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Suppression of DACH1 promotes migration and invasion of colorectal cancer via activating TGF-β-mediated epithelial-mesenchymal transition

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ABSTRACT

DACH1 has been found down-regulated in a variety of human cancers, but its clinical significance and functional roles in colorectal cancer (CRC) remain unknown. In this study, we identified DACH1 as a tumor suppressor in CRC. Suppression of DACH1 strikingly increased cell growth, migration and invasion potential of CRC cell line SW480. Expression analysis of a set of epithelial-mesenchymal transition (EMT) markers by RT-qPCR and western blot showed an increase in the expression of mesenchymal markers (vimentin and N-cadherin) and a reduction in the expression of epithelial marker (E-cadherin and γ-catenin). Furthermore, EMT characteristics in DACH1-downregulated CRC cells were abrogated by TGF-β inhibitor SB431542. DACH1 overexpression reduced TGF-β-induced EMT and inhibited SW480 cell invasion which can be reversed in the presence of TGF-β. Thus, our results suggest that DACH1 loss of function results in increased cell growth, motility and invasiveness through TGF-β-mediated EMT, and DACH1 loss of function has important therapeutic implications for targeted therapies of CRC.

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1. Introduction

DACH1, a Drosophila Dachshund homolog, is located in chromosome 13q22 whose function has not yet been fully elucidated. DACH1 has initially been identified in drosophila eye development, and is an essential member of retinal determination gene network [1]. Recently, it has been found DACH1 acts a tumor suppressor involved in multiple tumor types, such as lung, breast, endometrial, prostate, gastric, glioma and liver [2–8]. Furthermore, DACH1 loss of function correlates with poor survival in breast, endometrial, and prostate cancers [3–5].

Previous study has found that DACH1 inhibited TGF-β signaling pathway by binding to Smad4 in breast cancer cells [9] and DACH1 significantly repressed the activity of TGFβ in human CRC [10]. TGF-β is the most potent inducer of EMT in epithelial cancers [11]. During EMT, the epithelial protein level, such as E-cadherin and γ-catenin, are downregulated, while mesenchymal protein such as N-cadherin and vimentin are upregulated [12]. The preponderance of evidence indicates that inappropriate activation of EMT in cancer results in driving cancer cell migration, invasion, and ultimately metastasis [11]. Although much is known about the downstream molecular networks that signal EMT through TGF-β, the master regulatory genes on TGF-β mediated EMT are not well understood [13]. Therefore, we hypothesized that the loss of function of DACH1 might be critical in migration and invasion of CRC associated with TGF-β mediated EMT.

2. Materials and methods

2.1. Cell culture and reagents

Human CRC cell line SW480 and human embryonic kidney cell 293T were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). SW480 and 293T were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Transfection was carried out using Lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. TGF-β inhibitor SB431542 and TGF-β1 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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2.2. Plasmids construction

Synthetic shRNA constructs for knockdown of DACH1 were purchased from OriGene (Rockville, MD; cat no, TL313572). And they were prepared and stored according to the manufacturer's instructions. Four shRNA sequences targeting human DACH1 were used, with one scramble shRNA sequence as a negative control. The sequences targeting DACH1 were as follows: shDACH1-A 5′-CCAGAGAACTCTCACATCATGCCGCATTC-3′; shDACH1-B 5′-TCAGCCTACCTCCAGCATCTGTCACCATG-3′; shDACH1-C 5′-TTGCTTCTTATTCAAACTGTTGGTCATAT-3′; shDACH1-D 5′-TATTTGAGCCTGGTTTCAATGTGAGAACC-3′. shRNA lentiviral packaging were carried out according to the manufacturer's instructions. Full length DACH1 cDNA (GenBank accession number NM_080759.4) was cloned into the pcDNA3.1 vector (Addgene, Cambridge, MA, USA). The constructed plasmid was verified by DNA sequencing.

2.3. Proliferation assays

Cell growth was measured using (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay Kit (BioDev-Tech, Beijing, China). Five thousand cells per well were seeded onto 96-well plates for 1 day prior to addition of DMSO. After 24 h incubation, 10 μl MTT reagent was added to each well and incubated at 37 °C for 4 h. Then, 100 μl DMSO was added to each well and the plates were shaken for 10 min in the dark. The absorbance values measured at 490 nm on Day 0 were set as 100%, and the rest of measurements were then normalized relative to the value on Day 0. Cell proliferation was monitored for up to 5 days. All experiments were repeated three times.

2.4. Invasion assays

Cells (1×10^5) were resuspended in 100 μl DMEM and placed into Matrigel-coated Transwell inserts containing 8 μm filters (BD BioCoat; cat no, 354480) in triplicate. 500 μl of DMEM with 10% FBS was placed to lower chambers. Cell invasion was measured according to the manufacturer's instructions. Generally, after 24 h incubation, the cells on the upper surface of the filters were removed with a cotton swab. The invaded cells were fixed using methanol at room temperature for 10 min. The filters were stained with crystal violet and photographed (×10 magnification). The number of invaded cells were then counted in six independent fields and the results are presented as mean ± SD.

2.5. Wound healing assays

5×10^5 cells were seeded in 6-well plates per well. Cells were incubated for 24 h until approximately 90% confluence. The wounds were made by scraping with 200 μl pipette tip across the cell monolayer and marked. The wounded cells were further incubated with fresh medium changed every day. Photographs were taken just after scratching (0 h) and at the time point of 48 h.

2.6. RNA preparation and reverse transcription quantitative PCR (RT-qPCR)

Total RNA were extracted from cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol, and 1 μg for each were used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Amplification was done for 40 cycles using iQ SYBR Green Supermix on iCycler (Bio-Rad, Hercules, CA, USA). Gene specific primers sequences are described as pervious [14]. Primers were synthesized by Shanghai Invitrogen Biotechnology Company. All quantitations were normalized to an endogenous control β-actin. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to β-actin). The relative expression levels of samples are presented by a semilog plot.

2.7. Western blot

Standard western blotting was performed to measure the expression levels of proteins. Cultured CRC cells were lysed in ice-cold RIPA buffer (Cell Signaling, Danvers, MA, USA; cat no. 9806) containing one tablet EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 10 min. Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to polyvinylidene fluoride (PVDF) membrane. Primary antibodies used were DACH1, β-Tubulin (Abcam, Cambridge, UK), E-cadherin, N-cadherin (BD Transduction). Anti-mouse, anti-goat and anti-rabbit IgG peroxidase antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used for secondary antibody and enhanced chemiluminescence (ECL) solution (Thermo Fisher Scientific, Waltham, MA, USA) was used for detection.

2.8. Statistical analysis

For statistical analyses, the GraphPad Prism 5 Software (GraphPad, San Diego, CA, USA) was used. All data are expressed as mean with SDs. The statistical significance of differences between two groups was analyzed by two-tailed t-test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Loss of DACH1 leads to increased colorectal cancer cell growth, motility and invasiveness

DACH1 expression was discovered in the human CRC cell lines as published before [10]. To evaluate the effect of DACH1 in CRC carcinogenesis, we measured cell growth, migration and invasion after DACH1 downregulated. Through RNAi, four stable DACH1 knockdown (DACH1-KD) clones in the CRC cell line SW480 and negative control (MOCK) clone were generated. DACH1 in SW480 cells significantly decreased its expression (Fig. 1A) compared with control. shDACH1-D showed the most significant decrease among all the four shRNAs. To further support the results, parallel experiments were performed with shDACH1-A in all the following assays. As expected, shDACH1-A also showed statistical significance compared to control. For simplicity, data from shDACH1-A were not shown in the following results. DACH1-KD clone in the following figures referred to shDACH1-D. Significant increase on cell growth was observed in DACH1-KD clones (Fig. 1B). By wound healing assay, RNAi-mediated DACH1 downregulation strikingly increased cell motility of SW480 cells (Fig. 1C). Furthermore, DACH1 downregulation also increased SW480 cell invasiveness with about 98% increase of invasion compared with control (Fig. 1D).

3.2. DACH1 loss of function upregulates EMT markers

As this increase in motility and invasiveness was accompanied by significant phenotypic changes of cellular morphology (data not shown), we hypothesized that DACH1 loss of function might induce a mesenchymal program. Therefore, we performed RT-qPCR and western blotting respectively to the compare expression patterns of a panel of EMT markers (E-cadherin, γ-catenin, vimentin and N-cadherin) between control and DACH1-KD SW480 cells. This assay revealed that DACH1-KD cells displayed decreased expression of

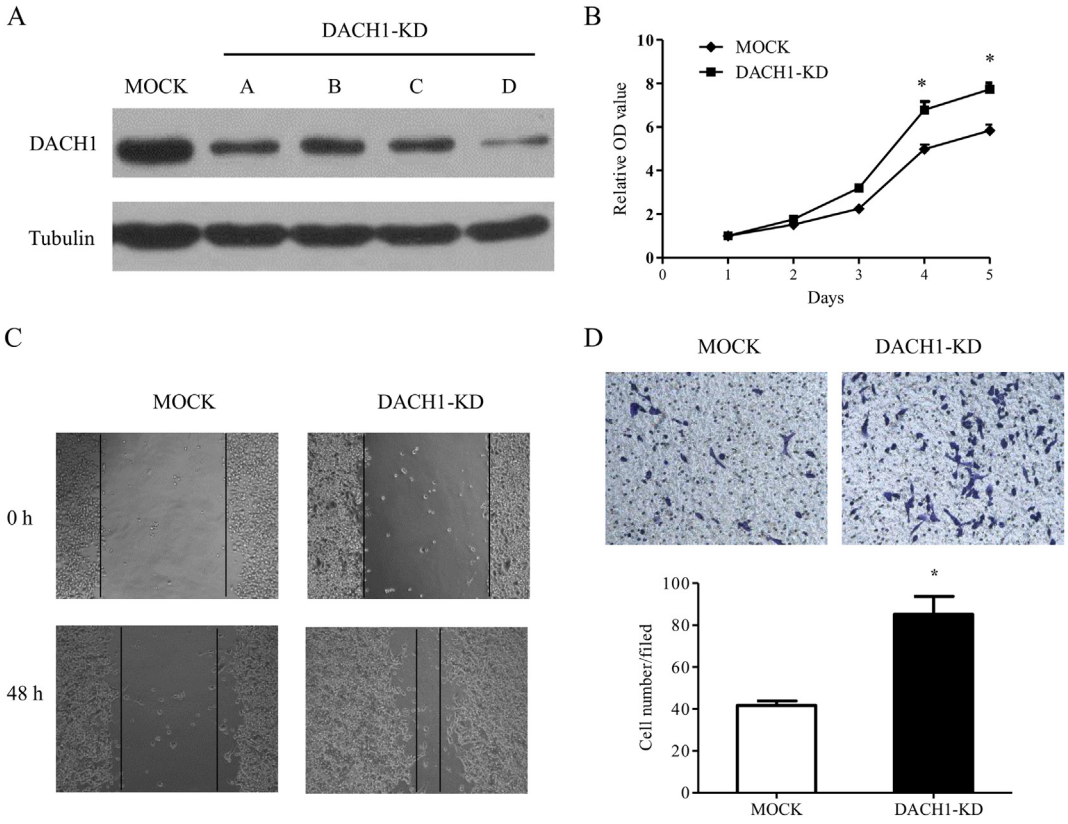

Fig. 1. Knockdown of DACH1 increases cell growth, motility and invasiveness. A. SW480 cells were infected by four DACH1 shRNA viruses and control, the protein level of DACH1 was detected by western blotting. B. The effect of DACH1 knockdown on cell growth was detected by MTT. *$P < 0.05$. C. The effect of DACH1 knockdown on cell migration was detected by wound healing assay at the indicated time point (left). D. The effect of DACH1 knockdown on cell invasion was detected by transwell assay. A representative result of three independent experiments and the statistic data were presented (down).

the epithelial markers E-cadherin and γ-catenin, whereas increased mesenchymal markers vimentin and N-cadherin (Fig. 2A). The changes in E-cadherin and N-cadherin expression were confirmed at the protein level (Fig. 2B). These results clearly suggest that DACH1 loss of function results in upregulated EMT markers with increased cell growth, migration and invasion characteristics.

3.3. *EMT characteristics in DACH1-downregulated colorectal cancer cells are abrogated by TGF-β inhibitor*

Previous work showed that TGF-β can induce EMT, we wanted to evaluate the effect of TGF-β inhibitor on DACH1 loss of function induced EMT. After treatment with SB431542, a TGF-βRI inhibitor, control clone showed no obvious difference in morphology, whereas DACH1-KD clones showed a prominent mesenchymal morphology. We examined mRNA levels of EMT markers by RT-qPCR of DACH1-KD and control clone in the presence of SB431542. Strikingly, mesenchymal markers vimentin and N-cadherin expression were significantly reduced and epithelial markers E-cadherin and γ-catenin were increased in DACH1-KD SW480 cells in the presence of SB431542 compared to control cells with or without SB431542 treatment (Fig. 3A). Furthermore, western blot analyses confirmed the results by RT-qPCR (Fig. 3B). Cell invasion assay also supported the changes of EMT markers. Treatment with SB431542 dramatically reduced the invasiveness of DACH1-KD cells

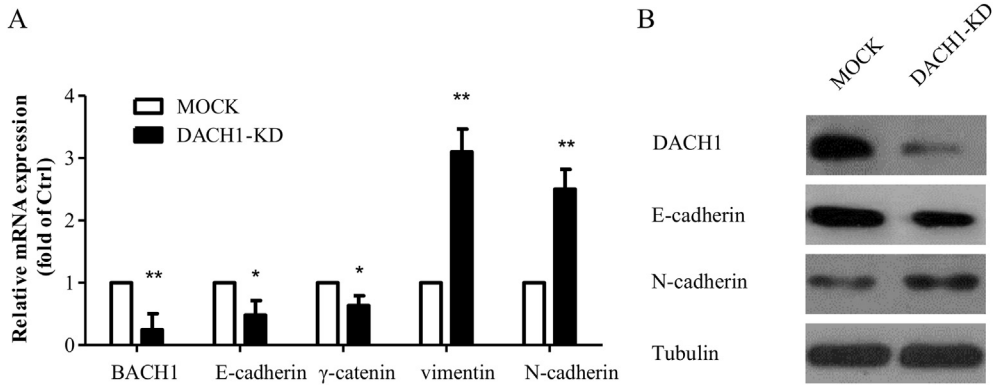

Fig. 2. Knockdown of DACH1 induces EMT. A. The mRNA expression levels of EMT markers were detected by RT-qPCR. *$P < 0.05$, **$P < 0.01$. B. Western blotting analysis of same samples used in A was shown. The protein level of DACH1, E-cadherin and N-cadherin was detected.

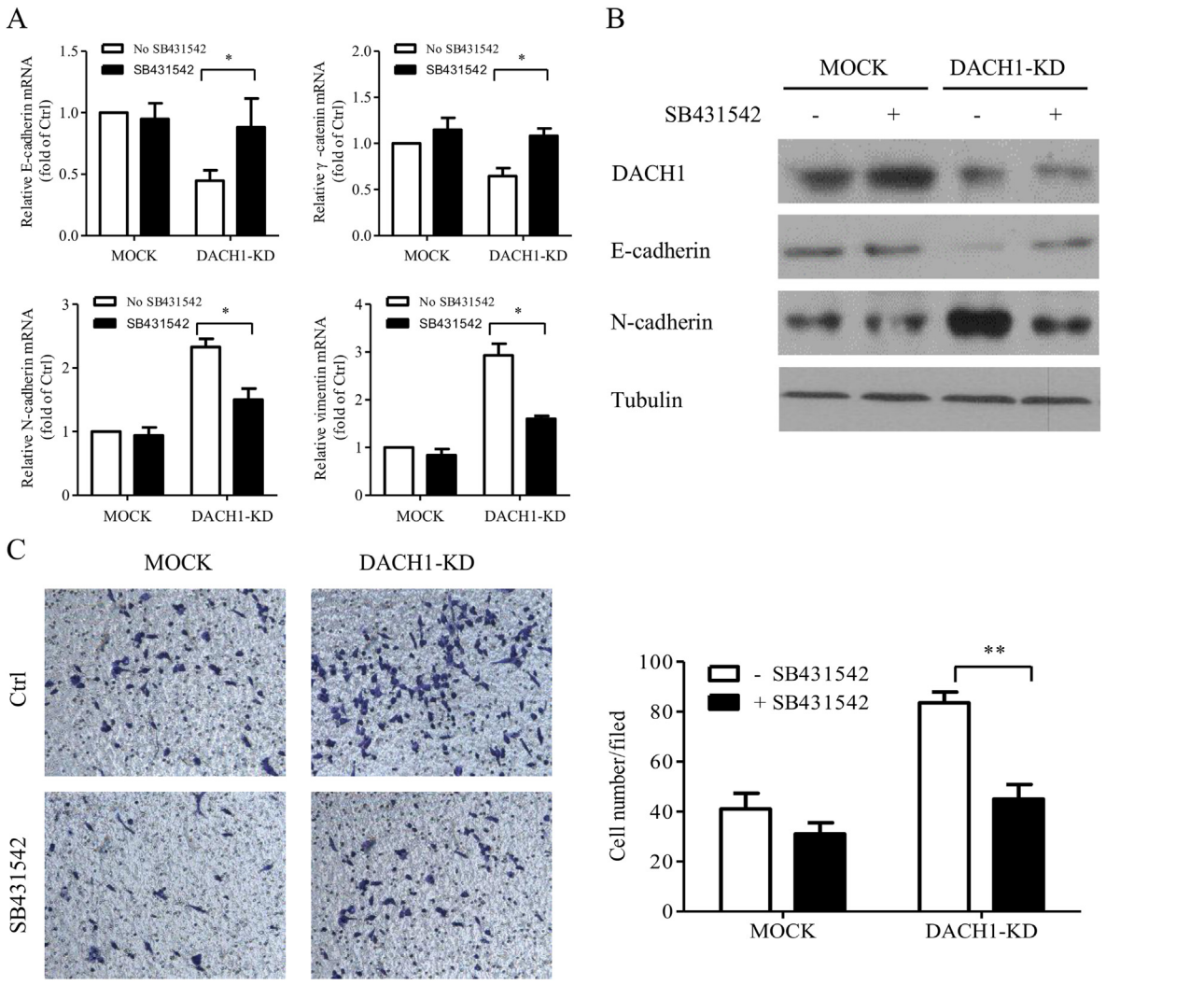

Fig. 3. DACH1 loss of function induced EMT can be blocked by TGF-β inhibitor. A. DACH1-KD and MOCK cells were treated with or without TGF-β inhibitor SB431542 (10 μM) for 3 days and mRNA expression levels of EMT markers were detected by RT-qPCR. $^{*}P < 0.05$. B. Western blotting analysis of same samples used in A was shown. The protein level of DACH1, E-cadherin and N-cadherin was detected. C. Transwell assay was performed after DACH1-KD and MOCK cells were treated with of without SB431542 (10 μM) for 3 days. A representative result of three independent experiments and the statistic data were presented (right). $^{**}P < 0.01$.

(Fig. 3C). Our data suggest that DACH1 loss of function induced EMT can be blocked in the presence of TGF-β inhibitor.

3.4. *DACH1 inhibits invasion of colorectal cancer cells by blocking TGF-β-mediated EMT*

We then investigated the role of DACH1 in the regulation of TGF-β-mediated EMT. As shown in Fig. 4A, overexpression of DACH1 increased E-cadherin and γ-catenin levels but decreased vimentin and N-cadherin level, suggesting a MET-like event was induced. Furthermore, overexpression of DACH1 reduced TGF-β-induced EMT (Fig. 4B). Because DACH1 knockdown increased the invasiveness of SW480 cells, we also determined whether DACH1 overexpression has the opposite effect. As expected, the invasion ability of DACH1 overexpression cells were decreased as examined by transwell assays (Fig. 4C). Moreover, after treatment with TGF-β, control clone showed no obvious difference in invasiveness, but DACH1 overexpressed cells showed a marked increase compared cells without TGF-β treatment (Fig. 4C). Thus, cumulative data from DACH1-KD and ectopic expression experiments show that DACH1 is an inhibitor of TGF-β-mediated EMT and loss of DACH1 expression promotes invasion of SW480 cells via activating TGF-β-mediated EMT.

4. Discussion

Identification of the genetic drivers of tumorigenesis and metastasis is essential for the design of targeted therapies aimed at the underlying pathways regulated by these genes. DACH1 has been found as an important factor in many cancer developments and tumorigenesis. Recent studies have found DACH1 methylation is associated with higher tumor stage and lymph node metastasis [15]. Because DACH1 expression was absent in the majority of tumors analyzed, we assume that DACH1 seems to behave as a classic tumor suppressor. When activated in cancer, EMT is thought to drive invasion and metastasis in breast cancer and other epithelial cancers [16].

In this study, we determined the role and potential mechanism of DACH1 in cell growth, migratory ability, invasiveness and EMT of CRC cell line SW480. DACH1 loss of function leads to increased CRC cell growth, motility and invasiveness. E-cadherin is one of the most important indicators of epithelial phenotype. In clinical

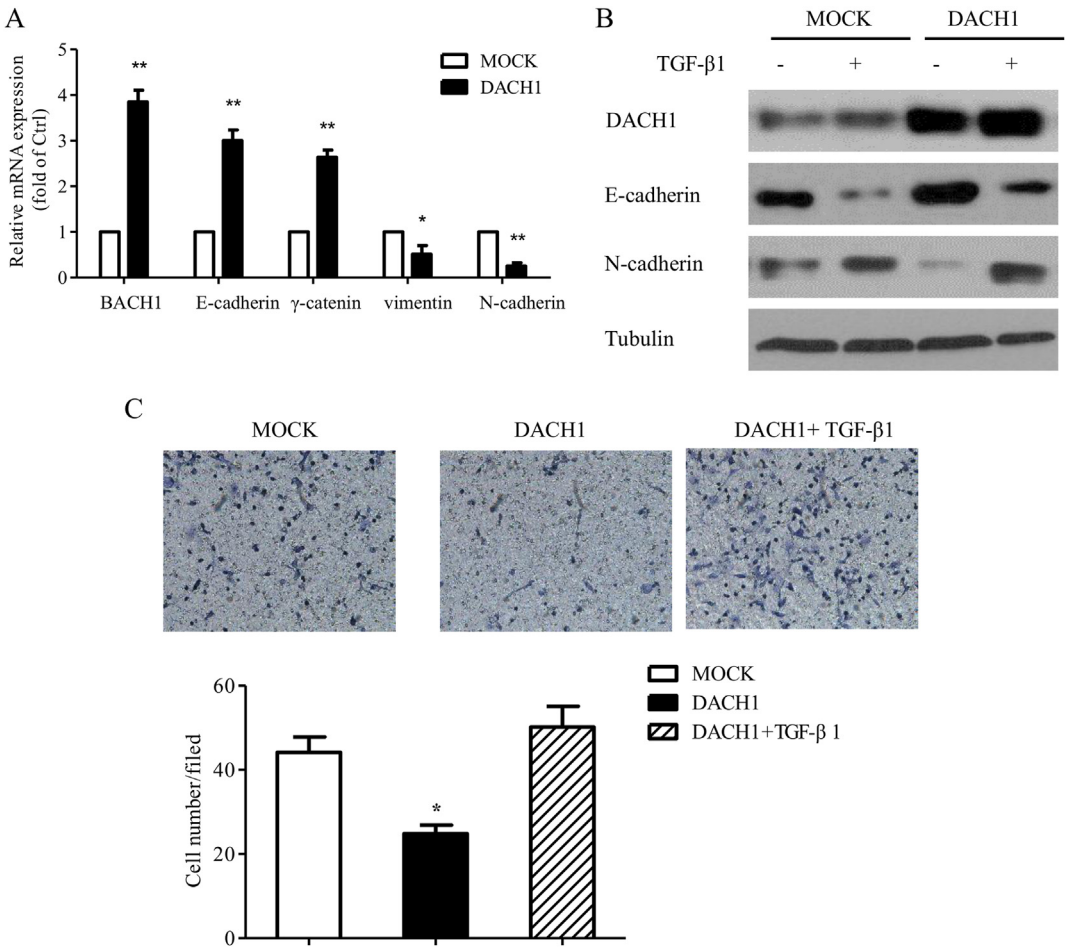

Fig. 4. Overexpression of DACH1 inhibits invasion by blocking TGF-β-mediated EMT. A. Ectopically expressed DACH1 and vector plasmid were transfected into SW480 cells. DACH1 overexpressed and MOCK cells were cultured with or without 2 ng/ml TGF-β1 for 48 h and then mRNA expression levels of EMT markers were detected by RT-qPCR. $^{*}P < 0.05$, $^{**}P < 0.01$. B. Western blotting analysis of same samples used in A was shown. C. DACH1 overexpressed cells were treated with or without TGF-β (2 ng/ml) for 48 h and the invasion ability was measured by transwell assay compared with control cells. A representative result of three independent experiments and the statistic data were presented (down). $^{*}P < 0.05$.

diagnosis, E-cadherin could be used as a prognostic factor in some types of cancers [17]. Our data indicate that loss of DACH1 in SW480 cells results in an EMT morphology with downregulation of E-cadherin and γ-catenin and upregulation of vimentin and N-cadherin, well-known markers of early EMT induction. Thus, DACH1 loss of function could play an early role in the initiation of EMT and could be an important biomarker of aggressive disease.

Remarkable, this induction can be blocked in the presence of TGF-β inhibitor. DACH1 loss of function in the presence of TGF-β inhibitor SB431542 results in increased E-cadherin and γ-catenin expression and reduced vimentin and N-cadherin expression as well as decreased invasiveness of SW480. Correspondingly, overexpression of DACH1 can shift the epithelial-mesenchymal balance toward the side of epithelial phenotype and decrease the intensity of EMT induced by TGF-β. Overexpression of DACH1 also inhibits the invasive growth of colorectal tumor cells which can be reversed in the presence of TGF-β.

In summary, our combined data provide critical evidence that DACH1 acts as a tumor suppressor in CRC as well as provide a novel evidence for its regulation of TGF-β-mediated EMT. Because TGF-β inhibitors are now being used in clinical trials [18], our data suggest that DACH1 could be an important biomarker to target those patients with CRC, showing DACH1 deletion or loss of function, for more effective treatment with specific targeted therapies aimed at TGF-β-mediated EMT.

Conflict of interest

None.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/.10.1016/j.bbrc.2015.03.032.

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