Tumor-suppressive microRNA-218 inhibits cancer cell migration and invasion via targeting of LASP1 in prostate cancer

Rika Nishikawa,1,2 Yusuke Goto,1,2 Shinichi Sakamoto,2 Takeshi Chiyomaru,2 Hideki Enokida,3 Satoko Kojima,4 Takashi Kinoshita,1 Noriko Yamamoto,1 Masayuki Nakagawa,3 Yukio Naya,4 Tomohiko Ichikawa2 and Naohiko Seki1

Key words
LASP1, microRNA, miR-218, prostate cancer, tumor suppressor

Correspondence
Naohiko Seki, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan.
Tel: +81-43-226-2971; Fax: +81-43-227-3442
E-mail: naoseki@faculty.chiba-u.jp

Funding information
KAKENHI (C) 24592590 and (B) 25293333.

Received January 16, 2014; Revised May 4, 2014; Accepted May 6, 2014
Cancer Sci 105 (2014) 802–811
doi: 10.1111/cas.12441

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death among men in developed countries.1–3 Most patients are initially responsive to androgen-deprivation therapy (ADT), but their cancers eventually become resistant to ADT and progress to castration-resistant prostate cancer (CRPC). CRPC is difficult to treat, and most clinical trials for advanced PCa have shown limited benefits, with disease progression and metastasis to the skeleton or other sites.2,3 Therefore, understanding the molecular mechanisms of CRPC and the metastatic pathways underlying PCa using genomic approaches would help to prevent and improve therapies for the disease.

The discovery of non-coding RNAs (ncRNAs) in the human genome was an important conceptual breakthrough in the post-genome sequencing era.4 Improved understanding of ncRNAs is necessary for continued progress in cancer research. MicroRNAs (miRNAs) are endogenous small ncRNA molecules (19–22 bases in length) that regulate the expression of protein-coding genes by repressing translation or cleaving RNA transcripts in a sequence-specific manner.5 Currently, 2578 human mature miRNAs are registered at miRBase release 20.0 (http://microrna.sanger.ac.uk/); miRNAs are unique in their ability to regulate multiple protein-coding genes. Bioinformatic predictions indicate that miRNAs regulate >30–60% of the protein-coding genes in the human genome.6,7

A significant amount of evidence suggests that miRNAs are aberrantly expressed in many human cancers and that they play significant roles in the initiation, development and metastasis of those cancers.8,9 Some highly expressed miRNAs can function as oncogenes by repressing tumor suppressors, whereas low-level miRNAs can function as tumor suppressors by negatively regulating oncogenes. It is believed that normal 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 miRNA-mediated oncogenic pathways.

Based on the foregoing discussion, we have constructed miRNA expression signatures using PCa clinical specimens and investigated the roles of miRNAs in PCa oncogenesis using differentially expressed miRNAs.10 Recently, we demonstrated that several miRNAs were downregulated in cancer tissues and that miR-1, miR-133a, miR-143 and miR-145 functioned as tumor suppressors by targeting several oncogenic genes.11,12 Based on our PCa miRNA signature, miR-218 was significantly downregulated, suggesting that this miRNA might be a candidate tumor suppressor in PCa cells. The aim of the present study was to investigate the functional significance of miR-218 in PCa and to identify novel miR-218-regulated cancer pathways and target genes involved in PCa oncogenesis and metastasis. Restoration of miR-218 in PCa cell lines (PC3 and DU145) revealed that this miRNA significantly inhibited cancer cell migration and invasion. Gene expression data and in silico analysis demonstrated that LIM and SH3 protein 1 (LASP1) is a potential target of miR-218 regulation. LASP1 is a cytoskeletal scaffold protein that plays critical roles in cytoskeletal organization and cell migration. Luciferase reporter assays showed that miR-218 directly regulated expression of LASP1. Moreover, downregulating the LASP1 gene significantly inhibited cell migration and invasion in cancer cells, and the expression of LASP1 was upregulated in cancer tissues. We conclude that loss of tumor-suppressive miR-218 enhanced cancer cell migration and invasion in PCa through direct regulation of LASP1. Our data on pathways regulated by tumor-suppressive miR-218 provide new insight into the potential mechanisms of PCa oncogenesis and metastasis.
was to investigate the functional significance of miR-218 in cancer cells and to identify novel miR-218-regulated genes that contributed to PCa oncogenesis and metastasis.

We found that restoration of mature miR-218 inhibited cancer cell migration and invasion, directly targeting LIM and SH3 protein 1 (LASP1). Downregulating the LASP1 significantly inhibited cell migration and invasion by cancer cells. Furthermore, LASP1-regulated novel molecular pathways were investigated through the use of si-LASP1-treated cells. Tumor-suppressive miR-218-LASP1-mediated cancer pathways might provide new insights into the potential mechanisms of PCa oncogenesis and metastasis.

Materials and Methods

Clinical prostate specimens. Seventeen radical prostatectomy specimens were obtained from patients with PCa who underwent treatment at Chiba University Hospital (Chiba, Japan) from 2009 to 2013. Seventeen paired samples of PCa and corresponding normal tissues were used for the present study. The samples considered normal were free of cancer cells as determined by pathologic examination. The patients’ backgrounds and clinico-pathological characteristics are summarized in Table 1. Before tissue collection, all patients provided written informed consent of tissue donation for research purposes. The protocol was approved by the Institutional Review Board of Chiba University.

Cell culture and RNA extraction. PC3 and DU145 cells, human PCa cells obtained from the American Type Culture Collection (Manassas, VA, USA), were maintained in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Total RNA was isolated from formalin-fixed paraffin-embedded (FFPE) samples with four 5-µm thick slices, using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time RT-PCR. The procedure for PCR quantification was described as previously (11–13). The TaqMan probes and primers for LASP1 (P/N: Hs01078815_m1 [Applied Biosystems, Foster City, CA, USA]) and for GUSB (the internal control; P/N: Hs00939627_m1 [Applied Biosystems]) were assay-on-demand gene expression products. The expression levels of miR-218 (Assay ID: 000521 [Applied Biosystems]) were analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay [Applied Biosystems]) and normalized to the expression of RNU48 (Assay ID: 001006 [Applied Biosystems]). All reactions were performed in triplicate, and each assay included negative control reactions that lacked cDNA.

Transfections with mature microRNA and siRNA. The following mature microRNA species were used in the present study: mature miRNA, Pre-miR miRNA Precursor (has-miR-218; P/N: AM17100 [Applied Biosystems]). The following siRNAs were used: Stealth Select RNAi siRNA, si-LASP1 (P/N: HSS105970 [Invitrogen, Carlsbad, CA, USA]) and negative control miRNA/siRNA (P/N: AM17111 [Applied Biosystems]). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen) as described previously. The transfection efficiencies of miRNA in PC3 and DU145 cells were confirmed based on downregulation of TWF1 (PTK9) mRNA following transfection with miR-1 as previously reported. (13)

Cell proliferation, migration and invasion assays. Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 96-well plates at 3 × 10³ cells per well. After 72 h, cell proliferation was determined with the XTT assay using a Cell Proliferation Kit II (Roche Molecular Biochemicals, Mannheim, Germany) as previously reported. (14,15) Cell migration was evaluated with a wound healing assay. Cells were plated in 6-well plates, and the cell monolayers were scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wounding were calculated from photomicrographs.

A cell invasion assay was carried out using modified Boyden chambers containing Transwell membrane filter inserts ( precoated with Matrigel) with 8 µm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA) at 2 × 10⁵ cells per well. Cells were transfected with 10 nM miRNA or siRNA by reverse transfection and plated in 10-cm dishes at 8 × 10⁵ cells per dish. After 48 h, the cells were collected, and 2 × 10⁵ cells were added to the upper chamber of each migration well.

Cells were allowed to invade for 48 h. After gentle removal of the non-migratory cells from the filter surface of the upper chamber, the cells that invaded into the lower chamber were fixed and stained with Diff-Quick (Sysmex Corporation, Kobe, Japan). The number of cells that migrated to the lower surface was determined microscopically by counting four areas of constant size per well. All experiments were performed in triplicate.

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. 50 µg protein lysates were separated on Mini-PROTEAN TGX Gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with mouse anti-LASP1 antibodies (1:250; HSA012072 [Sigma-Aldrich, St Louis, MO, USA]); anti-GAPDH antibodies (1:1000; ab8245 [Abcam, Cambridge, UK]) were used as an internal loading control.

Screening of miR-218 and LASP1 target genes using in silico analysis and gene expression data. Genes regulated by miR-218 were listed using the TargetScan database as described previously (14,15). To investigate the expression status of candidate miR-218-target genes in PCa clinical specimens, we examined gene expression profiles in the Gene Expression Omnibus (GEO database) (accession number: GSE29079). In addition, we performed gene expression analysis using miR-218-transfected PC3 cells and si-LASP1-transfected PC3 cells compared to control transfection cells. Oligo-microarray Human 60K (Agilent Technologies) was used for gene expression studies. Microarray procedures and data mining methods were as described previously (14,15).

Molecular pathway analysis using Kyoto Encyclopedia of Genes and Genomes pathways. To identify molecular signaling pathways regulated by miR-218 or LASP1 in PCa cells, in silico and gene expression data were adapted to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways categories by the GENECODIS program (http://genecodis.dacya.ucm.es). The strategy of the analysis procedure was described previously. (12)

Plasmid construction and dual-luciferase reporter assay. Partial wild-type sequences of the LASP1 3’-UTR or those with a deleted miR-218 target sites (positions 686-692, 1587-1593 and 2080-2087 of the LASP1 3’-UTR) were inserted between the XhoI–PmeI restriction sites in the 3’-UTR of the hRLuc gene in the psiCHECK-2 vector (C8021 [Promega, Madison, WI, USA]). The protocol for vector construction was as described previously. (16) The synthesized DNA was cloned into the psiCHECK-2 vector. PC3 cells were transfected with 50 ng of the vector and 10 nM miR-218 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 following the manufacturer’s protocol with the UltraVision Detection System (Thermo Scientific, Fremont, CA, USA). Primary rabbit polyclonal antibodies against LASP1 (Sigma-Aldrich) were diluted 1:500. The slides were treated with biotinylated goat antibodies.

Statistical analysis. The relationships between two groups and the numerical values obtained by real-time RT-PCR were analyzed using the paired t-test. The relationship among three variables and numerical values was analyzed using the Bonferroni-adjusted Mann–Whitney U-test. All analyses were performed using Expert StatView software (version 4, SAS Institute, Cary, NC, USA).

Results

Expression levels of miR-218 in prostate cancer specimens and cell lines. To validate our past miRNA profiling results, we evaluated the expression levels of miR-218 in 17 radical prostatectomy specimens. Quantitative stem-loop RT-PCR demonstrated that miR-218 expression was significantly lower in clinical PCa specimens and PCa cell lines (PC3 and DU145) compared with non-cancerous specimens (Fig. 1). The typical FFPE specimens used for expression analysis in this study are shown in Fig. S1.

Effects of restoring miR-218 on cell proliferation, migration and invasion activities in prostate cancer cell lines. To investigate the functional effects of miR-218, we performed gain-of-function studies using miRNA transfection of PC3 and DU145 cell lines.

The XTT assay demonstrated that cell proliferation was not inhibited in miR-218 transfectants in comparison with the mock or miR-control transfectant cells (Fig. 2a).

The Matrigel invasion assay demonstrated that cell invasion activity was significantly inhibited in miR-218 transfectants in comparison with the mock or miR-control transfectant cells (Fig. 2b).

The migration assay demonstrated that cell migration activity was significantly inhibited in miR-218 transfectants in comparison with the mock or miR-control transfectant cells (Fig. 2c).

| No. | PCa or non-PCa | Age | PSA (ng/mL) | Gleason score | Stage | T | N | M |
|-----|----------------|-----|-------------|---------------|-------|---|---|---|
| 1   | PCa            | 64  | 5.4         | 3 + 4         | C     | 3a| 0 | 0 |
| 2   | PCa            | 68  | 12.8        | 3 + 5         | C     | 3a| 0 | 0 |
| 3   | PCa            | 70  | 16.1        | 4 + 5         | B     | 2b| 0 | 0 |
| 4   | PCa            | 69  | 25.8        | 4 + 5         | B     | 2b| 0 | 0 |
| 5   | PCa            | 64  | 29.9        | 4 + 3         | B     | 2b| 0 | 0 |
| 6   | PCa            | 61  | 7.9         | 3 + 4         | C     | 3a| 0 | 0 |
| 7   | PCa            | 68  | 8.8         | 4 + 5         | B     | 2b| 0 | 0 |
| 8   | PCa            | 66  | 6.1         | 4 + 3         | B     | 2b| 0 | 0 |
| 9   | PCa            | 70  | 11.8        | 4 + 4         | C     | 3b| 0 | 0 |
| 10  | PCa            | 60  | 22.1        | 3 + 4         | B     | 2b| 0 | 0 |
| 11  | PCa            | 70  | 8.9         | 3 + 4         | B     | 2a| 0 | 0 |
| 12  | PCa            | 72  | 4.5         | 3 + 4         | B     | 2b| 0 | 0 |
| 13  | PCa            | 56  | 7.1         | 3 + 4         | C     | 3a| 0 | 0 |
| 14  | PCa            | 65  | 13.1        | 4 + 3         | B     | 2b| 0 | 0 |
| 15  | PCa            | 65  | 9.5         | 4 + 4         | B     | 2b| 0 | 0 |
| 16  | PCa            | 65  | 5.8         | 4 + 3         | B     | 2a| 0 | 0 |
| 17  | PCa            | 65  | 4.6         | 5 + 4         | B     | 2b| 0 | 0 |
Table 2. Candidate of putative miR-218 target genes

| Entrez gene ID | Symbol | Gene name | Location | Fold change | miR-218 transfectant | Conserved sites | Poorly conserved sites |
|---------------|--------|-----------|----------|-------------|---------------------|----------------|------------------------|
| 5652          | PRSS8  | Protease, serine, 8 | 16p11.2  | 1.58        | -2.08               | 0              | 1                      |
| 7163          | TPD52  | Tumor protein D52 | 8q21     | 1.52        | -2.22               | 2              | 1                      |
| 5591          | PRKDC  | Protein kinase, DNA-activated, catalytic polypeptide | 8q11     | 1.43        | -1.88               | 0              | 2                      |
| 22858         | ICK    | Intestinal cell (MAK-like) kinase | 6p12.1   | 1.41        | -2.28               | 2              | 1                      |
| 10893         | CCNI   | Cyclin I | 4q21.1   | 1.39        | -1.56               | 0              | 1                      |
| 8871          | SYNJ2  | Synaptojanin 2 | 6q25.3   | 1.39        | -1.77               | 0              | 1                      |
| 5054          | SERPINE1 | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 7q22.1   | 1.38        | -2.56               | 0              | 1                      |
| 23051         | ZHX3   | Zinc fingers and homeoboxes 3 | 20q12    | 1.33        | -1.74               | 0              | 3                      |
| 85414         | SLC45A3 | Solute carrier family 45, member 3 | 1q32.1   | 1.32        | -1.50               | 1              | 0                      |
| 80195         | TMEM25 | Transmembrane protein 254 | 10q22.3  | 1.31        | -2.01               | 1              | 0                      |
| 9342          | SNAP29 | Synaptosomal-associated | 22q11.21 | 1.30        | -1.91               | 0              | 1                      |
| 9289          | GPR56  | G protein-coupled receptor 56 | 16q13    | 1.30        | -2.23               | 0              | 1                      |
| 6745          | SSR1   | Signal sequence receptor, alpha | 1q24.3   | 1.30        | -1.63               | 1              | 0                      |
| 3927          | LASP1  | LIM and SH3 protein 1 | 17q11-q21.3 | 1.28 | -1.96               | 1              | 2                      |
| 54443         | ANLN   | Anillin, actin binding protein | 7p15-p14 | 1.26        | -1.82               | 0              | 1                      |
| 1824          | DSC2   | Desmocollin 2 | 18q12.1  | 1.25        | -2.33               | 0              | 1                      |
| 10447         | FAM3C  | Family with sequence similarity 3, member C | 7q31     | 1.24        | -1.92               | 1              | 0                      |
| 79443         | FYCO1  | FYVE and coiled-coil domain containing 1 | 3p21.31  | 1.24        | -1.62               | 1              | 0                      |
| 4008          | LMO7   | LIM domain 7 | 13q22.2  | 1.22        | -1.75               | 1              | 0                      |
| 272           | AMPD3  | Adenosine monophosphate deaminase 3 | 11p15    | 1.22        | -1.57               | 0              | 1                      |
| 9725          | TMEM63A | Transmembrane protein 63A | 1q42.12  | 1.21        | -1.64               | 0              | 1                      |
| 9917          | FAM208 | Family with sequence similarity 20, member B | 1q25     | 1.21        | -1.63               | 1              | 1                      |
| 114908        | TMEM123 | Transmembrane protein 123 | 11q22.1  | 1.19        | -1.69               | 1              | 0                      |
| 5781          | PTPN11 | Protein tyrosine phosphatase, non-receptor type 11 | 12p24    | 1.17        | -1.50               | 1              | 0                      |
| 5175          | PECAM1 | Platelet/endothelial cell adhesion molecule 1 | 17q23.3  | 1.16        | -1.89               | 0              | 2                      |
| 23788         | MTCH2  | Mitochondrial carrier 2 | 11p11.2  | 1.16        | -2.28               | 0              | 2                      |
| 79071         | ELOVL6 | ELOVL fatty acid elongase 6 | 4q25     | 1.15        | -2.30               | 0              | 1                      |
| 6645          | SNTB2  | Syntaxin, beta 2 (dystrophin-associated protein A1, 59 kDa, basic component 2) | 16q22.1  | 1.15        | -2.34               | 1              | 1                      |
| 10613         | ERLIN1 | ER lipid raft associated 1 | 10q24.31 | 1.14        | -1.62               | 0              | 1                      |
| 112939        | NACC1  | Nucleus accumbs associated 1, BEN and BTB (POZ) domain containing | 19p13.2  | 1.14        | -1.63               | 2              | 0                      |
| 54902         | TTC19  | Tetratricopeptide repeat domain 19 | 17p12    | 1.14        | -1.74               | 0              | 1                      |
| 55604         | LRRC16A | Leucine rich repeat containing 16A | 6p22.2   | 1.13        | -2.29               | 0              | 1                      |
| 26420         | MAPK9  | Mitogen-activated protein kinase 9 | 11       | 1.13        | -1.51               | 0              | 1                      |
| 9497          | SLC4A7 | Solute carrier family 4, sodium bicarbonate cotransporter, member 7 | 3p22     | 1.12        | -1.95               | 0              | 1                      |
| 10186         | LHFP   | Lipoma HMGB fusion partner | 13q12    | 1.12        | -1.79               | 2              | 0                      |
| 66008         | TRAK2  | Trafficking protein, kinesin binding 2 | 2q33     | 1.10        | -1.67               | 0              | 1                      |
| 54621         | VSIG10 | V-set and immunglobulin domain containing 10 | 12q24.23 | 1.09        | -2.06               | 1              | 2                      |
| 357           | SHROOM2 | Shroom family member 2 | Xp22.3   | 1.08        | -1.78               | 0              | 2                      |
| 41            | ACCN2  | Acid-sensing (proton-gated) ion channel 1 | 12q12    | 1.08        | -1.51               | 1              | 0                      |
| 3248          | HPGD   | Hydroxyprostaglandin dehydrogenase 15-(NAD) | 4q34-q35 | 1.02        | -2.54               | 1              | 1                      |
Identification of candidate genes targeted by miR-218. To gain further insight into the genes affected by miR-218, we analyzed a combination of in silico and gene expression data from PCa clinical specimens. First, we screened miR-218-targeted genes using the TargetScan database and identified 2940 genes. Next, we pared down the 2940 genes based on two kinds of gene expression data as follows: (i) upregulated genes determined by the gene expression dataset of PCa clinical specimens in GEO (accession number: GSE29079); and (ii) downregulated genes (log₂ ratio < -1.5) following miR-218 transfection of PC3 cells.

In this selection, we narrowed down the miR-218 target genes for the analysis from 40 genes (Table 2). Five genes (TPD52, ICK, ZHX3, LASP1 and VSIG10) were selected when we paid attention to the numbers of putative target sites of miR-218. Considering which genes are contributing to cancer cell migration and invasion among these five genes, we paid attention to cytoskeleton-regulated genes for control of cancer metastasis according to our previous studies. As a result of miR-218 target genes, we focused on the LIM and SH3 protein 1 (LASP1) gene, a cytoskeletal scaffold protein that has critical roles in cytoskeletal organization and cell migration. LASP1 was examined in further analyses.

LASP1 was a direct target of miR-218 in prostate cells. We performed quantitative real-time RT-PCR and western blotting in PC3 and DU145 cells to investigate whether LASP1 gene expression and LASP1 protein expression were reduced by restoration of miR-218. The mRNA and protein expression levels of LASP1/LASP1 were significantly repressed in miR-218 transfectants in comparison with mock or miR-control transfectants (Fig. 3a,b).

We performed luciferase reporter assays of PC3 to determine whether LASP1 mRNA had target sites for miR-218. The TargetScan database predicted that three putative miR-218-binding sites existed in the 3’-UTR of LASP1 (positions 686–692, 1587–1593 and 2080–2087 [Fig. 3c]). We used vectors encoding either the partial wild-type sequence of the 3’-UTR of LASP1 mRNA, including the predicted miR-218 target sites, or “deletion” vectors; that is, those lacking the miR-218 target sites. We found that the luminescence intensity was significantly reduced by transfection with miR-218 and two vectors carrying the wild-type 3’-UTR of LASP1 mRNA, including the predicted miR-218 target sites, or “deletion” vectors; that is, those lacking the miR-218 target sites. We found that the luminescence intensity was significantly reduced by transfection with miR-218 and two vectors carrying the wild-type 3’-UTR of LASP1 (positions 686–692 and 1587–1593), whereas transfection with deletion vectors (where nucleotides at positions 695–691 and 2080–2086 had been removed) blocked the decrease in luminescence.
These data suggest that miR-218 binds directly to two specific binding sites in the 3'-UTR of LASP1 mRNA.

Effects of downregulating LASP1 on cell proliferation, migration and invasion in prostate cancer cell lines. To investigate the functional role of LASP1 in PCa cells, we performed loss-of-function studies using si-LASP1 transfectants. First, we evaluated the knockdown efficiency of si-LASP1 treatments in PC3 and DU145. Quantitative real-time RT-PCR and western blotting indicated that the siRNA effectively downregulated LASP1 expression in both cell lines (Fig. 4).

The XTT assay demonstrated that cell proliferation was not inhibited in si-LASP1 transfectants in comparison with the mock or miR-control transfectant cells (Fig. 5a). The Matrigel invasion assay demonstrated that cell invasion activity was significantly inhibited in si-LASP1 transfectants in comparison with the mock or negative control transfectant cells (Fig. 5b). The migration assay demonstrated that cell migration activity was significantly inhibited in si-LASP1 transfectants in comparison with the mock or negative control transfectant cells (Fig. 5c).

Immunohistochemical detection of LASP1 in prostate cancer clinical specimens. We determined the expression levels of LASP1 in PCa specimens by immunohistochemical staining. LASP1 was strongly expressed in several cancer lesions, whereas no or low expression was observed in normal regions (Fig. 6). There was no significant correlation between LASP1 expression and various tested clinicopathological parameters (Gleason score and stages, data not shown).

Identification of novel molecular pathways regulated by LASP1 in prostate cancer cells. To investigate molecular pathways regulated by LASP1, a genome-wide gene expression analysis was performed in PC3 cells. A total of 1269 genes were downregulated in si-LASP1 transfection. Downregulated genes, both si-LASP1-transfectants and miR-218-transfectants (top 18 genes), are shown in Table 3. We also assigned the downregulated genes to KEGG pathways using the GeneCodis program (http://genecodis.cnb.csic.es) as described previously. A total of 21 pathways were identified as significantly enriched annotations (Table 4). We focused on the focal adhesion pathway and the genes categorized in this pathway are listed in Table 5.

Discussion

In early stage PCa, most patients initially respond to androgen deprivation therapy; however, many cases become refractory and progress to androgen-independent disease. Currently, there is no effective treatment for hormone-refractory PCa, with disease progression and metastasis to the skeleton or
other sites.\(^{(3)}\) Thus, new approaches to effective treatments of hormone-refractory PCa are necessary.

In cancer cells, aberrant expression of miRNAs can upset the tightly regulated system of miRNA-protein-coding RNA networks. Therefore, studies of differentially expressed miRNAs in cancer cells provide important information regarding the molecular mechanisms underlying oncogenesis and metastasis.

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**Fig. 4.** LASP1 mRNA and LASP1 protein expression levels were suppressed by si-LASP1 transfection of DU145 and PC3 cells. (a) LASP1 mRNA expression 72 h after transfection with si-LASP1. GUSB expression was used for normalization. (b) LASP1 protein expression 72 h after transfection with si-LASP1. GAPDH was used as a loading control. The ratio of LASP1/GAPDH expression was evaluated using ImageJ software (ver. 1.43; http://rsbweb.nih.gov/ij/index.html).

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**Fig. 5.** Effects of LASP1 downregulation by si-LASP1 on PCa cells (PC3 and DU145). (a) Cell proliferation determined with the XTT assay. (b) Cell migration activity determined with the wound healing assay. (c) Cell invasion activity determined with the Matrigel invasion assay. \(^*P < 0.001\).

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**Fig. 6.** Immunohistochemical staining of LASP1 in prostate clinical specimens. Differences in LASP1 expression were observed in cancer lesions and adjacent normal prostate tissues in the same fields: (a) patient number 6; and (b) patient number 16. Overexpression of LASP1 was observed in cancer lesions. In contrast, negative staining of LASP1 in normal prostate glands and stromal tissues (left panel, original magnification \( \times 50 \); right panel, original magnification \( \times 100 \)).
Table 3. Downregulated genes both si-LASP1 and miR-218-transfects in PC3 cells

| Entrez gene ID | Symbol | Gene name                                      | miR-218 transfectant | siLASP1 transfectant |
|---------------|--------|-----------------------------------------------|----------------------|----------------------|
| 10529         | NEBL   | Nebulette                                     | –2.03                | –3.79                |
| 54757         | FAM20A | Family with sequence similarity 20, member A  | –2.09                | –2.93                |
| 3131          | HLF    | Hepatic leukemia factor                      | –4.69                | –2.62                |
| 1381          | CRABP1 | Cellular retinoic acid binding protein 1      | –1.33                | –2.60                |
| 254552        | NUDT8  | Nudix (nucleoside diphosphate linked moiety X)-type motif 8 | –2.57                | –2.53                |
| 4312          | MMP1   | Matrix metallopeptidase 1 (interstitial collagenase) | –0.90                | –2.53                |
| 3927          | LASP1  | LIM and SH3 protein 1                        | –1.96                | –2.50                |
| 27151         | CPAMD8 | C3 and P2P-like, alpha-2-macroglobulin domain containing 8 | –0.56                | –2.49                |
| 3866          | KRT15  | Keratin 15                                    | –3.32                | –2.44                |
| 126823        | KLHDC9 | Kelch domain containing 9                    | –0.99                | –2.43                |
| 8722          | CTSF   | Cathepsin F                                  | –0.84                | –2.43                |
| 128434        | VSTM2L | V-set and transmembrane domain containing 2 like | 0.00                 | –2.35                |
| 3339          | HSPG2  | Heparan sulfate proteoglycan 2               | –2.23                | –2.28                |
| 6678          | SPARC  | Secreted protein, acidic, cysteine-rich (osteonectin) | –4.21                | –2.24                |
| 10669         | CGREF1 | Cell growth regulator with EF-hand domain 1   | –0.78                | –2.13                |
| 25890         | AB13BP | ABI family, member 3 (NESH) binding protein  | –2.58                | –2.12                |
| 11093         | ADAMTS13| ADAM metallopeptidase with thrombospondin type 1 motif, 13 | –0.67                | –2.10                |
| 2171          | FABP5  | Fatty acid binding protein 5 (psoriasis-associated) | –1.58                | –2.09                |

Table 4. Significantly enriched KEGG pathways regulated by si-LASP1 in PC3 cells

| Number of genes | P-value | Annotations                                      |
|-----------------|---------|--------------------------------------------------|
| 17              | 8E-08   | (KEGG) 04512: ECM-receptor interaction           |
| 16              | 7E-05   | (KEGG) 04142: Lysosome                          |
| 18              | 0.0001  | (KEGG) 05010: Alzheimer’s disease                |
| 15              | 0.0005  | (KEGG) 04910: Insulin signaling pathway          |
| 11              | 0.0006  | (KEGG) 04146: Peroxisome                        |
| 16              | 0.0008  | (KEGG) 04141: Protein processing in endoplasmic reticulum |
| 18              | 0.0008  | (KEGG) 04510: Focal adhesion                     |
| 8               | 0.001   | (KEGG) 00280: Valine, leucine and isoleucine degradation |
| 15              | 0.0018  | (KEGG) 00230: Purine metabolism                 |
| 9               | 0.0046  | (KEGG) 03320: PPAR signaling pathway            |
| 15              | 0.0054  | (KEGG) 05016: Huntington’s disease               |
| 22              | 0.0055  | (KEGG) 05200: Pathways in cancer                |
| 6               | 0.0059  | (KEGG) 00640: Propanoate metabolism             |
| 10              | 0.0137  | (KEGG) 05146: Amoebiasis                        |
| 6               | 0.0221  | (KEGG) 00480: Glutathione metabolism            |
| 11              | 0.0229  | (KEGG) 00190: Oxidative phosphorylation         |
| 11              | 0.023   | (KEGG) 05012: Parkinson’s disease               |
| 13              | 0.0266  | (KEGG) 04020: Calcium signaling pathway         |
| 8               | 0.0382  | (KEGG) 05222: Small cell lung cancer            |
| 8               | 0.0482  | (KEGG) 05322: Systemic lupus erythematosus      |
| 8               | 0.0492  | (KEGG) 05414: Dilated cardiomyopathy            |

KEGG, Kyoto Encyclopedia of Genes and Genomes.

To elucidate the molecular mechanisms underlying PCa, we have examined tumor-suppressive miRNAs, focusing on their regulated molecular targets and novel cancer pathways based on PCa expression signatures.\(^{10}\) Our recent studies of miRNA expression signatures showed that miR-218 was frequently reduced in other types of cancer tissues and it functioned as a tumor suppressor.\(^{17-19}\) Tumor-suppressive functions of miR-218 have been described by other research groups analyzing several types of cancers.\(^{20-25}\) Thus, miR-218 is a key molecule in the development of human cancers, making it important to understand the cancer molecular network that miR-218 regulates. The molecular mechanisms of miR-218 silencing in PCa cells are still unclear. The human genome database indicates that miR-218 is located in two different human chromosome loci (miR-218-1 at 4p15.31 and miR-218-2 at 5q35.1), and these miRNAs are embedded in the intronic regions of SLIT2 and SLIT3, respectively. Previous reports showed that expression of SLIT2 and SLIT3 were downregulated in several types of cancer cells through their promoter hypermethylation.\(^{20,24}\) Our preliminary examination showed that downregulation of SLIT2 and SLIT3 were observed in primary PCa tissues (Fig. S2). Furthermore, re-expression of SLIT2 and miR-218 were observed after 5-aza-2’-deoxycytidine treatment of PCa cell line (Fig. S3). Thus, our data and previous studies reveal that promoter hypermethylation of SLIT2 and SLIT3 regions drive PCa progression and downregulation of miR-218 in PCa cells.

To better understand PCa metastasis, we identified miR-218 target genes using in silico analysis. As determined by our laboratory and others, the targets of miR-218 include CAV1, LAMB3, ECOP, IKK-B, PXN, RICTOR, BIRC5 and ROBO1.\(^{17,18,20,22-25}\) Here, we focused on LASP1, a cytoskeletal scaffold protein. It plays critical roles in cytoskeletal organization and cell migration. LASP1 was initially identified in a cDNA library constructed from metastasized breast cancer cells. LASP1 encodes a LIM motif at its N-terminus and a src homology 3 (SH3) domain at its C-terminus.\(^{26,27}\) The C-terminal SH3 domain of LASP1 functions in protein–protein interactions such as vasodilator-stimulated phosphoprotein, palladin and zyxin.\(^{28-30}\) Zyxin might function as a messenger in the signal transduction pathway that mediates adhesion-stimulated changes in gene expression and might also modulate the cytoskeletal organization of actin bundles.\(^{30-32}\) In the present study, we performed genome-wide gene expression analysis using si-LASP1 transfectant PC3 cells to investigate LASP1-regulated molecular targets and pathways. Our data showed that zyxin was downregulated in LASP1-suppressed cells.
Zyxin is reportedly regulated by TGF-β and contributes to the epithelial–mesenchymal transition. Therefore, it will be important to analyze the molecular mechanisms of LASP1-zyxin signal transduction to better understand the metastasis of human cancer cells.

Overexpression of LASP1 has been reported in metastatic breast and ovarian cancers. Our group showed that LASP1 gene expression was elevated in bladder cancer and that downregulation of the LASP1 gene inhibited cancer cell migration and invasion, suggesting that LASP1 significantly contributes to cancer metastasis. This is the first report to show that overexpression of LASP1 in PCa clinical tissues might be involved in the metastasis of PCa. Our previous analysis of bladder cancer showed that tumor-suppressive miRNAs such as miR-1, miR-133a and miR-218 regulated LASP1. Recent studies demonstrated that LASP1 was regulated by miR-203 in breast cancer and esophageal cancer. Immunohistochemical staining demonstrated that the overexpression of LASP1 was detected in primary PCa tissues compared with non-PCa tissues. We also measured the expression of miR-218 status using the same FFPE tissues whether the downregulation of miR-218 was associated with upregulation of LASP1 in PCa. Our data showed that expression of miR-218 was significantly reduced in LASP1 overexpression PCa tissues in comparison to LASP1 low staining of non-PCa tissues (Fig. S4). Our data for primary PCa tissues and cell lines might be suggesting that downregulation of miR-218 caused upregulation of LASP1 in PCa cells. Improved understanding of tumor-suppressive miRNAs and their regulation of LASP1 signalling should shed light on PCa metastasis as well as delineate more effective strategies for future therapeutic interventions for this disease.

In conclusion, miR-218 was significantly downregulated in PCa clinical specimens and appeared to function as a tumor suppressor through regulation of oncogenic LASP1. Elucidation of the cancer pathways and target genes regulated by the tumor-suppressive miR-218 should provide new information on potential therapeutic targets in the treatment of PCa metastasis.

Acknowledgments
This study was supported by the KAKENHI (C), 24592590, and (B), 25293333.

Disclosure Statement
The authors have no conflict of interest.

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Supporting Information

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

Fig. S1. H&E staining of the formalin-fixed paraffin-embedded (FFPE) prostate specimens.

Fig. S2. Expression levels of SLIT2, SLIT3 and miR-218 in prostate cancer (PCa) tissues.

Fig. S3. Effects of 5-aza-2'-deoxycytidine (5-aza-dc) treatment of prostate cancer (PCa) cells.

Fig. S4. Expression levels of miR-218 in prostate cancer (PCa) tissues.