



Silencing effects of FOXD1 inhibit metastatic potentials of the PCa via N-cadherin – Wnt/ β -catenin crosstalk

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ABSTRACT

The elucidation of the mechanisms controlling the metastatic processes is important for the development of new treatment methods to prevent the progression of localized disease to metastasis. Forkhead box D1 (FOXD1) is a member of the FOX transcription factor family and has been reported to play an important role in the development and progression of various cancers. However, its role in prostate cancer (PCa) remains only partially understood. Therefore, we aimed to explore the effects on the associated regulatory signal pathway of FOXD1 in prostate cancer. To clarify the roles of FOXD1 in prostate cancer, we used siRNA to suppress its expression in 22Rv1 cells with relatively higher expression of FOXD1. The effects of FOXD1 silencing on cell proliferation, migration and invasion were determined. WST-1 assays were used to determine cell proliferation. Cell migration and invasion were evaluated through wound healing and transwell assays. The possible underlying mechanism of FOXD1 silencing on 22Rv1 was evaluated by determining the expression of proteins related to EMT and Wnt/ β -catenin signaling pathway. Our results showed that FOXD1 was highly expressed in prostate cancer cell lines -PC-3, DU145, LNCaP and 22Rv1- compared to normal prostate epithelial cell line RWPE-1. Additionally, silencing of FOXD1 significantly reduced proliferation, migration and invasion of 22Rv1 cells. Furthermore, silencing of FOXD1 decreased the expression of β -catenin and cyclin D1, which are involved in the Wnt/ β -catenin signaling pathway. However, it did not appear to affect the expression of EMT-related proteins other than N-cadherin. Our results suggest that silencing of FOXD1 suppresses metastatic potentials of the PCa via N-cadherin - Wnt/ β -catenin crosstalk. Therefore, the expression status of FOXD1 may be a new prognostic factor as well as a potential therapeutic target in prostate cancer treatment.

1. Introduction

According to the World Health Organization's (WHO) 2020 annual report, prostate cancer is the second most common cancer in men followed by lung cancer, and more than 375,000 people have died from this disease worldwide (World Health Organization-Cancer Profiles, 2020). Cancer metastasis but not the primary tumor is the primary cause of cancer-related deaths, including those from prostate cancer (Rycak et al., 2017). Current standard therapies such as radical prostatectomy or radiotherapy are often curative in the early stages of prostate cancer. However, once cancer has metastasized to distant organs, current standard therapies are only palliative (Hamilton and Theyer, 2013, Klusa et al., 2021). The epithelial-mesenchymal transition (EMT) is

suggested to be an important mechanism regulating the first steps in cancer metastasis (Radisky, 2005, Ribatti et al., 2020, Konac, 2017). Therefore, understanding the molecular mechanisms associated with EMT of prostate cancer is important for the development of new and effective treatment agents.

Forkhead box (FOX) proteins are a large family of transcription factors that play key roles in the regulation of important biological processes, including cell cycle progression, proliferation, differentiation, metabolism, aging, survival, apoptosis and DNA damage repair (Lam et al., 2013). FOXD1, also known as FKHL8 and FREAC-4, is a transcription factor belonging to the FOX family (Gomez and Duffield, 2014). FOXD1 is known to be involved in numerous biological functions such as cell cycle regulation, signal transduction, control of metabolism,

Abbreviations: EMT, epithelial-mesenchymal transition; FOX, forkhead box; FOXD1, forkhead box D1; PCa, prostate cancer; siRNA, small inhibitory RNA; WHO, world health organization; Wnt, wingless/integrated.

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stem cell niche maintenance, embryonic development and organogenesis (Qiu et al., 2021). Moreover, studies have shown that FOXD1 plays an important role in tumorigenesis, tumor progression and metastasis in various human cancers. It was found that the expression of FOXD1 increased in prostate cancer tissues and was associated with lymph node metastasis (van der Heul-Nieuwenhuijsen et al., 2009). It has also been reported that FOXD1 was involved in the development of various cancers, including non-small cell lung cancer (Nakayama et al., 2015), glioma (Cheng et al., 2016), colorectal cancer (Pan et al., 2018), pancreatic cancer (Wu, et al., 2020), breast cancer (Bai et al., 2020), liver cancer (Sun et al., 2021), cervical cancer (Zhang et al., 2021), and oral squamous cell carcinoma (Chen et al., 2021). These findings suggest that increased FOXD1 expression in tumor cells may affect biological functions, and lead to cell proliferation, apoptosis, invasion, and metastasis.

Wnt/ β -catenin signaling pathway plays a role in both normal prostate development and prostate cancer progression (Yu et al., 2009). Increased expression of wnt-1 and β -catenin has been reported in invasive prostate cancer cell lines and primary prostate cancer samples (Chesire et al., 2000, Chen et al., 2004). Wnt signaling prevents degradation of β -catenin and causes cytoplasmic/nuclear accumulation of β -catenin (Miller, 2002). Once β -catenin enters the nucleus, it functions as a transcription coactivator and activates target genes such as c-myc, cyclin D1, MMP7, uPA, and AR which are involved in proliferation, migration, invasion, and metastasis (Miller, 2002, Terry et al., 2006).

Nowadays, siRNAs are used as research subjects to study gene functions both *in vitro* and *in vivo*. siRNA-based therapeutics is also emerging as a new therapeutic class that is particularly attractive to undruggable targets for the treatment of cancer and other diseases (Dana et al., 2017). The number of siRNA-based therapeutics currently in development and undergoing clinical trials is growing rapidly. Only three siRNA therapeutics (Patisiran, Givosiran, and Lumasiran) have been approved by the FDA to date, but more are likely to join them in the coming years.

In this study, we first determined whether there was a difference in terms of FOXD1 expression levels in prostate cancer and prostate epithelial cell lines. Then, the effects of silencing FOXD1 on cell proliferation, migration and invasion were investigated in 22Rv1 prostate cancer cells with the highest FOXD1 expressions. In addition, the effects of FOXD1 silencing on the expression of some EMT proteins (E-cadherin, N-cadherin, Snail and Vimentin) and a number of proteins involved in the Wnt/ β -catenin pathway (β -catenin, c-myc and cyclin D1) were investigated.

2. Materials and methods

2.1. Cell culture

Human prostate cancer cell lines (PC-3, DU145, LNCaP and 22Rv1) and an immortalized normal prostate epithelial cell line (RWPE-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All of the prostate cancer cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (BIOSERA, Nuaille, France). PC-3 cells (passage 15–21), DU145 cells (passage 19–25), LNCaP cells (passage 20–25), and 22Rv1 cells (passage 9–20) were used in all experiments. RWPE-1 cells were cultured in a keratinocyte serum-free medium (K-SFM) kit (GIBCO, Thermo Fisher Scientific Inc., Waltham, MA, USA). Each 500 mL K-SFM medium was supplemented with 25 mg bovine pituitary extract (BPE) and 2.5 μ g of human recombinant epidermal growth factor (rEGF), and 1% penicillin/streptomycin (BIOSERA, Nuaille, France). The passage number of the RWPE-1 cell line used throughout the experiments was between 7 and 12. All cell lines were maintained at 37 °C with 5% CO₂ in a humidified incubator.

2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from human prostate cells using TRIzol reagent (Ambion) following producer's instructions. Total RNA of each sample was subsequently reverse transcribed to cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Inc., Waltham, MA, USA). Real-time PCR reactions were performed in Rotor-Gene Q5-plex HRM system (QIAGEN Benelux B.V. — Netherlands) using RealMOD Green W2 2x qPCR kit according to the manufacturer's protocol. The following primers were used in this study: FOXD1, forward 5-AAGAACCCGCTGGTGAAG-3 and reverse 5-GTCCAGTAGTTGCCCTTGC-3; GAPDH, forward 5-ACAACCTTGGTATCGTGAAGG-3 and reverse 5-GCCATCACGCCACAGTTTC-3. The relative gene expression levels were determined using the $2^{-\Delta\Delta C_t}$ method and the internal control to GAPDH (Livak and Schmittgen, 2001). All experiments were performed in triplicate.

2.3. Western blot

Total protein was extracted from cells using RIPA buffer (Sigma-Aldrich St. Louis, USA). 30 μ g of protein per lane were separated by 8%–12% SDS-PAGE and the proteins were transferred to PVDF membranes (Thermo Fisher Scientific Inc., Waltham, MA, USA). The PVDF membranes were blocked in bovine serum albumin. The PVDF membranes were incubated with an appropriate dilution of primary antibodies against FOXD1, E-cadherin, N-cadherin, vimentin, β -catenin, cyclin D1, c-myc and β -actin at 4 °C overnight. Then, the PVDF membranes were washed and incubated with a horseradish peroxidase (HRP) conjugate secondary antibody for 2 h at room temperature. The protein bands were visualized by ECL reagent.

2.4. Cell transfection

The SMARTpool siRNA against human FOXD1 (siFOXDX1) and negative control siRNA (siNC) were purchased from Dharmacon (Lafayette, CO, USA). Before transfection, 22Rv1 cells were seeded at a density of 5×10^5 cells per well in a six-well culture plate. After 24 h, 22Rv1 cells were transfected with siFOXDX1 or negative control siRNA using DharmaFECT transfection reagent according to the manufacturer's instructions. 22Rv1 cells were harvested 24 h or 48 h after transfection for mRNA and protein analysis. The efficiency of transfection was confirmed by qRT-PCR and western blot analysis.

2.5. Cell proliferation assay

Effect of FOXD1 silencing on cell proliferation was measured using WST-1 assay kit. Briefly, 22Rv1 cells transfected with siFOXDX1 or siNC were seeded at a density of 5×10^3 cells per well in 96 well plates, and then incubated for 24, 48, or 72 h, respectively. Following incubation, the culture medium was replaced with 100 μ l of complete medium. Subsequently, 10 μ l of WST-1 reagent was added to each well and incubated for 4 h at 37 °C under 5% CO₂ in a humidified incubator. Finally, the absorbance at 450 nm was measured with a microplate reader (SpektraMax M3 plate reader, Molecular Devices, USA).

2.6. Wound healing assay

22Rv1 cells were seeded into 6-well plates at 5×10^5 cells per well and cultured for 24 h. After 24 h of culture, 22Rv1 cells were transfected with siFOXDX1 or siNC and cultured to 90% confluency at 37 °C, 5% CO₂. At this point, a wound was made on the monolayer of 22Rv1 cells using a sterile 200 μ l pipette tip. After wound infliction, 22Rv1 cells were incubated at 37 °C, 5% CO₂ for 24 and 48 h. The size of the wound was photographed at 0, 24 and 48 h with an inverted microscope. The area of the wound was measured using ImageJ software and the distance of the wound closure calculated. 22Rv1 cells transfected with negative control siRNA or not transfected were used as controls.

2.7. Transwell assays

The transwell assays were performed to evaluate migration and invasion abilities of transfected or untransfected 22Rv1 cells. In cell invasion assay, transwell insert (Corning Costar, Thermo Fisher Scientific Inc., Waltham, MA, USA) membranes were coated with Matrigel (BD Biosciences, CA, USA), while in cell migration assay, transwell insert membranes were not coated. For both assays, untransfected cells and 22Rv1 cells transfected with siFOXD1 and siNC were suspended in serum-free RPMI 1640 medium. The cells (5×10^4 cells per well) were seeded in the upper compartment of transwell insert, while the lower chamber of the 24 well plate was filled with RPMI 1640 medium containing 10% FBS. After 48 h of incubation at 37 °C with 5% CO₂, cells remaining on the upper surface of the membrane were removed with a cotton swab. The cells on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with Giemsa. Subsequently, the cells were counted in six representative fields under an inverted microscope.

2.8. Statistical analysis

All statistical analyses were performed using SPSS 23.0 software (IBM). Statistical comparisons between the groups were analyzed with Student's *t*-test or one-way ANOVA. Results were presented as mean $\pm$ standard deviation and statistical significance was examined by calculating the *p* value. A *p* value < 0.05 (**p* < 0.05, ***p* < 0.01, ****p* < 0.001 and *****p* < 0.0001) was considered statistically significant.

3. Results

3.1. FOXD1 was highly expressed in human prostate cancer cell lines

We firstly studied the expression of FOXD1 in four human prostate cancer (22Rv1, LNCaP, DU145, PC-3) lines and a normal prostate epithelial cell line (RWPE-1) by qRT-PCR and Western blotting. The results of qRT-PCR demonstrated that the expression levels of FOXD1 mRNA were significantly higher in 22Rv1, DU145, PC-3 cell lines than those of in RWPE-1 cells (Fig. 1a). Although the increase in FOXD1 mRNA expression in LNCaP was higher compared to that in RWPE-1, this increase was not statistically significant (*p* > 0.05). On the other hand, Western blotting results demonstrated that the expression of FOXD1 protein was also notably higher in all prostate cancer cell lines than those in RWPE-1 cells (Fig. 1b). 22Rv1 cells among these prostate cells, which exhibited the highest expression of FOXD1 at both mRNA and protein levels, were selected for subsequent experiments.

3.2. Silencing of FOXD1 inhibited the proliferation of 22Rv1 cells

FOXD1 was silenced in 22Rv1 cells by transfection with siRNA to investigate the role of FOXD1 in prostate cancer. Subsequently, the transfection efficiency was verified by qRT-PCR and Western blotting analyses. After siFOXD1 transfection, mRNA levels of FOXD1 expression decreased in 22Rv1 cells in comparison to the siNC transfected cells and untransfected 22Rv1 cells, by 88% and 85% respectively (Fig. 2a). Western blot revealed the same results in consistence with the mRNA levels (Fig. 2b). Then, the effects of FOXD1 silencing on 22Rv1 cell proliferation were measured in 24, 48 and 72 h of post-transfection by using WST-1 assay. The results of WST-1 assay demonstrated that silencing of FOXD1 significantly inhibited the ability of the proliferation of 22Rv1 cells, when compared with the siNC and 22Rv1 groups (Fig. 2c).

3.3. Silencing of FOXD1 significantly decreased migration and invasion of 22Rv1 cells

Whether FOXD1 regulates migration and invasion of 22Rv1 cells was

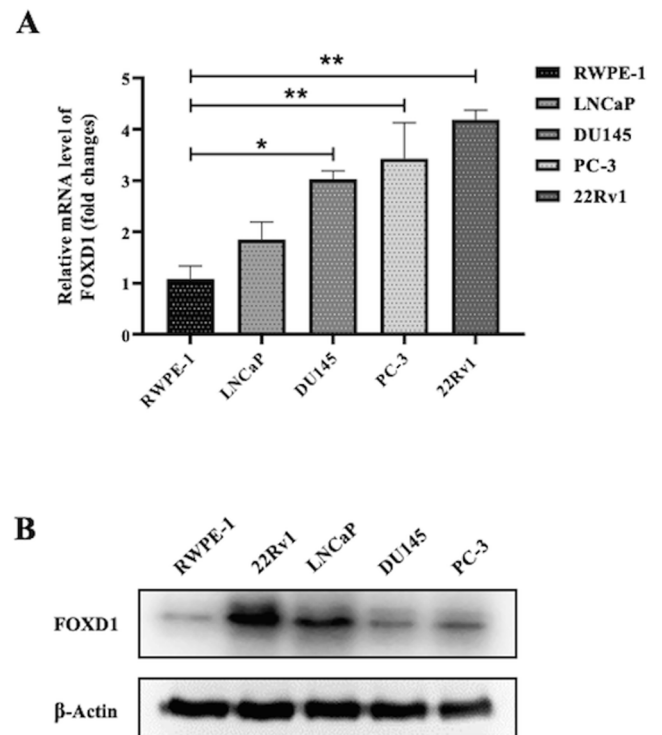


Fig. 1. FOXD1 was highly expressed in human prostate cancer cell lines. (A) qRT-PCR analysis of FOXD1 in four human prostate cancer cell lines (PC-3, DU145, LNCaP and 22Rv1) in comparison with the immortalized normal prostate epithelial cell line (RWPE-1). (B) Western blot analysis of FOXD1 in the same four human prostate cancer cell lines in comparison with RWPE-1. Data are represented as mean SD. **p* < 0.05, ***p* < 0.01.

studied to further determine the function of FOXD1 in prostate cancer. Cell migration was assessed by wound healing and transwell migration assays. While the percentage of migration was 9% in the cells treated with FOXD1 siRNA at 24 h, it was 26% in the negative control siRNA-treated group and 29% in the control group that did not receive FOXD1 siRNA. On the other hand, migration percentages at 48 h were calculated as 22% in the cells treated with FOXD1 siRNA, 39% in the negative control siRNA-treated group, and 46% in the control group without FOXD1 siRNA treatment. Briefly, the wound healing assay results showed that migration ability of 22Rv1 cells transfected with FOXD1 siRNA significantly decreased at 24 and 48 h after scratch treatment compared with the siNC and 22Rv1 groups (Fig. 3). On the other hand, the migration levels were 50% and 89% for the siFOXD1 group and the siNC group, respectively. The transwell migration assay consistently demonstrated that silencing of FOXD1 decreased the number of migrated cells of 22Rv1 (Fig. 4). Similarly, the results of the transwell invasion assay revealed that the invasive ability of 22Rv1 cells decreased in the siFOXD1 group compared with the siNC and 22Rv1 groups (Fig. 5). The invasion levels were 34% for the siFOXD1 group and 80% for the siNC group, respectively. Overall, these results suggested that silencing of FOXD1 decreased the migration and invasion abilities of 22Rv1 cells.

3.4. Silencing of FOXD1 did not affect the expression levels of some EMT protein markers

We analyzed whether silencing of FOXD1 caused a change in the expression levels of some EMT-related proteins in 22Rv1 cells in order to determine whether FOXD1 has a regulatory role in the EMT process. Silencing of FOXD1 had no effects on a number of EMT protein markers, such as vimentin, snail, E-cadherin when compared with the control groups (data not shown). However, silencing of FOXD1 in 22Rv1 cells

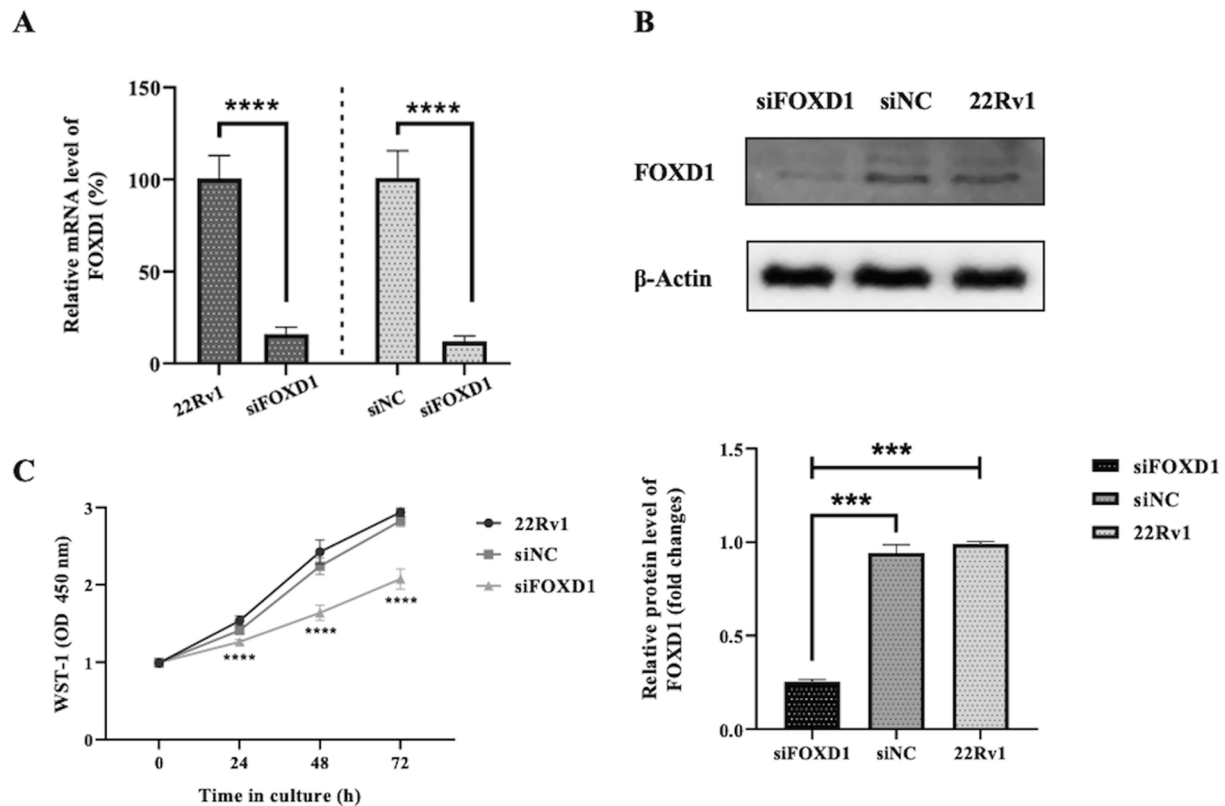


Fig. 2. Silencing of FOXD1 inhibited the proliferation of 22Rv1 cells. (A) 22Rv1 cells were transfected with siFOX1 and siNC for 24 h, respectively. The transfection efficiency was confirmed using qRT-PCR. (B) 22Rv1 cells were transfected with siFOX1 and siNC for 48 h, respectively. The transfection efficiency was confirmed using Western blot assay. (C) Cell proliferation was evaluated using the WST-1 assay. A statistically significant difference was found when the siFOX1 group was compared with the siNC and 22Rv1 control cells. Data are represented as mean SD. *** $p < 0.001$, **** $p < 0.0001$.

caused a statistically significant decrease (40%) in the N-cadherin protein expression level ($p < 0.001$) (Fig. 6).

3.5. Silencing of FOXD1 suppressed the activation of Wnt/ β -catenin signaling pathway in 22Rv1 cells

We also investigated whether silencing of FOXD1 in 22Rv1 cells had an effect on the expression of a number of proteins (β -catenin, c-myc and cyclin D1) involved in Wnt/ β -catenin pathway. Western blot analysis results showed that silencing of FOXD1 in 22Rv1 cells down-regulated the protein expression levels of β -catenin by 20% ($p < 0.001$) and those of cyclin D1 by 15% ($p < 0.01$), in comparison to the siNC and 22Rv1 groups (Fig. 6). However, c-myc expression level was not affected by the silencing of FOXD1 (data not shown).

4. Discussion

In the present study, we found that FOXD1 was highly expressed in human prostate cancer cell lines at both mRNA and protein levels. Furthermore, silencing of FOXD1 significantly decreased the cell proliferation, migration and invasion of 22Rv1 prostate cancer cells *in vitro*. Lastly, silencing of FOXD1 down-regulated the protein levels of N-cadherin, β -catenin and cyclin D1 in 22Rv1 cells.

So far, multiple studies have suggested that FOXD1 was aberrantly expressed in various cancers and that abnormal expression of FOXD1 may be linked to the progression of many types of cancers, and that this connection may be via different signaling pathways. For example, Zhao et al. (2015) reported that the mRNA and protein levels of FOXD1 in breast cancer samples were greatly higher than the adjacent normal tissues. They also stated that depletion of FOXD1 expression decreased the ability of cell proliferation of MDA-MB-231 cells and that FOXD1

promoted chemotherapeutic drug resistance by targeting p27. Gao and colleagues (2017) showed that FOXD1 expression was found to be significantly higher in glioma tissues compared to normal brain tissues. It has also been reported that silencing of FOXD1 has significantly reduced cell proliferation and migration in U251 and U87 glioma cells (Gao et al., 2017). Expression analyses of non-small cell lung cancer (NSCLC) specimens and lung cancer cell lines have shown that expression of FOXD1 was elevated in NSCLC specimens and cells. Knockdown of FOXD1 in lung cancer cell lines inhibited cell proliferation, migration and invasion (Li et al., 2018). In addition, patients with high levels of FOXD1 mRNA expression have been shown to have a poor prognosis (Nakayama et al., 2015). Other studies have demonstrated that in colorectal cancer (CRC), FOXD1 expression was significantly upregulated and knockdown of FOXD1 suppressed cell proliferation, migration and invasion by reversing the activation of polo-like kinase 2 (Plk2) expression (Han et al., 2018) and/or ERK 1/2 pathway (Pan et al., 2018). Han et al. (2018) also showed that high FOXD1 level was associated with tumor size (≥ 5 cm), TNM tumor stage (III + IV) and poor prognosis of CRC patients. It was revealed that FOXD1 expression in oral squamous cell carcinoma (OSCC) samples were significantly higher compared to normal samples (Chen et al., 2021). Knockdown of FOXD1 expression decreased cell proliferation, migration and invasion in OSCC cell lines by inhibiting the transcriptional activation of SNAI2. Consistent with the above-mentioned findings, we found that FOXD1 was highly expressed in prostate cancer cell lines, and silencing of FOXD1 significantly decreased proliferation, migration and invasion of 22Rv1 cells. Our results suggest that FOXD1 may be an oncogene in the development and progression of prostate cancer.

EMT is thought to be a critical process during the morphogenesis of multicellular organisms, but also one of the key processes involved in tumorigenesis, invasion and metastasis (Thiery, 2002, Thiery et al.,

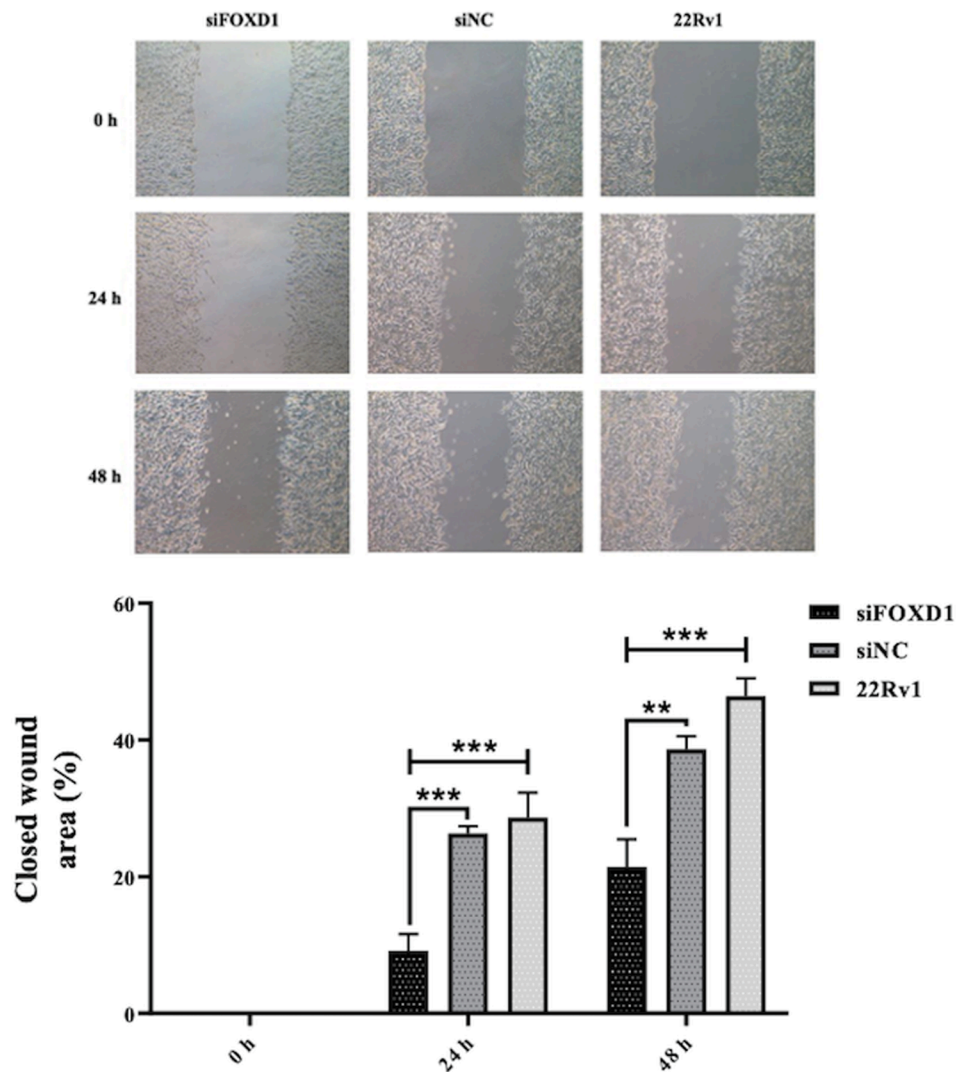


Fig. 3. Silencing of FOXD1 decreased migration of 22Rv1 cells. Cell migration was assessed by wound healing assay. Results from a representative experiment are shown. Data are represented as mean SD. ** $p < 0.01$, *** $p < 0.001$ compared with siNC and 22Rv1 control cells.

2009). The present study demonstrates that silencing of FOXD1 significantly decreases 22Rv1 prostate cancer cell migration and invasion. Moreover, silencing of FOXD1 decreases expression of EMT related protein N-cadherin, while no effects were observed in E-cadherin, Vimentin and Snail protein levels. Chen et al. (2021) found that FOXD1 overexpression dramatically reduced E-cadherin expression and increased N-cadherin, and Vimentin expression at both the protein and RNA levels in OSCC lines. Conversely, we showed in 22Rv1 cells -the cell line that overexpresses FOXD1- that silencing of FOXD1 had no effects on the expression of E-cadherin and Vimentin, but it caused a significant decrease in N-cadherin expression. These converse findings may have emerged due to the different etiopathogenesis mechanism of different cancer types due to different cell lines, as well as the fact that the expression between E-cadherin and N-cadherin will not always be inversely proportional. Loss of E-cadherin expression during EMT is generally thought to promote metastasis by allowing differentiation and invasion of cancer cells (Hanahan and Weinberg, 2011, Valastyan and Weinberg, 2011). However, some studies have reported that many metastatic tissues may have high levels of E-cadherin expression and that epithelial cells expressing E-cadherin can become invasive and metastasize in cancers without undergoing EMT (Bukholm et al., 2000, Lou et al., 2008, Hollestelle et al., 2013, Querzoli, et al., 2010). In addition, it has been reported that E-cadherin plays important roles in

the dissemination, intravasation and survival of cancer cells (Shamir et al. 2014, Rodriguez et al., 2012). In a previous study (Konac et al. 2017), we analyzed the expression levels of some EMT-related proteins in metastatic castration-resistant prostate cancer patients (mCRPC), benign prostatic hyperplasia (BPH) patients and healthy controls. There were no statistically significant differences, except for periostin, among EMT-related protein levels of patients who were classified according to Gleason scores and serum PSA levels. The significant increase in periostin expression in only the bone metastasis group may indicate that this protein is important in the adhesion of the tumor cell to the bone surface. According to the overall evaluation of the above findings, it seems like the metastasis-associated EMT process cannot be predicted using the expression pattern of a traditional EMT marker such as E-cadherin. It has been reported that in E-cadherin-positive cancer cell lines, overexpression of N-cadherin made cells more invasive both *in vitro* and *in vivo*, but there was no parallel reduction in E-cadherin expression (Nieman et al., 1999, Hazan et al., 2000). Results from these reports demonstrated that N-cadherin may promote cancer metastasis even in the presence of E-cadherin (Hazan et al., 2004). Therefore, our results suggest that the decrease in N-cadherin expression may have contributed to the decrease in the migration and invasion capacities of 22Rv1 cells. We showed that other than EMT, which only contributes through N-cadherin in the migration and invasion capacity of 22Rv1 cells, two

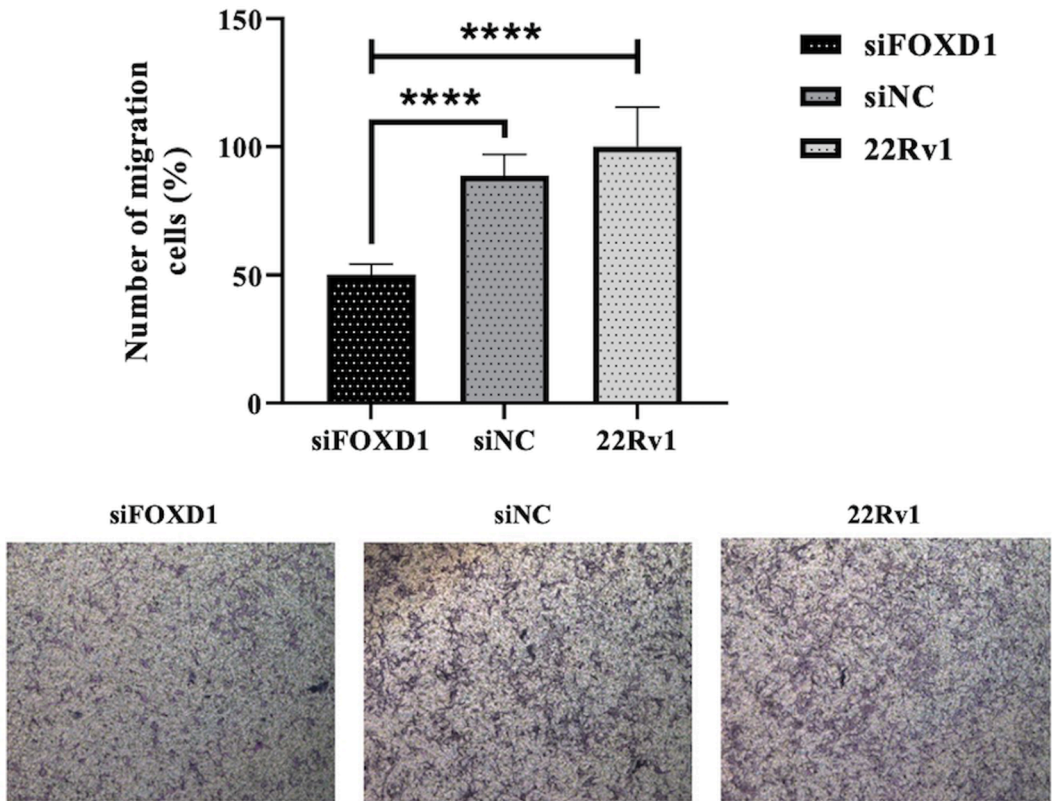


Fig. 4. Silencing of FOXD1 decreased migration of 22Rv1 cells. Cell migration was evaluated by transwell migration assay. Results from a representative experiment are shown. Data are represented as mean SD. **** $p < 0.0001$ compared with siNC and 22Rv1 control cells.

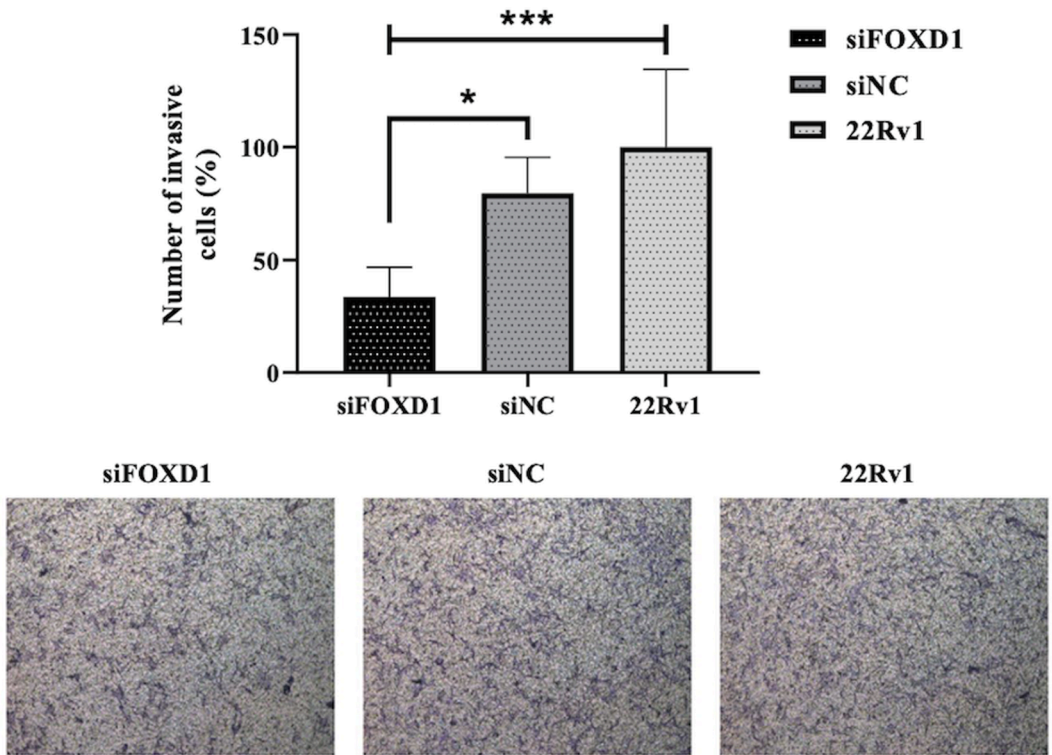


Fig. 5. Silencing of FOXD1 decreased invasion of 22Rv1 cells. Cell invasion was detected by transwell invasion assay. Results from a representative experiment are shown. Data are represented as mean SD. * $p < 0.05$, *** $p < 0.001$ compared with siNC and 22Rv1 control cells.

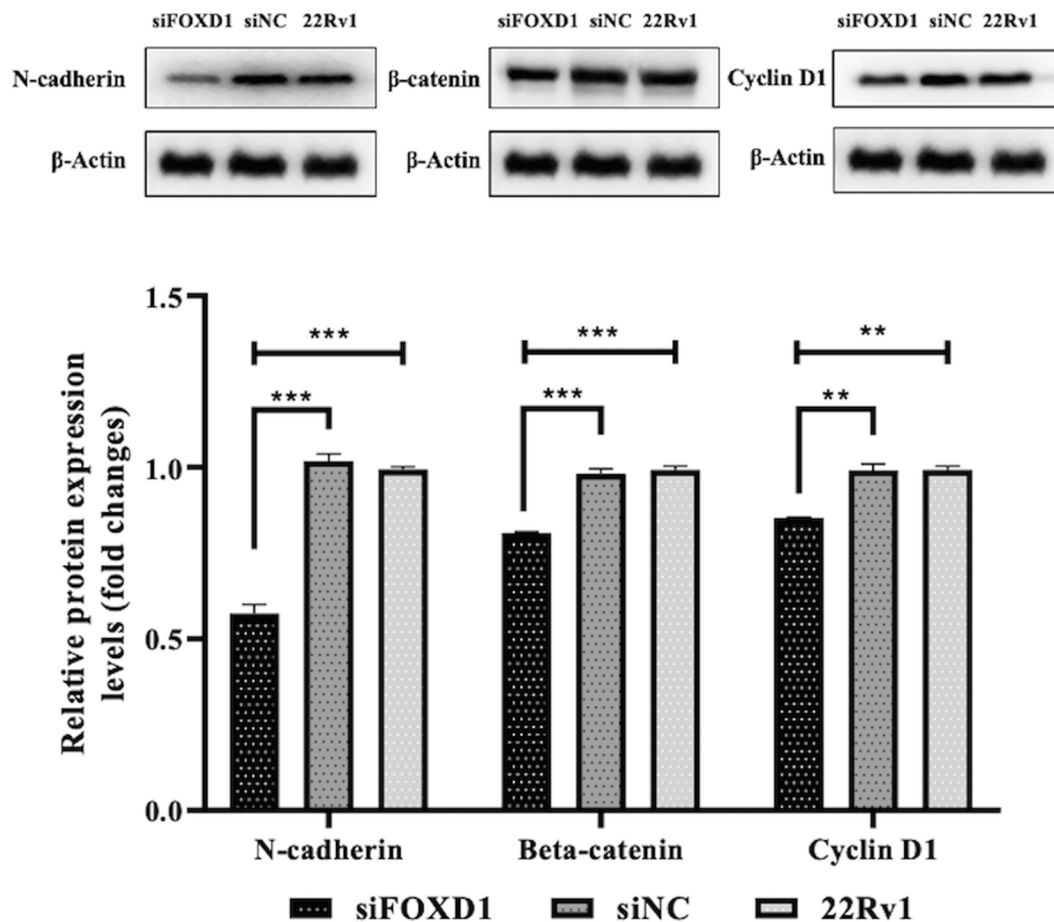


Fig. 6. Silencing of FOXD1 suppressed the activation of Wnt/ β -catenin signaling pathway in 22Rv1 cells. 22Rv1 cells were transfected with siFOXDX1 and siNC for 48 h. The protein expressions of N-cadherin, β -catenin, and cyclin D1 were detected using Western blot. The intensity of N-cadherin, β -catenin, and cyclin D1 bands were normalized to the intensity of their respective β -actin bands and quantified against each other. Data are represented as mean SD. ** $p < 0.01$, *** $p < 0.001$ compared with siNC and 22Rv1 control cells.

other genes that made a statistically significant contribution were β -catenin and cyclin D1. One mechanism involves the interaction of N-cadherin with β -catenin at the cell membrane, resulting in β -catenin sequestration, reduction of the cytosolic β -catenin pool and inhibition of Wnt signaling. Briefly, N-cadherin can modulate the β -catenin pool (Marie and Haÿ, 2013). We concluded that the silencing of FOXD1 caused a decrease in the expressions of N-cadherin and β -catenin, and this supports the aforementioned relationship between them.

Accumulating evidence indicates that many family members of the FOX transcription factors act as activators or inhibitors of Wnt in cancer cells (Moparthy et al., 2019; Koch, 2021). In doing so, they act in part by fine-tuning the Wnt signaling output in a tissue-specific and context-dependent manner. That is, FOX proteins that act as activators of the Wnt pathway generally promote tumorigenesis, while FOX proteins that act as inhibitors of this pathway are recognized as tumor suppressors (Koch, 2021). In the present study, we observed in 22Rv1 cells that silencing FOXD1 significantly reduced protein expression levels of β -catenin and cyclin D1 both of which are well-known Wnt target genes. However, we did not observe changes in c-myc expression between groups. A molecule that affects the subcellular localization or protein level of β -catenin directly regulates the Wnt transcriptional response (Nusse and Clevers, 2017). Therefore, we can say that FOXD1 is an activator that modulates β -catenin and there is a direct correlation between them. Our findings suggest that FOXD1 may regulate proliferation, migration and invasion of 22Rv1 cells, at least in part, via suppressing related proteins- β -catenin and cyclin D1- involved in the Wnt/ β -catenin signaling pathway.

Many of the studies in the context of carcinoma-associated EMT have causally associated increased cell migration and invasion behavior with EMT. However, conflicting findings have been reported as to whether a cell undergoing EMT would always cause an increase in migration and invasion behavior. Schaeffer and colleagues (2014) investigated whether the changes in migration and invasion are always positively associated with EMT, and reported that enhanced migration was not a phenotypic requirement of EMT (Schaeffer et al., 2014). In another study, Kang and colleagues (2016) observed that silencing of PSCA in DU145 prostate cancer cell reduced the metastatic potentials of DU145 cells and simultaneously induced EMT in DU145. Therefore, they reported that the decreased metastatic potential was independent of EMT (Kang et al., 2016). In this study, we observed that silencing of FOXD1 caused a significant decrease in the proliferation, migration and invasion abilities of 22Rv1 cells. However, not all EMT markers used in this study presented changes, so the decrease in the migration and invasion abilities of 22Rv1 cells were not solely dependent on EMT. Silencing of FOXD1 caused downregulation of N-cadherin, β -catenin and cyclin D1, pointing to an EMT-dependent process. On the other hand, silencing of FOXD1 did not affect the expression of E-cadherin, Vimentin, Snail, c-myc, suggesting that a second pathway independent of EMT may have contributed to the aggressive phenotype such as migration and invasion. Finally, since β -catenin and cyclin D1 are active in the Wnt pathway and N-cadherin can modulate β -catenin, we underlined the importance of further studies on the FOXD1 gene in siRNA-based therapeutics in PCa cells.

In conclusion, our results suggest that FOXD1 has the potential to

offer a novel target in context of gene-targeted prostate cancer therapies and to serve as a regulator of cell behavior. To our knowledge, this study is the first to provide evidence that silencing of FOXD1 reduced metastatic potentials of the 22Rv1 prostate cancer cells. However, whether FOXD1 participates in regulating EMT and stemness in metastatic PCa remains unknown. It should therefore be a major goal in preclinical siRNA-based therapeutics in PCa research to identify context-dependent pathway regulators, such as FOXD1, that can be safely targeted in human diseases. Understanding the mechanisms by which regulation of FOXD1 expression occurs may emerge as an independent prognostic factor and an innovative treatment strategy in terms of siRNA-based therapeutics, for patients with aggressive PCa.

CRedit authorship contribution statement

Cigdem Donmez: Conceptualization, Investigation, Methodology, Formal analysis. **Ece Konac:** Project administration, Conceptualization, Investigation, Methodology, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ece Konac reports financial support was provided by Gazi University. Ece Konac reports financial support was provided by Council of Higher Education of the Republic of Turkey. Ece Konac (Corresponding Author) is an Associate Editor in the GENE Journal.

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