miR-138-5p inhibits the metastasis of prostate cancer by targeting FOXC1

Dapeng Zhang
Chifengshi Hospital
Xiaodong Liu
Chifengshi Hospital
Qingwei Zhang
Chifengshi Hospital
Xin Chen (chenxin_uro@163.com)
Chifengshi Hospital https://orcid.org/0000-0001-6418-8178

Primary research

Keywords: miR-138-5p; FOXC1; Prostate cancer; Metastasis

Posted Date: March 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16546/v1

Version of Record: A version of this preprint was published on July 9th, 2020. See the published version at https://doi.org/10.1186/s12935-020-01386-6.

Abstract

Background: This study aimed to uncover the regulatory effect of miR-138-5p on the metastasis of PCa cells, and further explore the potential regulatory mechanisms via regulating FOXC1. Methods: 60 pairs tumor specimens from PCa patients were collected to determine the expression level of miR-138-5p by qRT-PCR. Subsequently, over-expression of miR-138-5p were established to explore the proliferation and metastasis of miR-138-5p in PCa cell lines was analyzed by CCK-8, Tranwell assay and Wounding healing assay, respectively. Bioinformatics analysis and luciferase reporter gene assay were performed to search for the target genes of miR-138-5p, and FOXC1 was selected. Finally, the biological role of miR-138-5p and FOXC1 in the progression of PCa was clarified by a series of rescue experiments. Results: The results of qRT-PCR revealed that miR-138-5p was lowly expressed in PCa tissues and cell lines. Besdies, these PCa patients with low-miR-138-5p had a higher Gleason score, lymph node metastasis, bone metastasis and poor prognosis of PCa, compared with the patients with high-miR-138-5p. Over-expression of miR-138-5p inhibited the viability, migratory and invasive capacities of PC-3 and DU-145 cells. Bioinformatics analysis and luciferase reporter gene assay suggested that FOXC1 was predicted to be the target of miR-138-5p. Moreover, FOXC1 level was negatively correlated to that of miR-138-5p in PCa tissues. Importantly, FOXC1 could reverse miR-138-5p mimic induced-inhibition of PCa malignant progression. Conclusions: Downregulated miR-138-5p was closely associated with Gleason score, distant metastasis and poor prognosis of PCa patients. In addition, miR-138-5p alleviated the malignant progression of PCa by targeting and downregulating FOXC1.

Background

Prostate cancer (PCa) is one of the most common malignancies all over the world, which is the leading cause of cancer-related deaths in the United States and European countries [1–3]. In recent years, the incidence and mortality rate of PCa in China has rapidly increased year by year, which has become a serious threat to human healthy [4, 5]. When prostate specific antigen (PSA) test was used primarily to screen for PCa before symptoms appear, the detection rate of PCa peaked in the early 1990s [6, 7]. So far, approximately 85% newly diagnosed PCa cases were limited to early-stage cancer [8]. Although PSA test greatly improves the early-stage diagnostic rate of PCa, its benefit in decreasing the mortality of PCa remains controversial [9, 10]. Like other malignancies, the malignant progress of PCa is a multi-step and multi-stage process, including inactivation of tumor suppressor genes and/or activation of proto-oncogenes [11, 12]. Currently, target therapy based on tumor-related miRNAs presents a promising application, and the results of these studies showed that miRNA had a good application prospect in the diagnosis, treatment, prognosis and other aspects of cancer, to provide new ideas for the pathogenesis of PCa [13, 14].

MiRNAs are small, endogenous non-coding RNAs that negatively regulate expressions of protein-coding genes at translational level [15, 16]. MiRNAs exert the biological function by degrading or inhibiting translation of mRNAs [16]. It is reported that miRNAs are extensively involved in affecting cellular behaviors and disease progression [17, 18]. Accumulating evidences have demonstrated the effects of

abnormally expressed miRNAs on the occurrence and progression of tumors [17, 19]. These certain miRNAs might be utilized for developing anti-tumor drugs or biological hallmarks [20]. About 30% human genome could be regulated by miRNAs, and most of human miRNAs (52%) locate on tumor-associated genomic regions or gene fragile sites [21, 22]. MiR-138-5p is a newly discovered cancer-related miRNA, which has been confirmed to be down-regulated in pancreatic cancer, colorectal cancer and other malignant tumors [21, 22]. However, its expression and function of miR-138-5p in PCa are rarely reported.

Bioinformatics analysis has been widely applied for analyzing genome to further uncover the gene expression pattern. By analyzing miR-138-5p profiling microarray, Forkhead box C1 (FOXC1) was selected to be the target genes of miR-138-5p. FOXC1 is an essential member of the forkhead box transcription factors and has been highlighted as an important transcriptional regulator of crucial proteins associated with a wide variety of carcinomas [25, 26]. Based on the above series of researches, this study aimed to elaborate the possible roles of miR-138-5p and FOXC1 in the progression of PCa, as well as the association with the clinical characteristics and prognosis of PCa patients, so as to bring a new idea for clinical treatment of PCa.

Materials And Methods

Patients and PCa tissue samples

A total of 60 PCa patients undergoing radical prostatectomy were enrolled in this study at my hospital. Tumor tissues and matched paracancerous tissues (5 cm away from tumor edge) were surgically resected and preserved within 5 min ex vivo. The clinical and pathological characteristics of PCa patients were collected for further analyses. The clinical stages of PCa were graded in accordance with International Federation of PCa staging criteria. All patients in this study had been fully signed the informed consent. In addition, this study has been approved by the Ethics Committee.

Cell lines and reagents

Prostate epithelial cell line (RWPE-1) and PCa cell lines (LNCaP, 22RV1, PC-3 and DU-145) were provided by ATCC, USA. Cells were cultured in RPMI 1640 or DMEM (Dulbecco'S modified MEM medium) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 ug/mL) at 37 °C with 5% CO_2.

Transfection

Negative control (NC mimic) and miR-138-5p overexpression sequence vectors (miR-138-5p mimic) were purchased from GenePharma (GenePharma, Shanghai, China). After cells were plated in 6-well plates and grown to a cell density of 50–70%, transfection was performed using Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer's instructions. After 48 hours, cells were collected for verification of transfection efficacy and subsequent experiments.

CCK-8 assay

The cells after 48 h of transfection were seeded in the 96-well plates with 2×10^3 cells per well. After cultured for 24 h, 48 h, 72 h and 96 h, these cells were added with CCK-8 Kit (Dojindo Laboratories, Japan). After incubation for 2 hours, the fluorescent absorbance at the optical density (OD) value of 450 nm of each sample was recorded for plotting the viability curves in the microplate reader.

Transwell assay

After 48 h of transfection, the cells were adjusted to a dose of 2.0×10^5/mL. 200 μL suspension was applied in the upper side of Transwell chamber (Millipore, MA, USA) inserted in a 24-well plate. In the bottom side, 700 μL of medium containing 10% FBS was applied. After 48 h of incubation, cells penetrated to the bottom side were fixed in 4% paraformaldehyde for 15 min, dyed with crystal violet for 20 min and counted using a microscope. The number of migratory cells was counted in 5 randomly selected fields per sample.

Wound healing assay

After 48 h of transfection, the cells were inoculated in 6-well plates and grown to 90% confluence. After the creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. After 24 hours, the wound closure was captured by a microscope.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), purified by DNase I treatment, and reversely transcribed into cDNA using Primescript RT Reagent (Takara, Otsu, Japan). The obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (Takara, Japan). GAPDH and U6 were used as internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Primer 5.0 was used for designing qRT-PCR primers. The following primers were used for qRT-PCR reactions:

miR-138-5p:

forward, 5'-GCGAGCTGGTGTTGTGAATC-3',

reverse, 5'-AGTGCAGGGTCCGAGGTATT-3';

U6:

forward, 5'-CTCGCTTCGGCAGCACA-3',

reverse, 5'-AACGCTTCACGAATTTGCGT-3';

FOXC1:

forward, 5'-CGGGTTGGAAAGGGATATTTA-3',

reverse, 5'-CAAAATGTTCTGCTCCTCTCG-3';

GAPDH:

forward, 5'-GAAATCCCATCACCATCTTCCAGG-3',

reverse, 5'-GAGCCCCAGCCTTCTCCATG-3'.

Western Blotting

The transfected cells were lysed using PRO-PREPTM lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4 °C. Total protein concentration was calculated by the PCaA Protein Assay Kit (Pierce, Rockford, Il, USA). Rabbit anti-human monoclonal antibodies against FOXC1 were purchased from Santa Cruz, USA; horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from Genscript. GAPDH was used as the internal reference control. Protein samples were separated by SDS-PAGE, transferred to PVDF membrane, and blocked with 5% skim milk powder for 1 h at room temperature. Primary antibodies were added for incubation overnight at 4 °C shaker. In the next day, the membrane was rinsed 3 times with TBST and incubated with second antibody for 1 h at room temperature. After that, the protein samples on the membrane were finally semi-quantitatively analyzed by alpha SP image analysis software.

Dual-luciferase reporter assay

3'-UTR of wild-type (WT) human FOXC1 gene, which contains a putative miR-138-5p binding DNA sequence, was amplified by PCR and inserted into a p-miR-reporter (Ambion, USA) to create a firefly FOXC1-WT luciferase vector. The putative miR-138-5p binding sequence on FOXC1 3'-UTR was then mutated to void miR-505 binding. The mutant (MUT) 3'-UTR was also inserted into p-miR-reporter to create a firefly FOXC1-MUT luciferase vector. Human HEK293T cells were transduced with NC mimic or miR-138-5p mimic, then cross-transfected with FOXC1-WT or FOXC1-MUT for 48 h. After that, relative luciferase activities were measured using a Dual-Luciferase Reporter assay (Promega, USA) according to the manufacturer’s protocol.

In vivo xenograft vectors

The Animal Ethics and Use Committee approved the tumor-forming experiment in nude mice. 8-week-old male nude mice were purchased from the animal center and randomly divided into two groups (5 in each group). The PC-3 cells with miR-138-5p mimic were injected subcutaneously into the axilla of mice. Tumor size was monitored every 5 days; Then, after 6 weeks, the mice were sacrificed. The tumor volumes were calculated using the following formula: tumor volume = (width 2 x length)/2.

Statistically analysis

GraphPad Prism 6 V6.01 was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the t-test. Kaplan-Meier curves were introduced for survival

analysis. Chi-square test was performed to evaluate the correlation between miR-138-5p level with pathological indexes of PCa patients. $P < 0.05$ was considered as statistically significant.

Results

miR-138-5p was down-regulated in PCa tissues and cell lines

Data from PCa patients of TCGA were complied for investigating the potential relevant miRNAs associated with PCa progression. We first focused insight into the expression level of miRNAs form TCGA database, and miR-138-5p with significant statistical difference was finally selected (Fig. 1A). In order to investigate the role of miR-138-5p in PCa development, qRT-PCR was performed to evaluate the expression of miR-138-5p in PCa tissues and cells. As showed in Fig. 1B, miR-138-5p was down-regulated in PCa tissues, compared with paracancerous normal tissues. Similarly, miR-138-5p was also down-regulated in PCa cell lines than that of Prostate epithelial cell line (RWPE-1) (Fig. 1E).

miR-138-5p expression was correlated with clinicopathologic characteristics and overall survival in PCa patients

The clinical and pathological characteristics and follow-up data of enrolled PCa patients were collected for further analyses. According to the median level of miR-138-5p, PCa patients were assigned into two groups as high-miR-138-5p level group and low-miR-138-5p level group. As shown in Table 1, the relationships between the expression of miR-138-5p and Age, Tumor size, Gleason score, Lymph node metastasis and Bone metastasis of PCa patients were analyzed. The results found that these PCa patients with low-miR-138-5p expression had a higher Gleason score, Lymph node metastasis and Bone metastasis of PCa, compared with the patients with high-miR-138-5p expression (Fig. 1C). In addition, Kaplan-Meier methods revealed the poor prognosis in PCa patients of low-miR-138-5p level group than that of high-miR-138-5p level group (Fig. 1D).

Table 1
Association of miR-138-5p expression with clinicopathologic characteristics of prostate cancer.

Parameters	Number of cases	miR-138-5p expression		P-value
		High (%)	Low (%)	
Age (years)				0.830
<60	24	14	10	
≥60	36	22	14	
Tumor size				0.526
<4 cm	28	18	10	
≥4 cm	32	18	14	
Gleason score				0.025
≤7	40	28	12	
>7	20	8	12	
Lymph node metastasis				0.009
No	37	27	10	
Yes	23	9	14	
Bone metastasis				0.109
No	35	24	11	
Yes	25	12	13	

miR-138-5p alleviated the proliferation and metastasis of PCa cell lines

To explore the biological function of miR-138-5p in PCa cell lines was analyzed by CCK-8 cell proliferation assay, Tranwell assay and Wounding healing assay, respectively. miR-138-5p overexpression vectors were successfully constructed in the PC-3 and DU-145 cell lines, respectively (Fig. 2A). It was found by the CCK-8 assay that the cell proliferation ability of miR-138-5p mimic was remarkably decreased in PCa cell lines, compared with NC mimic (Fig. 2B). Transwell assay revealed that the metastasis ability of PCa cells was significantly decreased in miR-138-5p mimic, compared with NC mimic (Fig. 2C). In addition, Wound healing assay showed that the overexpression of miR-138-5p could hinder the invasion and crawling ability of PCa cell lines (Fig. 2D). These results suggested that miR-138-5p could inhibited cell proliferation and metastasis in PCa.

Interaction of miR-138-5p and FOXC1

Potential target genes of miR-138-5p were predicted in the miRDB, TargetScan and StarBase (Fig. 3A). At last, the intersection contained 5 potential targets (FOXC1, SYT13, SIN3A, FOXP4 and KLF11). Among them, FOXC1 was the most differentially expressed one after transfection of miR-138-5p mimic in PC-3 cells (Fig. 3B). Western Blotting showed that the expression level of FOXC1 was significantly down-regulated in PC-3 and DU-145 cell lines after overexpressing miR-138-5p (Fig. 3C). In addition, the results of qRT-PCR also revealed the same trend (Fig. 3D). To further uncover the biological role of FOXC1 in PCa, we constructed pcDNA3.1-NC and pcDNA3.1-FOXC1. qRT-PCR found that miR-138-5p level was found to be significantly down-regulated in PCa cell lines transfected with pcDNA-FOXC1, compared to that transfected with pcDNA-NC (Fig. 3E). Luciferase reporter assay verified that miR-138-5p could indeed combine with FOXC1 through specific sequences (Fig. 3F & Fig. 3G). Additionally, a significant negative correlation was identified to detect the expression levels of miR-138-5p and FOXC1 in PCa tissues (Fig. 3H).

miR-138-5p negatively regulated FOXC1 to inhibit the malignant progression of PCa

To further explore the specific regulatory mechanisms in which miR-138-5p exactly regulated FOXC1 to inhibit malignant progression of PCa. Firstly, the overexpressed endogenous FOXC1 was established with a FOXC1 overexpressing plasmid, and an empty overexpressing plasmid NC, to transfect PCa cell lines with miR-138-5p mimic. qRT-PCR demonstrated that PCa cells transfected with pcDNA3.1-FOXC1 had significantly lower miR-138-5p expression levels than cells transfected with pcDNA3.1-NC (Fig. 4A). In addition, Western Blotting demonstrated that PCa cells transfected with pcDNA3.1-FOXC1 had significantly higher FOXC1 expression levels than cells transfected with pcDNA3.1-NC (Fig. 4B). Subsequently, overexpression of FOXC1 was demonstrated to be able to counteract the effects of miR-138-5p mimic on the metastasis of PCa cells by Tranwell assay and Wound healing assay (Fig. 4C&Figure 4D). Therefore, these results revealed that miR-138-5p could inhibit the malignant progression of PCa through modulating FOXC1.

Over-expression of miR-138-5p suppressed the PCa in vivo tumorigenicity

In an in vivo tumorigenicity assay, NC mimic or miR-138-5p mimic transduced PC-3 cells were subcutaneously inoculated into the abdominal compartments of athymic nu/nu mice for 6 weeks. The volumes of PC-3 xenografts were calculated weekly. It showed that, in vivo tumor growth was significantly suppressed by miR-138-5p over-expression ($P < 0.05$; Fig. 5A&Figure 5B). Subsequently, we validated the reduction of weight in tumor-forming tissues of nude mice injected with miR-138-5p mimic ($P < 0.05$; Fig. 5C). The results of qRT-PCR revealed that the overexpression of miR-138-5p in the tumor

tissues of nude mice could decrease miR-138-5p expression level (Fig. 5D). In addition, compared with NC mimic, FOXC1 expression dramatically decreased in the tumor tissues of nude mice with miR-138-5p mimic by Western Blotting (Fig. 5E). Immunohistochemistry showed that the level of miR-138-5p mimic-transduced PC-3 xenografts significantly decreased than NC mimic-transduced xenografts (Fig. 5F).

Discussion

The etiology of PCa is complex, and no clear conclusion about the pathogenesis is found [1, 7, 9]. Nowadays, the understanding of the pathogenesis and biological behavior of PCa still has great limitations [8, 9]. The development of PCa is a multi-factors process, influenced by a variety of biomolecules and regulated by signaling pathways [11, 12]. With the progress of the Human Genome Project, researches on molecular level have been extensively conducted to detect the differential expression profile of tumor genes, which is of great significance for exploring the molecular mechanism of the development of PCa, and finding the molecular biomarkers in the early diagnosis and prognosis of PCa [13, 14].

MiRNAs not only participate in normal physical biological processes, but also regulate tumor progression at transcriptional and post-transcriptional levels[15, 16]. It is estimated that over 30% of human genes and cellular processes are regulated or controlled by miRNAs[17, 20]. In the past, oncogenes or tumor-suppressor miRNAs were used to develop as drug targets for tumor treatment [15, 18, 20]. Nowadays, tumor-related miRNAs have been well concerned, exerting more crucial application in early-stage diagnosis, target therapy and effective prognosis of tumors [13, 14, 19]. As a member of tumor-associated miRNAs family, miR-138-5p is located on chromosome Xq38.13[24]. Previous researches showed that miR-138-5p could inhibit the malignant progress of several human tumors[23, 24]. In this study, miR-138-5p was down-regulated in PCa tissues and cell lines. Besides, these PCa patients with low-miR-138-5p expression had a higher Gleason score, Lymph node metastasis, Bone metastasis and poor prognosis of PCa, compared with the patients with high-miR-138-5p expression. Thus, the above results suggested that miR-138-5p might act as anti-tumor effect in the progression of PCa. In order to further investigate the biological function of miR-138-5p in PCa cell lines, CCK-8, Tranwell assay and Wounding healing assay were used to introduce that miR-138-5p mimic could inhibit the proliferation and metastasis of PCa cell lines. The above results provided a theoretical basis for revealing the mechanism of PCa development. Of course, the specific molecular mechanism of signal transduction in PCa need to further study.

Regulatory mechanism of miRNAs depends on the expressions and functions of their target genes[15, 20]. A miRNA degrades target mRNA or suppress its translation by base pairing with 3’UTR of the mRNA[15, 16]. The degree of base pairing decides the degradative or translation inhibitory effect of a miRNA, that is, complete base pairing leads to mRNA degradation; otherwise, translation inhibition is achieved[27, 28]. MiRNAs only account for only 1% of the whole human genome, but they are able to regulate more than 30% protein-encoding genes[29]. Multiple miRNAs could precisely regulate a single target gene[16, 22]. Bioinformatics analysis and luciferase reporter gene assay showed that FOXC1 was

the target gene of miR-138-5p through predicting in the miRDB, TargetScan and StarBase. FOXC1 level was up-regulated in PCa tissue and cell lines. Based on these findings, we hypothesized that miR-138-5p might act as a ceRNA in the progression of PCa and found that miR-138-5p negatively regulated FOXC1 expression in PCa tissue. In addition, qRT-PCR showed that compared with NC mimic, miR-138-5p mimic could decreased the level of FOXC1. In order to explore the associations between miR-138-5p and FOXC1 in the development of PCa, the overexpression of FOXC1 was found to reverse the metastasis ability of miR-138-5p mimic on PCa cells, thus promoting the malignant progression of PCa.

Conclusions

In summary, the down-regulated miR-138-5p was closely associated with Gleason score, Distant metastasis and poor prognosis of PCa patients. In addition, miR-138-5p alleviated the malignant progression of PCa by targeting and downregulating FOXC1.

Declarations

Acknowledgements

Not applicable.

Funding

Not applicable.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Ethics Approval and consent to participate

Ethical approval was provided by the Medical Ethics Committee of Chifengshi hospital.

Consent for Publication

All authors are responsible for the submission of this article and accept the conditions of submission.

Author Contributions

Protocol/project development: X C

Molecular biology experiment: DP Z and XD L

Data collection or management: DP Z and QW Z

Data analysis: XD L and QW Z

Manuscript writing/editing: X C and DP Z

Conflict of Interest

We declare that we have no conflict of interest.

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Figures

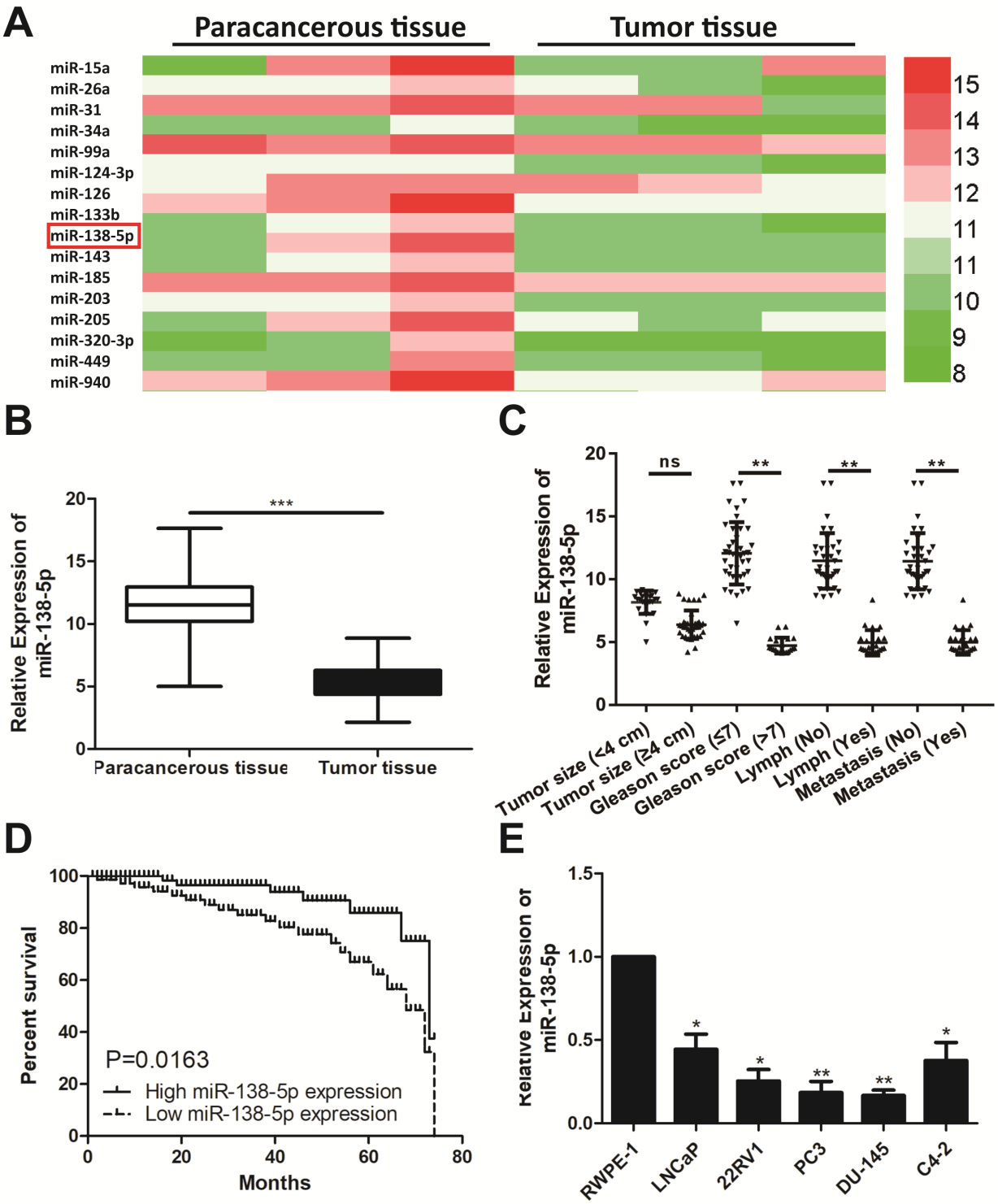

Figure 1

miR-138-5p is lowly expressed in PCa tissues and cell lines. (A). The heatmap of miRNAs expression profiles with PCa progression in TCGA database; (B). qRT-PCR was used to detect the expression level of miR-138-5p in PCa tissues and paracancerous tissues; (C). qRT-PCR was used to detect the difference expression of miR-138-5p in tissue samples of PCa patients with different clinicopathologic characteristics (Tumor size, Gleason score, Lymph node metastasis and Bone metastasis); (D). Kaplan-Meier survival curve of PCa patients based on miR-138-5p expression; (E). qRT-PCR was used to detect the expression level of miR-138-5p in PCa cell lines. Data are mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.

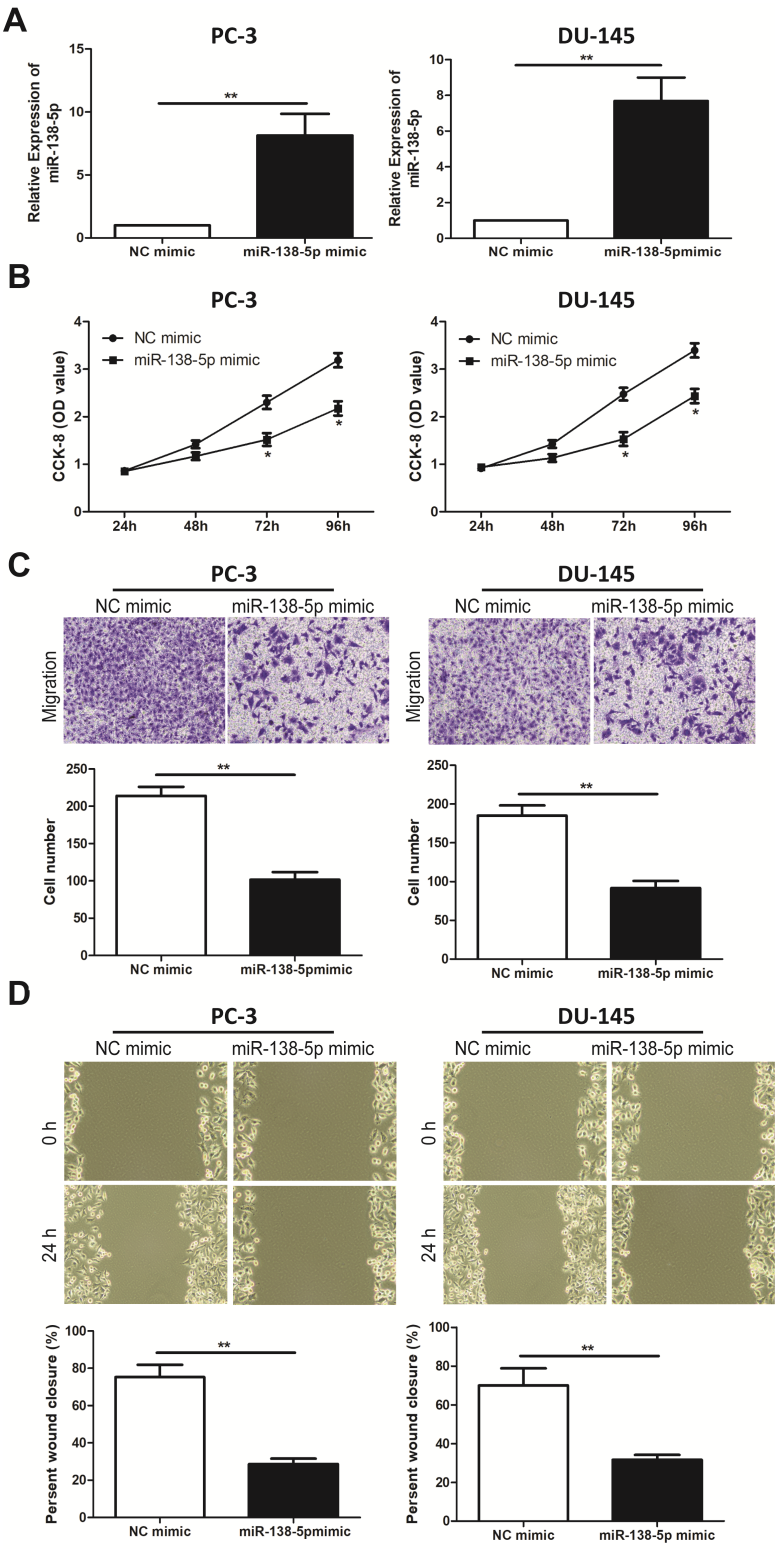

Figure 2

miR-138-5p inhibited cell proliferation and metastasis of PCa. (A). qRT-PCR was used to verify the transfection efficiency of miR-138-5p after transfection of NC mimic and miR-450b-3p mimic in PC-3 and DU-145 cell lines; (A). CCK-8 assay detected cell proliferation of PCa cell lines after transfection of NC mimic and miR-138-5p mimic; (B). Transwell assay detected the migration of PCa cell lines after transfection of NC mimic and miR-138-5p mimic (Magnification: 40X); (C). Wound healing assay detected

the invasion and crawling ability of PCa cell lines after transfection of NC mimic and miR-138-5p mimic (Magnification: 40X). Data are mean ± SD, *p < 0.05, **p < 0.01.

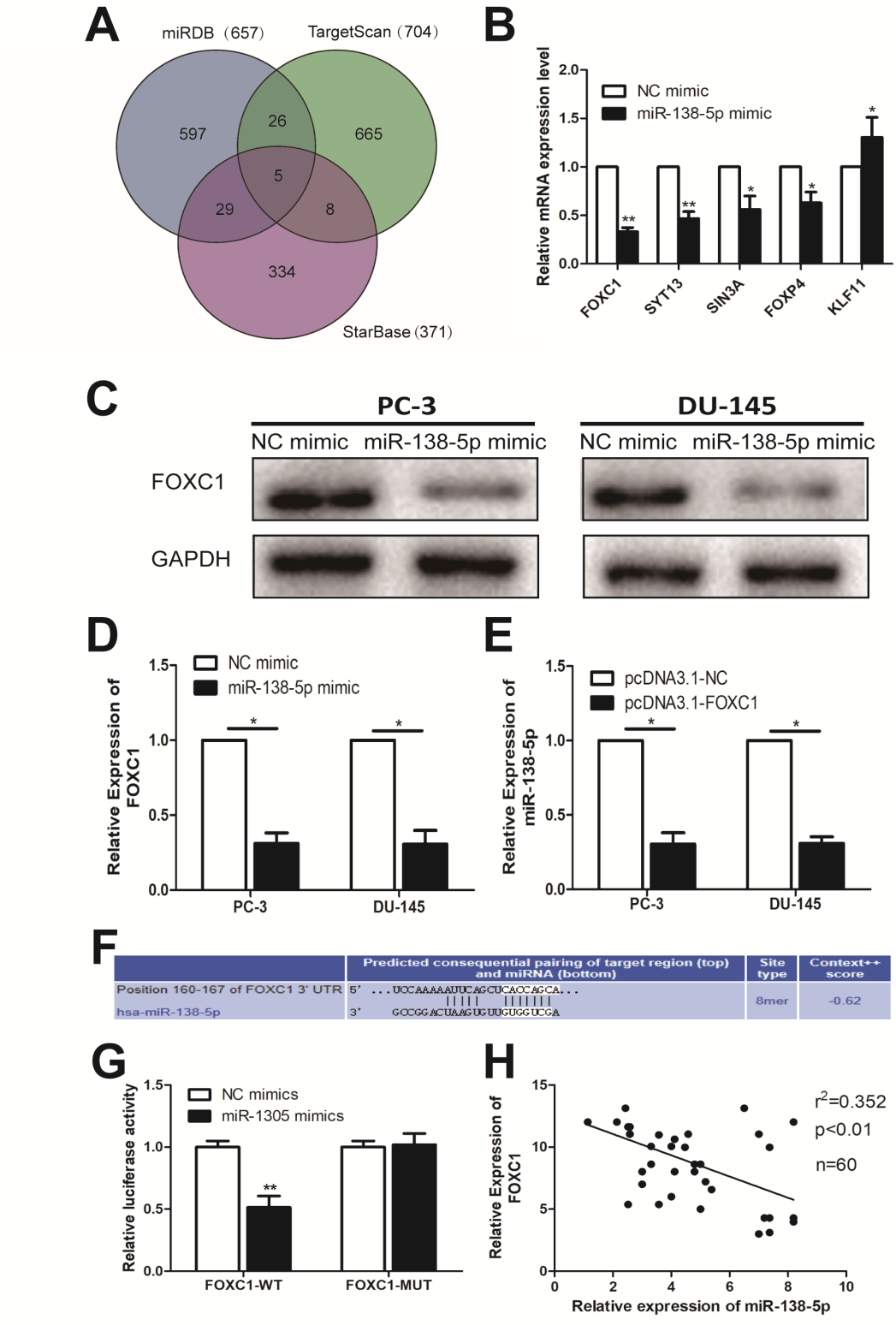

Figure 3

Interaction of miR-138-5p and FOXC1. (A). Bioinformatics analysis websites (miRDB, TargetScan and StarBase) showed the potential downstream target gene of miR-138-5p; (B). qRT-PCR was used to detect the differential expression of the potential downstream target gene of miR-138-5p in NC mimic and miR-

138-5p mimic, respectively; (C). Western Blotting verified the expression level of FOXC1 after transfection of NC mimic and miR-138-5p mimic PCa cell lines, respectively; (D). qRT-PCR verified the expression level of FOXC1 after transfection of NC mimic and miR-138-5p mimic PCa cell lines, respectively; (E). qRT-PCR verified the expression level of miR-138-5p after transfection of pcDNA3.1-NC and pcDNA3.1-FOXC1 in PC-3 and DU-145 cell lines, respectively; (F,G). Dual luciferase reporter assays demonstrated direct targeting of miR-138-5p to FOXC1; (H). A significant negative correlation between miR-138-5p and FOXC1 expression in PCa tissues. Data are mean ± SD, *p < 0.05, **p < 0.01.

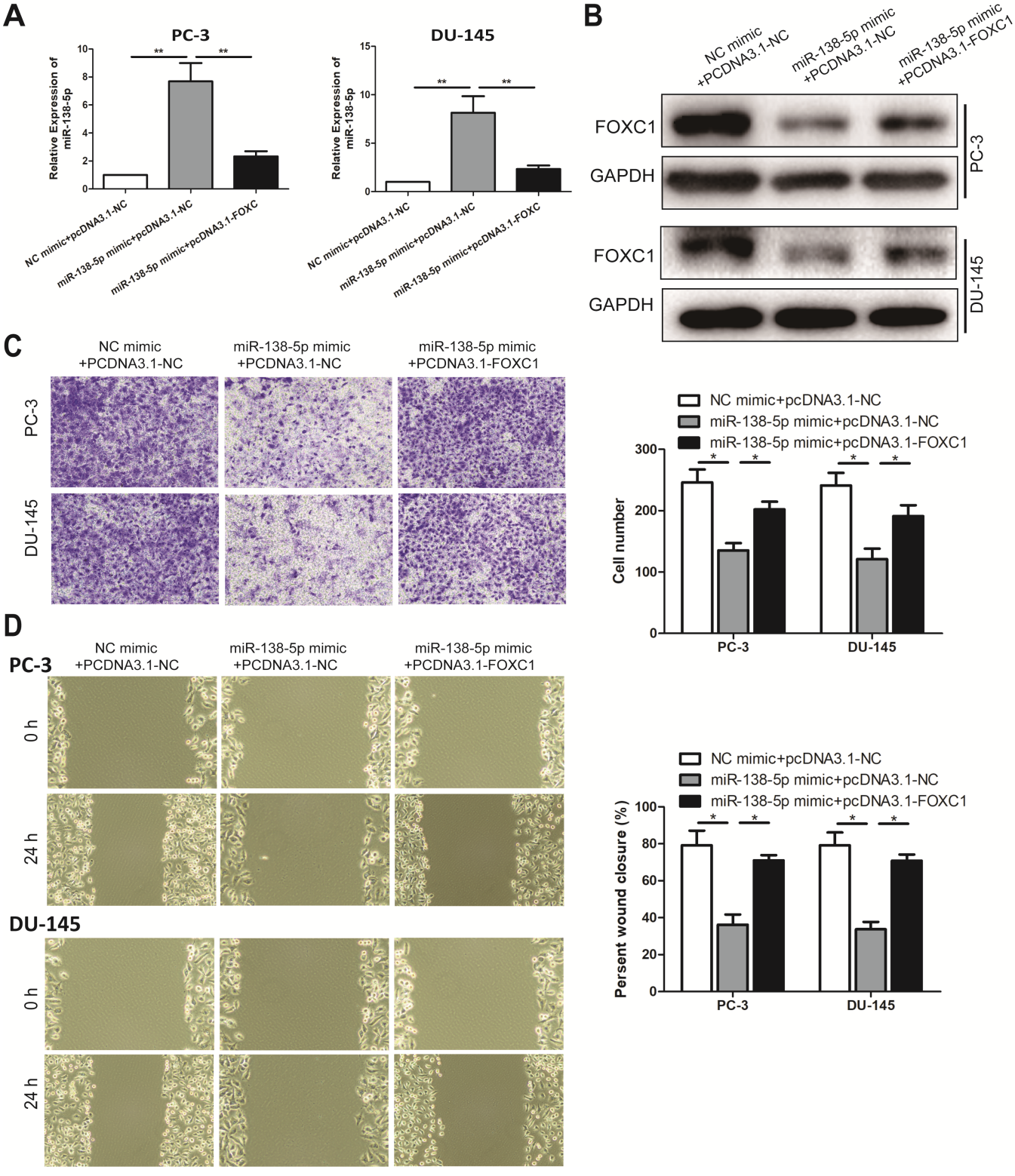

Figure 4

miR-138-5p negatively regulated the expression of FOXC1 in PCa cell lines. (A). The expression level of miR-138-5p in the co-transfected PCa cell lines of miR-138-5p and FOXC1 was detected by qRT-PCR; (B). The expression level of FOXC1 in the co-transfected PCa cell lines of miR-138-5p and FOXC1 was detected by Western Blotting; (C). Transwell assay detected the cell migration in the co-transfected PCa cell lines of miR-138-5p and FOXC1 (Magnification: 40X); (D). Wound healing assay was used to detect the invasion and crawling ability of PCa cell lines after co-transfection of miR-138-5p and FOXC1 (Magnification: 40X). Data are mean ± SD, *$p < 0.05$, **$p < 0.01$.

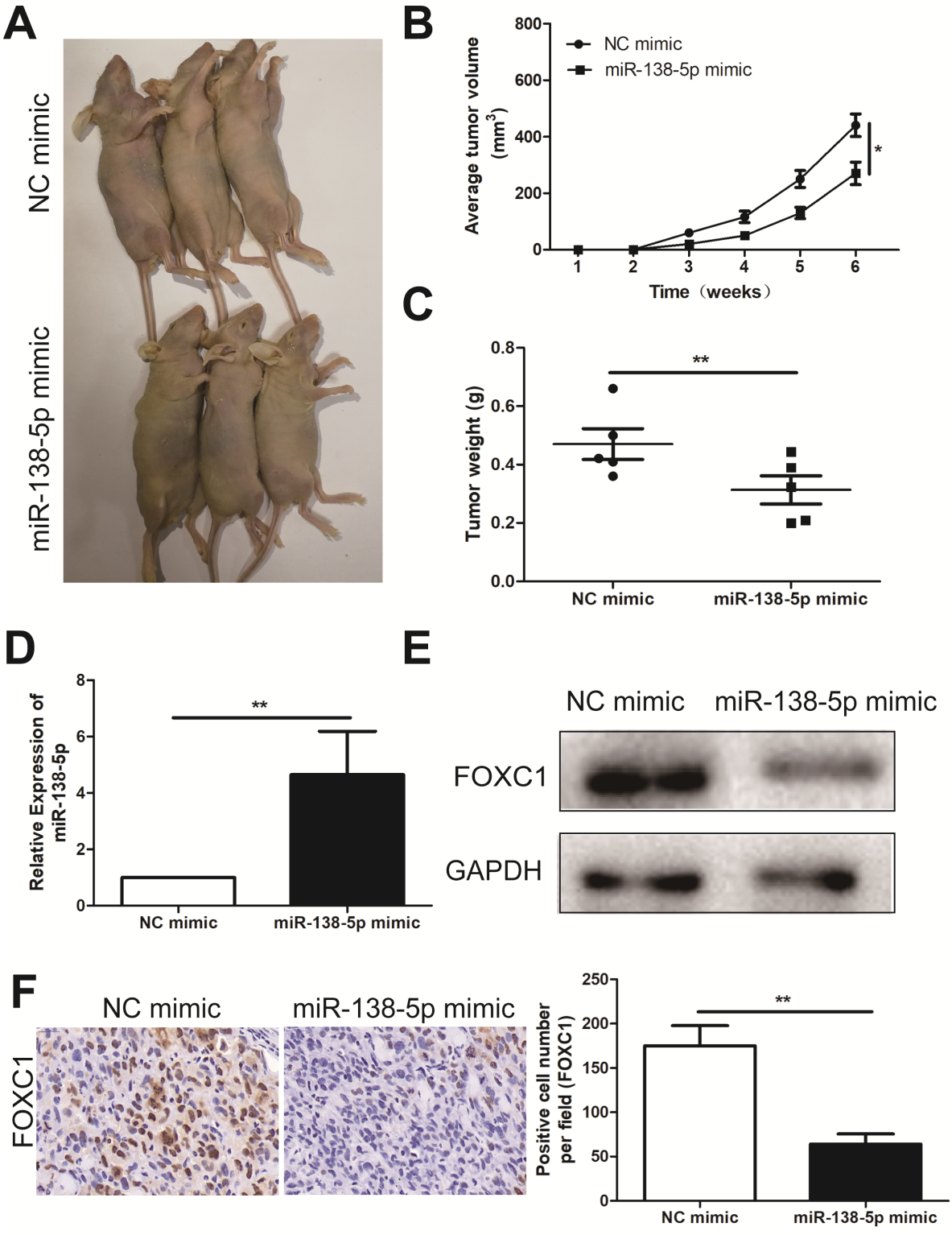

Figure 5

Over-expression of miR-138-5p inhibited tumorigenic ability in nude mice. (A,B). Tumor volume growth curves were calculated for different nude mice after injection of NC mimic and miR-138-5p mimic, respectively; (C). Tumor weight growth curves were calculated after injection of NC mimic and miR-138-5p mimic, respectively; (D) qRT-PCR was used to detect the expression level of miR-138-5p in the tumor-forming tissues of nude mice; (E) Western Blotting was used to detect the expression level of FZD4 in the

tumor-forming tissues of nude mice; (F). Immunohistochemistry was used to detect the expression level of FZD4 in the tumor-forming tissues of nude mice with Hep3B cell line (Magnification: 40X). Data are mean ± SD, *p < 0.05, **p < 0.01.