However, there have been few to date studies on autophagy and tumor cell migration and invasion. In breast cancer models, autophagy activation was related to the degradation of EMT regulators Snail and Twist [36].

One of the key mechanisms of autophagy regulation is the interaction between the autophagy regulatory protein Beclin1 and the anti-apoptotic protein family member Bcl-2 [37]. In the process of autophagy activation, autophagy-related proteins Beclin1 and Vps34 can form pro-autophagy complexes with Atg14L or UVRAG. It is known that the binding of Bcl-2 and Beclin1 can inhibit autophagy by disrupting Vps34 binding to Beclin1, meaning that Bcl-2 and Vps34 compete for binding to Beclin1. The dynamic regulation of Beclin1-Vps34 complex formation and autophagy induced by Bcl-2 is known to inhibit autophagy [38]. In this study, we demonstrated that δ-catenin could promote the migration and invasion of prostate cancer cells via Bcl-2-regulated autophagy. δ-Catenin suppressed autophagy by increasing β-catenin targeted Bcl-2 expression. This study clarified the mechanism by which δ-catenin promotes the migration and invasion of prostate cancer cells and provides a new perspective for the treatment of prostate cancer metastasis.

Materials and methods

Reagents

Antibodies against LC3 (sc-398822), Beclin1 (sc-48341), Vps34 (sc-365404), Bcl2 (sc-7382), Snail (sc-271977), and β-actin (sc-47778) were obtained from Santa Cruz Biotechnology (USA). Antibody against δ-catenin (611537) was purchased from BD Bioscience (USA). Antibody against β-catenin (C2206) was purchased from Millipore Sigma-Aldrich (USA).

Normal mouse (31430) and rabbit (31460) IgG-HRP secondary antibodies were purchased from Thermo Fisher Scientific (USA). Normal mouse IgG (sc-2025) was purchased from Santa Cruz Biotechnology. Rapamycin (HY-10219) and bafilomycin A1 (HY-100558) were purchased from MedChemExpress (USA). δ-Catenin siRNA#1 (145777) and δ-catenin siRNA#2 (145778) were purchased from Thermo Fisher Scientific. β-Catenin siRNA#1 (6225) and β-catenin siRNA#2 (6238) were purchased from Cell Signaling Technology (USA). Bcl-2 siRNA (sc-29214) was purchased from Santa Cruz Biotechnology (USA). MG132 (474790) was purchased from Millipore Sigma-Aldrich.

Plasmids

The δ-catenin wild type (WT) and β-catenin wild-type constructs have been previously described [39].

Cell culture

CWR22Rv-1 (22RV1), PC3, and LNCaP obtained from the American Type Culture Collection (USA) were cultured in RPMI1640 medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) in a 37°C and 5% CO_2 cell incubator. Rv/δ (Rv-1 transfected with mouse δ-catenin-green fluorescent protein (GFP)) [39] and Rv/C (Rv-1 transfected with GFP) were established and selected with G418 (200 μg/ml, Sigma-Aldrich) at 37°C in 5% CO_2. All experiments were performed with mycoplasma-free cells. Plasmid DNA and siRNA were used to transfect cells with Lipofectamine TM 2000 reagent (Invitrogen, USA) for 24 h. Before performing a series of experimental procedures, the RPMI1640 medium with 10% FBS was removed and replaced with a serum-free medium for 2 h, then the cells were treated as described.

Immunoblotting

Immunoblotting was performed as previously described [40]. Briefly, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane which was incubated with an appropriate primary antibody overnight. The blots were visualized using enhanced chemiluminescence (ECL from GE Healthcare, Germany). The bands were quantified using Quantity One Software (Bio-Rad, USA).

Immunoprecipitation

Rv-1, Rv/C and Rv/δ were lysed with buffer (137 mM NaCl, 20 mM Tris-HCl, 1% NP-40, 2 mM EDTA, 50 mM NaF, and 0.1 μM aprotinin). After incubating with primary antibodies and agarose beads overnight at 4°C and washing with lysate buffer 3 times, the immunoprecipitates were eluted with 2× sample buffer (4% SDS, 0.1 M Tris-HCl, 20% glycerol, 0.2% bromophenol blue, and 0.2 M dithiothreitol (DTT), pH6.8), separated and detected by immunoblotting. The cell lysates were also subjected to immunoprecipitation with the mouse IgG isotype control appropriate for the immunoglobulin type of the primary antibody.

Wound healing scratch assay

The wound healing scratch test was used to determine cell migration. Briefly, the cells were plated in 6-well plates and incubated in the medium with 2.5% FBS until grown to full confluency, then scraped by a sterile 100 μL pipette tip. Then the medium was replaced with phosphate-buffered saline (PBS) and the wound gap was photographed using a microscope (Nikon, Japan) at 0 h and 24 h. Quantitative analysis of cell migration in 3 independent experiments (n=3).

Transwell assay

The Transwell assay was used to evaluate cell invasion ability. Briefly, Matrigel was coated in the upper chamber. After 12 h, cells were placed in the upper chamber with serum-free medium, the medium containing 10% FBS was added to the lower chamber. After 24 h, the cells were fixed with 4% paraformaldehyde and then stained with Giemsa staining solution. The cells that migrated to the lower side of the membrane were counted under a light microscope (Nikon, Japan). Quantitative analysis of cell invasion in 3 independent experiments (n=3).

RNA isolation and qRT-PCR

Total RNA was extracted from the cells using Trizol (Invitrogen, USA) according to the manufacturer's protocol. Quantitative real-time poly-

merase chain reactions (qRT-PCR) were performed according to a previous study. The data were analyzed and normalized to GAPDH. The primer sequences were follows:

Bcl-2	F: 5'-GGGGTCATGTGTGTG-GAGAG-3'	R: 5'-CATCCCAGCCTCCGT-TATCC-3'
Snail	F: 5'-CCAGACCCACTCAGAT-GTCAAG-3'	R: 5'-GGGCAGGTATG-GAGAGGAAGA-3'
GAPDH	F: 5'-CCCTTCATTGACCT-CAACTACATG-3'	R: 5'-TGGGATTTCCATTGAT-GACAAGC-3'

Immunofluorescence assay

Immunofluorescence staining was performed as previously described [40]. Briefly, cells were cultured on a cover glass-bottom plate overnight at 37°C and 5% CO_2. After the treatment, the cells were fixed with 4% formaldehyde for 10 min. The cells were permeabilized with 0.2% Triton X-100 for 10 min at RT before blocking with 3% bovine serum albumin for 30 min at room temperature (RT). Next, the cells were incubated with primary the antibody, as indicated overnight at 4°C, and then incubated with fluorescent secondary antibody for 1 hour at RT. Subsequently, the fluorescent images were visualized by fluorescence microscopy.

Statistical analysis

The results are expressed as means ± SD, and an analysis of variance (ANOVA) with Bonferroni's test was used for the statistical analysis of multiple data comparisons. A *P*-value of 0.05 or less was considered significant.

Results

δ-Catenin increases the migration and invasion ability of prostate cancer cells

Our previous studies demonstrated that δ-catenin could increase the proliferation and growth of prostate cancer cells [9], but its role in cell migration and invasion is still unknown. Cell migration and invasion are two important processes in tumor metastasis and are closely related to the recurrence of cancer. Therefore, it is necessary to understand the mechanism of prostate cancer cell migration and invasion for cancer prognosis. At the beginning of this study, the ability of prostate cancer cells to migrate and invade was investigated. The 22RV1 cell line RV/δ stably expresses GFP-δ-catenin and the RV/C cell line stably expresses GFP. As shown in **Figure 1A** and **1B**, compared to RV/C and 22RV1, RV/δ showed stronger migration and invasion capabilities. Next, we transfected GFP-δ-catenin into 22RV1 and analyzed the migration and invasion ability after transfection. The different exposure time of δ-catenin protein in 22RV1 and PC3 were showed in Figure S6. The results suggested that δ-catenin overexpression increased the migration and invasion ability of 22RV1 cells (**Figure 1E** and **1F**). To further confirm our findings, we used other prostate cancer cell lines PC3 and LNCaP. PC3 and LNCaP cells were transfected with GFP-δ-catenin. As expected, the result was the same as in the 22RV1 cells (Figure S2A and S2C). Taken together, we concluded that δ-catenin could increase the migration and invasion ability of prostate cancer cells.

δ-Catenin inhibits autophagy in prostate cancer cells

Since autophagy was reported to exert vital effects in cancer metastasis and the inhibition of autophagy could promote cancer cell migration and invasion [26, 36], we wanted to investigate whether δ-catenin affected autophagy. The Western blot results suggested that the protein level of the autophagy-related protein LC3-II was lower in RV/δ cells (**Figure 1D**). After transfection with GFP-δ-catenin, the LC3-II protein level decreased in 22RV1 cells, PC3 cells, and LNCaP cells (**Figures 1G**, S2B and S2D). Furthermore, the immunofluorescence results suggested that the autophagy level was lower in RV/δ cells compared to RV/C cells (**Figures 1C** and S1). In summary, these results indicated that δ-catenin downregulated autophagy in prostate cancer cells.

To further confirm the role of δ-catenin in autophagy inhibition and invasion promotion, we chose two silencing RNAs to interfere with δ-catenin expression. As shown in **Figure 2**, the two δ-catenin siRNAs both increased autophagy and inhibited cell migration and invasion in RV/δ cells. Furthermore, δ-catenin siRNA also decreased autophagy in PC3 cells (Figure S3).

Rapamycin induces autophagy and decreases the migration and invasion ability in prostate cancer cells

We demonstrated that δ-catenin could promote migration and invasion and downregulate autophagy in prostate cancer cells. However, whether the ability of δ-catenin to increase

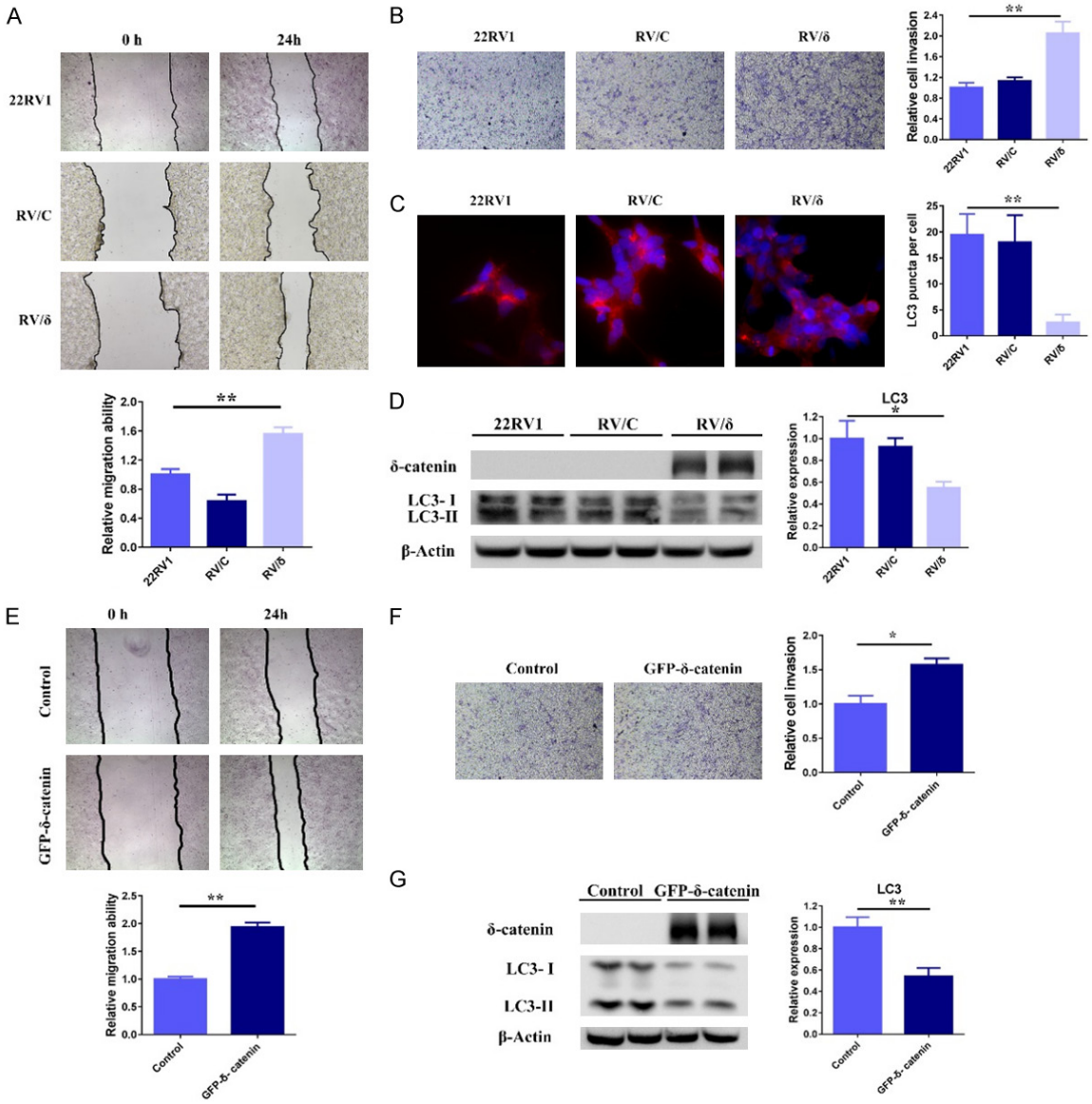

Figure 1. δ-Catenin increased migration and invasion ability and decreased autophagy in prostate cancer cells. A. The wound-healing scratch assay was used to detect the migration ability of 22RV1, RV/C, and RV/δ cells. Quantitative analysis of cell migration in 3 independent experiments (n=3). Magnification: 50×. B. The Transwell assay was used to analyze the invasion ability of 22RV1, RV/C, and RV/δ cells. Quantitative analysis of cell invasion in 3 independent experiments (n=3). Magnification: 100×. C. Immunofluorescence staining to determine LC3 puncta in 22RV1, RV/C, and RV/δ cells. Magnification: 500×. 22RV1 cells were transfected with GFP-control and GFP-δ-catenin. D. Western blot analysis of LC3 protein levels in 22RV1, RV/C, and RV/δ cells. The relative quantification of protein levels was analyzed using ImageJ software. E. The wound-healing scratch assay was used to detect the cell migration ability of each group. Quantitative analysis of cell migration in 3 independent experiments (n=3). Magnification: 50×. F. The Transwell assay was used to analyze the invasion ability of each group. Quantitative analysis of cell invasion in 3 independent experiments (n=3). Magnification: 100×. G. Western blot analysis of LC3 protein levels in each group. The relative quantification of protein levels was performed using ImageJ software. Data are shown as means ± SD of 3 independent experiments. $^{*}P<0.05$ and $^{**}P<0.01$.

migration and invasion was attributable to autophagy inhibition was still unclear. To clarify autophagy's role in prostate cancer cell migration and invasion, we treated three types of prostate cancer cells with the autophagy inducer rapamycin. As shown in **Figure 3C**, the LC3-II protein level increased in a dose-dependent manner after rapamycin treatment. Compared

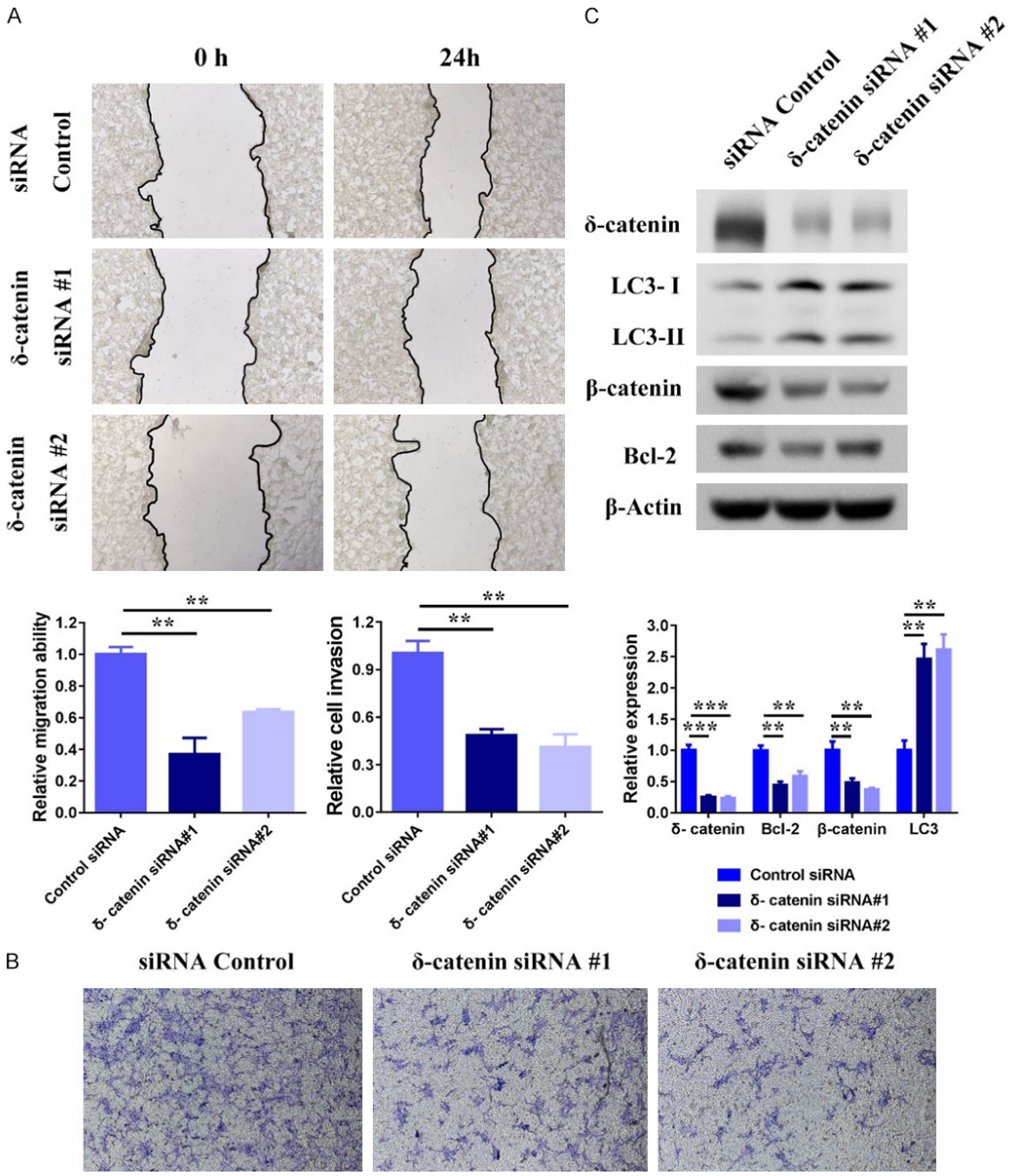

Figure 2. δ-Catenin siRNA disrupted migration and invasion ability and increased autophagy in RV/δ cells. RV/δ cells were transfected with control siRNA, δ-catenin siRNA#1, and δ-catenin siRNA#2. A. The wound-healing scratch assay was used to detect the cell migration ability of each group. Quantitative analysis of cell migration in 3 independent experiments (n=3). Magnification: 50×. B. The Transwell assay was used to analyze the invasion ability of each group. Quantitative analysis of cell invasion in 3 independent experiments (n=3). Magnification: 100×. C. Western blot analysis of LC3, β-catenin, and Bcl-2 protein levels in each group. The relative quantification of protein levels was performed using ImageJ software. Data are shown as means ± SD of 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

to 22RV1 and RV/C cells, RV/δ cells showed lower LC3-II expression after treatment with increasing rapamycin doses. The immunofluorescence results suggested that rapamycin increased LC3 puncta in 22RV1, RV/C, and RV/δ cells. However, the RV/δ cells showed fewer puncta both in normal conditions and in rapamycin treatment (**Figures 3A** and S4B).

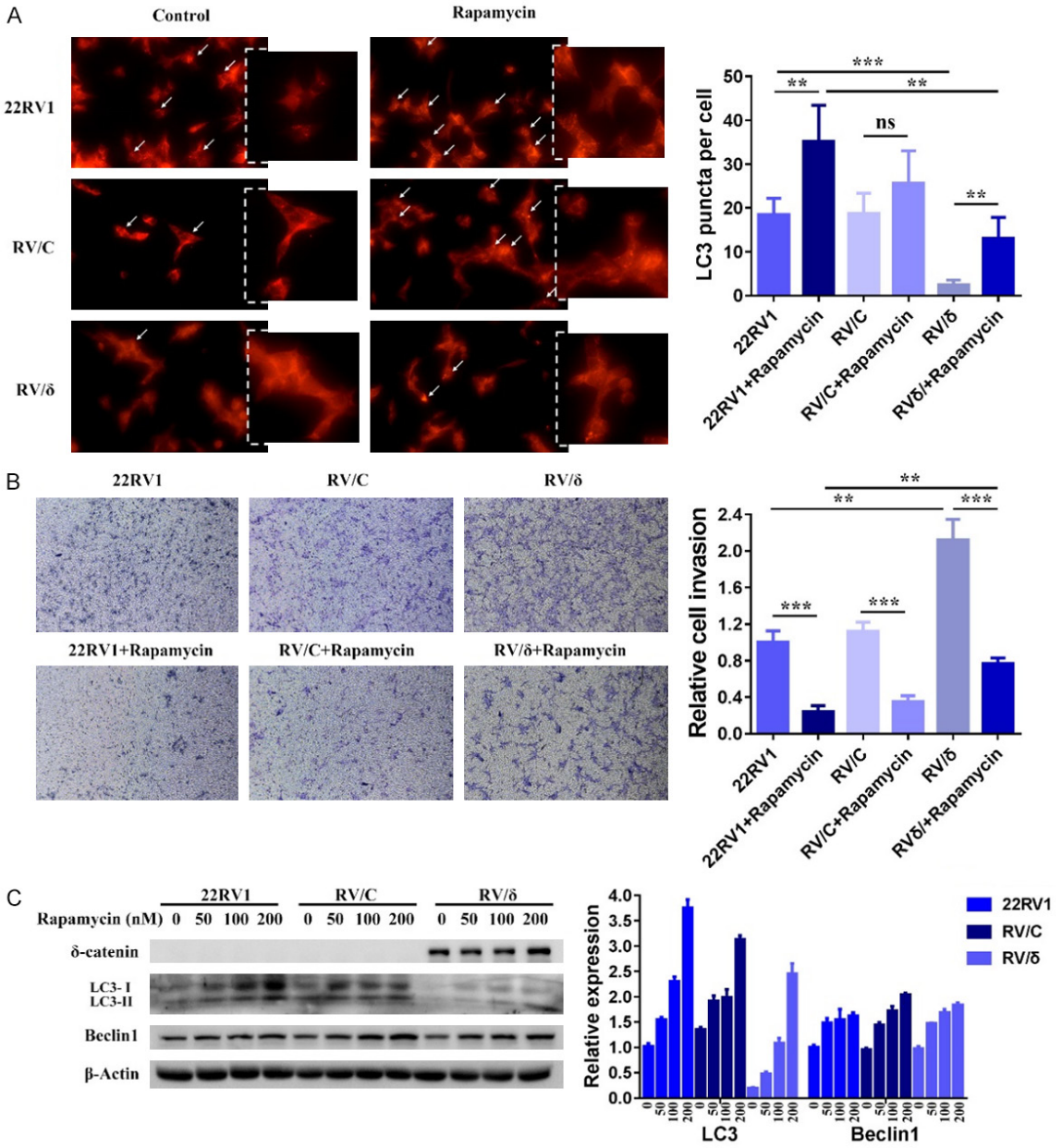

Figure 3. Rapamycin-induced autophagy decreased the migration and invasion of prostate cancer cells. A. 22RV1, RV/C, and RV/δ cells were treated with or without 100 nM rapamycin. Immunofluorescence staining was performed to determine LC3 puncta. Magnification: 500×. B. 22RV1, RV/C, and RV/δ cells treated with or without 100 nM rapamycin. The Transwell assay was used to analyze the invasion ability of each group. Magnification: 100×. C. 22RV1, RV/C, and RV/δ cells treated with 0, 50, 100, or 200 nM rapamycin. Western blots were used to analyze LC3 and Beclin1 protein levels in each group. Data are shown as means ± SD of 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

These results suggested that the effects of rapamycin in RV/δ were weakened. Next, we analyzed the invasion and migration ability with and without rapamycin treatment (**Figures 3B** and S4A). After rapamycin treatment, the migration and invasion ability of the three types of cells was drastically reduced, while the decrease in RV/δ cells was not as obvious compared to the other two types of cells. These results suggested that autophagy activation inhibited cell migration and invasion in prostate cancer cells. It is worth noting that RV/δ cells showed stronger migration and invasion tendencies and lower autophagy levels, and that

δ-catenin may play an important role in regulating autophagy, migration, and invasion. Beclin1 is a very important protein in autophagosome complex formation and is thought to be a decisive protein in the autophagy process [41]. However, the expression of Beclin1 was not decreased in RV/δ cells (**Figure 3C**). Therefore, other mechanisms could regulate autophagy.

δ-Catenin-downregulated autophagy is involved in Bcl-2-mediated disassociation of the Vps34-Beclin1 complex

The anti-apoptosis protein Bcl-2 was reported to serve as an autophagy inhibitory protein, and the mechanism involves binding to Beclin1 by competing with Vps34 [42]. To confirm this mechanism of autophagy inhibition by δ-catenin, we first used Western blots to detect Bcl-2 and Vps34 expression in 22RV1, RV/C, and RV/δ cells (**Figure 4A**). The Vps34 protein expression level did not change in the three cell lines, but Bcl-2 expression was higher in RV/δ cells. Treatment with increasing rapamycin doses did not change the protein expression levels. These results suggested that Bcl-2 could participate in the autophagy regulation of δ-catenin. To prove this, we performed an immunoprecipitation assay to analyze the binding relationship between Bcl2, Beclin1, and Vps34 in RV/C and RV/δ cells (**Figure 4B-D**). The two cell lines were treated with and without rapamycin. The immunoprecipitation assay revealed that the binding of Beclin1 and Vps34 decreased in RV/δ cells, whereas the association between Beclin1 and Bcl-2 increased. Treatment with rapamycin increased Beclin1 expression, thus increasing its binding to two partners. However, rapamycin did not affect the binding relationship between Bcl2, Beclin1, and Vps34 in RV/C and RV/δ cells. Taken together, these results suggested that RV/δ cells had higher Bcl-2 expression and that autophagy inhibition was involved in the mechanism by which Bcl-2 mediated the disassociation of the Vps34-Beclin1 complex.

To further confirm our finding that Bcl-2 participated in the autophagy regulation of δ-catenin, we used silencing RNA to interfere with Bcl-2. As shown in **Figure 4E**, Bcl-2 siRNA increased the LC3-II level in RV/δ cells but had little effect on RV/C cells. Bcl-2 siRNA did not affect Vps34 and Beclin1 expression, suggesting that Bcl-2 regulation was mainly dependent upon its binding with Beclin1. Next, we conducted an immunoprecipitation assay to confirm our assumption. Bcl-2 siRNA decreased the binding between Bcl-2 and Beclin1 and increased the binding between Beclin1 and Vps34, thus increasing autophagy (**Figure 4F**). Therefore, we could conclude that the δ-catenin downregulation of autophagy was attributed to δ-catenin increases in Bcl-2 and competition with Vps34 for binding to Beclin1.

δ-Catenin downregulates autophagy via β-catenin transcriptional regulation of Bcl-2

Next, we explored how δ-catenin decreased Bcl-2 and affected autophagy. β-catenin is a protein that is closely related to cancer growth [43]. In our previous study, δ-catenin was shown to increase β-catenin expression and promote prostate cancer cell proliferation [10]. Some studies have suggested that Bcl-2 could be a transcriptional target gene of β-catenin [44]. Therefore, we investigated whether the underlying mechanism was that δ-catenin could increase β-catenin and cause the transcription of Bcl-2. The Western blot results suggested that δ-catenin increased β-catenin expression in prostate cancer cells (**Figure 5A**). And δ-catenin siRNA decreased β-catenin and Bcl-2 protein expression (**Figure 2C**). To prove that β-catenin could upregulate Bcl-2 in prostate cancer cells, we transfected RV/δ cells with GFP-β-catenin. As shown in **Figure 5B**, **5C**, compared to RV/C cells, RV/δ cells showed higher Bcl-2 expression and GFP-β-catenin transfection increased Bcl-2 both at the protein and mRNA levels. Furthermore, in RV/δ cells, transfection with GFP-β-catenin showed a greater reduction in autophagy levels (**Figure 5B**). To confirm that the role of δ-catenin in autophagy inhibition was dependent upon β-catenin-regulated Bcl-2 expression, β-catenin siRNA was used. Bcl-2 expression was downregulated after transfection with β-catenin siRNA both in RV/C and RV/δ cells (**Figure 5D**). Consistent with these results, autophagy was upregulated in RV/δ cells. These data indicated that δ-catenin downregulated autophagy via the β-catenin transcriptional regulation of Bcl-2.

δ-Catenin increases migration and invasion ability via inhibiting autophagy-mediated Snail degradation

Many studies have confirmed that autophagy could act as a tumor inhibitor and affect tumor

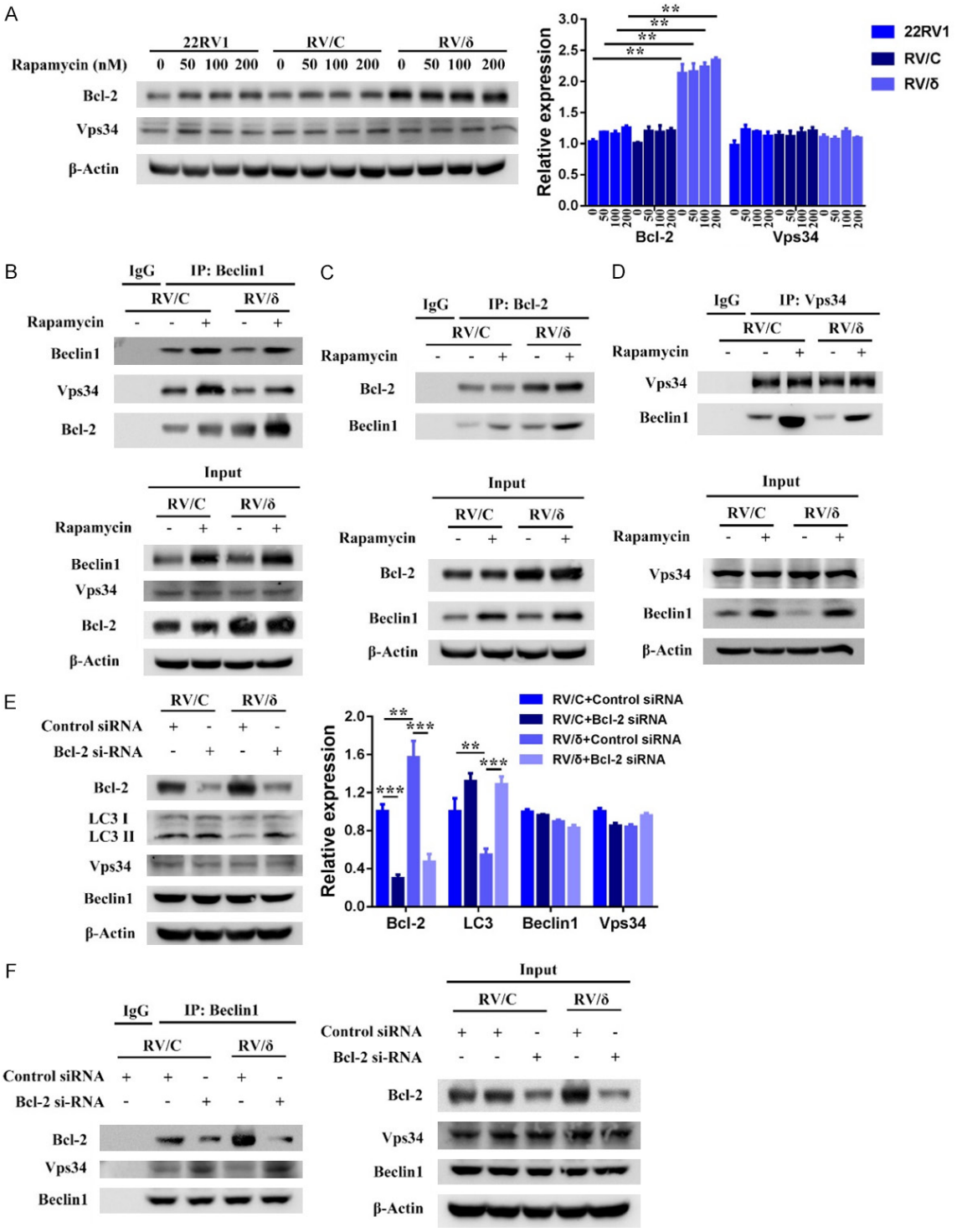

Figure 4. δ-Catenin downregulation of autophagy was involved in the Bcl-2-mediated disassociation of the Vps34-Beclin1 complex. A. 22RV1, RV/C, and RV/δ cells treated with 0, 50, 100, or 200 nM rapamycin. Western blots were used to analyze Bcl-2 protein levels in each group. Data are shown as means ± SD of 3 independent experiments. **P<0.01. B-D. RV/C and RV/δ cells were treated with or without rapamycin. The immunoprecipitation assay was used to analyze the interaction of Bcl-2 and Vps34 with Beclin1. Mouse IgG was used as the negative control. E. RV/C and RV/δ cells were transfected with control siRNA and Bcl-2 siRNA. Western blot analysis of LC3, Vps34, Beclin1, and Bcl-2 protein levels in each group. Data are shown as means ± SD of 3 independent experiments. **P<0.01, ***P<0.001. F. RV/C and RV/δ cells were transfected with control siRNA and Bcl-2 siRNA. The immunoprecipitation assay was used to analyze the interaction of Bcl-2 and Vps34 with Beclin1. Mouse IgG was used as the negative control.

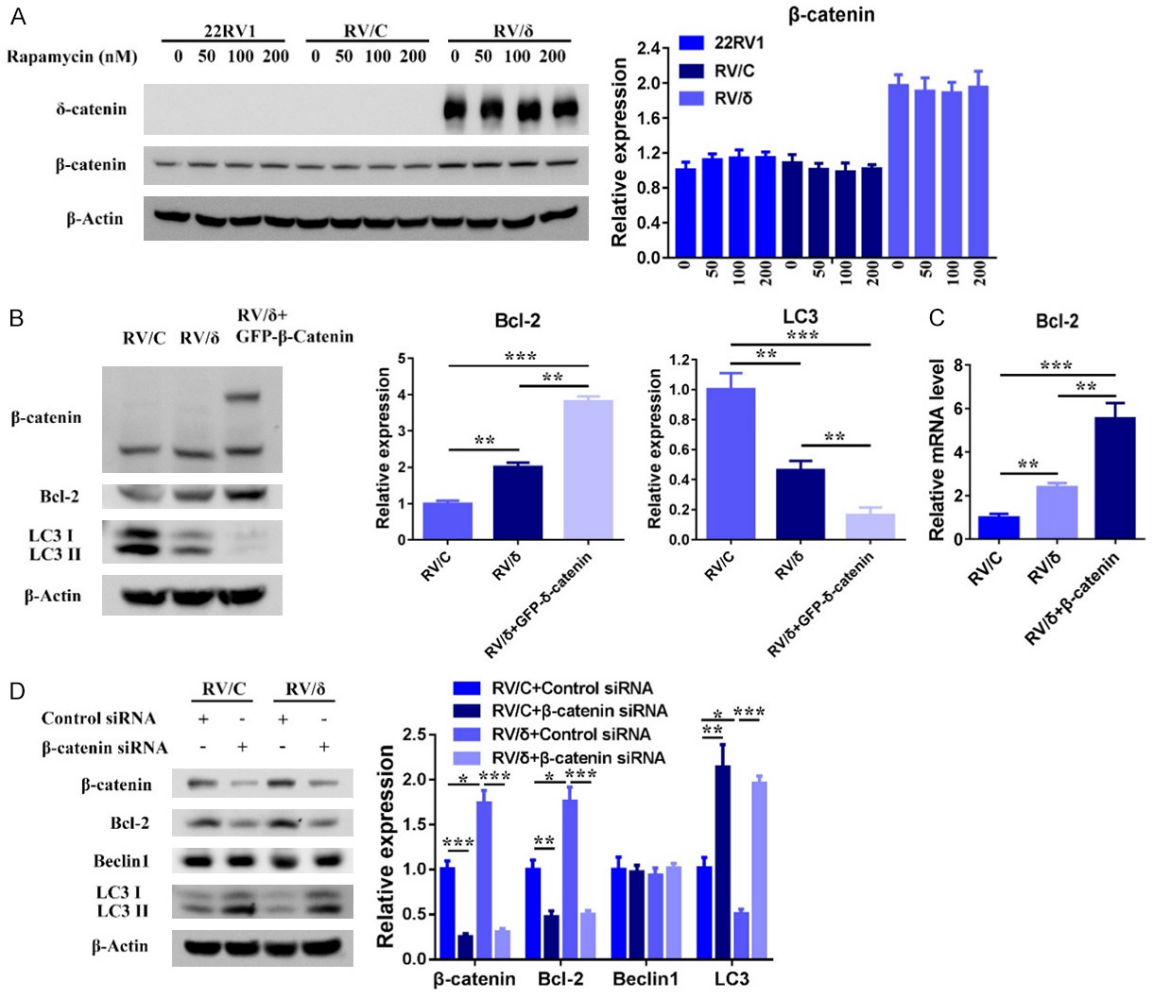

Figure 5. δ-Catenin downregulated autophagy via β-catenin transcriptional regulation of Bcl-2. A. 22RV1, RV/C, and RV/δ cells treated with 0, 50, 100, or 200 nM rapamycin. Western blots were used to analyze β-catenin protein levels in each group. B. RV/C cells were transfected with GFP control. RV/δ cells were transfected with GFP control and GFP-δ-catenin. Western blot analysis of LC3 and Bcl-2 protein levels in each group. C. QRT-PCR analysis of Bcl-2 mRNA levels. D. RV/C and RV/δ cells treated with β-catenin siRNA and control siRNA. Western blot analysis of LC3, β-catenin, Beclin1, and Bcl-2 protein levels in each group. Data are shown as means ± SD of 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

cell growth and metastasis [36, 45]. However, little is known about the role of autophagy in prostate cancer cell migration and invasion. Snail is a cell migration and invasion-related protein that controls EMT. Some studies have suggested that autophagy activation could degrade Snail [45]. Thus, we hypothesized that Snail degradation could participate in the δ-catenin's role in the promotion of prostate cancer cell migration and invasion by δ-catenin. We first detected Snail protein levels in RV/C and RV/δ cells and found that the expression was higher in RV/δ cells. Furthermore, GFP-δ-catenin transfection also increased Snail protein levels (**Figure 6B**). Correspondingly, compared to 22RV1 cells, δ-catenin-overexpressing cells showed a more mesenchymal morphologic phenotype (Figure S5). To further clarify the role of autophagy in Snail expression, we treated RV/C and RV/δ cells with the autophagy activator rapamycin and the autophagolysosome inhibitor bafilomycin. As shown in **Figure 6C**, rapamycin downregulated Snail in RV/C and RV/δ cells. However, treatment with bafilomycin rescued Snail protein expression. To confirm that δ-catenin's role in the regulation of Snail was dependent upon protein degradation rather than transcription, we conducted a qRT-PCR

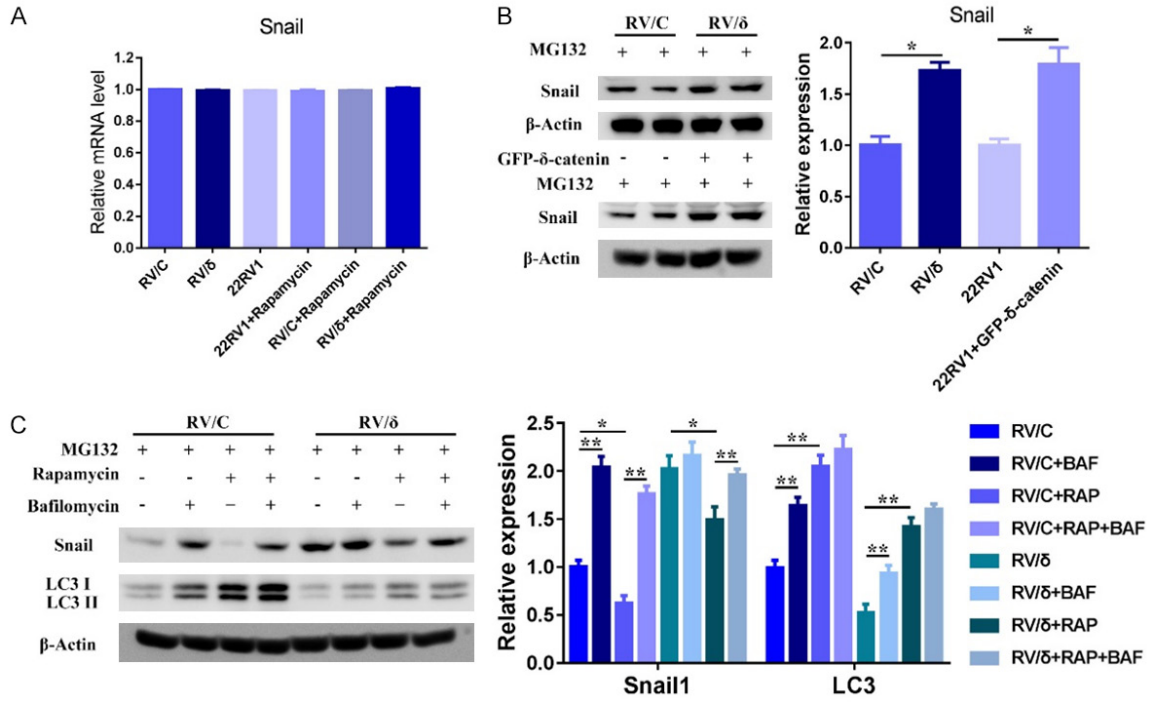

Figure 6. δ-Catenin increased migration and invasion ability via inhibiting autophagy-mediated Snail degradation. A. 22RV1, RV/C, and RV/δ cells were treated with or without 100 nM rapamycin. QRT-PCR analysis of Snail mRNA levels. B. RV/C and RV/δ cells were treated with 5 uM MG132. Western blot analysis of Snail protein levels. C. RV/C and RV/δ cells were treated with 100 nM rapamycin, 10 nM bafilomycin, and rapamycin plus bafilomycin. Western blot analysis of LC3 and Snail protein levels in each group. Data are shown as means ± SD of 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

assay. There was no mRNA change in 22RV1, RV/C, and RV/δ cells treated with or without rapamycin (**Figure 6A**). Taken together, δ-catenin increased migration and invasion ability via inhibiting autophagy-mediated Snail degradation.

Overall, this study demonstrated that δ-catenin could promote the cell migration and invasion of prostate cancer cells by increasing Snail through the β-catenin/Bcl-2-mediated down-regulation of autophagy.

Discussion

Prostate cancer is one of the most common tumors in men and is a major health problem worldwide [46]. Metastasis is the main cause of complications and death in male prostate cancer patients [47]. Despite treatment, the survival rate of men with metastatic prostate cancer is still not high [48]. In addition, androgen deprivation induces the expression of genes that induce EMT, which plays a role in cancer progression and metastasis [49]. However, the mechanism involved in this process is still unknown. Therefore, understanding the mechanism of prostate cancer metastasis is an urgent problem that needs to be solved. Migration and invasion are two key steps in tumor metastasis. Understanding the specific mechanisms of prostate cancer cell migration and invasion is particularly important for the treatment of prostate cancer metastasis.

δ-Catenin belongs to the p120ctn subfamily of armadillo proteins and was identified for the first time because of its interaction with presenilin-1. In recent years, a large number of studies have shown that it is overexpressed in a variety of tumors, including colorectal cancer, prostate cancer, lung cancer, and ovarian cancer. In particular, the expression of δ-catenin in prostate cancer is closely related to tumor progression, malignancy, mortality, and recurrence. This makes δ-catenin a promising target for the treatment of prostate cancer. Our previous studies showed that δ-catenin could promote prostate cancer cell growth and progression through different mechanisms. δ-Catenin could promote prostate cancer progression via

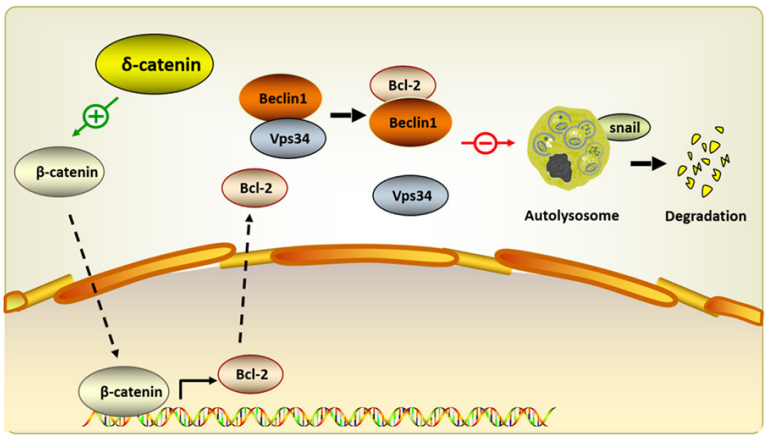

Figure 7. Schematic illustration of δ-catenin increasing migration and invasion in prostate cancer cells.

E-cadherin processing and activate β-catenin-mediated signaling [10]. δ-Catenin promoted prostate cancer cell growth and progression by altering cell cycle and survival gene profiles [8]. However, there are few studies on the effect of δ-catenin in prostate cancer cell migration and invasion.

In this study, we showed that δ-catenin could promote the migration and invasion of prostate cancer cells via Bcl-2-regulated autophagy. Autophagy is a major intracellular degradation system, which transports organelles and proteins into lysosomes for degradation. Autophagy is a double-edged sword for tumors. In ovarian cancer, autophagy induced by MARCH5 RNA promoted cell migration and invasion [50]. In contrast, autophagy induction impaired migration and invasion by reversing EMT in glioblastoma cells [45]. Consequently, the effect of autophagy on tumor cells migration and invasion depends upon the tumor type and the stage of tumor development. In the prostate cancer cells in this study, low autophagy levels showed stronger migration and invasion ability. Treatment with the autophagy activator rapamycin inhibited cell migration and invasion both in 22RV1 cells and the δ-catenin-overexpressing RV/δ cell line.

Beclin1, a Bcl-2 homology 3 (BH3) domain-only protein, is an essential initiator of autophagy [51]. Beclin1 recruits key autophagy-related proteins into the pre-autophagosomal structure to form a core complex composed of Beclin1, Vps34, and Vps15 [52]. Surprisingly, the expression of Beclin1 itself did not change in this study. Beclin1 has been shown to interact with anti-apoptotic Bcl-2 family members through its BH3 domain. This interaction prevents Beclin1 from assembling the pre-autophagosomal structure, thereby inhibiting autophagy [42]. Here, we proved that δ-catenin-regulated autophagy was related to the upregulation of the Bcl-2-Beclin1 complex.

Our previous study demonstrated that δ-catenin could increase β-catenin expression. Many studies have reported that Bcl-2 was one of the target genes of β-catenin. In this study, we proved that β-catenin could increase Bcl-2 mRNA and protein levels. Next, we used β-catenin siRNA to confirm the results. Therefore, we could conclude that the overexpression of δ-catenin increased β-catenin levels and activated the transcription of its target gene Bcl-2.

Snail is an EMT-related protein that plays an important role in migration and invasion. Our results also demonstrated that autophagy could degrade Snail and inhibit cell migration and invasion. However, Snail is a highly unstable protein, with a half-life of about 25 min, and its degradation has been reported to be triggered by glycogen synthase kinase 3β phosphorylation and mediated by the ubiquitin-proteasome system (UPS) [53]. In autophagy-competent cells, Snail is degraded by both autophagy and proteasomes. To inhibit proteasome degradation, we treated prostate cancer cells with the proteasome inhibitor MG132. After preventing the proteasome degradation of Snail, we could easily see the effect of the autophagosome-lysosomal system on the degradation of Snail was easily observed. As shown in **Figure 6**, the autophagy inducer accelerated the degradation of Snail protein and the Snail protein level was rescued when the autophagy-lysosome degradation systems were blocked. Combined with the unchanged mRNA levels, we concluded that δ-catenin increased migration and invasion ability via inhibiting autophagy-mediated Snail degradation.

In summary, based on our findings, we proposed a model for the δ-catenin suppression of

autophagy, in promoting prostate cancer cells migration and invasion (**Figure 7**). In this model, δ-catenin increases β-catenin, activating transcription of its target gene Bcl-2; increased Bcl-2 binding to Beclin1, and disrupts the Beclin1-Vps34 complex, inhibiting autophagy and decreasing Snail degradation by autophagolysosomes.

Acknowledgements

We thank Mengying Jiang and Zhili Liu for technical assistance. This study was supported by a grant (NRF-2019R1I1A3A01061094) from the National Research Foundation of Korea (NRF) funded by the Ministry of Science, Republic of Korea.

Disclosure of conflict of interest

None.

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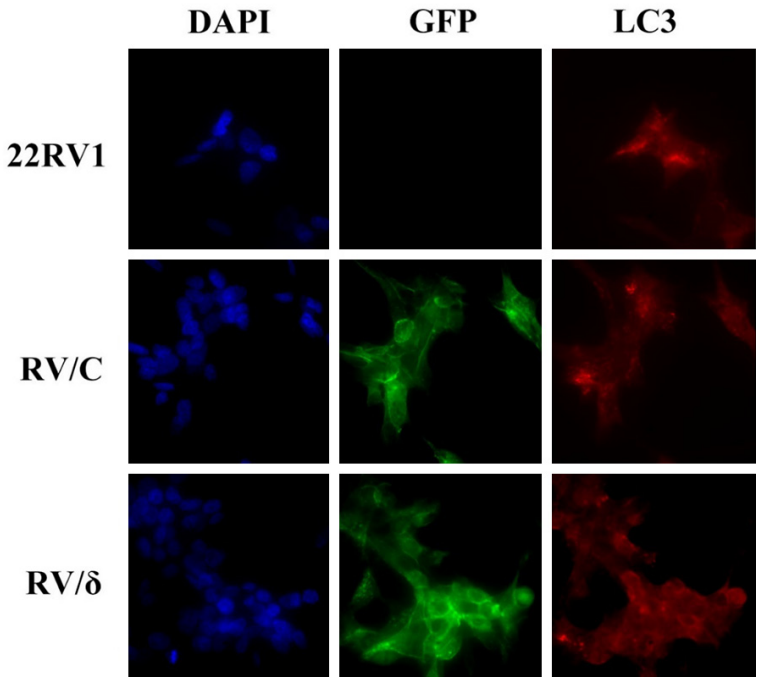

Figure S1. Immunofluorescence staining for LC3 puncta in 22RV1, RV/C, and RV/δ cells. Magnification: 500×.

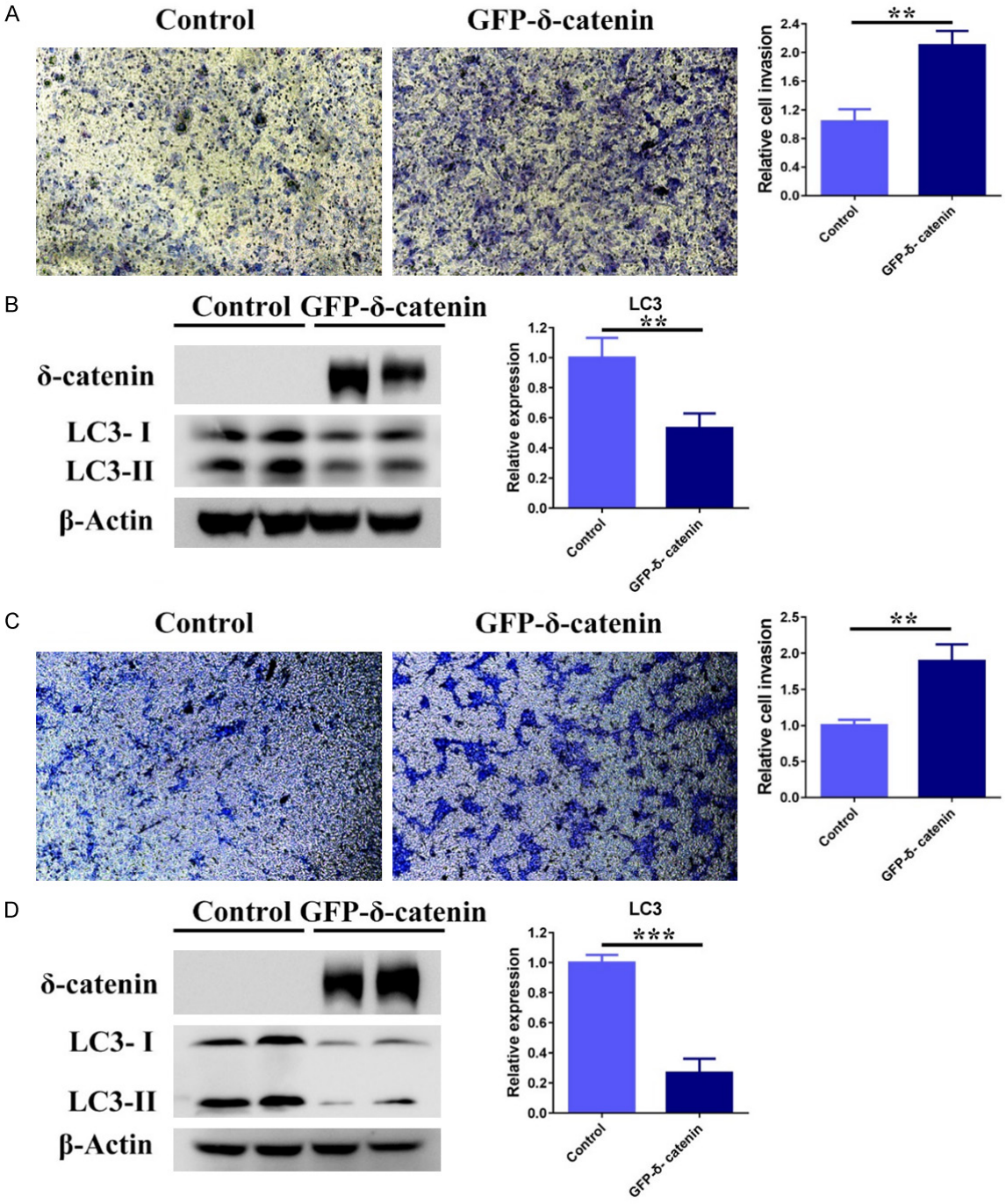

Figure S2. δ-Catenin increased migration and invasion ability and decreased autophagy in PC3 and LNCaP cells. A. PC3 cells were transfected with GFP-Control and GFP-δ-catenin. The Transwell assay was used to analyze the invasion ability of each group. Quantitative analysis of cell invasion in 3 independent experiments (n=3). Magnification: 100×. B. Western blot analysis of LC3 protein levels in each group. C. LNCaP cells were transfected with GFP-Control and GFP-δ-catenin. The Transwell assay was used to analyze the invasion ability of each group. Quantitative analysis of cell invasion in 3 independent experiments (n=3). Magnification: 100×. D. Western blot analysis of LC3 protein levels in each group. Data are shown as means ± SD of 3 independent experiments. $^{**}P<0.01$, $^{***}P<0.001$.

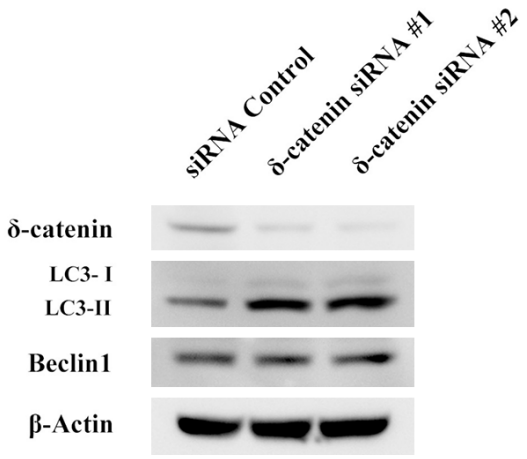

Figure S3. PC3 cells were transfected with control siRNA, δ-catenin siRNA#1, and δ-catenin siRNA#2. Western blot analysis of LC3 and beclin1 protein levels in each group.

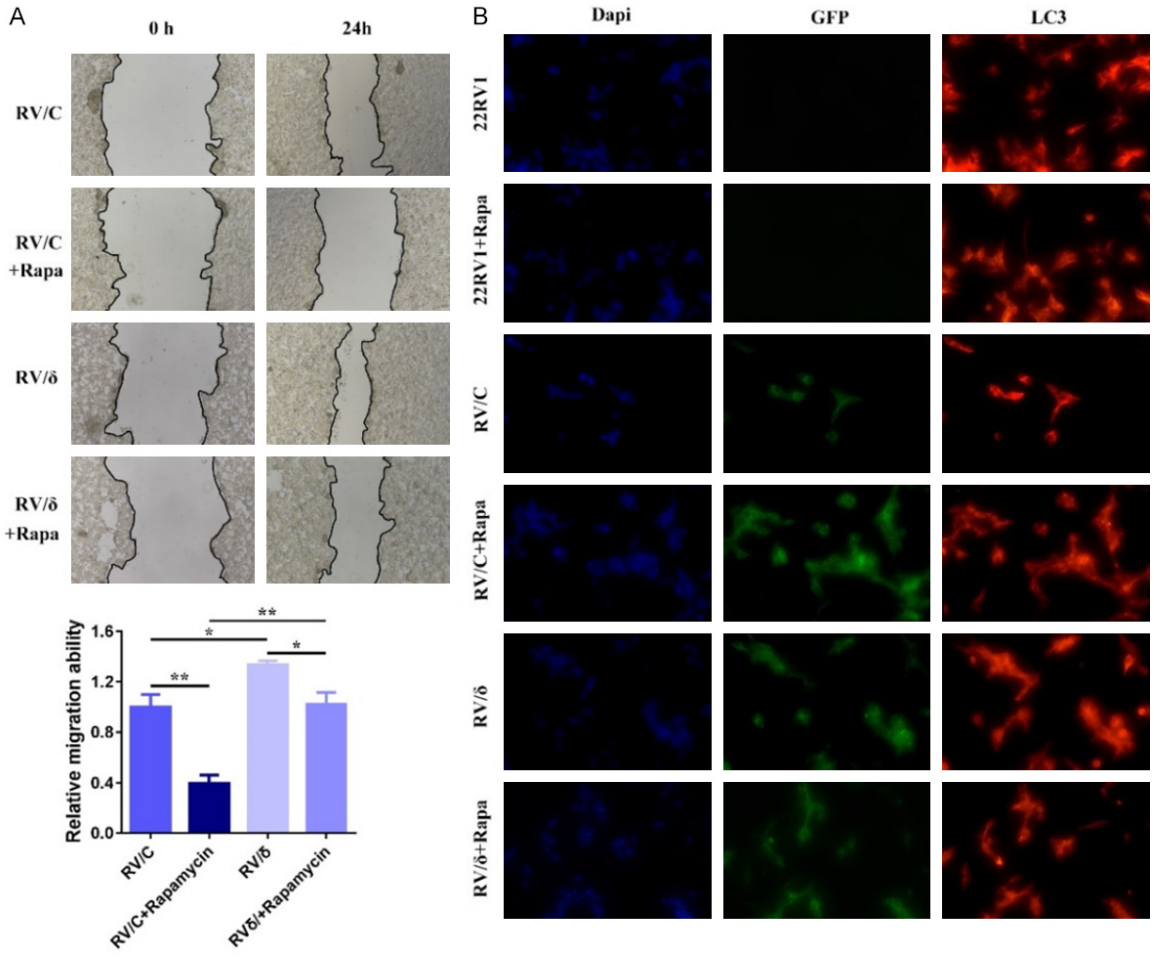

Figure S4. A. 22RV1, RV/C, and RV/δ cells were treated with or without 100 nM rapamycin. The Transwell assay was used to analyze the invasion ability of each group. Magnification: 100×. B. 22RV1, RV/C, and RV/δ cells were treated with or without 100 nM rapamycin. Immunofluorescence staining for LC3 puncta. Magnification: 500×. Data are shown as means ± SD of 3 independent experiments. $^{*}P<0.05$, $^{**}P<0.01$.

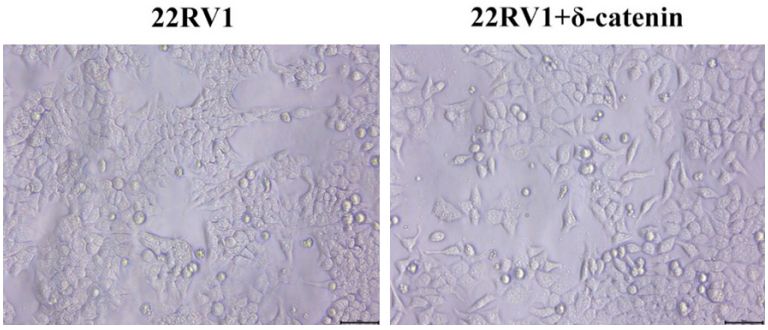

Figure S5. 22RV1 cells were transfected with GFP-Control and GFP-δ-catenin. After transfection, the cells were cultured 36 hours and photographed using a microscope. Magnification: 200×.

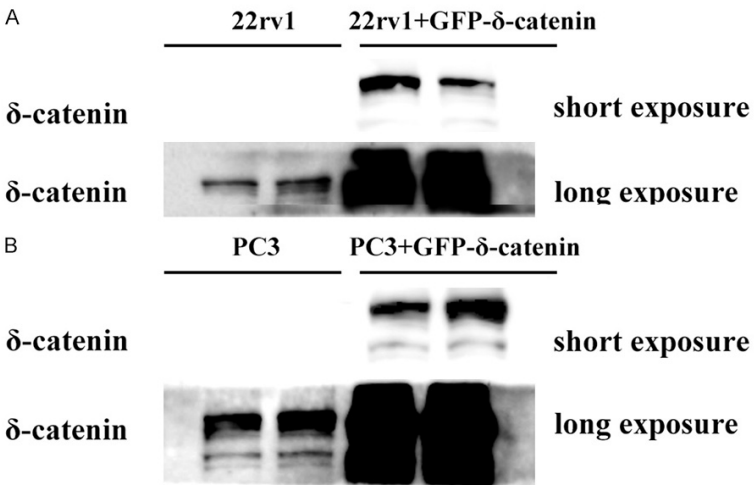

Figure S6. 22RV1 and PC3 cells were transfected with GFP-δ-catenin. Western blot analysis of δ-catenin protein levels in each group.