Tumor-suppressive *microRNA-223* inhibits cancer cell migration and invasion by targeting *ITGA3/ITGB1* signaling in prostate cancer

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Analysis of microRNA (miRNA) expression signatures in prostate cancer (PCa) and castration-resistant PCa has revealed that *miRNA-223* is significantly downregulated in cancer tissues, suggesting that *miR-223* acts as a tumor-suppressive miRNA by targeting oncogenes. The aim of this study was to investigate the functional roles of *miR-223* and identify downstream oncogenic targets regulated by *miR-223* in PCa cells. Functional studies of *miR-223* were carried out to investigate cell proliferation, migration, and invasion using PC3 and PC3M PCa cell lines. Restoration of *miR-223* significantly inhibited cancer cell migration and invasion in PCa cells. *In silico* database and genome-wide gene expression analyses revealed that *ITGA3* and *ITGB1* were direct targets of *miR-223* regulation. Knockdown of *ITGA3* and *ITGB1* significantly inhibited cancer cell migration and invasion in PCa cells by regulating downstream signaling. Moreover, overexpression of ITGA3 and ITGB1 was observed in PCa clinical specimens. Thus, our data indicated that downregulation of *miR-223* enhanced *ITGA3/ITGB1* signaling and contributed to cancer cell migration and invasion in PCa cells. Elucidation of the molecular pathways modulated by tumor-suppressive miRNAs provides insights into the mechanisms of PCa progression and metastasis.

Prostate cancer (PCa) is the most common cause of cancer in men, accounting for approximately one-quarter of all cancer cases in men.[1] Multiple treatment options are available for localized PCa, and the 5-year survival rate is nearly 100%; however, effective curative treatments for advanced PCa have not been developed.[2] Skeletal metastasis is one of the most common and important features of advanced PCa.[3] Metastatic PCa is initially treated with androgen-deprivation therapy; however, the cancer gradually becomes resistant to first-line androgen-deprivation therapy and progresses to castration-resistant PCa (CRPC).[2] Progression of PCa to metastatic disease significantly affects the survival of men with PCa. Therefore, developing a deeper understanding of the molecular mechanisms of metastatic pathways underlying PCa metastasis using novel approaches will facilitate the development of novel treatment options for advanced PCa.

Normal regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic microRNAs (miRNAs) in cancer cells. Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating the details of miRNA-mediated oncogenic pathways. Based on this background, we have identified tumor-suppressive miRNAs and the molecular targets and pathways regulated by these miRNAs on the miRNA expression signatures of PCa and CRPC specimens.[4–11] Recent studies of several miRNA signatures in PCa showed that *miR-223* expression is frequently reduced in cancer tissues compared to that in normal

prostate tissues,[4,12,13] suggesting that *miR-223* acts as a tumor suppressor in PCa.

Integrins are cell surface receptors for ECM proteins, and integrin-mediated signaling plays a key role in cell survival, proliferation, migration, and invasion in normal and malignant cells.[14,15] Studies have shown that silencing of these genes significantly inhibits cell migration and invasion in cancer cells through targeting its downstream signaling.

The aim of the present study was to investigate the functional significance of *miR-223* and to identify the molecular targets and downstream signaling pathways regulated by *miR-223* in PCa cells. Our data showed that restoration of mature *miR-223* inhibited cancer cell migration and invasion. Moreover, gene expression data and *in silico* database analysis showed that the genes coding for integrin A3 (*ITGA3*) and integrin B1 (*ITGB1*) were potential targets of *miR-223* regulation. The discovery that tumor-suppressive *miR-223* regulated integrin genes provides important insights into the potential mechanisms of PCa metastasis and suggests novel therapeutic strategies for the treatment of PCa.

Materials and Methods

Clinical prostate specimens and cell culture. Seventeen patients with PCa who had undergone radical prostatectomy at Chiba University Hospital (Chiba, Japan) from 2009 to 2013 and 29 patients with elevated prostate-specific antigen (PSA) who

Table 1. Characteristics of patients with prostate cancer (PCa) who had undergone radical prostatectomy (*n* = 17) and patients with elevated prostate-specific antigen (PSA) who had undergone transrectal needle biopsy (non-PCa) (*n* = 29) and their paired normal samples

No.	Procedure	PCa or non-PCa	Age, years	PSA, ng/mL	Gleason score	Stage	cT	cN	cM
1	Prostatectomy	PCa	64	5.43	3+4	III	3a	0	0
2	Prostatectomy	PCa	68	12.81	3+5	III	3a	0	0
3	Prostatectomy	PCa	70	16.06	4+5	III	3b	0	0
4	Prostatectomy	PCa	69	25.79	4+5	II	2a	0	0
5	Prostatectomy	PCa	64	29.93	4+3	II	2b	0	0
6	Prostatectomy	PCa	61	7.85	3+4	III	3a	0	0
7	Prostatectomy	PCa	68	8.78	4+5	II	2b	0	0
8	Prostatectomy	PCa	66	6.13	4+3	II	2b	0	0
9	Prostatectomy	PCa	70	11.75	4+4	III	3b	0	0
10	Prostatectomy	PCa	60	22.10	3+4	II	2b	0	0
11	Prostatectomy	PCa	70	8.88	3+4	II	2a	0	0
12	Prostatectomy	PCa	72	4.48	3+4	II	2b	0	0
13	Prostatectomy	PCa	56	7.12	3+4	III	3a	0	0
14	Prostatectomy	PCa	65	13.08	4+3	II	2b	0	0
15	Prostatectomy	PCa	65	9.53	4+4	II	2b	0	0
16	Prostatectomy	PCa	65	5.80	4+3	II	2a	0	0
17	Prostatectomy	PCa	65	4.59	5+4	II	2b	0	0
18	Prostatectomy	Non-PCa	64	5.43	–	–	–	–	–
19	Prostatectomy	Non-PCa	68	12.81	–	–	–	–	–
20	Prostatectomy	Non-PCa	70	16.06	–	–	–	–	–
21	Prostatectomy	Non-PCa	69	25.79	–	–	–	–	–
22	Prostatectomy	Non-PCa	64	29.93	–	–	–	–	–
23	Prostatectomy	Non-PCa	61	7.85	–	–	–	–	–
24	Prostatectomy	Non-PCa	68	8.78	–	–	–	–	–
25	Prostatectomy	Non-PCa	66	6.13	–	–	–	–	–
26	Prostatectomy	Non-PCa	70	11.75	–	–	–	–	–
27	Prostatectomy	Non-PCa	60	22.10	–	–	–	–	–
28	Prostatectomy	Non-PCa	70	8.88	–	–	–	–	–
29	Prostatectomy	Non-PCa	72	4.48	–	–	–	–	–
30	Prostatectomy	Non-PCa	56	7.12	–	–	–	–	–
31	Prostatectomy	Non-PCa	65	13.08	–	–	–	–	–
32	Prostatectomy	Non-PCa	65	9.53	–	–	–	–	–
33	Prostatectomy	Non-PCa	65	5.80	–	–	–	–	–
34	Prostatectomy	Non-PCa	65	4.59	–	–	–	–	–
35	Biopsy	PCa	65	989.00	4+5	IV	4	1	1
36	Biopsy	PCa	73	478.00	4+3	IV	3b	0	1
37	Biopsy	PCa	75	63.20	4+5	IV	4	1	1
38	Biopsy	PCa	79	95.60	4+5	IV	3b	1	1
39	Biopsy	PCa	69	248.00	4+4	IV	4	1	1
40	Biopsy	PCa	70	36.08	3+4	IV	3a	1	0
41	Biopsy	PCa	66	1338.00	4+5	IV	3a	1	0
42	Biopsy	PCa	81	332.00	4+5	IV	4	0	1
43	Biopsy	PCa	72	355.00	4+4	III	3a	0	0
44	Biopsy	PCa	65	208.00	4+4	IV	4	1	1
45	Biopsy	PCa	81	102.00	4+4	II	2	0	0
46	Biopsy	PCa	75	212.00	4+4	III	3b	0	0
47	Biopsy	PCa	73	12.90	4+4	IV	3b	1	0
48	Biopsy	PCa	58	11.40	4+4	IV	3b	1	0
49	Biopsy	PCa	73	22.70	4+4	III	3a	0	0
50	Biopsy	Non-PCa	54	5.44	–	–	–	–	–
51	Biopsy	Non-PCa	60	5.60	–	–	–	–	–
52	Biopsy	Non-PCa	67	5.93	–	–	–	–	–
53	Biopsy	Non-PCa	67	8.09	–	–	–	–	–
54	Biopsy	Non-PCa	60	14.00	–	–	–	–	–
55	Biopsy	Non-PCa	69	5.99	–	–	–	–	–
56	Biopsy	Non-PCa	56	8.44	–	–	–	–	–
57	Biopsy	Non-PCa	61	8.60	–	–	–	–	–
58	Biopsy	Non-PCa	62	35.50	–	–	–	–	–

Table 1 (Continued)

No.	Procedure	PCa or non-PCa	Age, years	PSA, ng/mL	Gleason score	Stage	cT	cN	cM
59	Biopsy	Non-PCa	57	5.19	–	–	–	–	–
60	Biopsy	Non-PCa	64	4.37	–	–	–	–	–
61	Biopsy	Non-PCa	60	5.68	–	–	–	–	–
62	Biopsy	Non-PCa	63	11.40	–	–	–	–	–
63	Biopsy	Non-PCa	65	13.20	–	–	–	–	–

–, Not applicable.

had undergone transrectal needle biopsy at Teikyo University Chiba Medical Center (Ichihara, Japan) from 2008 to 2013 were enrolled in this study. The patients' backgrounds are summarized in Table 1. As for prostatectomy specimens, 17 paired samples of PCa and corresponding normal tissues were obtained. For needle biopsy specimens, a pair of needle biopsy specimens was collected from the same region as from patients in this study, and one was subjected to pathological verification. The normal tissues were free of cancer cells, as determined by pathological examination. Before tissue collection, written informed consent of tissue donation for research purposes was obtained from patients. The protocol was approved by the Institutional Review Board of Chiba University and Teikyo University.

For *in vitro* analyses, we used human PCa cell lines PC3 and PC3M obtained from ATCC (Manassas, VA, USA). These cells were maintained in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

RNA extraction. Total RNA was extracted from formalin-fixed paraffin-embedded samples with four 5-μm-thick slices, using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Quantitative real-time RT-PCR. The procedure for PCR quantification was described previously.[11,16,17] *Taq*Man probes and primers for *ITGA3* (P/N: Hs01076873_ml), *ITGB1* (P/N: Hs00559595_ml), and *ACTB* (internal control; P/N: Hs01060665_gl) (all Applied Biosystems, Foster City, CA, USA) were assay-on-demand gene expression products. The expression levels of *miR-223* (assay ID: 002295; Applied Biosystems) were analyzed by *Taq*Man quantitative real-time RT-PCR (qRT-PCR) (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to the expression of *RNU48* (assay ID: 001006; Applied Biosystems). All reactions were carried out in triplicate, and each assay included negative control reactions that lacked cDNA.

Mature miRNA, siRNA, and plasmid vector transfection. The following miRNA and siRNAs were used: Pre-miR miRNA precursor (hsa-miR-223-3p; P/N: AM12301; Applied Biosystems), Stealth Select RNAi siRNAs; si-ITGA3 (cat. nos. HSS105529 and HSS179967; Invitrogen Carlsbad, CA, USA), si-ITGB1 (cat nos. HSS105559 and HSS105561; Invitrogen), and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax transfection reagent (Invitrogen) as described previously.[11,16,17]

For *ITGA3* or *ITGB1* rescue experiments, 2.5 g pF4A CMV Flexi-plasmid vector containing *ITGA3* or *ITGB1* (C848A; Promega, Madison, WI, USA) were transfected 24 h before miRNA transfection by using Lipofectamine 2000 transfection reagent (Life Technologies, Carlsbad, CA, USA).

Cell proliferation, migration, and invasion assays. Cell proliferation, migration, and invasion assays were carried out as previously described.[11,16,17]

Western blot analysis. Cells were harvested 72 h after transfection, and lysates were prepared. Next, 20 μg each cell lysate was separated on Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was carried out with rabbit anti-ITGA3 antibodies (HPA008572, 1:250 dilution; Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-ITGB1 antibodies (9699, 1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-focal adhesion kinase (FAK) antibodies (#3285, 1:1000 dilution; Cell Signaling Technology), anti-phospho-FAK (Tyr397) (D20B1) rabbit mAbs (#8556, 1:1000 dilution; Cell Signaling Technology), anti-Akt (pan) (11E7) rabbit mAbs (#4685, 1:1000 dilution; Cell Signaling Technology), anti-phospho-Akt (Ser473) (D9E) XP rabbit mAbs (#4060, 1:1000 dilution; Cell Signaling Technology), anti-Src (32G6) rabbit mAbs (#2123, 1:1000 dilution; Cell Signaling Technology), anti-phospho-Src family (Tyr416) (D49G4) rabbit mAbs (#6943, 1:1000 dilution; Cell Signaling Technology), anti-p44 /42 MAPK (also known as ERK1/2) antibodies (#9102, 1:1000 dilution; Cell Signaling Technology), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit mAbs (#4370, 1:1000 dilution; Cell Signaling Technology), and anti-GAPDH antibodies (ab8245, 1:1000 dilution; Abcam, Cambridge, UK) as a loading control. The membranes were washed and incubated with anti-rabbit IgG HRP-linked antibodies (#7074; Cell Signaling Technology). Complexes were visualized with Clarity Western ECL Substrate (Bio-Rad).

Screening of *miR-223* target genes using *in silico* analysis and genome-wide gene expression analysis. To identify *miR-223* target genes, we used *in silico* analysis and genome-wide gene expression analysis, as described previously.[18,19] The strategy for selection of *miR-223* target genes is shown in Figure S1.

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the *ITGA3/ITGB1* 3′-UTR or those with deleted *miR-223* target sites were inserted between the *Xho*I–*Pme*I restriction sites in the 3′-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega). The synthesized DNA was cloned into the psiCHECK-2 vector. PC3 cells were transfected with 50 ng vector and 10 nM *miR-223* using Lipofectamine 2000 (Invitrogen). The activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (E1910; Promega). Normalized data were calculated as the ratio of *Renilla/*firefly luciferase activities.

Immunohistochemistry. A total of 17 radical prostatectomy specimens were used (Table 1). Tissue specimens were immunostained using an Ultra-Vision Detection System
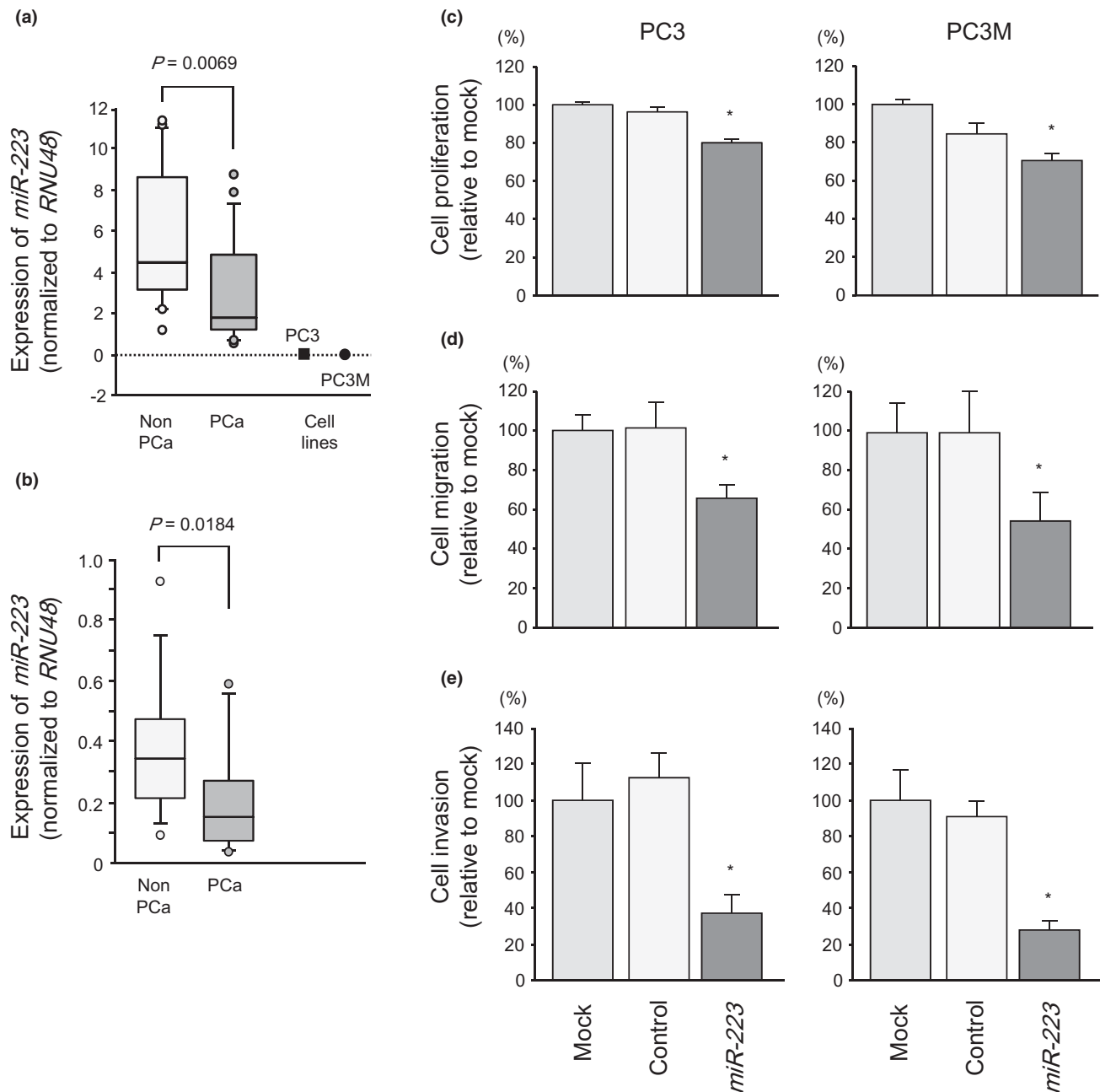
Fig. 1. Expression levels of *miR-223* in prostate cancer (PCa) clinical specimens and PCa cell lines PC3 and PC3M. (a, b) Quantitative real-time RT-PCR showed that the expression levels of *miR-223* were significantly lower in PCa tissues and PCa cell lines than in normal prostate tissues. *RNU48* was used as an internal control. Effects of *miR-223* transfection on PCa cell lines PC3 and PC3M. (c) Cell proliferation was determined by XTT assays 72 h after transfection with *miR-223* (10 nM). (d) Cell migration activity was determined by wound-healing assays 48 h after transfection with *miR-223* (10 nM). (e) Cell invasion activity was determined by Matrigel invasion assays 48 h after transfection with *miR-223* (10 nM). *$P < 0.05$.

(Thermo Scientific, Fremont, CA, USA) according to the manufacturer's instructions. Primary rabbit polyclonal antibodies against ITGA3 (HPA008572; Sigma-Aldrich) were diluted 1:15, and primary mouse mAbs against ITGB1 (ab3167; Abcam) were diluted 1:100. The slides were treated with biotinylated goat antibodies.

A tissue microarray containing 60 PCa specimens, 10 prostatic intraepithelial neoplasias, and 10 prostatic hyperplastic samples was obtained from Provitro (Berlin, Germany) (Cat.

#401 2209, Lot #146.1 P020212, 26-46). Detailed information on all cancer specimens can be found at http://www.provitro. com/fileadmin/provitro-data/TMA/4012209.pdf. Immunostaining was evaluated according to the scoring method described previously.[11]

Statistical analysis. The relationships between two groups and the numerical values obtained by real-time RT-PCR were analyzed using the Mann–Whitney *U*-test. The relationships among three variables and numerical values were analyzed

Table 2. Significantly enriched pathways following *miR-223* transfection in PC3 and PC3M prostate cancer cells

KEGG entry number	Annotation	P-value	Genes
4512	ECM–receptor interaction	0.0001809	SDC4, **ITGA3**, LAMC2, **ITGB1**, CD44
4010	MAPK signaling pathway	0.0002460	MAP3K13, AKT3, PRKCA, MKNK2, GNG12, RRAS2, STMN1, FLNB
4810	Regulation of actin cytoskeleton	0.0003455	ABI2, **ITGA3**, TMSB4X, GNG12, ARPC5L, RRAS2, **ITGB1**
5130	Pathogenic *Escherichia coli* infection	0.0003558	PRKCA, TUBA1B, ARPC5L, **ITGB1**
4141	Protein processing in endoplasmic reticulum	0.0004903	ERLEC1, UFD1L, HSPH1, RRBP1, TRAM1, HSP90B1
5200	Pathways in cancer	0.0009929	PPARD, EPAS1, **ITGA3**, AKT3, PRKCA, LAMC2, **ITGB1**, HSP90B1
4530	Tight junction	0.0013334	MPP5, AKT3, PRKCA, EPB41L3, RRAS2
4510	Focal adhesion	0.0014889	**ITGA3**, AKT3, PRKCA, LAMC2, **ITGB1**, FLNB
4120	Ubiquitin-mediated proteolysis	0.0015753	UBE2A, UBE3B, CDC27, UBE2L3, TRIM37
5222	Small-cell lung cancer	0.0018785	**ITGA3**, AKT3, LAMC2, **ITGB1**
4970	Salivary secretion	0.0019618	ATP1B1, KCNMA1, ITPR3, PRKCA
4972	Pancreatic secretion	0.0032883	ATP1B1, KCNMA1, ITPR3, PRKCA
5010	Alzheimer's disease	0.0035642	NDUFA5, ITPR3, BACE1, NDUFA3, PSEN1
4978	Mineral absorption	0.0039263	ATP1B1, MT1E, STEAP2

using the Bonferroni-adjusted Mann–Whitney *U*-test. All analyses were carried out using Expert StatView (version 5; SAS Institute Inc., Cary, NC, USA).

Results

Expression levels of *miR-223* in PCa clinical specimens and cell lines. To validate our past PCa miRNA signature results, we evaluated the expression levels of *miR-223* in 34 radical prostatectomy specimens and 29 transrectal needle biopsy specimens. The expression levels of *miR-223* were significantly lower in PCa clinical specimens than in non-cancerous specimens ($P = 0.0069$, Fig. 1a; $P = 0.0184$, Fig. 1b). The *miR-223* expression was also reduced in PC3 and PC3M cells compared with that in non-cancerous specimens.

However, it was not able to assess the correlation between *miR-223* expression and clinicopathological characteristics due to the limited number of clinical specimens. The cohort study with increased number of specimens will be important in the future.

Effects of *miR-223* restoration on cell proliferation, migration, and invasion in PCa cells. To investigate the functional effects

(a) Anti-ITGA3 Ab

Patient No.6   Patient No.16

(b) Anti-ITGB1 Ab
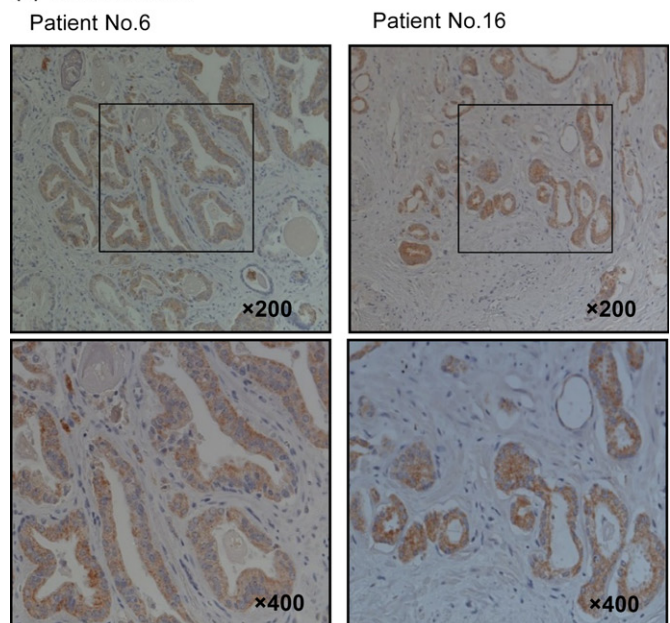
Patient No.6   Patient No.16
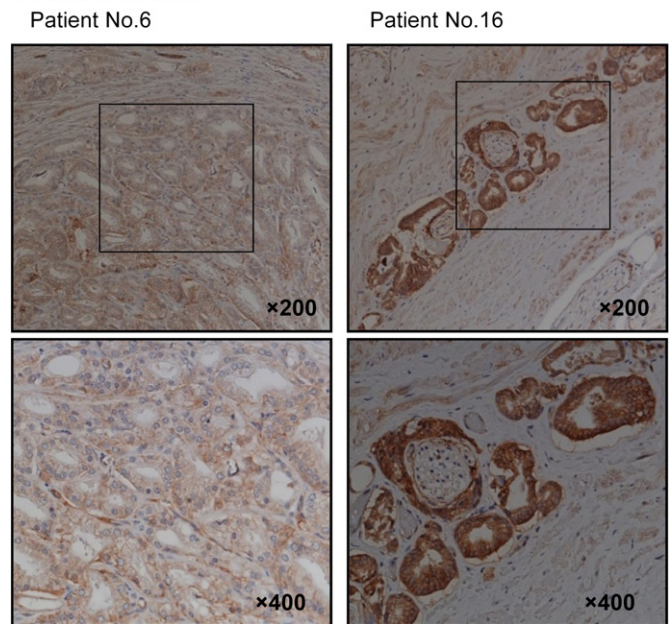
Fig. 2. Immunohistochemical staining of integrin A3 (ITGA3) and integrin B1 (ITGB1) in clinical specimens of prostate cancer and normal prostate tissues. (a) ITGA3 was expressed more strongly in several cancer lesions than in normal tissues. (b) ITGB1 was also overexpressed in cancer lesions.

of *miR-223*, we carried out gain-of-function studies using miRNA transfection of PC3 and PC3M cells.

The XTT assays revealed significant inhibition of cell proliferation in PC3 and PC3M cells transfected with *miR-223* in comparison with mock- or control-transfected cells (PC3, $P < 0.0001$; PC3M, $P = 0.0002$; Fig. 1c).

Similarly, wound healing assays showed significant inhibition of cell migration activities in *miR-223* transfectants compared with that in mock- or miR-control-transfected cells (PC3, $P = 0.0176$; PC3M, $P < 0.0001$; Fig. 1d).
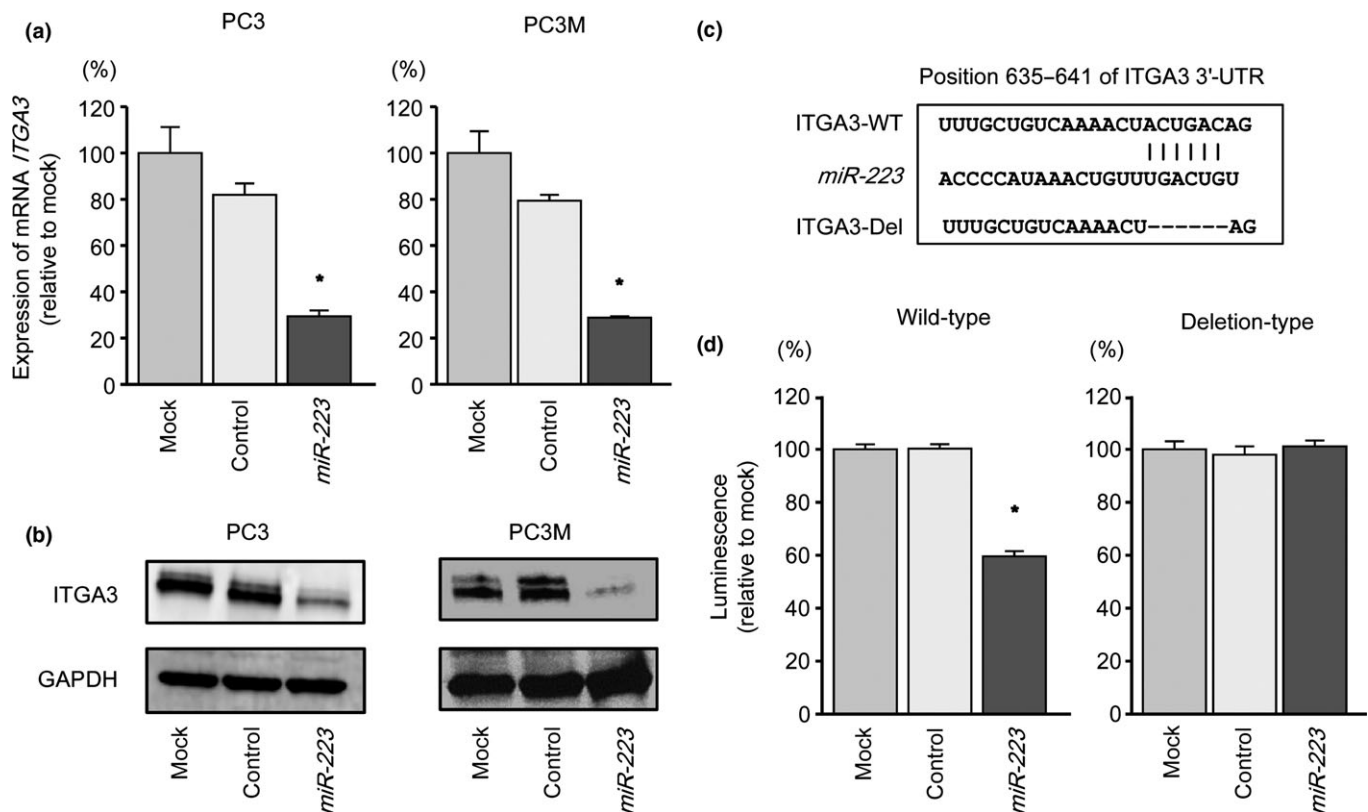
Fig. 3. Expression of the gene encoding integrin A3 (*ITGA3*) was suppressed by transfection of prostate cancer cell lines PC3 and PC3M with *miR-223*. (a) *ITGA3* mRNA expression was evaluated by quantitative RT-PCR 72 h after transfection with *miR-223* (10 nM). *ACTB* was used as an internal control. *$P < 0.01$. (b) *ITGA3* protein expression was evaluated by Western blotting 72 h after transfection with *miR-223* (10 nM). GAPDH was used as a loading control. (c) *miR-223* binding site in the 3'-UTR of *ITGA3* mRNA. (d) Luciferase reporter assays in PC3 cells using vectors encoding a putative *miR-223* target site at position 635–641 of *ITGA3* 3'-UTR. *Renilla* luciferase values were normalized to firefly luciferase values. *$P < 0.0001$.

Moreover, Matrigel invasion assays revealed that *miR-223* transfection significantly inhibited cell invasion. The number of invaded cells was significantly reduced in *miR-223*-transfected cells (PC3, $P < 0.0001$; PC3M, $P < 0.0001$; Fig. 1e).

Identification of candidate target genes of *miR-223* in PCa cells. To identify target genes of *miR-223*, we carried out a combination of *in silico* and oligomicroarray analyses. First, we undertook genome-wide gene expression analysis using PC3 and PC3M cells. A total of 936 genes that were downregulated (average log2 ratio < −0.5) following *miR-223* transfection as compared with expression levels in miR-control-transfected cells were selected. Next, we screened these genes using the TargetScan database and found that 247 genes had putative target sites for *miR-223* in their 3'-UTRs. Finally, we categorized these genes into KEGG pathways using GeneCodis analysis; 14 pathways were identified as significantly enriched (Table 2).

Because *miR-223* significantly inhibited cancer cell migration and invasion, we focused on three annotations: ECM–receptor interaction, regulation of actin cytoskeleton, and focal adhesion. These three annotations all included *ITGA3* and *ITGB1*; therefore, these genes were subjected to further analysis.

Immunohistochemical detection of ITGA3 and ITGB1 in PCa clinical specimens. Next, we examined the expression levels of ITGA3 and ITGB1 in PCa specimens by immunohistochemical staining. ITGA3 and ITGB1 were strongly expressed in several cancer tissues, while low expression was observed in normal tissues (Fig. 2). To gain further insights into whether the upregulation of ITGA3 and ITGB1 correlates with clinico-pathological features, we used tissue microarray (Table S1). Immunostaining was evaluated according to the scoring method as described previously.[11] Each case was scored on the basis of the intensity and area of staining. Both upregulation of ITGA3 and ITGB1 were confirmed in the PCa tissue compared with non-PCa tissue (Fig. S4, $P = 0.0294$ and $P = 0.0003$). The expression levels of ITGA3 or ITGB1 were not associated with clinicopathological features of PCa in this analysis.

ITGA3 and ITGB1 were directly regulated by *miR-223*. We then carried out qRT-PCR and Western blotting to confirm that restoration of *miR-223* resulted in downregulation of *ITGA3* and *ITGB1* in PC3 and PC3M cells. The mRNA and protein expression levels of ITGA3 and ITGB1 were significantly repressed in *miR-223* transfectants compared with mock or miR-control transfectants (PC3, $P = 0.0051$; PC3M, $P = 0.0029$; Fig. 3) (PC3, $P = 0.0014$; PC3M, $P = 0.0017$; Fig. 4).

We used luciferase reporter assays in PC3 cells to determine whether *ITGA3* and *ITGB1* mRNAs were directly regulated by *miR-223*. The TargetScan database predicted that putative *miR-223*-binding sites existed in the 3'-UTR of *ITGA3* (position 635–641; Fig. 3c) and of *ITGB1* (position 255–261;
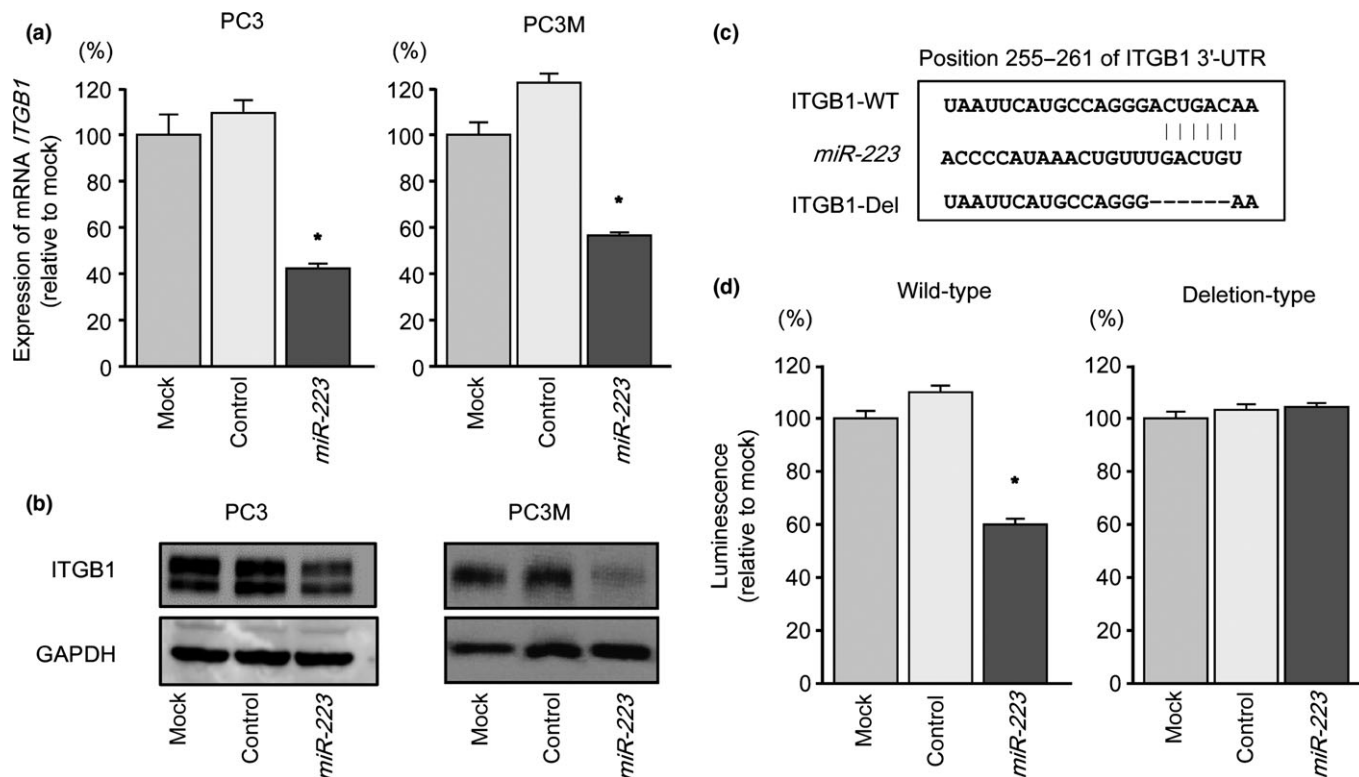
Fig. 4. Expression of the gene encoding integrin B1 (*ITGB1*) was suppressed by transfection of prostate cancer cell lines PC3 and PC3M with *miR-223*. (a) *ITGB1* mRNA expression was evaluated by quantitative RT-PCR 72 h after transfection with *miR-223* (10 nM). *ACTB* was used as an internal control. *$P < 0.005$. (b) *ITGB1* protein expression was evaluated by Western blotting 72 h after transfection with *miR-223* (10 nM). GAPDH was used as a loading control. (c) *miR-223* binding site in the 3′-UTR of *ITGB1* mRNA. (d) Luciferase reporter assays in PC3 cells using vectors encoding a putative *miR-223* target site at position 255–261 of the *ITGB1* 3′-UTR. *Renilla* luciferase values were normalized to firefly luciferase values. *$P < 0.0001$.

Fig. 4c). We used vectors encoding either the partial wild-type sequence of the 3′-UTR of *ITGA3* or *ITGB1* mRNA, including the predicted *miR-223* target sites, or deletion vectors lacking the *miR-223* target sites. We found that the luminescence intensities were significantly reduced by transfection with *miR-223* and vectors carrying the wild-type 3′-UTR of *ITGA3* and *ITGB1*, whereas transfection with deletion vectors blocked the decrease in luminescence ($P < 0.0001$ and $P < 0.0001$, respectively; Figs. 3d,4d). These data suggested that *miR-223* bound directly to specific sites in the 3′-UTRs of *ITGA3* and *ITGB1* mRNA.

Effects of silencing *ITGA3* and *ITGB1* on cell proliferation, migration, and invasion in PCa cells. To investigate the functional role of *ITGA3* and *ITGB1* in PCa cells, we carried out loss-of-function studies using *si-ITGA3* and *si-ITGB1* transfectants. First, we evaluated the knockdown efficiency of *si-ITGA3* and *si-ITGB1* transfection in PC3 cells. Quantitative RT-PCR and Western blotting indicated that *si-ITGA3* transfection effectively downregulated *ITGA3* expression and that *si-ITGB1* transfection effectively downregulated *ITGB1* expression in PC3 cells ($P < 0.0005$ and $P < 0.0001$, respectively; Fig. 5a,b).

In functional assays, cell proliferation, migration, and invasion assays indicated that cancer cell proliferation, migration, and invasion activity were significantly inhibited in *si-ITGA3*-transfected PC3 cells in comparison with mock- or *si-control*-transfected PC3 cells ($P < 0.0001$, $P < 0.0001$, and

$P < 0.0001$, respectively; Fig. 5c–e). Similarly, transfection with *si-ITGB1* significantly inhibited cancer cell proliferation, migration, and invasion ($P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively; Fig. 5f–h).

We also evaluated the knockdown efficiency of *si-ITGA3* and *si-ITGB1* transfection in PC3M cells. Cancer cell proliferation, migration, and invasion activity were significantly inhibited in *si-ITGA3*- and *si-ITGB1*-transfected cells in comparison with mock- or *si-control*-transfected cells (Fig. S2).

Effects of cotransfection of *ITGA3/miR-223* or *ITGB1/miR-223* in PC3 cell line. To confirm that *ITGA3/miR-223* or *ITGB1/miR-223* mediated the antitumor effect in PCa cells, we coexpressed *ITGA3* and *miR-223* or *ITGB1* and *miR-223* in PCa cells. *ITGA3* or *ITGB1* rescue studies indicated that cell migration and invasion properties were rescued by *ITGA3* or *ITGB1* transfectants compared with cells with restored *miR-223* only (Fig. S3). These data suggested that the role of ITGA3 or ITGB1 protein was modulated by *miR-223* and the effects of migration and invasion in PCa cells.

Effects of *ITGB1* knockdown on *ITGB1* downstream signaling. We analyzed the effects of *ITGA3/ITGB1* knockdown on downstream signaling in PC3 cells using *si-ITGA3* and *si-ITGB1* transfectants. To examine the effects of knockdown on *ITGB1*-mediated survival pathways, the phosphorylation of FAK (Tyr 397), SRC (Tyr 416), AKT (Ser 473), and ERK1/2 (Thr 202/Tyr 204) was examined. Knockdown of *ITGB1* greatly reduced the phosphorylation of FAK, SRC, AKT,
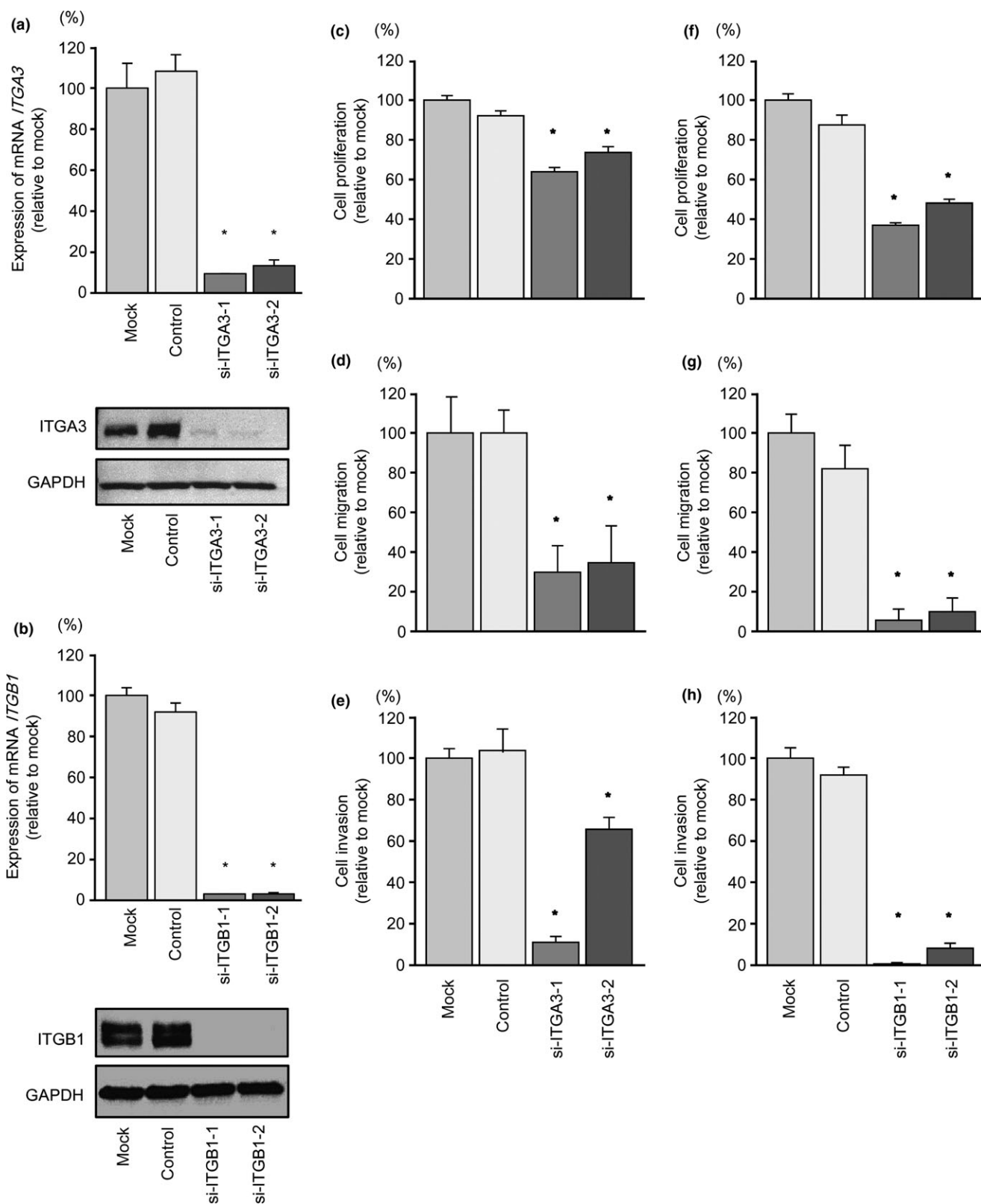
Fig. 5. Expression levels of the genes encoding integrins A3 (*ITGA3*) and B1 (*ITGB1*) were suppressed by transfection of PC3 prostate cancer cells with *si-ITGA3* and *si-ITGB1*. (a, b) *ITGA3* and *ITGB1* mRNA expression levels were evaluated by quantitative RT-PCR 72 h after transfection with *si-ITGA3* and *si-ITGB1* (10 nM). *ACTB* was used as an internal control. *P < 0.0005. ITGA3 and ITGB1 expression levels were evaluated by Western blotting 72 h after transfection with *si-ITGA3* and *si-ITGB1* (10 nM). GAPDH was used as a loading control. (c–e) Effects of *ITGA3* silencing on PC3 cells. (c) Cell proliferation was determined by XTT assays. (d) Cell migration activity was determined by wound-healing assays. (e) Cell invasion activity was determined by Matrigel invasion assays. (f–h) Effects of silencing *ITGB1* on PC3 cells. (f) Cell proliferation was determined by XTT assays. (g) Cell migration activity was determined by wound-healing assays. (h) Cell invasion activity was determined by Matrigel invasion assays. *P < 0.0001.
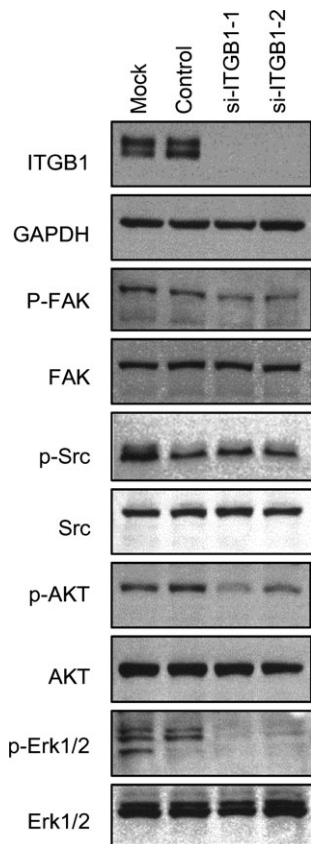
Fig. 6. Effects of the knockdown of the gene encoding integrin B1 (*ITGB1*) on ITGB1 downstream signaling. Knockdown of *ITGB1* in PC3 cells reduced the phosphorylation of FAK (Tyr 397), SRC (Tyr 416), AKT (Ser 473), and ERK1/2 (Thr 202/Tyr 204).

and ERK1/2 (Fig. 6). In contrast to knockdown of *ITGB1*, knockdown of *ITGA3* did not cause changes in the phosphorylation levels of these proteins (data not shown).

Discussion

A growing body of evidence has indicated that normal RNA regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating novel miRNA regulatory networks in PCa cells. Based on this, we constructed the miRNA expression signatures of PCa and CRPC using clinical specimens and identified tumor-suppressive miRNAs regulating novel oncogenic pathways.[5–12] Castration-resistant PCa is difficult to treat using currently available therapies, and most clinical trials for advanced PCa have shown limited benefits, with disease progression and metastasis to the bone or other sites.[3] Thus, understanding the molecular mechanisms of the metastatic signaling pathways underlying PCa using current genomic approaches would help to improve therapies for and prevention of the disease. Our miRNA signatures in PCa revealed that *miR-223* was significantly downregulated in naïve PCa and CRPC tissues. In the present study, we validated the downregulation of *miR-223* in PCa clinical specimens. Additionally, we focused on *miR-223* and investigated the functional roles of *miR-223* and *miR-223*-regulated novel oncogenic pathways in PCa.

In this study, we evaluated the low expression of *miR-223* and the effects of *miR-223* regulation on metastatic signaling pathways in PC3 and PC3M cells, two representative PCa cell lines. PC3M cells were derived from a liver metastasis in a nude mouse bearing a spleen explant of PC3 cells; these cells have increased metastatic ability compared with PC3 cells.[20,21] Restoration of *miR-223* significantly inhibited cancer cell migration and invasion in both PC3 and PC3M cells, suggesting that *miR-223* acts as a tumor-suppressive miRNA in PCa cells. The tumor-suppressive function of *miR-223* has been reported in various other types of cancers and is thought to occur through regulation of several oncogenic genes.[22,23] Previous reports have shown that *IGF-1R* is directly regulated by *miR-223* and that restoration of *miR-223* inhibits cell proliferation through suppression of downstream phosphoinositol 3-kinase/AKT/mammalian target of rapamycin pathways.[24,25] Moreover, overexpression of *miR-223* suppresses cell proliferation in colorectal cancer, cervical cancer, and hepatoma cells by targeting forkhead box transcriptional factor 1.[26] These findings indicate that *miR-223* is downregulated in cancer cells and functions as a tumor-suppressive miRNA by targeting oncogenic genes.

In contrast to our data, a recent report showed that expression of *miR-223* is upregulated in PCa tissues and cell lines, including LNCaP, DU145, and PC3 cells.[27] Moreover, knockdown of *miR-223* using antisense oligonucleotides leads to G_0/G_1 arrest and induces apoptosis in LNCaP, DU145, and PC3 cells.[27] Another study showed that overexpression of *miR-223* in primary gastric carcinoma is associated with poor metastasis-free survival.[28] Interestingly, overexpression of *miR-223* has been shown to result in acquisition of invasive ability in non-metastatic gastric cancer cells.[28] Thus, these studies of *miR-223* indicate that this miRNA has opposing roles as a tumor suppressor or oncogene in different types of cancer cells. Therefore, it is necessary to investigate the molecular targets and RNA networks regulated by *miR-223* in different types of cancers. Our recent data of CRPC miRNA expression signatures showed that *miR-223* was significantly reduced in CRPC specimens.[29] Downregulation of *miR-223* in PCa specimens has been reported by another group based on deep sequencing analysis.[12] These data strongly suggest that *miR-223* is frequently reduced in PCa tissues and acts as a tumor suppressor.

Recent bioinformatic predictions have indicated that miRNAs regulate more than 30–60% of protein-coding genes in the human genome.[30,31] Improving our understanding of *miR-223*-mediated targets and signaling pathways in PCa may provide important insights into the mechanisms of PCa metastasis. We used a combination of genome-wide gene expression analysis and *in silico* analysis to identify novel molecular targets and pathways regulated by tumor-suppressive miRNAs in cancer cells.[4–11] Using this strategy, we identified several pathways and targets regulated by *miR-223* in PCa cells. Among them, we further investigated the *ITGA3* and *ITGB1* genes, which are involved in focal adhesion and ECM receptor interaction according to KEGG pathway analysis. Integrins are a large family of cell surface receptors composed of specific two subunits (α and β) that bind to ECM components. Most types of cells require integrin-mediated signaling pathways for proliferation, migration, invasion, and survival.[14,15] ITGA3 and ITGB1 are components of a specific subset of integrins and are expressed PCa cells.[14,15,32–34]

In this study, we confirmed the upregulation of ITGA3 and ITGB1 in PCa clinical specimens. We showed that *ITGA3* and

ITGB1 were directly regulated by *miR-223* and that silencing of *ITGA3* or *ITGB1* significantly inhibited cancer cell migration and invasion in PC3 and PC3M cells. Moreover, our present data showed that knockdown of *ITGB1* inhibited downstream signals contributing to cell adhesion and invasion. Overexpression of ITGB1 has been observed in several cancer types in previous studies.[35,36] Additionally, a recent study showed that ITGB1 is activated in highly metastatic PCa cells, but not in PCa cells with low metastatic potential or normal prostate epithelial cells.[37] Both ECM ligands and Talin-1 have been shown to induce the activation of *ITGB1* in cancer cells.[37,38] Interestingly, our recent studies showed that tumor-suppressive *miR-29s* and *miR-218* are frequently downregulated in cancer cells, including PCa cells, and cause upregulation of ECM components.[9,17,39] Moreover, expression of Talin-1 is increased in metastatic tissues compared with that in primary prostate tumors.[40] Thus, these studies indicate that aberrant expression of miRNAs disrupts tightly regulated integrin-mediated signaling in the prostate and promotes cancer cell migration and invasion.

In conclusion, downregulation of *miR-223* was validated in PCa clinical specimens, and these miRNAs were shown to function as tumor suppressors in PCa. To the best of our knowledge, this is the first report showing that tumor-suppressive *miR-223* directly targeted *ITGA3* and *ITGB1* in PCa cells. Moreover, these integrins were upregulated in PCa clinical specimens and contributed to cancer cell migration and invasion. Metastatic progression of PCa significantly impacts the survival of men with prostate cancer. Suppression of *ITGA3/ITGB1* signaling in PCa cells may have applications in the development of novel therapies for PCa.

Acknowledgments

Disclosure Statement

The authors have no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Strategy for selecting target pathways regulated by *miR-223* in prostate cancer cells.

Fig. S2. Effects of *ITGA3* and *ITGB1* silencing on PC3M prostate cancer cells.

Fig. S3. Effects of cotransfection of *ITGA3/miR-223* or *ITGB1/miR-223* in PC3 prostate cancer cell line.

Fig. S4. Immunohistochemical staining of integrin A3 (ITGA3) or integrin B1 (ITGB1) in prostate cancer by tissue microarray.

Table S1. Clinical characteristics and immunohistochemistry scores of integrins A3 and B1 (ITGA3/ITGB1) in tissue microarray.