Direct regulation of *LAMP1* by tumor-suppressive *microRNA-320a* in prostate cancer

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Abstract. Advanced prostate cancer (PCa) metastasizes to bone and lymph nodes, and currently available treatments cannot prevent the progression and metastasis of the disease. Therefore, an improved understanding of the molecular mechanisms of the progression and metastasis of advanced PCa using current genomic approaches is needed. Our miRNA expression signature in castration-resistant prostate cancer (CRPC) revealed that *microRNA-320a* (*miR-320a*) was significantly reduced in cancer tissues, suggesting that *miR-320a* may be a promising anticancer miRNA. The aim of this study was to investigate the functional roles of *miR-320a* in naïve PCa and CRPC cells and to identify *miR-320a*-regulated genes involved in PCa metastasis. The expression levels of *miR-320a* were significantly reduced in naïve PCa, CRPC specimens, and PCa cell lines. Restoration of mature *miR-320a* in PCa cell lines showed that *miR-320a* significantly inhibited cancer cell migration and invasion. Moreover, we found that lysosomal-associated membrane protein 1 (*LAMP1*) was a direct target of *miR-320a* in PCa cells. Silencing of *LAMP1* using siRNA significantly inhibited cell proliferation, migration, and invasion in PCa cells. Overexpression of LAMP1 was observed in PCa and CRPC clinical specimens. Moreover, downstream pathways were identified using si-*LAMP1*-transfected cells. The discovery of tumor-suppressive *miR-320a*-mediated pathways may provide important insights into the potential mechanisms of PCa metastasis.

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer-related death among men in developed countries (1). Most patients with PCa are initially responsive to androgen deprivation therapy; however, their cancers eventually become resistant to androgen-deprivation therapy and progress to castration-resistant prostate cancer (CRPC). Several novel treatments have recently been developed for patients with advanced PCa. These treatments, however, cannot completely control the progression and metastasis of PCa (2). Therefore, more effective treatment strategies, based on current genomic approaches, are required. Moreover, because it is difficult to obtain clinical specimens of CRPC, there are very few reports describing the genomic analysis of CRPC.

Small non-coding RNAs known as microRNAs (miRNAs) have been shown to regulate gene expression by repressing translation or cleaving RNA transcripts in a sequence-dependent manner (3). At present, a substantial amount of evidence has suggested that miRNAs are aberrantly expressed in many human cancers, including PCa, and play significant roles in human oncogenesis, metastasis, and drug resistance (4-7). Several reports have shown that miRNAs regulate ~30-60% (or more) of the protein-coding genes in the human genome by bioinformatics predictions (7,8). Thus, normal regulatory mechanisms of RNA networks can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells. We propose that identification of aberrantly expressed miRNAs in clinical specimens is an important first step toward elucidating the details of RNA networks in cancer cells.

To identify novel RNA networks in PCa, we have previously constructed miRNA expression signatures using androgen-dependent PCa clinical specimens (9,10). Using this signature, we have identified tumor-suppressive miRNA-mediated PCa oncogenic pathways (11-16). More recently, we constructed miRNA expression signatures using CRPC clinical specimens and reported that the miRNA cluster *miR-221/miR-222*

functions as a tumor suppressor in PCa and CRPC cells (17). Moreover, *miR-223* expression has been shown to be significantly reduced in PCa and CRPC specimens and to act as a tumor suppressor by targeting integrin-A3/B1 oncogenic signaling (18). The CRPC expression signature reported by our laboratory has provided important information needed to elucidate novel RNA networks in CRPC cells.

In this study, we focused on *miR-320a* because downregulation of *miR-320a* was observed in our two miRNA signatures for androgen-dependent PCa and CRPC clinical specimens (9,17). The aim of this study was to investigate the functional significance of *miR-320a* and to identify the molecular targets and pathways mediated by *miR-320a* in PCa and CRPC cells. Our data showed that restoration of mature *miR-320a* in PC3 and DU145 PCa cells significantly inhibited cancer cell migration and invasion. Direct regulation of lysosomal-associated membrane protein 1 (*LAMP1*) by *miR-320a* was observed in PCa cells. Silencing of *LAMP1* using specific small interfering RNA (siRNA; si-*LAMP1*) significantly inhibited cell proliferation, migration, and invasion in PCa cells. The discovery of tumor-suppressive *miR-320a*-mediated molecular pathways provides new insights into the potential mechanisms of metastasis in PCa and suggests novel therapeutic strategies for the treatment of this disease.

Materials and methods

Clinical PCa specimens, cell lines, and RNA isolation. Clinical prostate specimens (PCa and normal prostate tissues) collected by needle biopsy or autopsy were obtained from patients admitted to Teikyo University Chiba Medical Center Hospital from 2008 to 2013. Twenty-nine prostate samples [PCa, n=15; non-cancerous prostate tissues (non-PCa), n=14] were obtained by transrectal prostate needle biopsy from patients with elevated serum prostate-specific antigen (PSA) levels, and eight metastatic PCa samples were obtained from patients who died of CRPC. The backgrounds of the patients are summarized in Table I. All patients provided written informed consent for tissue donation for research purposes. The protocol was approved by the Institutional Review Boards of Chiba University and Teikyo University.

Human prostate cancer cells (PC3 and DU145 cells) and normal prostate cells (RWPE-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). PC3 and DU145 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. RWPE-1 cells were cultured in keratinocyte serum-free medium containing 5 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract.

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol as described previously (14,17,18).

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The expression levels of *miR-320a* were analyzed by TaqMan quantitative real-time PCR (assay ID: 0000542; Applied Biosystems, Foster City, CA, USA) and normalized to the expression of *RNU48* (assay ID: 001006; Applied Biosystems). TaqMan probes and primers for *LAMP1* (P/N: Hs00174766_m1; Applied Biosystems), *GAPDH* (the internal control; P/N: Hs02758991_m1; Applied Biosystems) and *GUSB* (the internal control; P/N: Hs00939627_m1; Applied Biosystems) were assay-on-demand gene expression products. The procedure for PCR quantification was described previously (14,17,18).

Transfection with mature miRNA and siRNA. The following mature miRNA species were used in this study: mature miRNA and Pre-miR miRNA Precursor (*hsa-miR-320a*; P/N: 4427975; Applied Biosystems). The following siRNAs were used: Stealth Select RNAi siRNA, si-*LAMP1* (cat no. HSS180593, HSS180594; Invitrogen) and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). Transfection procedures and transfection efficiencies of miRNA in PC3 and DU145 cells were described previously (14,17,18).

Cell proliferation, migration, and invasion assays. PC3 and DU145 cells were transfected with 10 nM miRNAs or siRNAs by reverse transfection. Cell proliferation was determined by XTT assay using a Cell Proliferation Kit II (Roche Applied Sciences, Tokyo, Japan). Cell migration activity was analyzed using uncoated Transwell polycarbonate membrane filters. Cell invasion was evaluated using modified Boyden chambers containing Transwell-precoated Matrigel membrane filter inserts. These assays were performed as described previously (14,17,18).

Selection of putative target genes regulated by miR-320a in PCa cells. To identify *miR-320a* target genes, we used *in silico* analysis and genome-wide gene expression analysis. First, we used TargetScan Release 7.0 (http://www.targetscan.org/) to search for genes containing the *miR-320a* seed sequence in the 3'-untranslated region (UTR). Next, to identify upregulated genes in clinical PCa specimens, we analyzed a publicly available gene expression dataset in the GEO database (GEO accession no. GSE29079). Finally, we attempted to identify *miR-320a* target genes using *miR-320a*-transfected PC3 cells. A SurePrint G3 Human GE 60K v3 microarray (Agilent Technologies, Santa Clara, CA, USA) was used for genome-wide expression analysis of *miR-320a* transfectants compared with mock-transfected PC3 cells (GEO accession no. GSE77790). As a result, genes fulfilling the three following conditions were listed: containing putative *miR-320a* target sites, upregulated in PCa clinical specimens, and downregulated by *miR-320a* restoration.

Immunohistochemical staining and scoring. A tissue microarray containing a total of 71 prostate samples, 51 PCa specimens, 10 prostatic intraepithelial neoplasia (PIN) samples, and 10 normal prostate samples was obtained from Provitro (Berlin, Germany; cat. no. 4012209, lot no. 146.1P.020212.27). Table III shows the characteristics of patients included in the tissue microarray. Four specimens were used as CRPC tissues (Table I, nos. 31 and 34-36). Tissue specimens were immunostained following the manufacturer's protocol with the Ultra-Vision Detection System (Thermo Scientific, Fremont, CA, USA). Primary rabbit polyclonal antibodies against androgen receptor (AR; 1:50, ab9474; Abcam, Cambridge, UK), antibodies against PSA (1:500, HPA000764;

Table I. Patient characteristics.

No.	Diagnosis	Age (years)	PSA (ng/ml)	Gleason score	cT	cN	cM
1	Non-PCa	54	5.4	-	-	-	-
2	Non-PCa	60	5.6	-	-	-	-
3	Non-PCa	67	5.9	-	-	-	-
4	Non-PCa	67	8.1	-	-	-	-
5	Non-PCa	60	14	-	-	-	-
6	Non-PCa	69	6	-	-	-	-
7	Non-PCa	56	8.4	-	-	-	-
8	Non-PCa	61	8.6	-	-	-	-
9	Non-PCa	62	35.5	-	-	-	-
10	Non-PCa	57	5.2	-	-	-	-
11	Non-PCa	64	4.4	-	-	-	-
12	Non-PCa	60	5.7	-	-	-	-
13	Non-PCa	63	11.4	-	-	-	-
14	Non-PCa	65	13.2	-	-	-	-
15	PCa	65	277	4+5	4	1	1
16	PCa	73	478	4+3	3b	0	1
17	PCa	75	1,000	4+5	4	1	1
18	PCa	79	63.2	4+5	3b	1	1
19	PCa	69	95.6	4+4	4	1	1
20	PCa	70	248	3+4	3a	1	0
21	PCa	66	36.1	4+5	3a	1	0
22	PCa	81	1,338	4+5	4	0	1
23	PCa	72	102	4+4	3a	0	0
24	PCa	65	212	4+4	4	1	1
25	PCa	81	11.4	4+4	2	0	0
26	PCa	75	22.7	4+4	3b	0	0
27	PCa	73	467	4+4	3b	1	0
28	PCa	58	482	4+4	3b	1	0
29	PCa	73	76.4	4+4	3a	0	0
30	CRPC liver metastasis	64	4,100	4+5	3b	0	1b
31	CRPC lymph node metastasis						
32	CRPC bone metastasis						
33	CRPC bone metastasis	75	4,690	4+5	3b	0	0
34	CRPC lung metastasis						
35	CRPC liver metastasis	78	3,850	3+5	4	0	1b
36	CRPC dural metastasis						
37	CRPC bone metastasis						

PSA, prostate-specific antigen; PCa, prostate cancer; CRPC, castration-resistant prostate cancer.

Sigma-Aldrich, St. Louis, MO, USA), and antibodies against LAMP1 (1:1,000; #9091; Cell Signaling Technology, Danvers, MA, USA) were used for immunochemistry. The slides were treated with biotinylated goat antibodies (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The IHC score was the sum of the score of the weighted intensity and extension of cancerous area. The intensity of staining was graded from 0 to 3 as follows: 0, negative staining; 1, mild staining; 2, moderate staining; and 3, intense staining. The area of staining in the cancerous area was graded from 0 to 3 as follows: 0, no staining of cells in any microscopic field; 1, <30% of malignant cells stained; 2, 30-60% of malignant cells stained; and 3, >60% of malignant cells stained. The procedure was carried out as described previously (14,17,18).

Western blotting. Cells were harvested 72 or 96 h after transfection, and lysates were prepared. Cell lysates (20 μg protein) were separated on Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was carried out with rabbit anti-LAMP1 antibodies (1:2,000; #9091; Cell Signaling Technology); anti-GAPDH antibodies (1:4,000; ab8245;

Table II. Candidate target genes regulated by *miR-320a* in PCa.

Gene symbol	Gene name	PC3 *miR-320a* transfectant	No. of conserved sites	No. of poorly conserved sites	GEO fold change
CNOT6	CCR4-NOT transcription complex, subunit 6	-1.4261212	1	4	0.539992
VDAC1	Voltage-dependent anion channel 1	-1.2843819	1	0	0.609059
LAMP1	Lysosomal-associated membrane protein 1	-0.8793476	1	0	0.623045
TPD52	Tumor protein D52	-0.8678817	1	0	0.606609
SERBP1	SERPINE1 mRNA binding protein 1	-0.6758766	1	0	0.709015
HELZ	Helicase with zinc finger	-0.65864474	2	1	0.675884
MYEF2	Myelin expression factor 2	-0.6576734	1	1	0.597199
FUS	Fused in sarcoma	-0.6464454	1	1	0.568564
NEDD4L	Neural precursor cell expressed, developmentally downregulated 4-like	-0.6353359	1	0	1.241135
FAM117B	Family with sequence similarity 117, member B	-0.62850374	1	4	0.729023
NUFIP2	Nuclear fragile X mental retardation protein interacting protein 2	-0.60588837	3	1	0.775047
ZC3H7B	Zinc finger CCCH-type containing 7B	-0.5742502	2	1	0.571856
RANBP2	RAN binding protein 2	-0.55872875	1	1	0.562369
FBXO28	F-box protein 28	-0.52747154	1	0	0.591344

Abcam) were used as an internal loading control. Membranes were washed and incubated with anti-rabbit IgG horseradish peroxidase (HRP)-linked antibodies (7074; Cell Signaling Technology). Complexes were visualized with Clarity Western ECL Substrate (Bio-Rad). The procedure was performed as described previously (14,17,18).

Plasmid construction and dual-luciferase reporter assays. The partial wild-type sequence of the *LAMP1* 3'-UTR or that with deletion of the *miR-320a* target site was inserted between the *Xho*I-*Pme*I restriction sites in the 3'-UTR of the *hRluc* gene in the psiDHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for the dual-luciferase reporter assay was described previously (14,17,18).

Statistical analysis. The relationships between 2 groups and the numerical values obtained by RT-PCR were analyzed using Mann-Whitney U tests. The relationships among more than three variables and numerical values were analyzed using Bonferroni-adjusted Mann-Whitney U tests. All analyses were performed using Expert StatView (version 5; SAS Institute Inc., Cary, NC, USA).

Results

Expression levels of miR-320a in PCa specimens and cell lines. First, we evaluated the expression of *miR-320a* in clinical prostate specimens and cell lines. The median PSA level of patients with normal prostate tissues was 7.05 ng/ml (range, 4.4-35.5 ng/ml), and that of patients with PCa was 212 ng/ml (range, 11.4-1338 ng/ml). Of the patients with PCa, only 4 patients had organ-confined disease (Table I). The expression levels of *miR-320a* were significantly lower in cancer tissues than in non-cancerous tissues (P<0.0001; Fig. 1). In PC3 and DU145 cells, expression levels of *miR-320a* were relatively low compared with those in clinical specimen and were lower than those in normal prostate cells (RWPE-1 cells; Fig. 1).

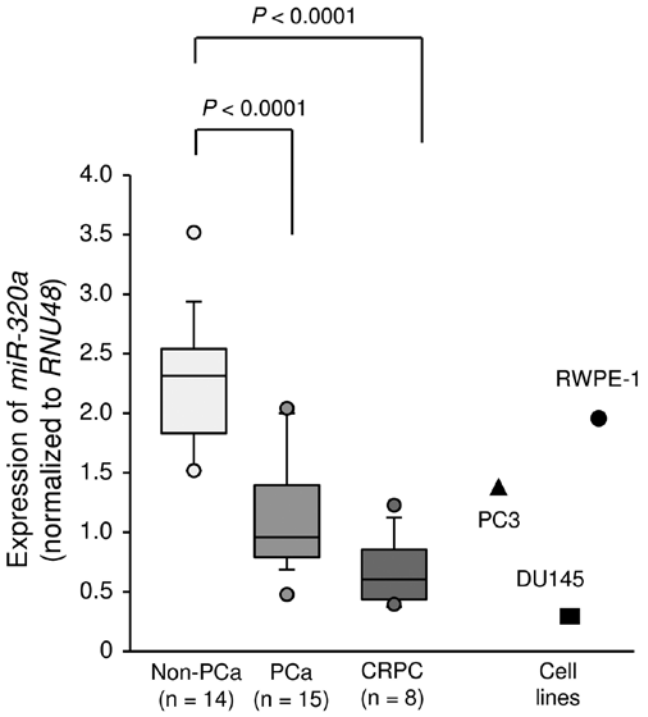

Figure 1. Expression levels of *miR-320a* in clinical prostate cancer specimens and cell lines. Expression levels of *miR-320a* were determined by RT-PCR. RNU48 was used for normalization.

Effects of miR-320a restoration on the proliferation, migration, and invasion of PCa cells. To investigate the functional effects of miR-320a, we performed gain-of-function studies

Table III. Characteristics of patients included in the tissue microarray analysis.

No.	Diagnosis	Age (years)	Gleason score	Stage pT	Stage pN
1	PCa	64	4+3	3b	0
2	PCa	67	3+4	2b	0
3	PCa	58	3+4	2b	0
4	PCa	63	7	3b	0
5	PCa	65	3+3	2b	0
6	PCa	61	4+4	3b	x
7	PCa	62	3+4	2b	x
8	PCa	66	4+4	2b	x
9	PCa	61	3+4	3a	x
10	PCa	74	4+3	2b	x
11	PCa	54	3+4	2c	x
12	PCa	68	3+4	3a	0
13	PCa	58	3+4	3a	0
14	PCa	65	3+3	2a	0
15	PCa	77	3+4	4	0
16	PCa	58	3+4	3a	0
17	PCa	50	4+3	2b	0
18	PCa	53	3+3	2b	0
19	PCa	59	4+5	3a	0
20	PCa	70	2+3	2b	0
21	PCa	65	5+4	3a	0
22	PCa	57	3+5	2b	0
23	PCa	68	4+4	2b	0
24	PCa	58	3+3	2b	0
25	PCa	63	3+4	2b	0
26	PCa	56	3+4	2b	0
27	PCa	63	5+3	3a	0
28	PCa	64	3+5	3a	0
29	PCa	60	3+4	2b	0
30	PCa	60	3+3	3a	0
31	PCa	57	3+2	2b	0
32	PCa	50	3+3	2a	0
33	PCa	68	3+3	3a	0
34	PCa	65	3+4	3b	1
35	PCa	69	5+5	3a	1
36	PCa	51	2+3	2b	0
37	PCa	62	3+3	3a	0
38	PCa	61	3+4	3a	0
39	PCa	53	4+4	3b	1
40	PCa	56	4+3	2b	0
41	PCa	59	2+3	2b	0
42	PCa	61	3+4	2b	0
43	PCa	62	3+4	3b	1
44	PCa	66	3+3	3a	0
45	PCa	62	3+3	2b	0
46	PCa	56	3+3	2b	0
47	PCa	58	3+3	3a	0
48	PCa	66	5+4	3a	0
49	PCa	55	3+4	3a	0
50	PCa	67	2+3	2b	0
51	PCa	61	3+5	2b	0
52	PIN	59	-	-	-
53	PIN	58	-	-	-
54	PIN	62	-	-	-
55	PIN	51	-	-	-
56	PIN	58	-	-	-
57	PIN	68	-	-	-
58	PIN	64	-	-	-
59	PIN	56	-	-	-
60	PIN	61	-	-	-
61	PIN	51	-	-	-
62	Normal	70	-	-	-
63	Normal	63	-	-	-
64	Normal	62	-	-	-
65	Normal	81	-	-	-
66	Normal	67	-	-	-
67	Normal	76	-	-	-
68	Normal	66	-	-	-
69	Normal	69	-	-	-
70	Normal	63	-	-	-
71	Normal	71	-	-	-

PCa, prostate cancer; PIN, prostatic intra-epithelial neoplasia.

using miRNA transfection into PC3 and DU145 cells. XTT assays revealed that cell proliferation was not inhibited in *miR-320a* transfectants in comparison with that in mock- or miR-control-transfected PC3 and DU145 cells (Fig. 2A). However, cell migration and invasion activities were significantly inhibited in *miR-320a* transfectants in comparison with those in mock- or miR-control-transfectants ($P<0.0001$; Fig. 2B and C).

Identification of candidate target genes of miR-320a in PCa cells. To identify target genes regulated by *miR-320a*, we performed a combination of *in silico* analysis and genome-wide gene expression analysis. First, we screened genes having putative 3'-UTR sites matching the seed sequence of *miR-320a*. Using the TargetScan online program, 5,188 genes with putative target sites for *miR-320a* were selected. Next, to choose upregulated genes in PCa clinical specimens compared with normal prostatic tissue, the genes were analyzed with available gene expression data from GEO (accession no. GSE29079), which compared 48 normal and 47 PCa tissue samples. As a result, 713 genes upregulated ($\log_2$ ratio >0.5) in PCa tissue were selected. To gain further insight into which genes were

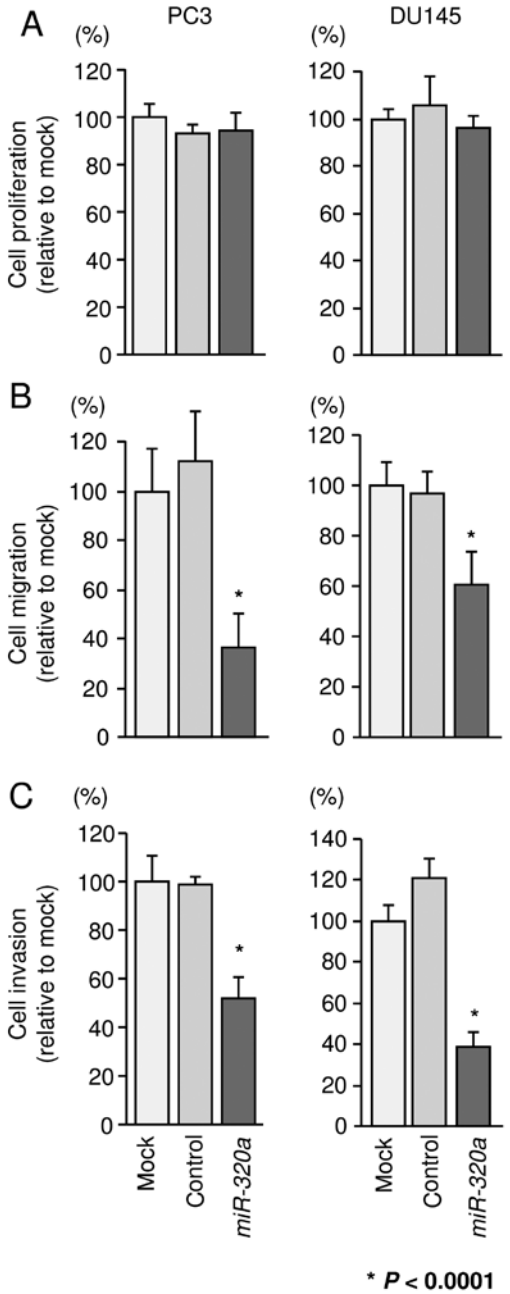

Figure 2. Effects of *miR-320a* restoration on the proliferation, migration, and invasion of PCa cells. (A) Cell proliferation was determined 72 h after transfection using XTT assays. (B) Cell migration activity was determined 48 h after transfection using Boyden chamber migration assays. (C) Cell invasion activity was determined 48 h after transfection using Matrigel invasion assays. *P<0.0001. Experiments were performed triplicate. Bars indicate SDs.

affected by tumor-suppressive *miR-320a* in PCa, we carried out genome-wide gene expression analysis using a microarray comparing *miR-320a*-transfected and mock-transfected PC3 cells (deposited in the GEO database, accession no. GSE77790). Consequently, a total of 14 candidate genes were identified as target genes of *miR-320a* (Table II). Of these, we focused on the *LAMP1* gene for further analyses because the functional significance of *LAMP1* in PCa had not been reported. A flow chart outlining our strategy for identification of candidate target genes of *miR-320a* is shown in Fig. 3.

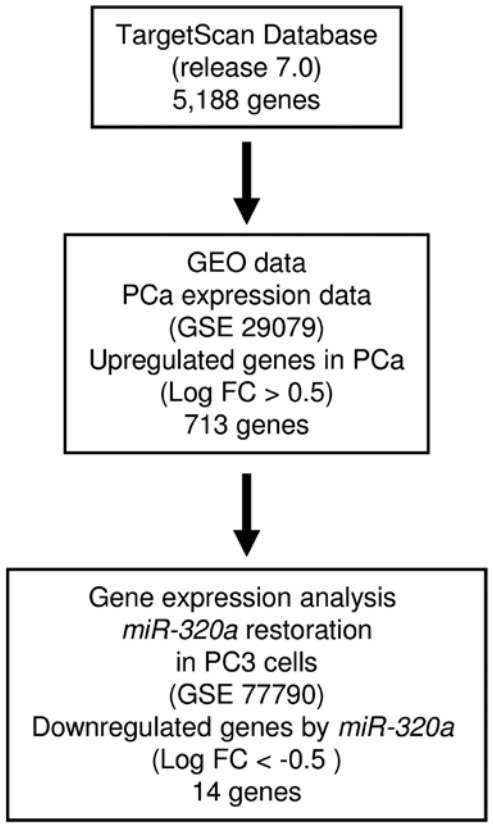

Figure 3. Strategy for identification of candidate target genes of *miR-320a*. TargetScan Release 7.0 was used to identify genes containing the *miR-320a* seed sequence in the 3'-UTR. Next, we analyzed the gene expression dataset using the GEO database (GEO accession no. GSE29079). Finally, we attempted to identify *miR-320a* target genes using *miR-320a*-transfected PC3 cells (GEO accession no. GSE77790).

LAMP1 was strongly expressed in clinical PCa/CRPC specimens. To analyze whether LAMP1 was upregulated in PCa clinical specimens, we carried out immunohistochemical staining of PCa, PIN, prostatic hyperplasia (non-PCa), and CRPC tissues. Using a tissue microarray, we confirmed that LAMP1 expression was significantly increased in PCa specimens compared with that in non-PCa specimens (Fig. 4B; P<0.0001). Representative images of IHC staining for *LAMP1* in the tissue microarray are shown in Fig. 4A. In CRPC specimens, we also found strong expression of LAMP1 (Fig. 5).

miR-320a directly regulated LAMP1 in PCa cells. Next, we performed quantitative RT-PCR and western blotting to analyze whether restoration of *miR-320a* in PC3 and DU145 cells reduced the expression of LAMP1. As shown in Fig. 6A and B, the expression levels of LAMP1 were significantly repressed by *miR-320a* transfection in comparison with those in mock- or miR-control-transfected cells.

Next, to determine whether *LAMP1* mRNA was directly regulated by *miR-320a*, we performed luciferase reporter assays. We used vectors encoding either the partial wild-type sequence of the 3'-UTR of *LAMP1*, including the predicted *miR-320a* target site (position 692-699 of the *LAMP1* 3'-UTR), or deletion vectors lacking this position. We found that the luminescence intensities were significantly reduced

Table IV. Genes downregulated by silencing of *LAMP1* in PC3 cells.

Gene symbol	Gene name	Log_2(si-*LAMP1*/mock)
LAMP1	Lysosomal-associated membrane protein 1	-5.447
ANLN	Anillin, actin binding protein	-2.699
KIF2C	Kinesin family member 2C	-2.610
KIF14	Kinesin family member 14	-2.595
AXL	AXL receptor tyrosine kinase	-2.594
CCNA2	Cyclin A2	-2.581
SPC25	SPC25, NDC80 kinetochore complex component	-2.543
NEK2	NIMA-related kinase 2	-2.507
ESCO2	Establishment of sister chromatid cohesion N-acetyltransferase 2	-2.478
MYL10	Myosin, light chain 10, regulatory	-2.464
HMGB2	High mobility group box 2	-2.440
HIST2H3A	Histone cluster 2, H3a	-2.432
MZT1	Mitotic spindle organizing protein 1	-2.416
CCNE2	Cyclin E2	-2.404
NUF2	NUF2, NDC80 kinetochore complex component	-2.403
CENPF	Centromere protein F, 350/400 kDa	-2.378
RRM2	Ribonucleotide reductase M2	-2.377
CDK1	Cyclin-dependent kinase 1	-2.319
SKA1	Spindle and kinetochore associated complex subunit 1	-2.301
PBK	PDZ binding kinase	-2.293
TOP2A	Topoisomerase (DNA) IIα 170 kDa	-2.274
CASC5	Cancer susceptibility candidate 5	-2.233
lnc-AKR1C2-3	lnc-AKR1C2-3:2	-2.224
NUSAP1	Nucleolar and spindle associated protein 1	-2.212
CEP55	Centrosomal protein 55 kDa	-2.201
UTS2	Urotensin 2	-2.188
SKA3	Spindle and kinetochore associated complex subunit 3	-2.185
HIST1H3B	Histone cluster 1, H3b	-2.184
G3BP2	GTPase activating protein (SH3 domain) binding protein 2	-2.174
SPC248	SPC24, NDC80 kinetochore complex component	-2.170
CENPA	Centromere protein A	-2.135
GNB4	Guanine nucleotide binding protein (G protein), β polypeptide 4	-2.131
MKI67	Marker of proliferation Ki-67	-2.119
TK1	Thymidine kinase 1, soluble	-2.118
MKI67	Marker of proliferation Ki-67	-2.115
GLUD2	Glutamate dehydrogenase 2	-2.102
TYMS	Thymidylate synthetase	-2.099
E2F8	E2F transcription factor 8	-2.087
LOC255187	Uncharacterized LOC255187	-2.076
KIF4A	Kinesin family member 4A	-2.073
CREG2	Cellular repressor of E1A-stimulated genes 2	-2.073
FAM83D	Family with sequence similarity 83, member D	-2.068
HIST1H1D	Histone cluster 1, H1d	-2.063
DTL	Denticleless E3 ubiquitin protein ligase homolog (*Drosophila*)	-2.062
FAM72D	Family with sequence similarity 72, member D	-2.054
KIF15	Kinesin family member 15	-2.042
ANXA1	Annexin A1	-2.022
FAM72A	Family with sequence similarity 72, member A	-2.020
NMU	Neuromedin U	-2.019
BIRC5	Baculoviral IAP repeat containing 5	-2.019
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	-2.016
E2F2	E2F transcription factor 2	-2.015
RAB31	RAB31, member RAS oncogene family	-2.010

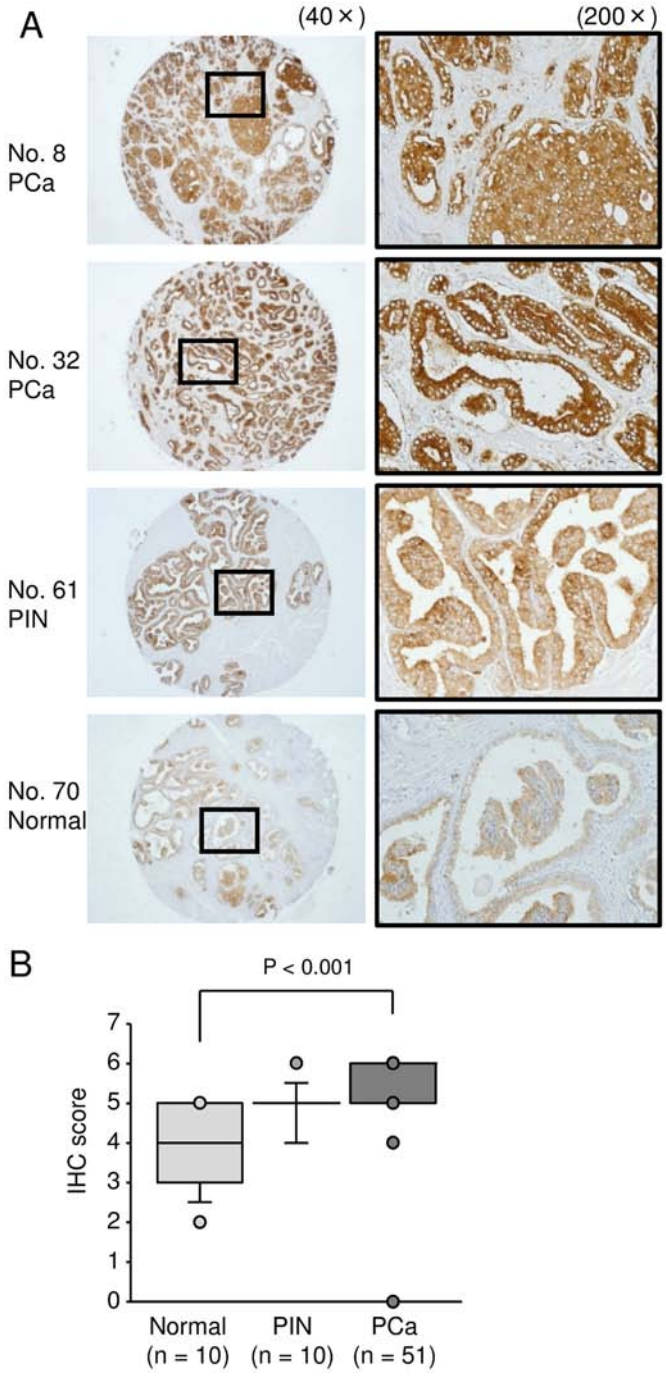

Figure 4. Expression of LAMP1 in clinical PCa specimens using a tissue microarray. (A) Representative immunohistochemical staining for LAMP1 in a prostate cancer tissue microarray (Table III; nos. 8, 32, 61 and 70). LAMP1 was strongly expressed in PCa. (B) Comparison of immunohistochemical staining of LAMP1 in prostate cancer tissues using IHC scores. LAMP1 expression was significantly higher in PCa tissues than in normal prostate tissues.

by transfection with *miR-320a* and the vector carrying the wild-type 3'-UTR of *LAMP1*, whereas transfection with the deletion vector blocked the decrease of luminescence in PC3 and DU145 cells. These data suggested that *miR-320a* bound directly to this site in the 3'-UTR of *LAMP1* (Fig. 6C).

Effects of silencing LAMP1 on cell proliferation, migration, and invasion in PCa cell lines. To investigate the functional role of LAMP1, we performed loss-of-function studies using si-*LAMP1* transfectants. First, we evaluated the knockdown efficiency of si-*LAMP1* transfection in PC3 and DU145 cells. Real-time PCR and western blotting indicated that two siRNAs (si-*LAMP1*-1 and si-*LAMP1*-2) could effectively reduce the expression of LAMP1 in PC3 and DU145 cells (Fig. 7A and B).

Using these two siRNAs, we carried out functional analyses. XTT assays demonstrated that cell proliferation was inhibited only in PC3 cells by si-*LAMP1* transfection (Fig. 7C). Cell migration and invasion activities were significantly inhibited in si-*LAMP1* transfectants in both PC3 and DU145 in comparison with mock or negative control transfectants (Fig. 7D and E).

Downstream genes affected by silencing of LAMP1 in PCa cells. To further investigate which genes are modulated by *LAMP1* signaling, we performed genome-wide gene expression analysis using si-*LAMP1* in PC3 cells. A SurePrint G3 Human GE 60K v3 microarray was used for genome-wide expression analysis. The raw data were submitted to the GEO database (accession no. GSE77790). In this study, we selected significantly downregulated genes by si-*LAMP1*-1 or si-*LAMP1*-2 transfection [Log_2(si-*LAMP1*/mock) <-2.0]. *LAMP1* was the most significantly downregulated gene, indicating the reliability of this array analysis. Genes significantly downregulated by silencing of *LAMP1* are shown in Table IV.

Discussion

Metastatic PCa initially responds to androgen-deprivation therapy; however, the cancer cells gradually acquire resistance to first-line androgen-deprivation therapy and consequently progress to CRPC. There are no effective treatments for preventing progression of metastasis in patients with CRPC, and this condition significantly affects the survival rates of men with advanced PCa (2,19). Therefore, developing a deeper understanding of the molecular mechanisms of metastatic pathways in advanced PCa will facilitate the development of novel treatment options for the disease. Recently, many studies have shown that aberrantly expressed miRNAs disrupt normally regulated RNA networks in cancer cells, and these events trigger cancer development, progression, and metastasis (4,10). Based on this background, we have identified tumor-suppressive miRNAs that specifically block cancer cell migration and invasion in PCa cells (9,11-18,20).

Our recent studies indicated that the *miR-221/miR-222* cluster is significantly downregulated in PCa and CRPC specimens and suppresses cancer cell aggressiveness by targeting *Ecm29*, a scaffold protein that links the 26S proteasome to motor proteins (17). Moreover, we showed that tumor-suppressive *miR-223* inhibits cancer cell migration and invasion by targeting *ITGA3* and *ITGB1* (18). Interestingly, knockdown of *ITGB1* significantly inhibits downstream oncogenic signaling pathways, including the focal adhesion kinase (FAK), AKT, and extracellular signal-regulated kinase (ERK) pathways (18). Thus, suppression of ITGA3/ITGB1 signaling in PCa cells may have applications in the development of novel therapies for PCa.

In this study, we focused on *miR-320a* because our miRNA signatures of hormone-naïve PCa and CRPC showed

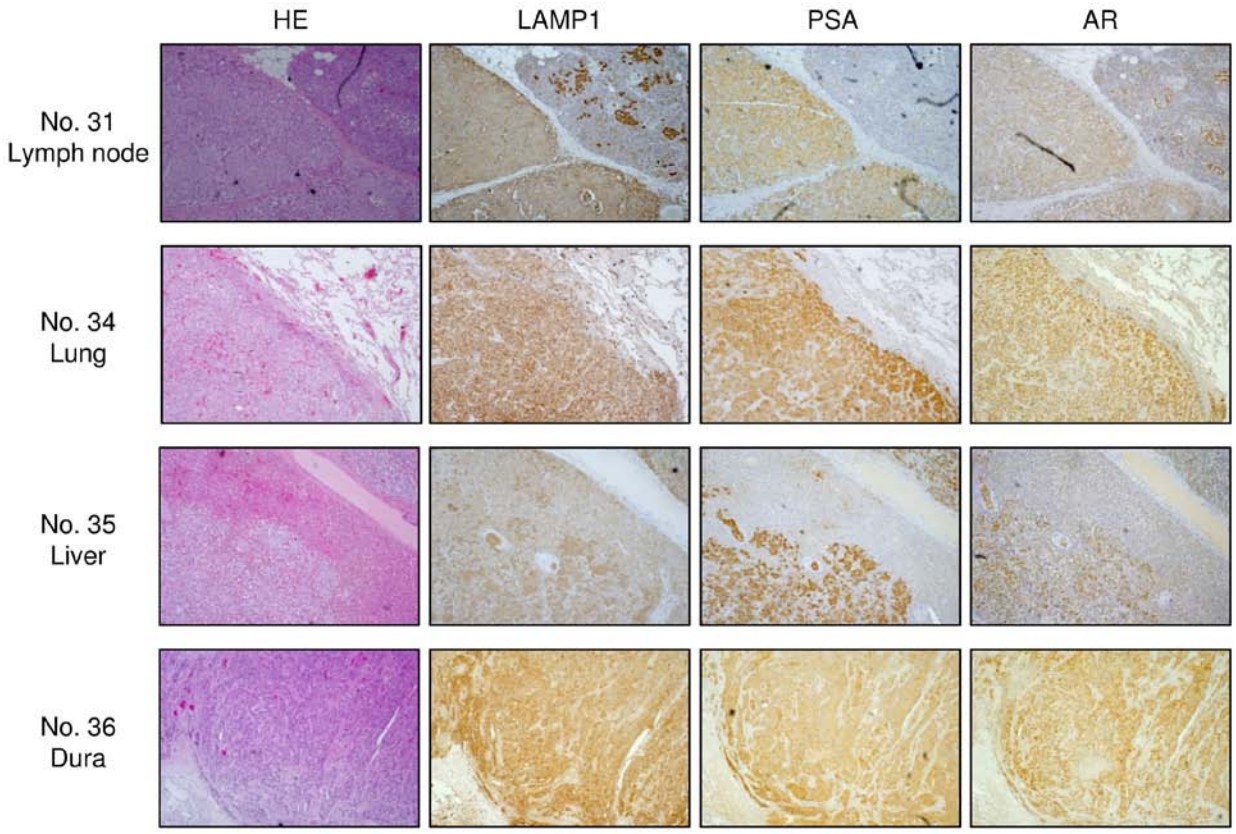

Figure 5. Expression of LAMP1 in clinical CRPC specimens. Representative immunostaining for LAMP1 in CRPC clinical specimens. PSA and AR were used to confirm prostate cancer metastasis.

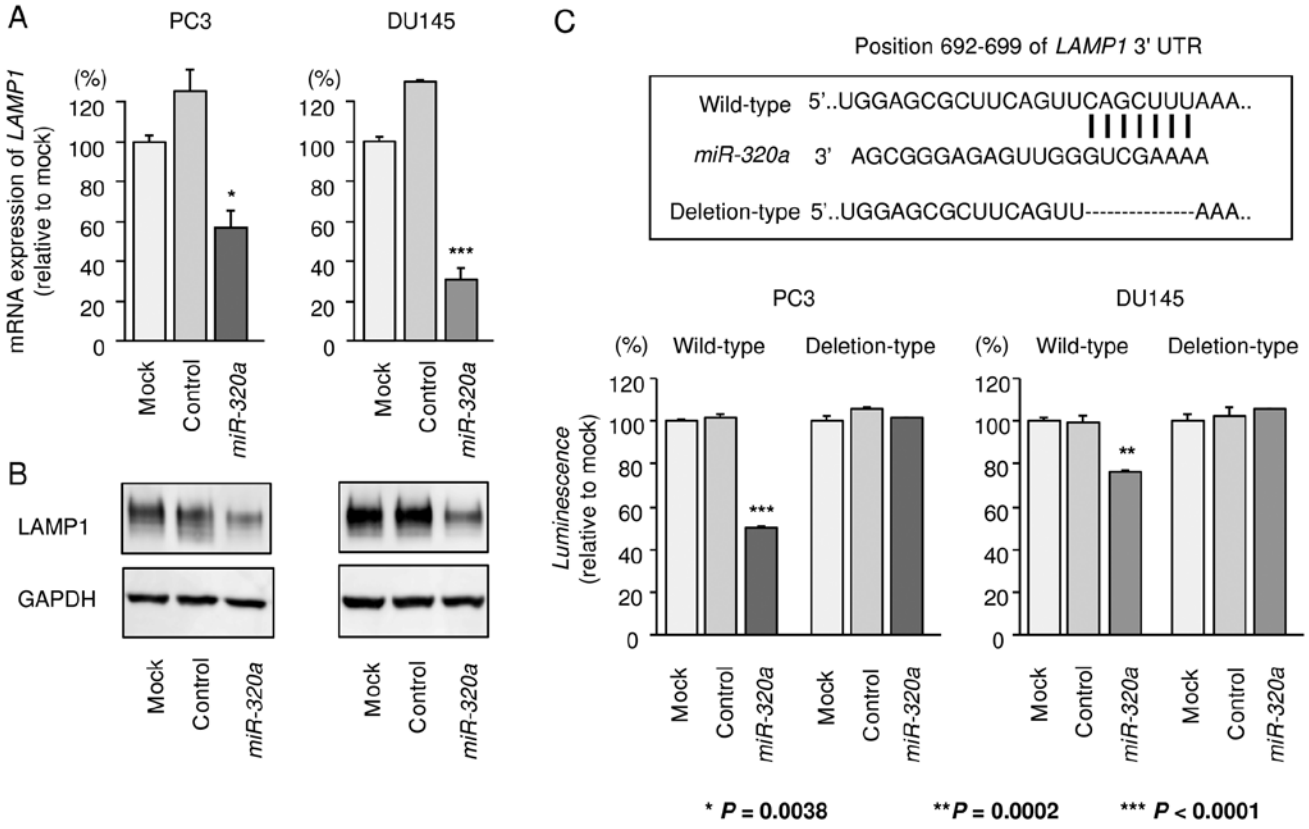

Figure 6. Direct regulation of *LAMP1* by *miR-320a*. (A) *LAMP1* mRNA expression was determined at 96 h after transfection of *miR-320a*. *GAPDH* was used as an internal control. (B) LAMP1 protein expression 72 h after transfection with *miR-320a*. GAPDH was used as a loading control. (C) Luciferase reporter assays using a vector encoding the putative *miR-320a* target site in the *LAMP1* 3'-UTR (wild-type and deletion constructs). *P=0.0038, **P=0.0002, and ***P<0.0001. Experiments were performed in triplicate. Bars indicate SDs.

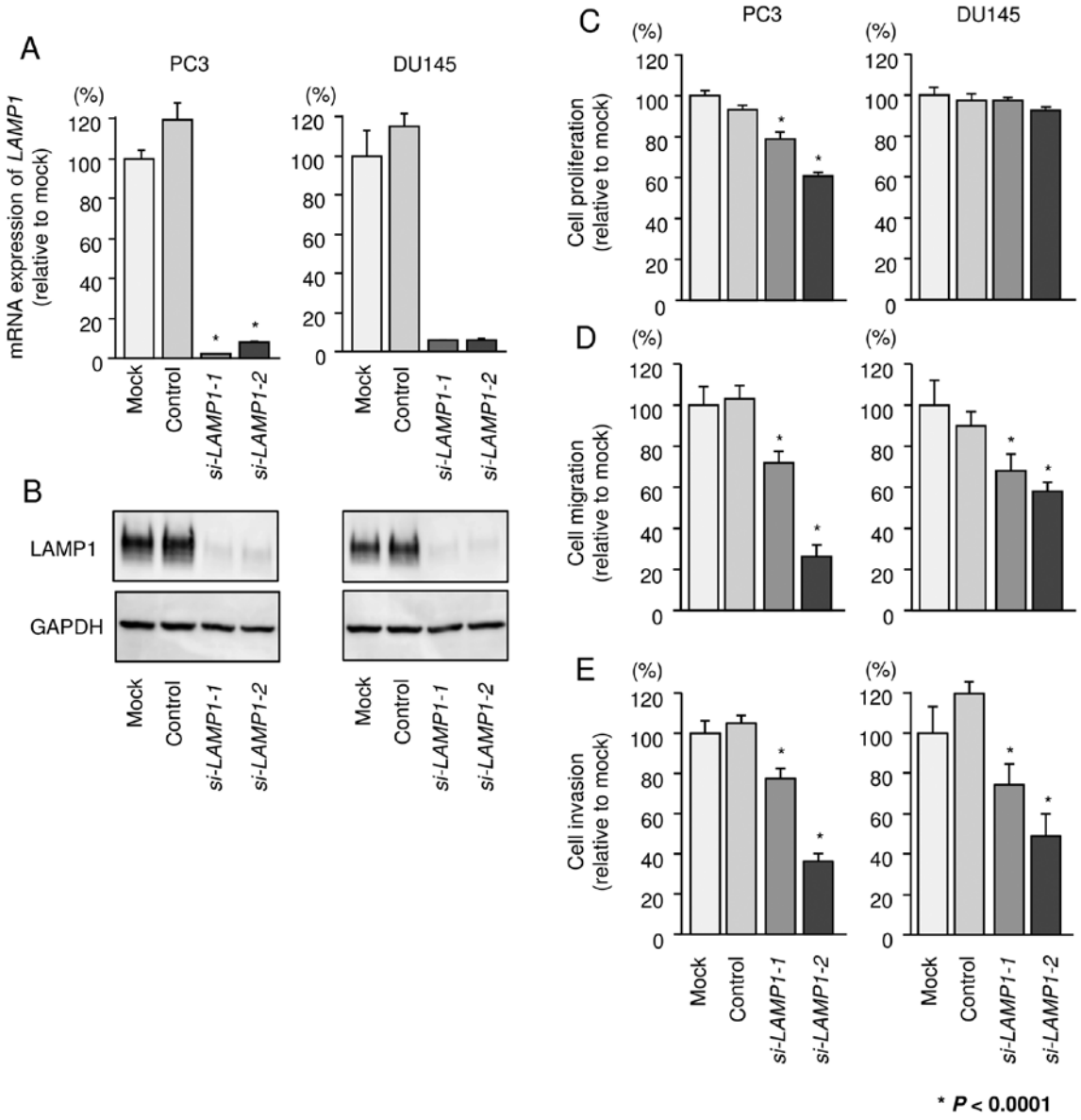

Figure 7. Effects of silencing *LAMP1* on cell proliferation, migration, and invasion in PCa cell lines. (A) *LAMP1* mRNA expression was determined at 72 h after transfection with si-*LAMP1*. *GAPDH* was used as an internal control. (B) LAMP1 protein expression was evaluated by western blotting at 72 h after transfection with si-*LAMP1*. GAPDH was used as a loading control. (C) Cell proliferation was determined by XTT assays. (D) Cell migration activity was determined by Boyden chamber migration assays. (E) Cell invasion activity was determined by Matrigel invasion assays. *P<0.0001. Experiments were performed in triplicate. Bars indicate SDs.

that *miR-320a* was significantly downregulated in cancer tissues (9,17). We validated the low expression of *miR-320a* in hormone-naïve PCa and CRPC clinical specimens. Restoration of *miR-320a* significantly inhibited cancer cell migration and invasion in both PC3 and DU145 cell lines, suggesting that *miR-320a* acted as a tumor-suppressive miRNA in PCa cells.

The anticancer effects of *miR-320a* have been reported in PCa and various other types of cancers (21-23). For example, overexpression of *miR-320* inhibits β-catenin expression and the expression of cancer stem cell markers, such as CD44, Oct-4, and CD133, in PCa cells (21). β-catenin is a multifunctional protein that regulates cell adhesion and functions as a transcriptional coactivator by interacting with several transcriptional factors (21,22,24). Accumulating evidence has shown that β-catenin colocalizes with AR, and these events are more frequently observed in CRPC than in hormone-naïve PCa (25). Therefore, β-catenin/AR signaling may be potential therapeutic target for the management of CRPC. Another study demonstrated that *miR-320a* expression is reduced in primary salivary adenoid cystic carcinoma (SACC) with metastasis compared with that in SACC without metastasis (26). Overexpression of *miR-320a* inhibits the adhesion, invasion, and migration of SACC cells through regulation of *ITGB3* (26). Chronic myeloid leukemia (CML) is a myeloproliferative disease involving the BCR/ABL fusion protein. Interestingly, a recent study showed that *miR-320a* expression is reduced in K562 cells and CML cancer stem cells. Additionally, restoration of *miR-320a* inhibits K562 cell migration, invasion, and proliferation and promotes apoptosis by targeting the BCR/ABL oncogene (27). These studies indicated that

continuous analysis of tumor-suppressive *miR-320a*-regulated oncogenic pathways may provide insights into novel RNA networks in cancer cells.

To better understand PCa progression and metastasis, we identified *miR-320a* target genes using a combination of *in silico* and genome-wide gene expression analyses. Recent miRNA studies in our laboratory have utilized this strategy to identify novel molecular targets and pathways regulated by tumor-suppressive miRNAs in several cancers, including PCa (17,18). A total of 14 putative target genes of *miR-320a* were identified in this study. Among these genes, we focused on the *LAMP1* gene because few reports have described the role of this target in PCa. Here, we found that downregulation of *LAMP1* inhibited the migration and invasion of cancer cells. Additionally, overexpression of LAMP1 was observed in hormone-naïve PCa and CRPC clinical specimens.

LAMP1 is a heavily glycosylated lysosomal membrane protein that is expressed mainly in the endosome-lysosomal membrane of cells (28,29). High expression of LAMP1 on the plasma membrane has been observed in several metastatic cell lines, including human melanoma, colon carcinoma, fibrosarcoma, and myelomonocytic leukemia cell lines (30-33). Previous studies have indicated that cell surface glycosylation in cancer cells differs from that in normal cells (30). Additionally, the expression of β1,6 branched N-oligosaccharides on the cell surface in several human cancers has been shown to be correlated with the malignant potential of the cells (34). LAMP1 acts as a major carrier of β1,6 branched N-oligosaccharides in B16 melanoma cells (31). A recent study also showed that LAMP1 expression on the cell surface enhances lung metastasis by providing ligands for galectin-3 (Gal-3), a member of the multifunctional β-galactoside binding lectin family (31). Gal-3 has high affinity for β-1,6-N-acetylglucosamine branched glycans. This interaction mediates binding of the lectin to many glycoproteins in the cell membrane, such as cadherin, integrins, and growth factor receptors, and these events may enhance and promote cancer cell growth and metastasis (35). Several studies have demonstrated that Gal-3 plays pivotal roles in cancer progression and aggressiveness by regulating cell proliferation, apoptosis, invasion, and metastasis (35,36). Moreover, overexpression of Gal-3 promotes PCa cell progression, and its heterogeneous expression may be associated with different PCa subtypes (37). These studies indicate that cell surface expression of LAMP1 may affect Gal-3 activation and mediate multiple stages of cancer progression and metastasis.

In this study, we identified LAMP1-mediated cancer pathways by using genome-wide gene expression analysis of si-*LAMP1*-transfected cells. Our data showed that several genes known to contribute to cancer cell aggressiveness, including *ANLN, AXL, CCNA2* and *CENPF* (16,38-40), were involved in LAMP1 downstream pathways. The identification of these novel molecular targets mediated by the *miR-320a/LAMP1* axis may lead to a better understanding of PCa progression and metastasis.

In conclusion, downregulation of *miR-320a* was observed in PCa and CRPC clinical specimens, and this miRNA was shown to function as a tumor-suppressive miRNA in PCa cells. *LAMP1* was directly regulated by tumor-suppressive *miR-320a* and contributed to cancer cell aggressiveness. The identification of novel molecular pathways regulated by the *miR-320a/LAMP1* axis may lead to a better understanding of PCa progression and metastasis.

Acknowledgements

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