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Tumour-suppressive *microRNA-24-1* inhibits cancer cell proliferation through targeting *FOXM1* in bladder cancer

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ABSTRACT

Here, we found that *microRNA-24-1* (*miR-24-1*) is significantly reduced in bladder cancer (BC) tissues, suggesting that it functions as a tumour suppressor. Restoration of mature *miR-24-1* inhibits cancer cell proliferation and induces apoptosis. Forkhead box protein M1 (*FOXM1*) is a direct target gene of *miR-24-1*, as shown by genome-wide gene expression analysis and luciferase reporter assay. Overexpressed *FOXM1* is confirmed in BC clinical specimens, and silencing of *FOXM1* induces apoptosis in cancer cell lines. Our data demonstrate that the *miR-24-1–FOXM1* axis contributes to cancer cell proliferation in BC, and elucidation of downstream signalling will provide new insights into the molecular mechanisms of BC oncogenesis.

1. Introduction

Bladder cancer (BC) is the fifth most common tumour in developed countries [1]. BCs are classified into 'non-muscle-invasive tumours' and 'muscle-invasive tumours'. The five-year survival rate for patients with muscle-invasive tumours is about 60%, even though that is close to 90% for patients with non-muscle-invasive BC (NMIBC) [2]. Patients with NMIBC tend to have a high rate of recurrence, and some cases become muscle invasive at recurrence [3]. Recently, no effective chemotherapeutics have been found for advanced BC by clinical trials [4,5]; therefore, it is desired that novel prognostic markers and effective treatments based on RNA network analyses.

The discovery of non-coding RNAs (ncRNAs) was an incredible breakthrough in the post-genome era [6]. MicroRNAs (miRNAs) are endogenous small ncRNAs (19–22 base pairs in length) repressing protein-coding genes by binding their 3 prime untranslated region (UTR) [7]. Numerous studies have shown that miRNAs are aberrantly expressed in various human malignancies and that they have important roles in the initiation, development, and metastasis of those malignancies [8–11]. Moreover, normal regulations can be destroyed by tumour-suppressive or oncogenic miRNAs in malignancies [12]. Therefore, detection of aberrantly expressed miRNAs is an important first step to elucidate miRNA-mediated oncogenic pathways.

Recently, miRNA expression studies of human malignancies revealed that *microRNA-24-1* (*miR-24-1*) was aberrantly expressed in several types of cancer tissues [13–19]. However, the functional role of *miR-24-1* in BC is unclear. The purpose of this study was to investigate the functional significance of *miR-24-1* and to identify the target genes regulated by the miRNA in BC. We found that restoration of mature *miR-24-1* inhibited cancer cell proliferation. In silico database analysis showed that forkhead box protein M1 (*FOXM1*) was a potential target gene of *miR-24-1*. *FOXM1* has been reported to modulate drug sensitivity and resistance in various tumour types [20]. Elucidating the cancer pathways and targets regulated by the tumour-suppressive *miR-24-1–FOXM1* axis will extend new insights of BC oncogenesis.

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2. Materials and methods

2.1. Clinical specimens and cell culture

The tissue specimens for quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) were collected from patients with BC ($n = 46$) who had received cystectomy or transurethral resection of bladder tumour (TUR-BT) at Kagoshima University Hospital between 2003 and 2013. Normal bladder epithelia ($n = 16$) were derived from patients with non-cancerous disease. The specimens were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer tumour-node-metastasis (TNM) classification and histologically graded [21]. Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were obtained from all patients. The patients' backgrounds and clinicopathological characteristics are summarised in Table 1.

We used two human BC cell lines: BOY, which was established in Kagoshima University [22] from an Asian male patient (age 66 years) diagnosed with stage III BC with lung metastasis; and T24, an invasive BC cell line obtained from the American Type Culture Collection. Both cell lines were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

2.2. Tissue collection and RNA extraction

Tissues were immersed in RNAlater (Ambion, Austin, TX, USA) and stored at −20 °C until RNA extraction. Total RNA, including miRNA, was extracted using the mirVana™ miRNA isolation kit (Ambion) following the manufacturer's protocol.

2.3. Quantitative real-time RT-PCR (qRT-PCR)

TaqMan probes and primers for *FOXM1* (P/N: Hs01073586_m1; Applied Biosystems, Foster City, CA, USA) were assay-on-demand gene expression products. We followed the manufacturer's protocol for PCR conditions. Stem-loop RT-PCR (TaqMan MicroRNA Assays; P/N: 000402 for *miR-24-1*; Applied Biosystems) was used to quantify miRNAs according to previously published conditions [23]. To normalise the data for quantification of *FOXM1* mRNA and the miRNA, we used human *GUSB* (P/N: Hs99999908_m1; Applied Biosystems) and *RNU48* (P/N: 001006; Applied Biosystems), respectively, and the $\Delta\Delta Ct$ method was employed to calculate the fold change.

Table 1
Characteristic of patients.

Bladder cancer (BC)		
Total number		46
Median age (range), years		70.5 (47–91)
Gender		
	Male	37
	Female	9
Stage		
	pTa	4
	pT1	10
	pT2	14
	pT3	6
	pT4	3
	Unknown	9
Grade		
	Low	14
	High	29
	Unknown	3
Operation		
	TUR-BT	30
	Total cystectomy	16
Normal bladder epithelium (NBE)		
Total number		16

2.4. Transfection with mature miRNA and small interfering RNA (siRNA)

As described elsewhere [24], the BC cell lines were transfected with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) with 10 nM mature miRNA. Pre-miR and negative-control miRNA (Applied Biosystems) were used in gain-of-function experiments, whereas *FOXM1* siRNA (cat No. HSS103713; Invitrogen) and negative-control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA, USA) were used in loss-of-function experiments. Cells were seeded in six-well plates for protein extraction (2×10^5 cells per well) and apoptosis (1×10^5 cells per well), in 24-well plates for well), and in 96-well plates for XTT assays (3×10^3 cells per well).

2.5. Cell proliferation assays

Cell proliferation was determined using XTT assays performed according to the manufacturer's instructions (Roche Applied Sciences, Tokyo, Japan), as described previously [25].

2.6. Flow cytometry

BC cell lines were transiently transfected with transfection reagent only (mock), miR-control, *miR-24-1*, si-control, or si-*FOXM1* in 6-well tissue culture plates, as described earlier [25]. Cells were harvested by trypsinization 72 h after transfection and washed in cold phosphate-buffered saline (PBS). For apoptosis assay, double staining with FITC-Annexin V and propidium iodide (PI) was carried out using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer's recommendations and immediately analysed within 1 h by flow cytometry (CyAn ADP analyzer; Beckman Coulter, Brea, CA, USA). Cells were sorted into viable cells, dead cells, early apoptotic cells, and apoptotic cells using Summit 4.3 software (Beckman Coulter), and the percentages of early apoptotic and apoptotic cells from each experiment were then compared.

Cells for the cell cycle analysis were stained with PI using the Cycletest™ PLUS DNA Reagent Kit (BD Biosciences) following the protocol and analysed by CyAn ADP analyzer. The percentages of the cells in the G1, S and G2/M phase were counted and compared.

Experiments were performed in triplicate.

2.7. Western blot analysis

Cells were harvested 72 h after transfection, and lysates were prepared. Thirty micrograms of protein were separated by NuPAGE on 4–12% bis–tris gels (Invitrogen) and transferred to PVDF membranes. Immunoblotting was carried out with diluted (1:1000) anti-FOXM1 antibodies (#5436; Cell Signaling, Danvers, MA, USA), anti-PARP antibodies (#9542; Cell Signaling), anti-Cleaved PARP antibodies (#5625; Cell Signaling) and anti-GAPDH antibodies (MAB374; Chemicon, Temecula, CA, USA). Specific complexes were visualised with an echochemiluminescence (ECL) detection system (GE Healthcare, Little Chalfont, UK).

2.8. Plasmid construction and dual-luciferase reporter assays

Partial wild-type sequences of the *FOXM1* 3′ untranslated region (UTR) or those with a deleted *miR-24-1* target site (positions

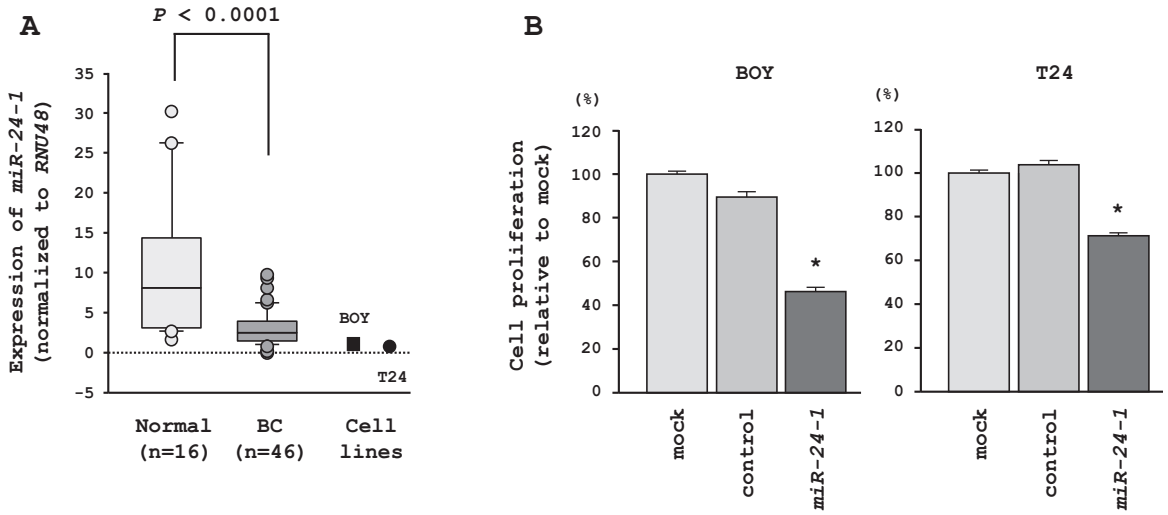

Fig. 1. Expression levels of *miR-24-1* in BC clinical specimens and cell lines and effects of restoring *miR-24-1* on cell proliferation. (A) *miR-24-1* expression levels in BC clinical specimens (BC and normal bladder epithelia) and BC cell lines (BOY and T24) were measured by qRT-PCR. Data were normalized to the expression of the *RNU48* gene. (B) Cell proliferation was determined by XTT assay 72 h after transfection with 10 nM *miR-24-1*. $^{*}P < 0.05$.

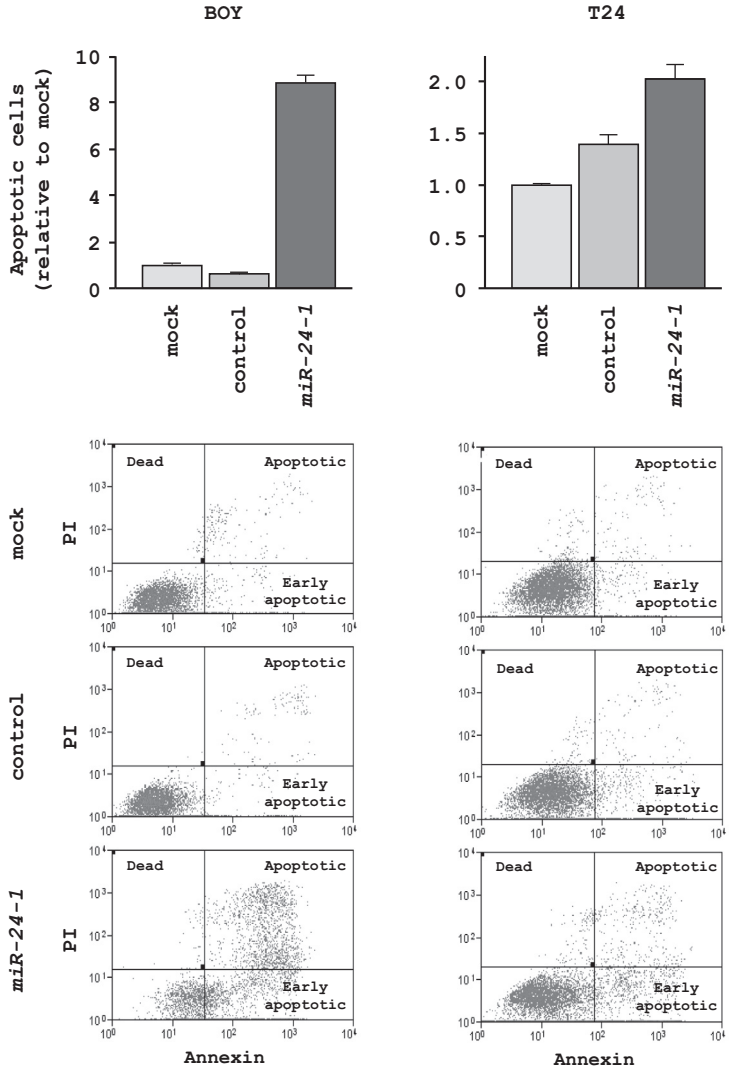

Fig. 2. Effects of *miR-24-1* transfection on apoptosis in BC cell lines. Ratio of the apoptotic cell fractions (early apoptotic + apoptotic cells) in *miR-24-1* transfectants in comparison with mock and miR-control transfectants. Data for the apoptotic cell fractions were expressed as the relative value of the average expression of the mock transfectant.

908–914 of the *FOXM1* 3′UTR) were inserted between the XhoI and PmeI restriction sites in the 3′UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The synthesised DNA was cloned into the psiCHECK-2 vector. T24 cells were transfected with 15 ng of the vector and 10 nM *miR-24-1* using Lipofectamine 2000 (Invitrogen). The activities of firefly and renilla luciferases in cell lysates were determined with a dual-luciferase assay system (E1960; Promega). Normalised data were calculated as the ratio of renilla/firefly luciferase activities.

2.9. Statistical analysis

Relationships between 2 or 3 variables and numerical values were analysed using the Mann–Whitney *U* test or Bonferroni-adjusted Mann–Whitney *U* test. Expert StatView software, version 4, was used in these analyses.

3. Results

3.1. miR-24-1 expression in BC specimens

The expression level of *miR-24-1* was evaluated in normal bladder epithelia ($n = 16$) and BC tissues ($n = 46$). *miR-24-1* expression was significantly lower in tumours than in non-cancerous tissues ($P < 0.0001$; Fig. 1A). We could not find significant relationship between any of the clinicopathological parameters (i.e., tumour stage or grade) and *miR-24-1* expression (data not shown). In BC cell lines (BOY and T24) also exhibited low expression of *miR-24-1* compared to normal bladder epithelia (Fig. 1A).

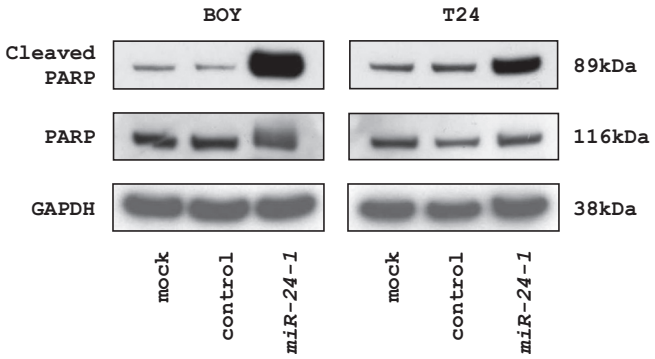

Fig. 3. Western blot analysis for apoptotic markers in BC cell lines. Protein expression of cleaved PARP and PARP was evaluated by Western blotting 72 h after transfection with *miR-24-1*. GAPDH was used as a loading control.

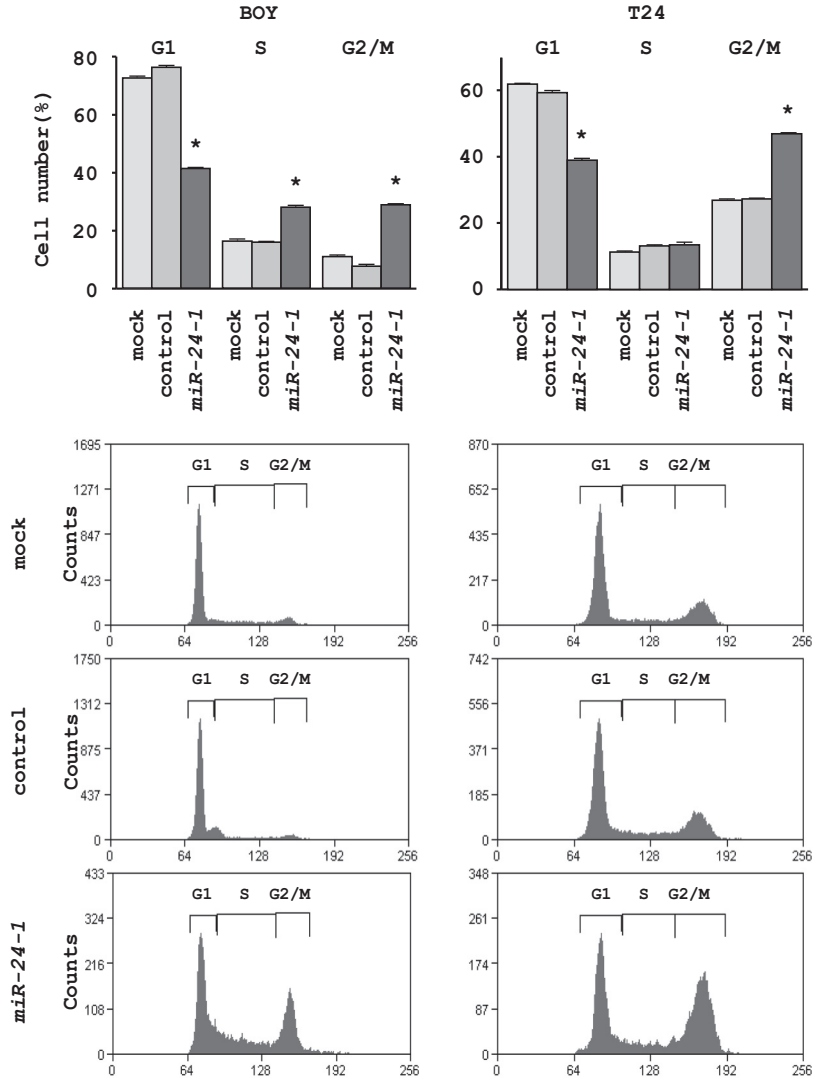

Fig. 4. Effects of *miR-24-1* transfection on cell cycle in BC cell lines. Upper: the bar charts represent the percentage of the cells in the G1, S, or G2/M phase. $^{*}P < 0.05$. Lower: typical results of cell cycle analysis of mock, miR-control, or *miR-24-1* transfectants.

3.2. Effects of miR-24-1 transfection on cell proliferation in BC cell lines (BOY and T24)

In order to investigate *miR-24-1* function, gain-of-function studies were performed by using BOY and T24 cell lines transfected with the miRNA. XTT assays demonstrated that significant inhibition of cell proliferation was found in *miR-24-1* transfectants in comparison with mock or miR-control transfectants (Fig. 1B).

3.3. Effects of miR-24-1 transfection on apoptosis and cell cycle in BC cell lines

Because *miR-24-1 transfection* inhibited cell proliferation in BC cell lines, we hypothesised that *miR-24-1* induction may induce apoptosis and/or cell cycle arrest. Hence, we performed flow cytometry and analysed the numbers of apoptotic cells following restoration of *miR-24-1* expression. The apoptotic cell fractions (apoptotic and early apoptotic cells) were significantly larger in *miR-24-1* transfectants than in mock or miR-control transfectants (Fig. 2, upper panel). Fig. 2 (lower panel) representatively showed that the apoptotic and early apoptotic fractions (upper right and lower right in the quadrant images) were larger in *miR-24-1* transfectants than in mock transfectants or the control. The induction of apoptosis by *miR-24-1* was confirmed by Western blotting. As shown in Fig. 3, the protein expression levels of Cleaved PARP was significantly increased in *miR-24-1* transfectants compared with the counter parts. In terms of the cell cycle distribution, number of cells in the G2/M phase was significantly larger in *miR-24-1* transfectants than in mock or miR-control transfectants (Fig. 4).

3.4. Identification of candidate miR-24-1 target genes in BC cells

To identify genes targeted by *miR-24-1*, in silico analysis was performed by using the TargetScan program. We found 4321 genes having putative target sites for *miR-24-1* in their 3′UTRs. We examined gene expression signatures in Gene Expression Omnibus (GEO) database to evaluate upregulated genes in BC specimens. Among the 4321 genes, 2061 genes were significantly upregulated in 90 BCs compared to 6 normal bladder epithelia (accession numbers, GSE11783 and GSE31684). Among them, 42 genes were markedly upregulated (fold change >10) and were considered as promising candidate oncogenes regulated by *miR-24-1* in BC (Table 2). We spotlighted the *FOXM1* gene because it was the top in the list.

3.5. Direct regulation of FOXM1 by miR-24-1

Next, qRT-PCR and Western blot analysis were performed to confirm that restoration of *miR-24-1* resulted in FOXM1 downregulation in BOY and T24 cells. The expression levels of FOXM1

Table 2
Forty-two highly expressed genes putatively regulated by *miR-24-1* in clinical BC specimens (fold change >10).

Entrez Gene ID	Gene symbol	Expression	Fold change	Description	*P*-value
2305	FOXM1	up	94.05	Forkhead box M1	4.38E-05
4751	NEK2	up	71.54	NIMA-related kinase 2	4.38E-05
9928	KIF14	up	48.65	Kinesin family member 14	4.38E-05
1116	CHI3L1	up	46.52	Chitinase 3-like 1 (cartilage glycoprotein-39)	2.49E-04
6362	CCL18	up	42.41	Chemokine (C-C motif) ligand 18	7.80E-05
23397	NCAPH	up	32.80	Non-SMC condensin I complex, subunit H	4.98E-05
55355	HJURP	up	31.25	Holliday junction recognition protein	4.38E-05
54972	TMEM132A	up	27.55	Transmembrane protein 132A	4.38E-05
2069	EREG	up	26.91	Epiregulin	1.28E-04
993	CDC25A	up	22.25	Cell division cycle 25A	4.38E-05
6374	CXCL5	up	20.63	Chemokine (C-X-C motif) ligand5	5.67E-05
7849	PAX8	up	19.55	Paired box 8	4.67E-05
55784	MCTP2	up	19.17	Multiple C2 domains, transmembrane 2	5.67E-05
30820	KCNIP1	up	16.71	kV channel interacting protein 1	7.32E-05
4320	MMP11	up	16.40	Matrix metallopeptidase 11 (stromelysin 3)	4.38E-05
51514	DTL	up	16.22	Denticleless E3 ubiquitin protein ligase homolog (Drosophila)	4.38E-05
990	CDC6	up	15.25	Cell division cycle 6	4.38E-05
57863	CADM3	up	15.06	Cell adhesion molecule 3	4.98E-05
9982	FGFBP1	up	14.67	Fibroblast growth factor binding protein1	4.38E-05
140766	ADAMTS14	up	13.29	ADAM metallopeptidase with thrombospondin type 1 motif, 14	2.09E-04
56937	PMEPA1	up	13.08	Prostate transmembrane protein, androgen induced 1	2.35E-04
343035	RD3	up	12.79	Retinal degeneration 3	7.80E-05
23293	SMG6	up	12.75	Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)	4.67E-05
5676	PSG7	up	12.67	Pregnancy specific beta-1-glycoprotein 7 (gene/pseudogene)	6.87E-05
43	ACHE	up	12.25	Acetylcholinesterase	6.87E-05
3589	IL11	up	12.09	Interleukin 11	2.65E-04
90381	C15orf42	up	11.98	Chromosome 10 open reading frame, human C15orf42	1.54E-04
4680	CEACAM6	up	11.85	Carcinoembryonic antigen-related cell adhesion molecule 6	3.15E-04
157570	ESCO2	up	11.31	Establishment of sister chromatid cohesion N-acetyltransferase 2	1.21E-04
388228	SBK1	up	10.71	SH3-binding domain kinase 1	2.47E-03
1824	DSC2	up	10.63	Desmocollin2	4.99E-04
55771	PRR11	up	10.62	Proline rich 11	4.38E-05
6664	SOX11	up	10.56	SRY (sex determining region Y)-box 11	2.35E-04
4319	MMP10	up	10.56	Matrix metallopeptidase 10 (stromelysin 2)	3.54E-04
113730	KLHDC7B	up	10.51	Kelch domain containing 7B	8.31E-05
2538	G6PC	up	10.45	Glucose-6-phosphatase, catalytic subunit	6.05E-05
55723	ASF1B	up	10.43	Anti-silencing function 1B histone chaperone	4.38E-05
3832	KIF11	up	10.39	Kinesin family member 11	4.38E-05
85285	KRTAP4-1	up	10.28	Keratin associated protein 4-1	2.21E-04
8061	FOSL1	up	10.26	FOS-like antigen 1	1.45E-04
51397	COMMD10	up	10.25	COMM domain containing 10	7.32E-05
113235	SLC46A1	up	10.12	Solute carrier family 46 (folate transporter), member 1	4.38E-05

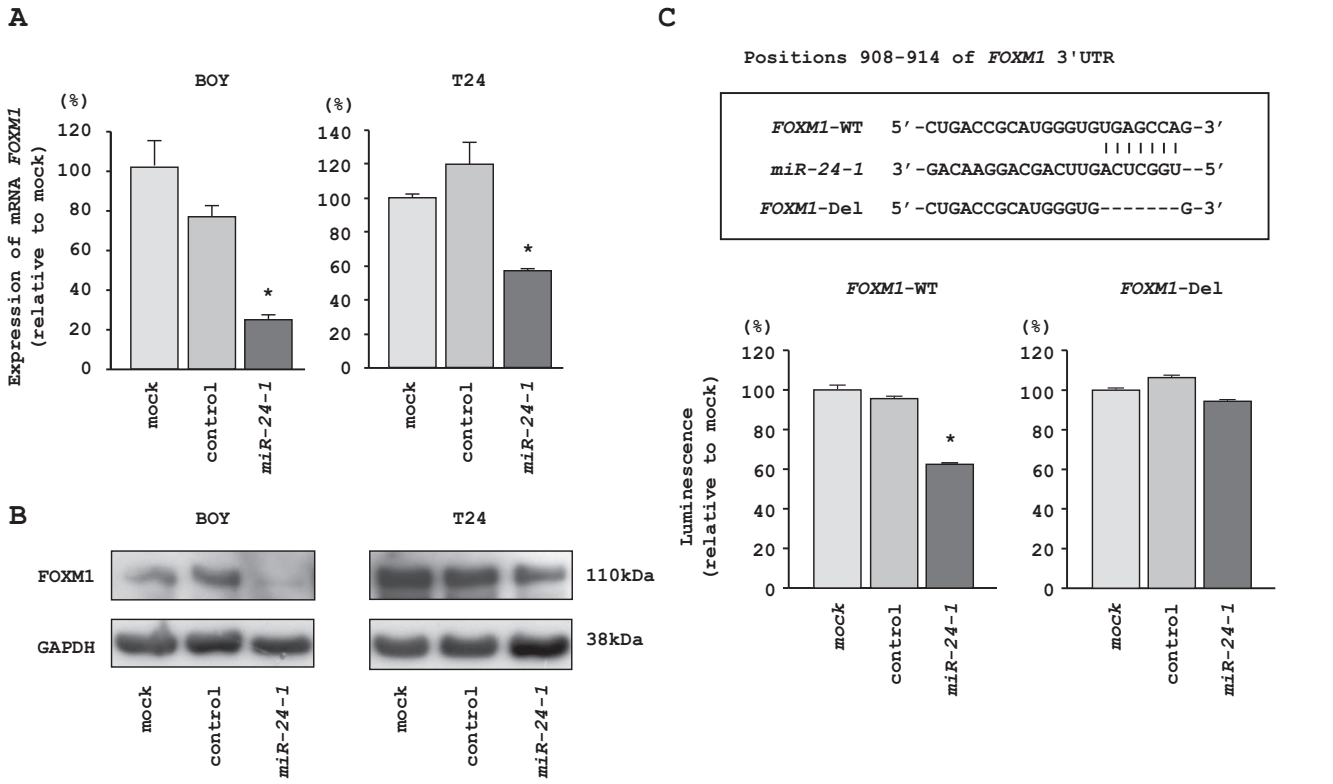

Fig. 5. Direct regulation of *FOXM1* by *miR-24-1*. (A) *FOXM1* mRNA expression was evaluated by qRT-PCR 72 h after transfection with *miR-24-1*. *GAPDH* was used as an internal control. (B) FOXM1 protein expression was evaluated by Western blotting 72 h after transfection with *miR-24-1*. GAPDH was used as a loading control. (C) *miR-24-1* binding sites in the 3′UTR of *FOXM1* mRNA. Luciferase reporter assay using a vector encoding putative *miR-24-1* target sites at positions 908–914 for both wild-type (WT) and deletion (Del) constructs. Renilla luciferase values were normalised to firefly luciferase values. $^{*}P < 0.05$.

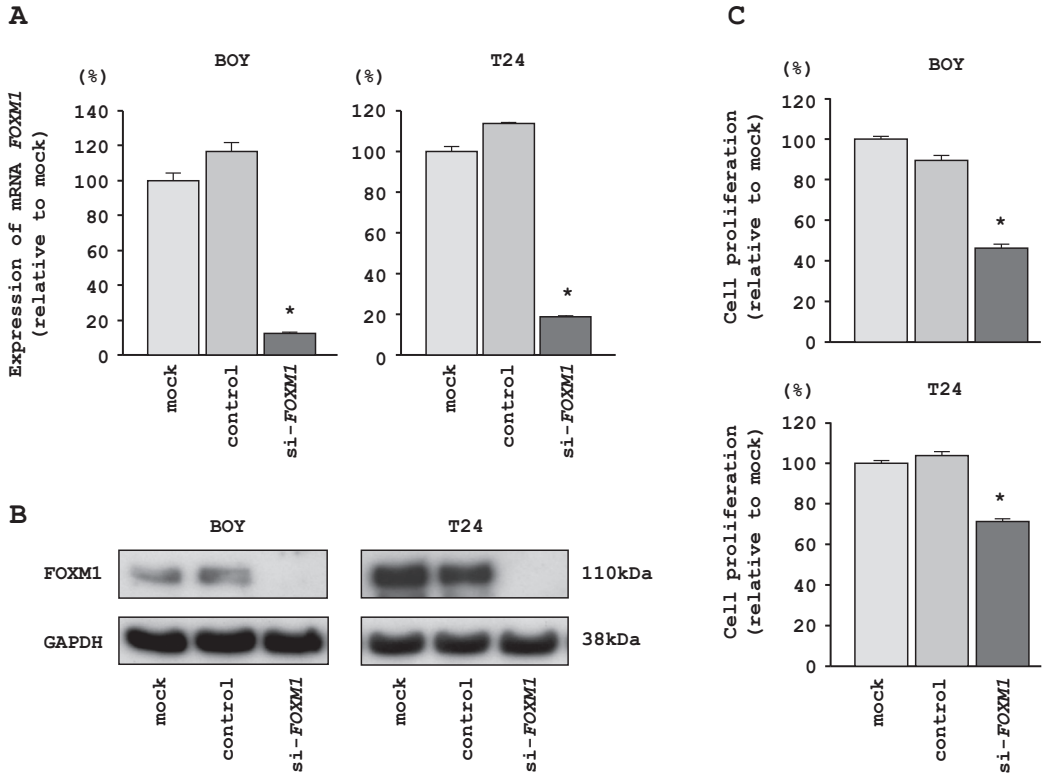

Fig. 6. Silencing of *FOXM1* mRNA and protein expression by si-*FOXM1* transfection in BC cells and effects of silencing *FOXM1* on cell proliferation. (A) *FOXM1* mRNA expression was measured 72 h after transfection with si-FOXM1. *GUSB* was used as an internal control. (B) FOXM1 protein expression was measured by Western blotting 72 h after transfection with si-FOXM1. GAPDH was used as a loading control. (C) Cell proliferation was measured by the XTT assay. $^{*}P < 0.05$.

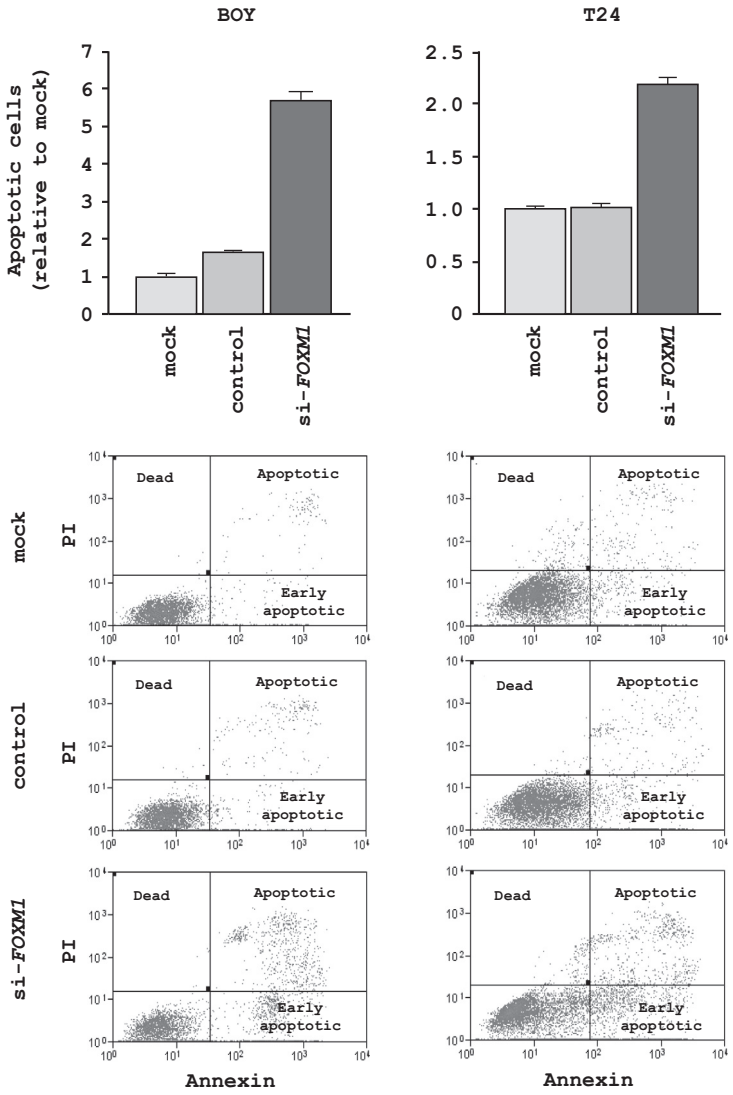

Fig. 7. Flow cytometry analysis of si-*FOXM1* transfection in BC cell lines. Apoptotic cells increased 72 h after transfection of cells with 10 nM si-FOXM1.

mRNA and protein were significantly suppressed in *miR-24-1* transfectants compared to mock or miR-control transfectants ($P < 0.005$, Fig. 5A and B).

We then carried out luciferase reporter assays in T24 cells in order to find out whether *FOXM1* was directly regulated by *miR-24-1*. The TargetScan database predicted that there is one binding site for *miR-24-1* in the 3′UTR of *FOXM1* (positions 908–914, Fig. 5C). We employed vectors encoding wild-type sequence of the 3′UTR of *FOXM1* mRNA in partial where the predicted *miR-24-1* target sites were located. We found that the luminescence intensity was significantly reduced by cotransfection with *miR-24-1* and the vector carrying the wild-type 3′UTR of *FOXM1*, whereas transfection with the deletion vector (positions 908–914 had been removed) blocked the decrease in luminescence ($P = 0.001$, Fig. 5C). These data suggested that *miR-24-1* bound directly to specific site in the 3′UTR of *FOXM1* mRNA.

3.6. Effects of FOXM1 knockdown on cell proliferation and apoptosis in BC cell lines

To investigate the functional role of *FOXM1*, loss-of-function studies were carried out by using si-*FOXM1* transfectants. First, the knockdown efficiency of si-*FOXM1* transfection was evaluated in BOY and T24 cells. Western blotting and qRT-PCR showed that si-*FOXM1* effectively downregulated FOXM1 expression in BOY and T24 cells (Fig. 6A and B). XTT assays showed that cell proliferation was inhibited in si-FOXM1 transfectants compared to mock or si-control transfectants in BOY and T24 cells (Fig. 6C).

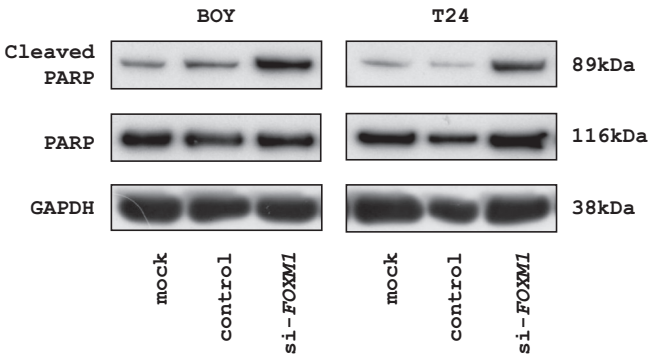

Fig. 8. Western blot analysis of apoptotic markers in BC cell lines. Protein expression of cleaved PARP and PARP was evaluated by Western blotting 72 h after transfection with si-*FOXM1*. GAPDH was used as a loading control.

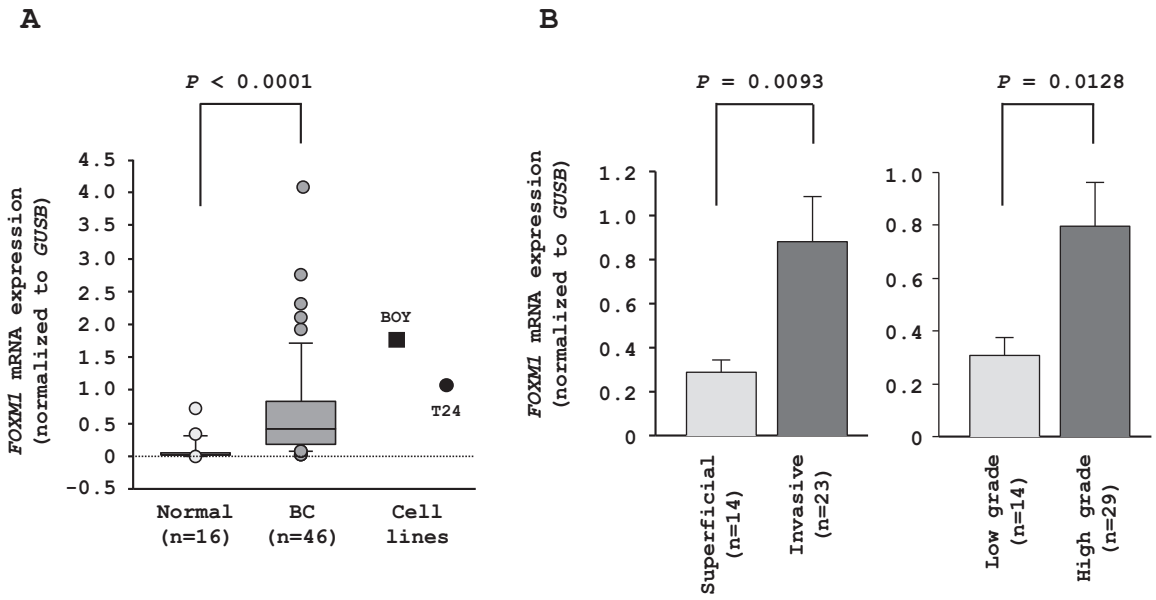

Fig. 9. mRNA expression of *FOXM1* in BC clinical specimens and cell lines and association of *FOXM1* expression with clinicopathological parameters. (A) *FOXM1* mRNA expression levels in BC clinical specimen (BC and normal bladder epithelia) and BC cell lines (BOY and T24) were determined by qRT-PCR. *GUSB* was used as an internal control. (B) *FOXM1* expression was significantly higher in patients with invasive BC compared to patients with superficial BC. *FOXM1* expression was significantly higher in patients with high-grade BC than in patients with low-grade BC.

The apoptosis assays using flow cytometry demonstrated that the ratio of apoptotic cell number in si-*FOXM1* transfectants was larger than those in mock and si-control transfectants 72 h after transfection (Fig. 7, upper panel). As shown in the representative quadrant images in Fig. 7 (lower panel), the apoptotic and early apoptotic fractions were greater in si-*FOXM1* transfectants than in mock transfectants or si-control. Therefore, transient transfection with si-*FOXM1* induced apoptosis in BC cell lines. To confirm the induction of apoptosis by *si-FOXM1*, We performed Western blotting. The protein expression levels of Cleaved PARP was significantly increased in *si-FOXM1* transfectants in comparison with mock or si-control transfectants (Fig. 8).

3.7. Expression of FOXM1 in BC clinical specimens

qRT-PCR analysis showed that *FOXM1* mRNA expression levels were significantly higher in 46 clinical BC specimens than in 16 non-BC specimens ($P < 0.0001$, Fig. 9A). To determine whether the levels of *FOXM1* mRNA in tumour tissues correlated with clinicopathological factors, we analysed *FOXM1* expression levels in human tumour samples. *FOXM1* expression increased from the superficial to invasive stage ($P = 0.0093$, Fig. 9B). *FOXM1* expression also increased from low-grade to high-grade tumours ($P = 0.0128$, Fig. 9B). These results suggested that *FOXM1* was significantly upregulated in BC and may be a putative oncogene in BC.

4. Discussion

Aberrant expression of miRNAs disrupts the tightly regulated RNA networks in cancer cells, and failure of these networks causes cancer development and metastasis [12]. Therefore, strategies to identify differentially expressed miRNAs and miRNA-regulated cancer pathways are being developed as a new direction in cancer research in the post-genome sequencing era. Therefore, in previous works, we have reported that tumour-suppressive miRNAs mediate novel molecular targets and pathways in several types of cancers [26–29]. In this study, we focused on *miR-24-1* because the functional role of *miR-24-1* was controversial in the past miRNA studies [13–19]. In the current study, *miR-24-1* was significantly downregulated in BC tissues compared with normal bladder epithelia. Our data showed that restoration of *miR-24-1* significantly inhibited cancer cell proliferation and induced apoptosis as well as cell cycle G2 arrest, suggesting its tumour-suppressive function in BC cells. The tumour-suppressive role of *miR-24-1* has been reported in previous studies of other cancer types, such as colorectal cancer, laryngeal squamous cell carcinoma, and prostate cancer [13–15].

Contrary to these reports, overexpression of *miR-24-1* has been observed in several types of cancers and has been shown to function as an oncogene [16–19]. In glioma cells, upregulation of *miR-24-1* is a common event; moreover, downregulation of *miR-24-1* inhibited glioma cell proliferation both in vitro and in vivo [30]. These data are contradictory to our present data in BC. It is difficult to explain the differences in the tumour-suppressive and oncogenic functions of *miR-24-1* in different cancer types. Elucidation of the molecular mechanisms of aberrant *miR-24-1* expression in each cancer type is necessary. *miR-24-1* might regulates different molecular targets and networks in different cancers, which may explain our contradictory results.

miRNAs have unique ability to regulate multiple protein-coding genes. Bioinformatic predictions suggest that miRNAs regulate 30–60% of the protein-coding genes in human [8]. Reduced expression of tumour-suppressive miRNAs may cause overexpression of oncogenes in cancer cells. To better understand BC oncogenesis, we identified *miR-24-1* target genes using in silico analysis. Recent miRNA studies in our laboratory have utilised this strategy to identify novel molecular targets and pathways regulated by tumour-suppressive miRNAs in several cancers, including BC [26–29].

In this study, we identified *FOXM1* as a target of tumour-suppressive *miR-24-1* and validated the direct binding of this miRNA to the 3′UTR using luciferase reporter assays. *FOXM1* is a member of the *FOX* family of transcription factors, and overexpression of *FOXM1* has been reported in many types of human cancers [31]. Our present data showed *FOXM1* was upregulated in BC

Table 3
Significantly enriched annotations (downstream genes of FOXM1), $P < 1.0E-03$.

Number of genes	Annotations	KEGG entry number	*P*-value	Genes
16	Focal adhesion	4510	2.16.E-09	*MET, ACTN1, ITGA6, PDGFC, ITGA3, CCND1, FLNA, SPP1, CAPN2, CAV1, ARHGAP5, AKT1, CAV3, PIK3CD, BCL2, PPP1CC*
9	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	5412	2.12.E-07	*ACTN1, ITGA6, ITGA3, SLC8A1, TCF7, DMD, LMNA, DAG1, GJA1*
13	Endocytosis	4144	6.11.E-07	*MET, ADRB2, F2R, TGFBR2, DNM2, EHD1, CAV1, EHD4, CAV3, RAB31, ARRB1, LDLR, AP2A1*
7	Colorectal cancer	5210	8.83.E-06	*BIRC5, TGFBR2, CCND1, TCF7, AKT1, PIK3CD, BCL2*
8	Apoptosis	4210	8.70.E-06	*PRKAR2B, FAS, CAPN2, IRAK1, IL1RAP, AKT1, PIK3CD, BCL2*
11	Chemokine signaling pathway	4062	1.54.E-05	*PREX1, CXCL2, CXCL3, GNB4, CXCL1, ADCY3, PLCB3, AKT1, PIK3CD, RASGRP2, ARRB1*
14	Pathways in cancer	5200	3.85.E-05	*MET, ITGA6, BIRC5, TGFBR2, ITGA3, CCND1, FAS, TCF7, AKT1, WNT5B, WNT10B, PIK3CD, BCL2, PPARG*
7	ECM-receptor interaction	4512	6.51.E-05	*SDC1, SDC4, ITGA6, ITGA3, SPP1, CD44, DAG1*
7	Dilated cardiomyopathy	5414	9.41.E-05	*ITGA6, ITGA3, SLC8A1, ADCY3, DMD, LMNA, DAG1*
7	Gap junction	4540	8.76.E-05	*PDGFC, TUBB2B, TUBB2A, ADCY3, PLCB3, TUBB6, GJA1*
7	Chagas disease (American trypanosomiasis)	5142	2.21.E-04	*TGFBR2, FAS, SERPINE1, IRAK1, PLCB3, AKT1, PIK3CD*
5	Endocrine and other factor-regulated calcium reabsorption	4961	2.85.E-04	*DNM2, SLC8A1, PLCB3, ATP1B3, AP2A1*
5	Pathogenic *Escherichia coli* infection	5130	4.51.E-04	*TUBB2B, TUBB2A, KRT18, TUBB6, ARHGEF2*
5	Inositol phosphate metabolism	562	5.80.E-04	*PI4KA, IMPA2, PLCB3, PIK3CD, PLCD3*
5	Acute myeloid leukemia	5221	5.80.E-04	*CCND1, TCF7, AKT1, PIK3CD, PIM1*
6	Salivary secretion	4970	5.35.E-04	*ADRB2, ATP2B4, KCNN4, ADCY3, PLCB3, ATP1B3*
6	Small cell lung cancer	5222	5.02.E-04	*ITGA6, ITGA3, CCND1, AKT1, PIK3CD, BCL2*
6	Prostate cancer	5215	6.43.E-04	*PDGFC, CCND1, TCF7, AKT1, PIK3CD, BCL2*
6	Hypertrophic cardiomyopathy (HCM)	5410	4.41.E-04	*ITGA6, ITGA3, SLC8A1, DMD, LMNA, DAG1*

Table 4
Upregulated genes involved in the annotations on Table 3.

Entrez Gene ID	Gene symbol	Expression	Fold change	Description	*P*-value
6696	SPP1	up	35.94	Secreted phosphoprotein 1	0.00105
5054	SERPINE1	up	23.16	Serpin peptidase inhibitor, clade E	0.00105
332	BIRC5	up	17.27	Baculoviral IAP repeat containing 5	0.00105
2919	CXCL1	up	12.82	Chemokine (C–X–C motif) ligand 1	0.00105
2921	CXCL3	up	12.26	Chemokine (C–X–C motif) ligand 3	0.00111
2920	CXCL2	up	7.83	Chemokine (C–X–C motif) ligand 2	0.00174
5331	PLCB3	up	3.46	Phospholipase C, beta 3	0.00105
5292	PIM1	up	3.22	Pim-1 oncogene	0.00195
3675	ITGA3	up	3.04	Integrin, alpha 3	0.01138
595	CCND1	up	2.53	Cyclin D1	0.01227
3654	IRAK1	up	2.40	Interleukin-1 receptor-associated kinase 1	0.00111
3949	LDLR	up	2.23	Low density lipoprotein receptor	0.01660
160	AP2A1	up	2.00	Adaptor-related protein complex 2, alpha 1 subunit	0.04582
207	AKT1	up	1.88	v-Akt murine thymoma viral oncogene homolog 1	0.00132
6385	SDC4	up	1.76	Syndecan 4	0.00908
10938	EHD1	up	1.56	EH-domain containing 1	0.00752
9181	ARHGEF2	up	1.46	Rho/Rac guanine nucleotide exchange factor (GEF) 2	0.00840
4000	LMNA	up	1.38	Lamin A/C	0.02865
1605	DAG1	up	1.32	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	0.04294

tissues, confirming previous studies. *FOXM1* is the master regulator of a member of genes that are critical for cell cycle progression, such as *Aurora B*, *Cyclin B1*, and *CDC25B* [32]. Interestingly, *FOXM1* also stimulates the expression of *SKP2* and *CKS1*, which are involved in the proteolysis of $p27^{kip1}$ and in G_1/S progression [33]. Our previous study of urothelial carcinoma showed that *SKP2* and *CKS1* expression correlated with tumour stage, grade, and progression [34]. Overexpression of *FOXM1* has been reported in many types of human cancers [35], and our present data in BC confirmed the results of these previous studies. Therefore, down-regulation of *miR-24-1* in BC cells causes overexpression the *FOXM1-SKP2/CSK1* axis and promotes aggressive cancer behaviour via regulation of $p27^{kip1}$ degradation.

Furthermore, we investigated the molecular mechanisms downstream of *miR-24-1–FOXM1* signalling using si-*FOXM1* transfectants (Table 3). Our results showed that *SPP1*, *SERPINE1*, *BIRC5*, and *CXCLs* were potential targets of *miR-24-1–FOXM1* signalling in BC cells (Table 4). Among these, our recent study showed that the tumour-suppressive *miR-195/497* cluster directly regulated *BIRC5* in BC cells [28]. *BIRC5* is an inhibitor of apoptosis (IAP) and is preferentially expressed in many malignancies, including BC [36]. Several studies have shown that *BIRC5* is directly regulated by *FOXM1* [37,38], indicating that the *miR-24-1–FOXM1-BIRC5* axis contributes substantially to BC oncogenesis.

5. Conclusions

miR-24-1 was frequently downregulated in clinical BC specimens and appeared to function as a tumour suppressor through regulating the oncogenic *FOXM1* transcription factor. Elucidating cancer pathways mediated through the tumour-suppressive *miR-24-1–FOXM1* axis should provide novel insights into oncogenic mechanisms in BC and new therapeutics for BC treatment.

Disclosure statement

All authors have no conflicts of interest.

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