European Review for Medical and Pharmacological Sciences 2018; 22: 8664-8674

Podocalyxin-like, targeted by miR-138, promotes colorectal cancer cell proliferation, migration, invasion and EMT

Y. XU, Z.-G. PAN, L. SHU, Q.-J. LI

Department of Gastroenterology, Huai'an First People's Hospital, Nanjing Medical University, Huai'an, Jiangsu, China

Abstract. – OBJECTIVE: Emerging evidence has shown that Podocalyxin-like (PODXL) plays an important role in the development and progression of several tumors, including colorectal cancer (CRC). However, its potential role in CRC is still not documented. The present study aimed to explore biological functions and molecular mechanisms in CRC development.

PATIENTS AND METHODS: Microarray data were downloaded from TCGA datasets and statistically analyzed. RT-PCR was performed to detect the expression of PODXL and miR-138. Lost-function assay was used to explore the roles of PODXL on CRC behavior. Bioinformatics tools were used to identify the upstream miRNAs and the relationship between PODXL and miR-138 was detected via Dual-Luciferase assay, Western blot and rescue experiments.

RESULTS: PODXL expression was significantly up-regulated in both CRC tissues and cell lines. *In vitro* experiments showed the knockdown of PODXL suppressed reduces CRC tumor growth, metastasis and EMT, and promoted apoptosis. Moreover, PODXL was predicted and confirmed to be a target of miR-138. In addition, ectopic expression of PODXL significantly reversed the suppression of cell proliferation and metastasis caused by the miR-138 over-expression.

CONCLUSIONS: We provided important evidence that PODXL, targeted by miR-138, acted as a tumor promoter in CRC by suppressing CRC cells proliferation and metastasis, which may provide a novel potential target for diagnostic and therapeutic applications in CRC.

Key Words:
PODXL, Colorectal cancer, MiR-138, Proliferation, Metastasis.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths and the second most common type of cancer, with high rates of incidence and disease-related mortality and morbidity[1,2]. In China, CRC has rapidly increased in recent years[3]. Most colorectal cancers are due to old age, with only a small number of patients due to underlying genetic disorders[4]. Surgery is the most effective treatment for CRC. However, the effectiveness of surgery is limited for patients with advanced stages[5]. Although great developments have been made in diagnostic and therapeutic modalities, the overall survival rate of CRC patients remains unsatisfactory[6,7]. Therefore, a better understanding of the tumorigenesis is critical to find effective methods for early diagnosis and treatment of CRC. Podocalyxin-like protein (PODXL), encoded by the PODXL gene belonging to the CD34 family and initially identified in podocytes of renal glomeruli, is usually expressed on the apical surface of glomerular epithelial cells[8,9]. As a well-known stem cell marker, PODXL is associated with stem cell marker CD34[10]. According to the Human Protein Atlas consortium, PODXL is widely expressed in human cells, tissues and organs. Although the biological function of PODXL remains largely unclear, more and more evidence shows a positive association between PODXL and tumor progression[11]. The dysregulation of PODXL was reported in several different tumors, such as prostate cancer[12], breast cancer[13] and oral squamous cell carcinoma cell[14]. Interestingly, in CRC, it was reported that high PODXL expression was significantly associated with poor overall survival and may be used as a potential prognostic biomarker for CRC patients[15]. However, the biological function and potential mechanism of PODXL in CRC progression remain largely unclear. In this study, we reported that PODXL expression was significantly up-regulated in CRC tissues and cell lines by analyzing data from TCGA datasets

Corresponding Author: Qian-jun Li, MD; e-mail: aple2338@sina.com

and performing RT-PCR. Then, a series of *in vitro* experiments were performed to determine the potential biological function of PODXL in CRC behaviors. In addition, the upstream mechanism by which PODXL was regulated was studied by exploring the potential functional miRNAs which can regulate gene expression post-transcriptionally by inhibiting translation or inducing target mRNAs degradation. The present work contributed to a better understanding of the role of PODXL in CRC progression.

Patients and Methods

Cell Lines and Cell Transfection

In this study, we examined the PODXL expression in four CRC cell lines (SW480, SW1116, LOVO and HCT116), and one human colorectal mucosa cell line, FHC. The FHC and SW480 cell lines were obtained from Chengdu Biotechnology Co., Ltd. (Chengdu, Sichuan, China), and the other three cancer cell lines (SW1116, LOVO and HCT116) as well as 293T cell line were purchased from Fuheng Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium at 37˚C with 5% CO_2. Moreover, 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1×penicillin/streptomycin solution (Biodragon, Beijing, China) were also added into the medium. The study was approved by the Ethics Committee of Huai'an First People's Hospital, Nanjing Medical University. A transfection reagent, Poly-Jet Transfection kit (Signagen, Jinan, Shandong, China), was utilized to transfect plasmid, siRNAs or miRNA mimic into cells. The miR-138 mimic and negative control (NC) mimic were purchased from Shanghai Biotechnology Co., Ltd. (Shanghai, China). Small interfering RNAs (siRNAs) which were specific targeting PODXL (PODXL-siRNA#1 and PODXL-siRNA#2), as well as negative control siRNAs (Control-siRNA), were obtained from Suzhou Biotechnology (Suzhou, Jiangsu, China). The PODXL overexpressing plasmid was constructed by GeneCreate Co., Ltd. (Wuhan, Hubei, China).

Quantitative Real Time-PCR (qRT-PCR)

A total RNA extraction kit (HuiJia Biotechnology, Xiamen, Fujian, China) was applied to isolate total RNA from CRC cells used in this study. For PODXL mRNA examination, a One-Step EvaGreen qRT-PCR-iCycler kit (ChunduBio, Wuhan, Hubei, China) was applied. The qRT-PCR assays were performed on a Real Time-PCR system. GAPDH was used as an internal control of PODXL detection. For miRNA detection, a Mir-X miRNA qRT-PCR SYBR detection kit (Bioneeds Biotechnology, Guangzhou, Guangdong, China) was employed. The expression levels of miR-138 were normalized to U6. All the primers were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assays

After the HCT116 and LOVO cells were transfected with PODXL siRNAs, plasmid or miRNA mimics, the cells were first digested, collected and then cultured in 96-well plates (2000 cells/well) for 24 h. Subsequently, 10 µl CCK-8 solution (Biolite, Tianjin, China) was added into each well of the plates. The HCT116 and LOVO cells were then incubated at 37°C for 1 h. Afterward, a SuPerMax-3100Plus microreader (Spectrum Technologies, Shanghai, China) was applied to examine the absorbance of 450 nm.

Colony Formation Assays

HCT116 and LOVO cells were first transfected with siRNAs, miRNA mimics or PODXL overexpressing plasmids. Then, the treated HCT116 and LOVO cells were collected and seeded into 6-well plates (500 cells/well). Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium was utilized to maintain the cells for appropriate two weeks. Finally, 0.1% crystal violet solution (Mery-

Table I. The primer sequences for PCR.

Names	Sequences (5'-3')
PODXL: Forward	TCCCAGAATGCAACCCAGAC
PODXL: Reverse	GGTGAGTCACTGGATACACCAA
miR-138: Forward	CTGAACCCAGGTACAAAGCAG
miR-138: Reverse	CAAGAACAGAAGGGAGAGGC
GAPDH: Forward	CGAGCCACATCGCTCAGACA
GAPDH: Reverse	GTGGTGAAGACGCCAGTGGA

No statistically significant differences were found between the two groups for any of the parameters reported above.

er Chemical Technology, Shanghai, China) was employed to stain the cell colonies of HCT116 and LOVO cells. The images of the cell colonies were taken by a microscope (MF53, Mshot, Guangzhou, Guangdong, China).

Western Blot Analysis

Total protein was extracted from PODXL siRNAs, miR-138 mimic or plasmids transfected HCT116 and LOVO cells using a Protein Extraction kit (BestBio, Shanghai, China). Then, equal amounts of protein (20 μg) were resolved on 8-12% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane (ZikerBio, Guangdong, China), and probed at 4°C overnight with primary antibodies. The anti-PODXL, anti-caspase-3, anti-caspase-9, anti-N-Cadherin antibodies were obtained from Abcam Co., Ltd. (Abcam, Cambridge, MA, USA). The anti-Vimentin, anti-β-Catenin, anti-cyclin D1 and GAPDH were purchased from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA). After the membranes were incubated with secondary antibodies, an ABSIN enhanced ECL Reagent kit (Thermo Scientific, Waltham, MA, USA) was applied to examine the protein bands. Besides, the optical density of the protein bands was analyzed by the Image J software (Bethesda, MD, USA).

Dual-Luciferase Reporter Assay

The 3' untranslated regions (3'-UTR) of PODXL mRNA containing wild-type (PODXL WT) or mutant (PODXL MUT) binding sequence of miR-138 were constructed into the pGL3 basic vector by Hanbio Biotechnology Co., Ltd. (Pudong, Shanghai, China). Then, 293T cells were plated in 24-well plates for 24 h and co-transfected with 0.5 μg PODXL WT or PODXL MUT with 80 nM of miR-138 mimics or control mimics. Finally, the Luciferase activity was evaluated by a SuperLight Dual-Luciferase Reporter kit (Hypercyte, Beijing, China).

Cell Apoptosis Assays

A cell apoptosis assay kit (Kanglang Biotechnology, Shanghai, China) was employed to assess the apoptosis of HCT116 and LOVO cells. Briefly, the HCT116 and LOVO cells transfected with PODXL siRNAs, miR-138 mimic or plasmids were digested and resuspended in the binding buffer. Then, the cells were incubated with propidium iodide (PI) as well as Annexin V for 10-15 min. Finally, a CytoFLEX LX flow cytometer (Beckman-Coulter Inc., Fullerton, CA, USA) was applied to examine the apoptotic rates of HCT116 and LOVO cells.

Transwell Assays

HCT116 and LOVO cells were first transfected with PODXL siRNAs, miR-138 mimic or plasmids. After 48 h, the treated cells were digested and placed in an appropriate volume of RPMI-1640 medium without FBS in the upper chamber of a transwell insert (Millipore, Billerica, MA, USA) which was pre-coated with 80 μl Matrigel. In addition, the lower chambers were added 350 μl RPMI-1640 complete medium (containing 20% FBS) as chemoattractant. After 24 h, 0.1% crystal violet solution (Meryer Chemical Technology, Shanghai, China) was applied to stain the invaded HCT116 and LOVO cells on the lower sides of the chambers. After washing with PBS three times, a microscope (MF53, Mshot, Guangzhou, Guangdong, China) was utilized to observe the invasive cells.

Wound Healing Assays

The PODXL siRNAs, miR-138 mimic or plasmids were first transfected into the HCT116 and LOVO cells. Subsequently, the treated cells (70 μl; 5×10^5 cells per ml) were collected and seeded into the culture inserts of an Ibidi 3.5cm μ-dish (BioMars, Beijing, China). After 24 h, a sterilized tweezer was utilized to remove the culture inserts. The wounded areas were observed and photographed using a microscope (MF53, Mshot, Guangzhou, Guangdong, China) at 0 h and 24 h.

Statistical Analysis

The Student's *t*-test was applied for two-group analysis, while one-way ANOVA was conducted followed by the Tukey's test when analyzing more than two groups. All the values are shown as the mean ± standard error of the mean (SEM). All the statistical analyses in our experiments were performed by the Statistical Package for Social Sciences (SPSS 19.0) software (Chicago, IL, USA). Differences were considered statistically significant at $p<0.05$.

Results

Comprehensive Bioinformatics Analysis Indicated that PODXL Was Highly Expressed in CRC Tissues and Cell Lines

To identify abnormally expressed genes in CRC, we analyzed microarray expression profiles. TCGA datasets were selected and analyzed

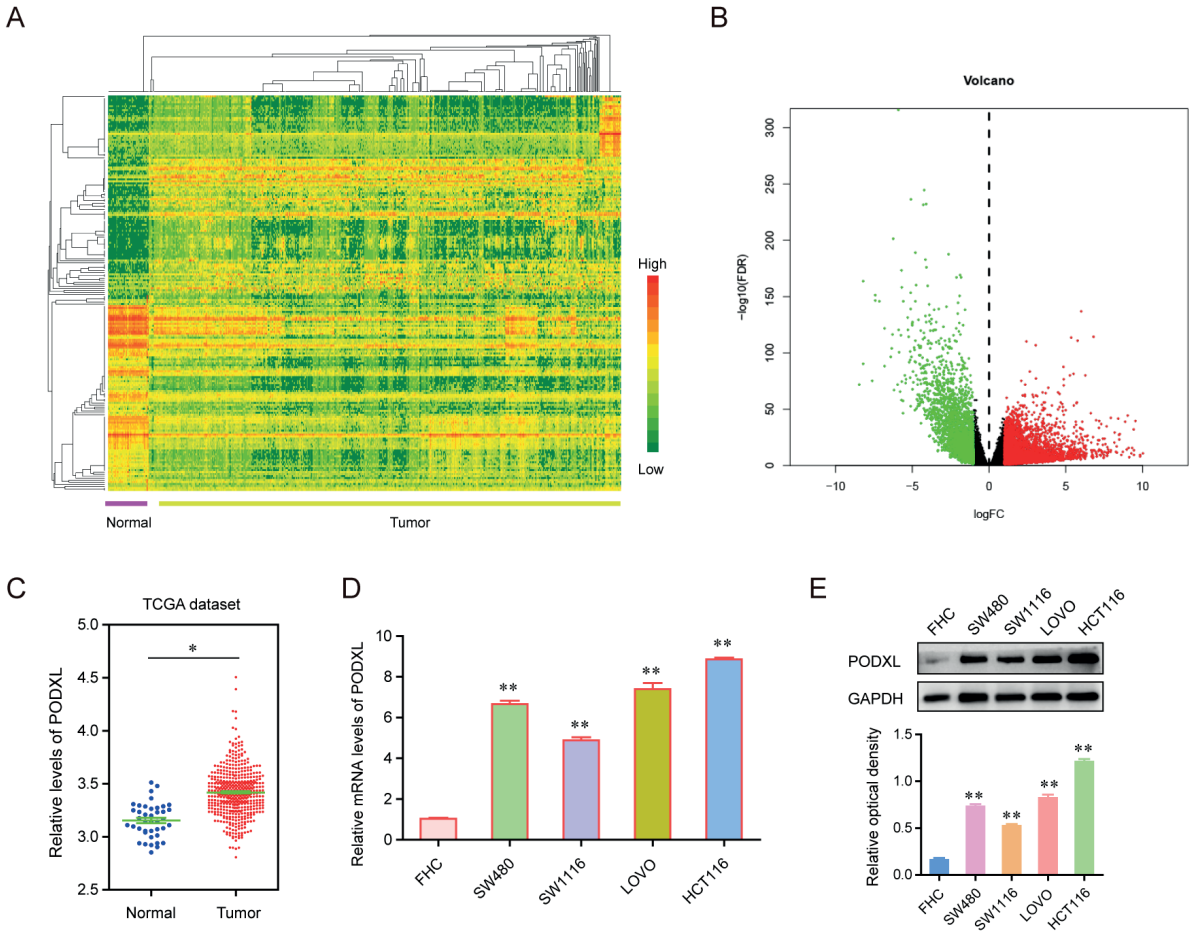

Figure 1. Bioinformatics analysis of differentially expressed genes in CRC. ***A,*** Partial miRNAs expression profiles of CRC tissues in TCGA datasets. ***B,*** Volcano plot of the aberrantly expressed genes between CRC and colorectal tissues. ***C,*** The expression of PODXL in CRC tissues and normal colorectal tissues in TCGA datasets. ***D,*** qRT-PCR analysis of PODXL expression in four CRC cell lines (SW480, SW1116, LOVO and HCT116) and FHC. ***E,*** Western blot analysis of PODXL expression in four CRC cell lines (SW480, SW1116, LOVO and HCT116) and FHC. **p*<0.05, ***p*<0.01.

for consistently aberrant genes between CRC and normal colorectal tissues. As shown in Figure 1A and 1B, a large number of genes were identified to be aberrantly expressed in CRC. Among these differentially expressed genes, PODXL was one of the most up-regulated genes (Figure 1C). Next, we performed RT-PCR to detect the expression levels of PODXL in CRC cell lines, finding that PODXL expression was significantly up-regulated in four CRC cell lines (SW480, SW1116, LOVO and HCT116) compared to FHC (Figure 1D). In addition, the results of Western blot also showed that the levels of PODXL proteins were significantly up-regulated in four CRC cell lines (Figure 1E). Our results, together with previous studies, indicated that PODXL, a highly expressed gene, may contribute to the development and progression of CRC.

Change in PODXL Expression Affected the Proliferation and Apoptosis of CRC Cells

To investigate the biological influence of PODXL on the proliferation and apoptosis of CRC cells, we employed siRNAs against PODXL (PODXL-siRNA#1 and PODXL-siRNA#2) to repress PODXL expression with knockdown efficiency higher than 70% in HCT116 and LOVO cells (Figure 2A). The growth curves examined by CCK-8 assays demonstrated that the knockdown of PODXL remarkably inhibited the proliferation of HCT116 and LOVO cells (Figure 2B and C). In addition, the silence of PODXL notably attenuated the clonogenic abilities of HCT116 and LOVO cells (Figure 2D). Furthermore, the results of flow cytometry revealed that the transfection of PODXL siRNAs significantly accelerated the apoptotic rates of HCT116 and LOVO cells (Fig-

ure 2E). Given that the silence of PODXL induced cell apoptosis, we next performed Western blot to examine the change of apoptosis-related molecules such as caspase-3 and caspase-9 in CRC cells. The results confirmed that the transfection of PODXL siRNAs led to dramatically decreased protein levels of caspase-3 and caspase-9 in HCT116 and LOVO cells (Figure 2F and G). Overall, these data indicated that PODXL served as an essential modulator in regulating the development of CRC cells.

Silence of PODXL Suppressed the Migratory and Invasive Abilities of CRC Cells

To investigate whether PODXL influenced the metastatic potentials of CRC cells, we next examine the invasive and migratory capabilities of HCT116 and LOVO cells after transfection with PODXL siRNAs. The transwell invasion assays suggested that transfection of PODXL siRNAs significantly reduced the invaded HCT116 or LOVO cells (Figure 3A and B). Additionally, the cell migratory abilities evaluated by wound healing assays were remarkably decreased after HCT116 and LOVO cells were transfected with PODXL siRNAs (Figure 3C and D). Since PODXL affected the invasion and migration of CRC cells, we next conducted Western blot to detect the alteration of epithelial-mesenchymal transition related molecules such as N-cadherin and Vimentin. As the data presented in Figure 3E and F, the silence of PODXL significantly reduced the protein levels of N-cadherin as well as Vimentin in both HCT116 and LOVO cells. Col-

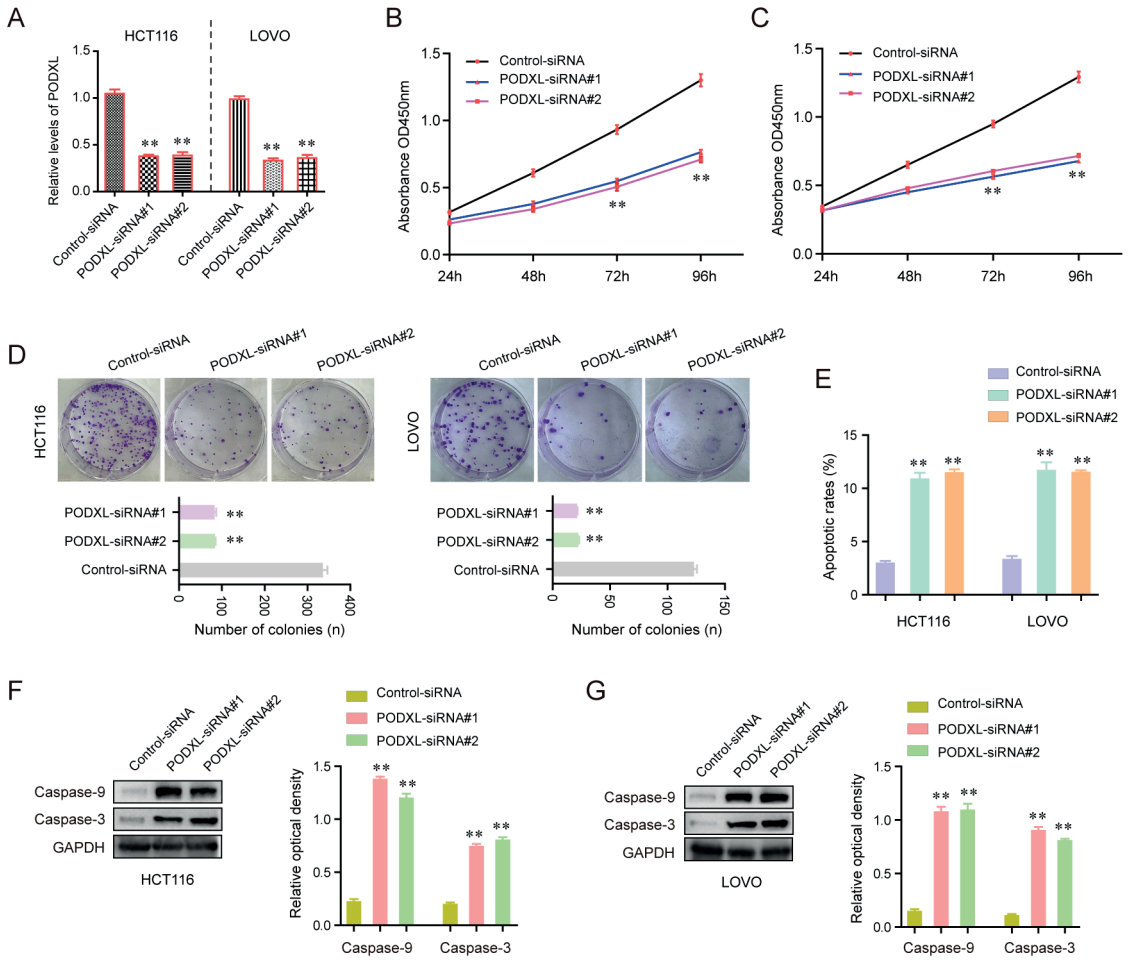

Figure 2. PODXL knockdown suppressed the proliferation and accelerated the apoptosis of HCT116 and LOVO cells. ***A,*** The relative mRNA levels of PODXL in HCT116 and LOVO cells transfected with PODXL siRNAs (PODXL-siRNA#1 and PODXL-siRNA#2) or negative control siRNAs (Control-siRNA). ***B, C,*** Transfection of PODXL siRNAs reduced the cellular growth of HCT116 and LOVO cells. ***D,*** Silence of PODXL reduced colony formation abilities of HCT116 and LOVO cells. ***E,*** The cells apoptosis was detected by flow cytometry. ***F, G,*** PODXL knockdown elevated the protein levels of caspase-3 and caspase-9. **p*<0.05, ***p*<0.01.

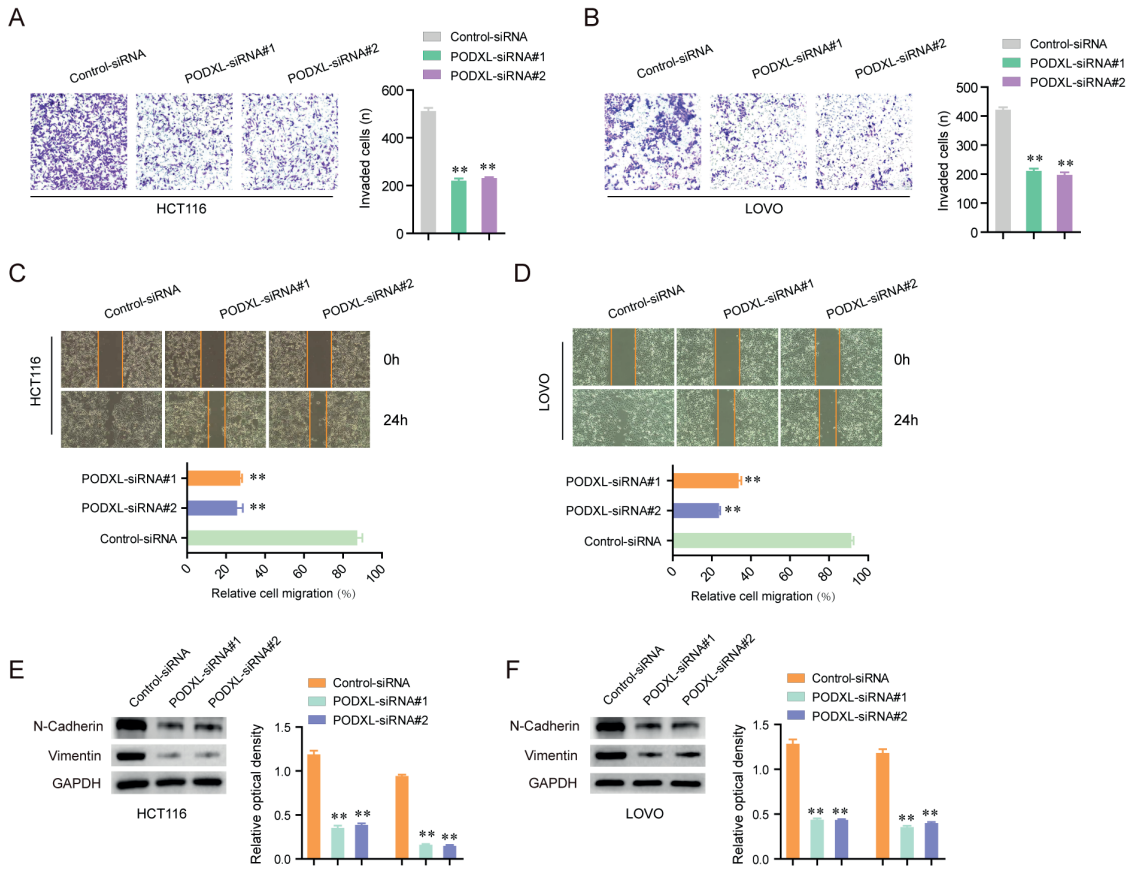

Figure 3. Silence of PODXL repressed the invasion and migration of HCT116 and LOVO cells. ***A, B,*** The transfection of PODXL siRNAs remarkably reduced the invasive abilities of HCT116 and LOVO cells. ***C, D,*** The knockdown of PODXL significantly reduced the migration of HCT116 and LOVO cells. ***E, F,*** The transfection of PODXL siRNAs significantly decreased the protein levels of N-cadherin and Vimentin. *p<0.05, **p<0.01.

lectively, our data revealed that PODXL played important roles in modulating the metastatic potentials of CRC cells.

PODXL Was a Direct Downstream Target of MiR-138

A plethora of studies had reported the correlation between miRNAs and the mRNAs of corresponding genes. Hence, we next applied bioinformatics analysis using "miRDB" (http://www.mirdb.org/) to predict the interaction between PODXL and potential miRNAs. Among these predicted miRNAs, we focused on miR-138 and the complementary binding site between miR-138 and PODXL were shown in Figure 4A. To further validate that PODXL was a direct downstream target of miR-138, we carried out Dual-Luciferase reporter assays in 293T cells. First, we constructed Luciferase reporter plasmids which contained the wild-type 3'UTR of PODXL (PODXL WT) and its matched mutant sequence (PODXL MUT). Then, we transfected PODXL WT or PODXL MUT plasmids with negative control (NC) mimic or miR-138 mimic into 293T cells. The results revealed that the co-transfection of miR-138 mimic and PODXL WT remarkably reduced the Luciferase activities, through transfecting miR-138 mimic with PODXL MUT did not change the Luciferase activities in 293T cells (Figure 4B). Besides, the results of qRT-PCR and Western blot assays demonstrated that the transfection of miR-138 mimic significantly reduced both the mRNA and protein levels of PODXL in HCT116 and LOVO cells (Figure 4C and D). Moreover, we performed Western blot assays to detect the protein levels of β-Catenin as well as cyclin D1, which were involved in the Wnt/β-Catenin signaling. As the data presented in Figure 4E and F, the transfection of miR-138 mimic resulted in a notably decreased protein level of β-Catenin and cyclin D1

in HCT116 and LOVO cells. Therefore, these data clearly proved that PODXL was a direct downstream target of miR-138 in CRC cells and miR-138 suppressed the activity of the Wnt/β-Catenin signaling.

Enhanced Expression of PODXL Abrogated the Suppressive Effects of MiR-138 on the Proliferation and Invasion of CRC Cells

Since the above findings validated that miR-138 could directly target PODXL, we next aimed to explore the ability of PODXL to change the effects of miR-138 on cell proliferation and invasion. First, we employed pcDNA3.1 vector to construct the overexpressing plasmid of PODXL. Then, we observed that the co-transfection of miR-138 mimic and PODXL overexpressing plasmid significantly impeded the inhibitory effects of miR-138 on PODXL expression (Figure 5A). In addition, CCK-8 assays demonstrated that the transfection of miR-138 mimic led to a markedly decreased proliferative rates of HCT116 and LOVO cells, while the re-introduc-

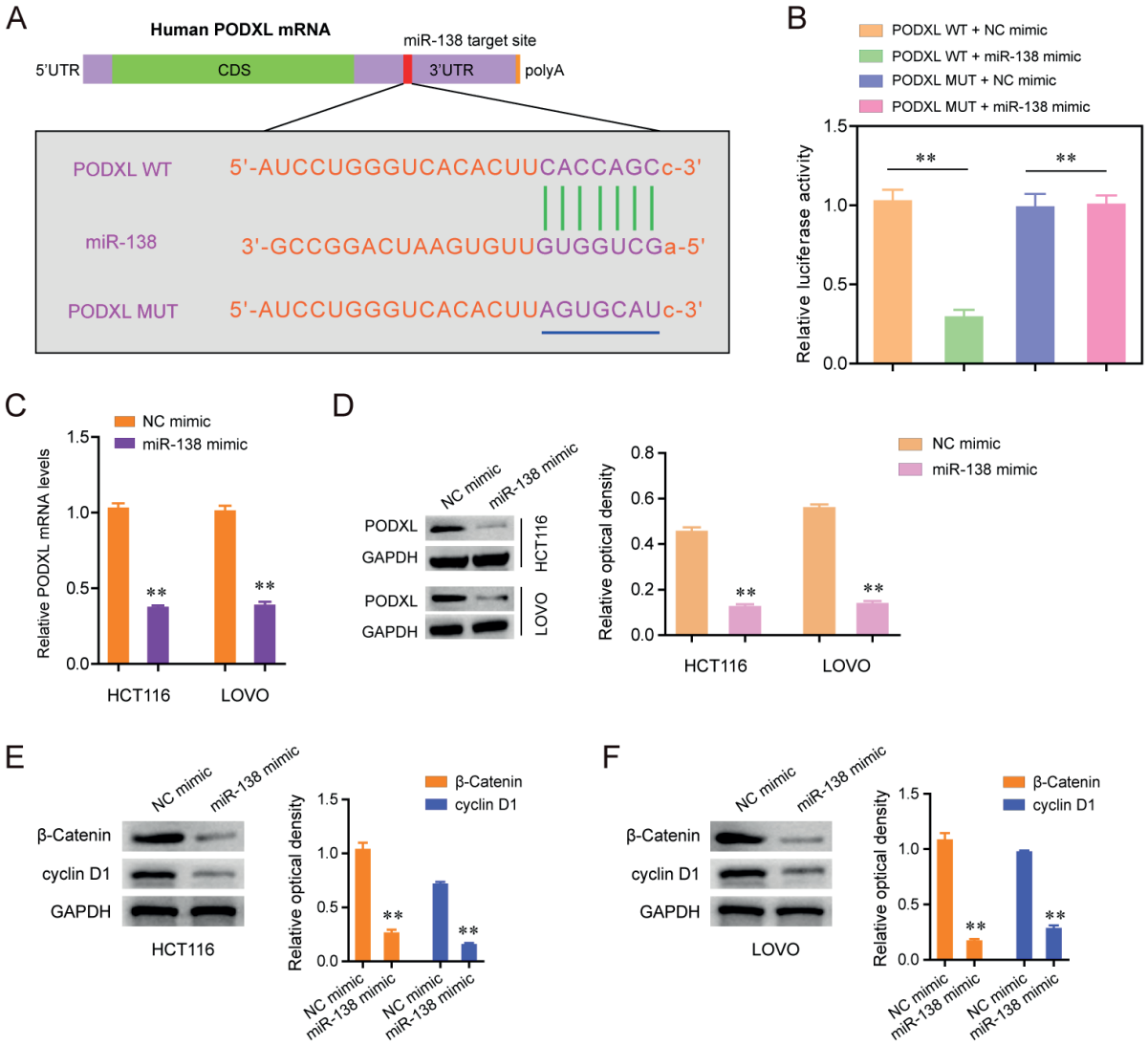

Figure 4. MiR-138 directly targeted PODXL. ***A,*** The putative and mutated miR-138 binding sequences in the 3'-UTR of PODXL mRNA were predicted by "miRBD". ***B,*** The Luciferase activity of 293T cells after co-transfecting with PODXL WT or PODXL MUT plasmids as well as negative control (NC) mimic or miR-138 mimic. ***C, D,*** The relative mRNA or protein levels of PODXL in HCT116 and LOVO cells transfected with NC mimic or miR-138 mimic. ***E, F,*** The relative protein levels of β-Catenin and cyclin D1 in HCT116 and LOVO cells transfected with NC mimic or miR-138 mimic. *$p<0.05$, **$p<0.01$.

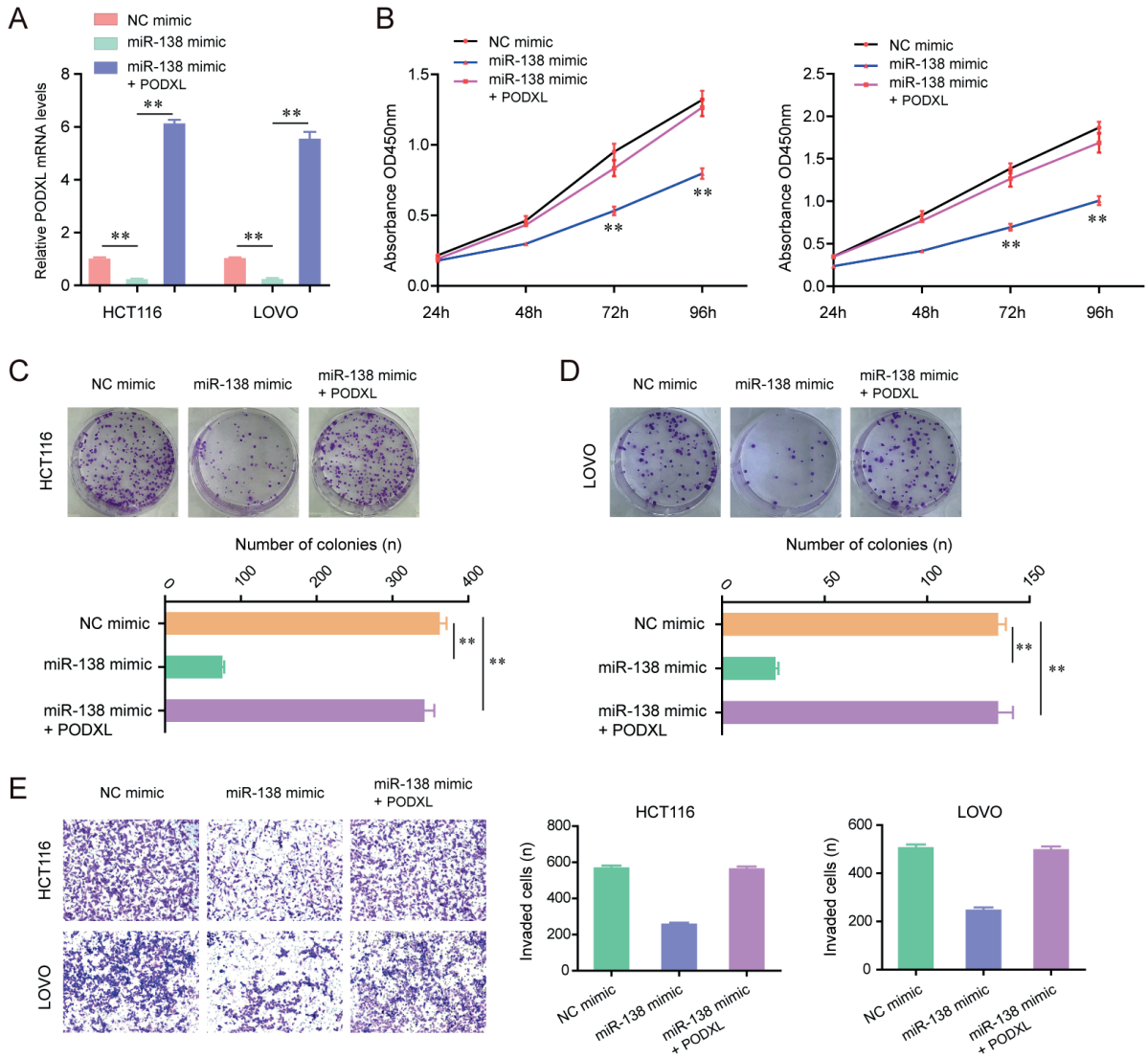

Figure 5. Transfection of PODXL impeded the inhibitory effects of miR-138 on cell proliferation and invasion. ***A,*** The qRT-PCR assays examined the relative mRNA levels of PODXL in HCT116 and LOVO cells transfected with NC mimic, miR-138 mimic or co-transfected with miR-138 mimic and PODXL overexpressing plasmids. ***B,*** Cell proliferation was determined by CCK-8 assays. ***C, D,*** Re-introduction of PODXL reversed the clonogenic formation capacities of HCT116 and LOVO cells. ***E,*** Co-transfection of miR-138 mimic and PODXL overexpressing plasmids abrogated the suppressive effects of miR-138 on the invasiveness of HCT116 and LOVO cells. **p*<0.05, ***p*<0.01.

tion of PODXL reversed the growth curve of cell proliferation (Figure 5B). Similarly, the number of HCT116 and LOVO cell colonies which was reduced by miR-138 were also elevated by overexpressing PODXL (Figure 5C and D). Furthermore, co-transfection of miR-138 mimic and PODXL overexpressing plasmid resulted in a remarkable increase of invasive cell number, which indicated that PODXL notably reversed the suppressive effects of miR-138 on cell invasion (Figure 5E). Hence, these data confirmed that the overexpression of PODXL could effectively impair the inhibitory effects of miR-138 on CRC cell proliferation and invasion.

Discussion

CRC is one of the most common causes of cancer mortality not only in China but also worldwide, and its incidence decreased in China over the last 20 years[16,17]. Most colorectal cancers are

due to old age and lifestyle factors[18]. For CRC patient at an early stage, routine treatments, such as surgery, chemotherapy and radiation therapy are widely used and the effects are ideal[19,20]. However, for metastatic CRC patients, treatment effects of these methods are limited and the current approach for these patients favors the use of combination cytotoxic therapy[21,22]. However, the prognosis of metastatic CRC patients remains very poor. Targeted therapies based on a genic mutation in subgroups of CRC have already provided some treatment ideas[23]. Thus, the identification of the key molecules involved in CRC progression and understanding of potential mechanism must be amenable to both early diagnosis and development of targeted drugs. PODXL is a newly characterized oncogenic protein. Several studies have reported the oncogenic function of PODXL. For instance, Lin et al[24] reported that PODXL is highly expressed in breast cancer cells and metastatic breast cancer tissues and its knockdown plays a negative role in metastasis by the activation of the Rac1/Cdc42 signaling. In addition, similar data were reported by Snyder et al[25]. Zhang et al[26] found that the levels of PODXL were up-regulated in gastric cancer and significantly associated with positive lymph node metastasis. Additionally, the knockdown of PODXL suppressed metastasis of gastric cancer cells *in vitro* and *in vivo*. Of note, investigations indicated that PODXL was highly expressed in CRC and have great potential to be prognostic biomarkers for CRC because of its close association with long-term survival scholars. However, the biological function of PODXL remains largely unclear. In this work, we first analyzed the TCGA datasets and found that many genes were dysregulated in CRC tissues. Importantly, PODXL was one of the most up-regulated genes in CRC. Indeed, several previous studies had reported that PODXL was overexpressed in CRC tissues and cell lines. The online microarray data was consistent with the results of PCR by other authors. Then, we performed a lost-function assay to explore the potential role of PODXL in CRC cells, finding that the knockdown of PODXL suppressed proliferation of CRC cells and promoted apoptosis by regulating Caspase-9 and Caspase-3. We also found that knocking PODXL led to an increasing ability of migration and invasion. At the same time, the results of Western blot showed that the oncogenic role of PODXL in migration and invasion of CRC cells may be mediated by promoting EMT progression, which has been indicated to be involved in the metastasis of cancer cells. Our findings showed that PODXL acted as an oncogene in CRC progression. MicroRNAs (miRNAs), endogenous non-coding RNAs of ~22 nucleotides, can regulate gene expression by binding specific sites at the 3' untranslated region (UTR) of target mRNAs[29]. It has been confirmed that alterations in miRNAs are involved in tumor development, progression and metastasis[30,31]. MiR-138 is an important tumor-related miRNA. Its expression, function and potential mechanism in various tumors have been well studied[32-34]. Interestingly, miR-138 plays different or opposite roles according to the types of tumors. In CRC, many works have frequently reported that miR-138 served as a tumor suppressor and closely associated with clinical progression of CRC patients[35,36]. In this paper, the results of miRNA target analysis tools PicTar and TargetScan 6.2 showed that PODXL may be a direct target of miR-138, which was confirmed by a Luciferase reporter assay and Western blot. More importantly, rescue experiments confirmed that PODXL overexpression abolishes the suppression induced by the miR-138 mimic. To our best knowledge, our findings first showed that PODXL, targeted by miR-138, contributed to the development and progression of CRC.

Conclusions

We identified high expression of the oncogene PODXL in CRC. Functionally, PODXL, targeted by miR-138, promoted CRC cells proliferation, migration, invasion and EMT. These findings suggest that targeting PODXL may provide a promising strategy for treating CRC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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