

The function of FAK/CCDC80/E-cadherin pathway in the regulation of B16F10 cell migration

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Received October 20, 2016; Accepted March 16, 2018

DOI: 10.3892/ol.2018.9159

Abstract. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase involved in the development and progression of cancer. However, the regulatory role of FAK in cell migration remains unclear. The aim of the present study was to investigate the mechanism underlying the regulation of melanoma cell migration by FAK. The effect of FAK knockdown on gene expression in B16F10 cells was examined by gene chip analysis. The expression levels of coiled-coil domain containing 80 (CCDC80) and epithelial (E)-cadherin were analyzed by reverse transcription quantitative polymerase chain reaction and western blotting. Wound healing and transwell assays were used to monitor B16F10 cell migration. It was identified that the knockdown of FAK increased the expression levels of CCDC80 and E-cadherin, while the overexpression of CCDC80 elevated E-cadherin expression. Concurrently, upregulation of CCDC80 inhibited the migration of B16F10 cells, and downregulation of CCDC80 promoted the migration of B16F10 cells. The clinical data from the Oncomine database also revealed that the mRNA level of FAK was increased while the mRNA levels of CCDC80 and E-cadherin were decreased in patients with melanoma compared with normal controls. Taken together, the results of the present study suggest that

the regulation of B16F10 melanoma cell migration by FAK is potentially mediated by CCDC80.

Introduction

Malignant melanoma is one of the most aggressive malignancies in humans, and is responsible for 60-80% of skin cancer-associated mortalities (1). As revealed in 2013, the 5-year survival rate of patients with metastatic malignant melanoma is ~14%, and its incidence has been increasing in the white population over the previous 2 decades (1). However, the mechanisms leading to the malignant transformation of melanocytes remains poorly understood. As a non-receptor tyrosine kinase, focal adhesion kinase (FAK) is widely expressed in various human tissues and cell types, including mesenchymal cells, neuronal cells, platelets, lymphocytes and erythrocytes (2,3). Overexpressed in a wide range of types of cancer (4), FAK promotes tumor cell migration and invasion (5). FAK is also involved in various signaling pathways that promote tumor growth and metastasis (6). However, the precise mechanisms underlying the regulatory role of FAK in cell migration remain to be elucidated.

Coiled-coil domain containing 80 (CCDC80) has been suggested to be a multifunctional protein among vertebrates (7). Previous studies have demonstrated that the expression of CCDC80 was downregulated in thyroid carcinomas, papillary carcinomas and colorectal and pancreatic cell lines (8-10). Ectopic expression of CCDC80 inhibits cancer cells growth *in vitro*, indicating that CCDC80 may be a candidate target for tumor therapy (10). In addition, CCDC80 also modulates the apoptotic pathways (10-12). However, the role of CCDC80 as a possible tumor suppressor remains unclear.

In the present study, the mechanism underlying the regulation of B16F10 cell migration by FAK was investigated. The knockdown of FAK promoted the expressions of CCDC80 and E-cadherin, and suppressed the migration of B16F10 cells. Meanwhile, CCDC80 was demonstrated to inhibit the migration of B16F10 cells, and elevate the level of E-cadherin. Clinical data for patients with melanoma from the Oncomine Cancer Microarray database were also analyzed and compared with the experimental data.

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Abbreviations: FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; NC, normal control; NCOA3, nuclear receptor coactivator-3; RT-qPCR, quantitative real time polymerase chain reaction; siFAK cell line, stable interference of FAK in B16F10 cells; siNC cell line, negative control for siFAK cell line

Key words: focal adhesion kinase, coiled-coil domain containing 80, epithelial cadherin, melanoma, migration

Materials and methods

Biological chemicals and antibodies. Blasticidin was obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The primary antibody against E-cadherin (cat. no.: 610181) was purchased from BD Biosciences (Franklin Lakes, NJ, USA), and the primary antibodies against extra-cellular-signal-regulated kinase (ERK; cat. no.: 4695S) and phosphorylated ERK (p-ERK; cat. no.: 4377S) were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The primary antibody against tubulin (cat. no.: sc-32293) was obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA).

Cells and cell culture. Using small interfering (si)RNA, the stable interference of FAK in B16F10 cells [American Type Culture Collection (ATCC), Manassas, VA, USA] (siFAK) and siFAK negative control (siNC) cell lines were constructed by the State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University (Nanjing, Jiangsu). These cells, alongside B16F1 cells (ATCC), were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Wisent, Inc., St. Bruno, QC, Canada) supplemented with 10% fetal bovine serum (FBS; Life Technologies; Thermo Fisher Scientific, Inc.) and 3 mg/l⁻¹ blasticidin.

Gene chip analysis. siFAK and siNC cells were lysed in 1 ml TRIzol[®] reagent (Life Technologies; Thermo Fisher Scientific, Inc.), and gene chip analysis was performed by CapitalBio Corporation (Beijing, China).

Migration assays. Cell migration was monitored by wound healing and transwell assays. For the wound healing assay, B16F10 cells that were transfected with pcDNA3.1-CCDC80 or pcDNA3.1 plasmid or siCCDC80 RNA were seeded at an initial density of 2x10⁵ cells/well, and cultured (37°C, 5% CO₂) in DMEM supplemented with 10% FBS overnight. Following culture (37°C, 5% CO₂) of the cells in serum-free DMEM for an additional 24 h, a micro-pipette tip was used to create a wound in the monolayer of cells. Wound closures were observed by phase-contrast microscopy (magnification, x40; Olympus Corporation, Tokyo, Japan), and digital images were captured at the interval times of 0 and 24 h (Photoshop CS6, Adobe Systems, Inc., San Jose, CA, USA). The percent migration rate was calculated using the formula: (1-T/C) x100%, where T and C represent the scrape distance at the indicated treatment time (24 h) and at the previous treatment time (0 h), respectively.

For the transwell assay, 1x10⁵ cells per insert (8-μm pore size) were incubated (37°C) in serum-free DMEM for 24 h in the upper chamber, with DMEM with 10% FBS in the lower chamber. Then, cells inside the inserts were removed with cotton swab, while cells on the underside of the inserts were fixed in 10% formalin for 20 min (room temperature) and stained at room temperature for 30 min (1% crystal violet). Cells from 5 random microscopic fields/insert were counted in triplicate at magnification, x200.

Total RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from siNC and siFAK cell lines using TRIzol[®]

Table I. Gene chip analysis of the FAK, CCDC80 and E-cadherin gene expression.

Gene	Fold change (siFAK vs. siNC)
FAK	-2.12
CCDC80	1.72
E-cadherin	1

The fold change (siFAK to siNC) is a relative value. Fold change >0 suggests that the expression of the gene is upregulated in siFAK cells, compared with that in the siNC cells. Fold change <0 signifies that the expression is downregulated. si, small interfering; FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; E-cadherin, epithelial cadherin.

reagent (Life Technologies; Thermo Fisher Scientific, Inc.). RNA quantity (absorbance at wavelength 260 nm, A260) and purity (A260/A280) were determined by BioPhotometer[®] (Eppendorf, Hamburg, Germany). First-strand cDNA was synthesized with 1.5 μg of total RNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, Basel, Switzerland). The thermocycler conditions were as follows: 95°C for 10 min, then 94°C for 10 sec and 60°C for 1 min, for 40 cycles. Data were normalized using GAPDH. The gene-specific primers were synthesized by Nanjing GenScript (Nanjing, China), and were as follows: CCDC80 forward, 5'-GATCCTGGAGCAGCCTCTGG-3'; CCDC80 reverse, 5'-ACATGGCTTCCAGCCTGACC-3'; nuclear receptor coactivator-3 (NCOA3) forward, 5'-TTCGCC TAGTCCACTCATCC-3'; NCOA3 reverse, 5'-GTGGACTCC GAGATCGGTAA-3'; E-cadherin forward, 5'-GTGGGTCAG GAAATCACATC-3'; E-cadherin reverse, 5'-CTCCAAATC CGATACGTGATC-3'; GAPDH forward, 5'-TGAAGCAGG CATCTGAGGG-3'; GAPDH reverse, 5'-CGAAGGTGGAAG AGTGGGAG-3'.

RNA and plasmid transfection. CCDC80 (siCCDC80) and negative control (NC) siRNA were synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). The PcDNA3.1-CCDC80 plasmid was donated by Professor Kolligs (University of Munich, Munich, Germany). Cells were transfected with the siRNA (100 pmol) or plasmid (1.5 μg) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer, and cultured for 48 h prior to harvesting and subsequent experimentation. The sequences of the siRNA were as follows: siCCDC80 forward, 5'-GAUGAGUAUGCAGGAU AUGUU-3'; siCCDC80 reverse, 5'-CAUAUCCUGCAUACU CAUCUU-3'; siNC forward, 5'-UUCUCCGAACGUGUC ACGUTT-3'; siNC reverse, 5'-ACGUGACACGUUCGGAGA ATT-3'. The 2^{-ΔΔCq} method was used for quantification (13).

Western blot analysis. Cells were lysed in lysis buffer (Beyotime, Institute of Biotechnology, Haimen, China), and following staining with 0.1 g/l G-250 at room temperature for 1 min, protein determination was performed by measuring

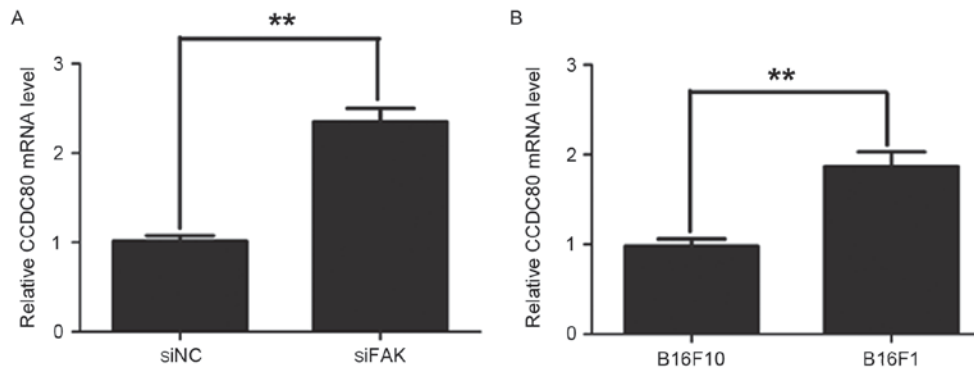


Figure 1. Downregulation of FAK promoted the gene transcription of CCDC80. (A) The mRNA levels of CCDC80 in siNC and siFAK cells were examined by RT-qPCR. (B) The mRNA levels of CCDC80 in B16F10 and B16F1 cells were examined by RT-qPCR. **P<0.01. FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; RT-qPCR, reverse transcription quantitative polymerase chain reaction; si, small interfering.

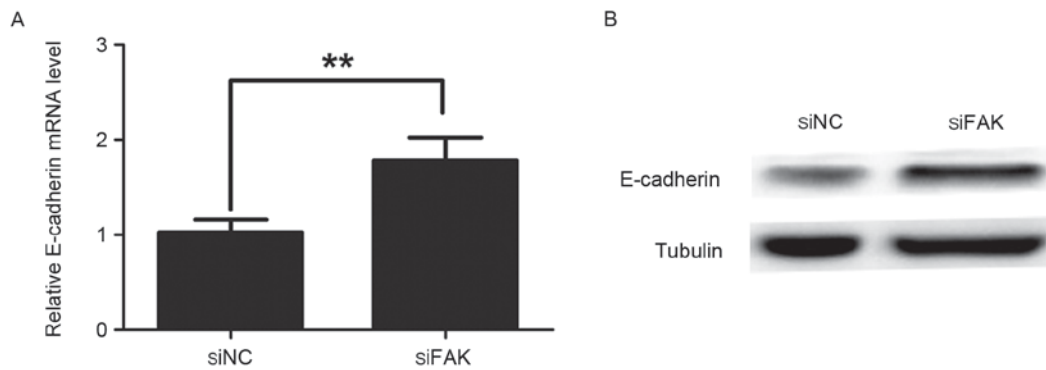


Figure 2. FAK regulated E-cadherin expression. (A) The mRNA levels of E-cadherin in siNC and siFAK cells were examined by reverse transcription quantitative polymerase chain reaction. (B) The protein levels of E-cadherin in siNC and siFAK cells were revealed by western blotting. **P<0.01. FAK, focal adhesion kinase; si, small interfering; E-cadherin, epithelial cadherin.

absorbance at a wavelength of 595 nm using the BioPhotometer® (Eppendorf, Hamburg, Germany). Total proteins (30 µg) were fractionated using SDS-PAGE (8%) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in 1X PBS Tween-20 buffer (5% tween-20) at room temperature for 1 h. and then incubated with the aforementioned primary antibodies (1:1,000) at room temperature for 1 h. Horseradish peroxidase-conjugated anti-mouse (cat. no.: 7076S; 1:2,000 dilution) or anti-rabbit IgG (cat. no.: 7074S; 1:2,000 dilution) from Cell Signaling Technology Inc. were used as the secondary antibodies at room temperature, and the protein bands were detected using an enhanced chemiluminescence detection system (Tanon Science and Technology Co., Ltd., Shanghai, China).

Analysis gene expression by Oncomine. The Oncomine Platform provides robust, peer-reviewed analysis methods that compute gene expression signatures, clusters and gene-set modules, extracting biological observations from the data. Following the successful registration of an Oncomine username, Oncomine (www.oncomine.org, last access date August 2016) was used to analyze the genes, terms searched were 'CCDC80', 'FAK' and 'E-cadherin'.

Statistical analysis. The data are presented as the mean ± standard deviation. Analysis of two groups was

performed using Student's t-test and comparisons of multiple groups was conducted using one-way analysis of variance with Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference. Software GraphPad Prism5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. A total of 3 replicates were performed for each experiment.

Results

Knockdown of FAK increases the expression levels of CCDC80 and E-cadherin in B16F10 cells. To explore the role of FAK in B16F10 melanoma progression, siFAK and siNC cell lines were constructed. It has been established previously that compared with those in the siNC cells, the mRNA and protein levels of FAK in siFAK cells were decreased markedly (14). In the present study, the gene chip assay revealed that the mRNA level of FAK was decreased, while the mRNA levels of CCDC80 and E-cadherin were increased in the siFAK cells compared with the siNC cells (Table I). The RT-qPCR assay also indicated that the CCDC80 mRNA level was significantly increased in the siFAK cells compared with that in the siNC cells (Fig. 1A). Previously, it was demonstrated that the expression level of FAK was lower in B16F1 cells, which exhibit low rates of metastasis, as compared with that in highly metastatic B16F10 cells (14). Therefore, the

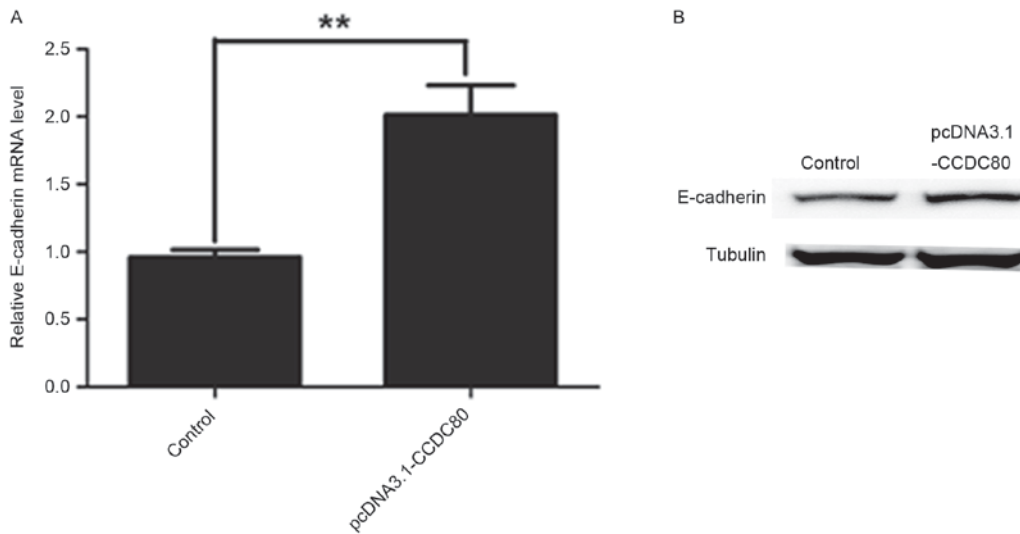


Figure 3. CCDC80 promoted E-cadherin expression. (A) The plasmid pcDNA3.1-CCDC80 was transfected into B16F10 cells, and the mRNA level of E-cadherin was examined by reverse transcription quantitative polymerase chain reaction. (B) The plasmid pcDNA3.1-CCDC80 was transfected into B16F10 cells, and the protein level of E-cadherin was examined by western blotting. ** $P < 0.01$. CCDC80, coiled-coil domain containing 80; si, small interfering; E-cadherin, epithelial cadherin.

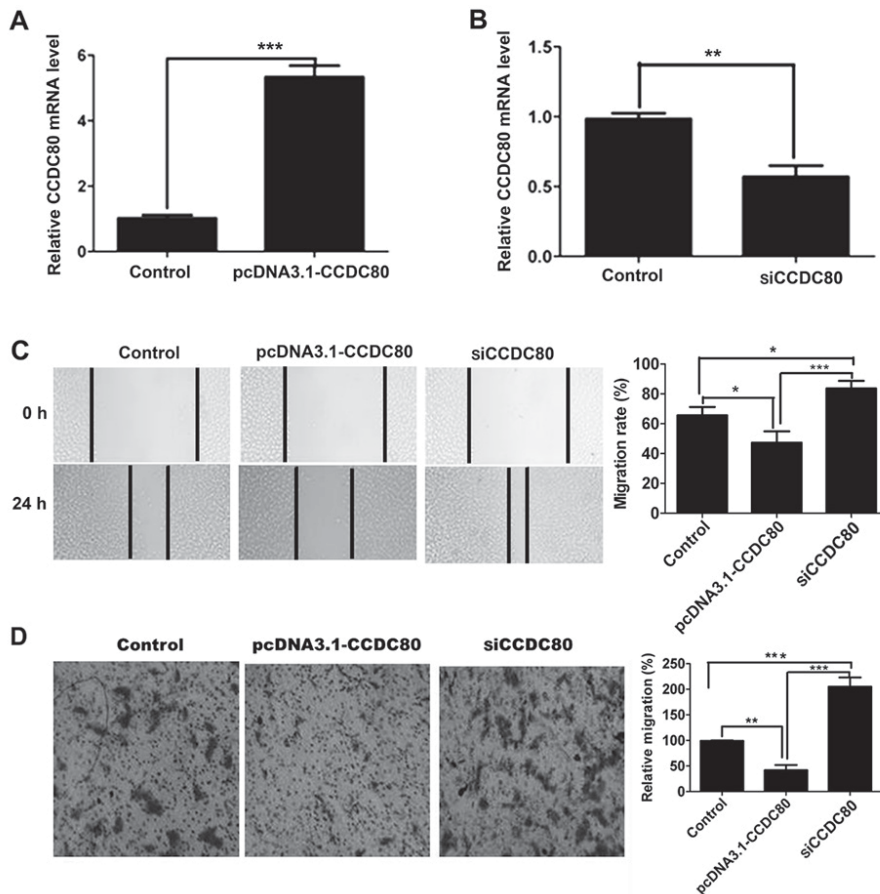


Figure 4. CCDC80 inhibited B16F10 cell migration. (A) The pcDNA3.1-CCDC80 plasmid was transfected into B16F10 cells, and the mRNA levels of CCDC80 were analyzed by RT-qPCR. (B) CCDC80 siRNA was transfected into B16F10 cells, and the mRNA levels of CCDC80 were analyzed by RT-qPCR. (C) The effect of CCDC80 on B16F10 cell migration was revealed by wound healing assay. (D) The effect of CCDC80 on B16F10 cell migration was also revealed by transwell assay. The migrated cell number was normalized to that of the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CCDC80, coiled-coil domain containing 80; RT-qPCR, reverse transcription quantitative polymerase chain reaction; si, small interfering.

present study examined the CCDC80 mRNA level in B16F10 and B16F1 cells. It was identified that the CCDC80 mRNA

level was increased in the B16F1 cells compared with that in the B16F10 cells (Fig. 1B). Concomitantly, RT-qPCR and

Table II. mRNA levels of FAK, CCDC80 and E-Cadherin in the Riker melanoma dataset.

Gene	Fold change (Melanoma vs. normal)
FAK	2.12
CCDC80	-3.23
E-cadherin	-2.68

The fold change (Melanoma vs. Normal) is a relative value. Fold change >0 suggests that the expression of the gene is upregulated in melanoma tissues compared with that in the normal control tissues. Fold change <0 signifies that the expression is downregulated. FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; E-cadherin, epithelial cadherin.

Table III. mRNA levels of FAK, CCDC80 and E-Cadherin in the Haqq melanoma dataset.

Gene	Fold change (Melanoma vs. normal)
FAK	1.36
CCDC80	-2.39
E-cadherin	-5.32

The fold change (Melanoma vs. Normal) is a relative value. Fold change >0 suggests that the expression of the gene is upregulated in melanoma tissues compared with that in the normal control tissues. Fold change <0 signifies that the expression is downregulated. FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; E-cadherin, epithelial cadherin.

Table IV. mRNA levels of FAK, CCDC80 and E-Cadherin in the Critchley-Thorne melanoma dataset.

Gene	Fold change (Melanoma vs. normal)
FAK	-1.01
CCDC80	1.05
E-cadherin	1.20

The fold change (Melanoma vs. Normal) is a relative value. Fold change >0 suggests that the expression of the gene is upregulated in melanoma tissues compared with that in the normal control tissues. Fold change <0 signifies that the expression is downregulated. FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; E-cadherin, epithelial cadherin.

western blotting assays also indicated that the expression of E-cadherin was significantly increased in the siFAK cells compared with the siNC cells (Fig. 2A and B). These data suggest that FAK inhibits the expression levels of CCDC80 and E-cadherin in B16F10 cells.

CCDC80 promotes the expression of E-cadherin. E-cadherin is a suppressor of tumor invasion and metastasis (15,16). Loss

Table V. Gene chip analysis of FAK and NCOA3 gene expression.

Gene	Fold change (siFAK vs. siNC)
FAK	-2.12
NCOA3	-1.09

The fold change (siFAK to siNC) is a relative value. Fold change <0 suggests that the expression of the gene is downregulated in the siFAK cells compared with that in the siNC cells. FAK, focal adhesion kinase; NCOA3, nuclear receptor coactivator-3; si, small interfering.

of E-cadherin expression promotes metastatic tumor dissemination and predicts poor prognosis (17). A previous study indicated that an increase in E-cadherin expression inhibited cancer cell migration (18). Additionally, CCDC80 was demonstrated to maintain a normal E-cadherin expression rate in thyroid cancer (8). As demonstrated in Figs. 1 and 2, knock-down of FAK promoted the expression levels of CCDC80 and E-cadherin in the B16F10 cells. However, it was not clear whether CCDC80 regulated E-cadherin expression in B16F10 cells. In the present study, it was identified that the overexpression of CCDC80 elevated E-cadherin expression in B16F10 cells at mRNA and protein levels (Fig. 3A and B). These data indicated that CCDC80 promoted E-cadherin expression in B16F10 cells.

CCDC80 inhibits B16F10 cell migration. As CCDC80 promoted E-cadherin expression, it may be involved in the regulation of B16F10 cell migration. To explore the role of CCDC80 in cell migration, the expression level of CCDC80 was upregulated or downregulated in B16F10 cells by transfection of pcDNA3.1-CCDC80 plasmids or siCCDC80 molecules, respectively (Fig. 4A and B). The results from the wound healing assay indicated that the upregulation of CCDC80 inhibited B16F10 cell migration, while the downregulation of CCDC80 promoted B16F10 cell migration compared with the control (Fig. 4C). The transwell assay also suggested that the upregulation of CCDC80 inhibited B16F10 cell migration compared with the control (Fig. 4D). Taken together, these results demonstrated that CCDC80 may inhibit B16F10 cell migration.

mRNA levels of FAK, CCDC80 and E-cadherin in patients with melanoma. As aforementioned, it was identified that FAK regulates E-cadherin expression via CCDC80, and CCDC80 may regulate B16F10 cell migration. Consequently, the mRNA levels of FAK, CCDC80 and E-cadherin in human melanoma were analyzed using the Oncomine Cancer Microarray database (www.oncomine.org). As revealed by melanoma datasets from Riker *et al* and Haqq *et al* (data from www.oncomine.org), the expression level of FAK was upregulated, while the expression levels of CCDC80 and E-cadherin were downregulated in human melanoma samples compared with the normal controls (Tables II and III). In the Critchley-Thorne melanoma dataset (data from www.oncomine.org), the mRNA level of FAK was decreased, while the mRNA levels of CCDC80 and E-cadherin were upregulated (Table IV). The results from human melanoma datasets are consistent with the data from



Figure 5. Proposed model for the signal pathway regulated by FAK in melanoma cells. FAK, focal adhesion kinase; CCDC80, coiled-coil domain containing 80; E-cadherin, epithelial cadherin.

the siFAK cells, suggesting that the regulation of B16F10 melanoma cell migration by FAK is potentially mediated by CCDC80 (Fig. 5).

Discussion

Malignant melanoma is one of the most aggressive types of malignancy (19-21), and the incidence of melanoma is increasing more rapidly compared with any other type of cancer globally (22). In the present study it was demonstrated that FAK is overexpressed and may serve a key role in melanoma progression, the role of FAK in B16F10 cell migration was investigated and the underlying mechanism was additionally explored. It was identified that the knockdown of FAK promoted the expression levels of CCDC80 and E-cadherin, and suppressed the migration of B16F10 cells. Concurrently, CCDC80 inhibited the migration of B16F10 cells, and increased the levels of E-cadherin. In the present study, only the role of CCDC80 in B16F10 cell migration was examined. As a potential target for tumor therapy, the roles of CCDC80 in cell proliferation and apoptosis require additional investigation.

However, it remains to be elucidated how FAK regulates CCDC80 and E-cadherin in B16F10 cells. As demonstrated in Fig. 1A, the RT-qPCR assay indicated that the CCDC80 mRNA level was significantly increased in siFAK cells, suggesting that FAK regulates CCDC80 expression at the transcriptional level. It has been revealed that NCOA3 is overexpressed in primary cutaneous melanoma, and that NCOA3 is a negative regulator of CCDC80 (23). When the expression of NCOA3 was silenced in MCF-7 cells, there was a significant

increase in the expression level of CCDC80 (12). Additionally, it was suggested that ERK may modulate NCOA3 (24). An increased phosphorylation of ERK was accompanied by an increased expression of NCOA3 in MCF7-YB-1 cells (25). Preliminary data indicated that the protein level of p-ERK was decreased in siFAK cells (data not shown). In addition, the results from the gene chip assay (Table V) and RT-qPCR assay (data not shown) revealed that the mRNA level of NCOA3 was downregulated in siFAK cells. The data suggest that FAK may regulate CCDC80 via the ERK/NCOA3 signaling pathway.

There are also certain indications of how CCDC80 regulates E-cadherin expression. As revealed in Fig. 3A, the overexpression of CCDC80 increased the mRNA level of E-cadherin, indicating that CCDC80 regulates E-cadherin expression at the transcriptional level. A previous study demonstrated that the silencing of CCDC80 decreases the mRNA level of peroxisome proliferator-activated receptor γ (PPAR γ), suggesting that PPAR γ is a downstream target of CCDC80 (14). Preliminary data from the present study indicated that FAK knockdown promoted PPAR γ expression, and that PPAR γ knockdown decreased E-cadherin expression (data not shown). Therefore, it was hypothesized that CCDC80 promotes E-cadherin expression via PPAR γ . However, additional studies are required to provide complete characterization of this interaction.

In conclusion, the data of the present study suggest that FAK potentially regulates B16F10 cell migration by the CCDC80/E-cadherin pathway. These results may assist in melanoma treatment by the identification of a novel biomarker for diagnosis.

Acknowledgements

The authors would like to thank Mrs. Min Lu and Mr Tongyang Zhu (The State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, China) for their assistance with experimental apparatus and materials.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (grant nos.: 81421091, 30425009, 30330530, 31200572, 81121062 and 31071196), the Natural Science Foundation of Jiangsu province (grant no.: BE2013630) and Bureau of Science and Technology of Changzhou (grant no.: CE20135013).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZCH undertook study design; GP and YL were responsible for study design and conducting the experiments. WL designed the primers, GP wrote the manuscript and LJ contributed substantially to the analysis and interpretation of data for the work and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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