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

（癌抑制型 microRNA-218 は LASP1 を制御して前立腺癌細胞の転移を抑制する）

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

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Abstract

Our recent studies of the microRNA (miRNA) expression signature in PCa indicated that *miRNA-218* (*miR-218*) was significantly downregulated in clinical specimens, suggesting that *miR-218* might act as a tumor-suppressive miRNA in PCa. The aim of this 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) was 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 insights into the potential mechanisms of PCa oncogenesis and metastasis.

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

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer death among men in developed countries.[1] 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 improve therapies for and prevention of 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, 2,578 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, 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 this study 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 this 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% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Total RNA was isolated from Formalin-Fixed Paraffin-Embedded (FFPE) sample with four 5μm 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] 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 miRNA and siRNA

The following mature miRNA species were used in this 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 10nM miRNA or siRNA by reverse transfection and plated in 96-well plates at 3×10^3 cells per well. After 72h, 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 (0h) and the residual gap length 24h 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 8μm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA, USA) at 2×10^5 cells per well. Cells were transfected with 10nM miRNA or siRNA by reverse transfection and plated in 10cm dishes at 8×10^5 cells per dish. After 48h, the cells were collected, and 2×10^5 cells were added to the upper chamber of each migration well.

Cells were allowed to invade for 48h. 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 72h after transfection, and lysates were prepared. 50μg 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 the Gene Expression Omnibus (GEO) database (accession number: GSE29079). Also, 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) was used for gene expression studies. Microarray procedures and data mining methods were 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'-untranslated region (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 described previously.[16] The synthesized DNA was cloned into the psiCHECK-2 vector. PC3 cells were transfected with 50ng of the vector and 10nM *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, St. Louis, MO) 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 Inc., Cary, NC, USA).

Results

The 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 that 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).

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 2,940 genes. Next, we pared down the 2,940 genes based on two kinds of gene expression data as follows: 1) upregulated genes determined by the gene expression data set of PCa clinical specimens in GEO (accession number: GSE29079), 2) downregulated genes ($\log_2$ 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.[13,14] 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 (Figs. 3A and 3B).

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* (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 ($P < 0.001$, Fig. 3C). These data suggested that *miR-218* bounds directly to specific two 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*/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 1,269 genes were downregulated in *si-LASP1* transfection. Downregulated genes both *si-LASP1*-transfectants and *miR-218*-transfectans (top 18 genes) were shown in Table 3. We also assigned the down-regulated genes to KEGG pathways using the GeneCodis program (http://genecodis.cnb.csic.es) as described previously.[12,15] A total of 21 pathways were identified as significantly enriched annotations (Table 4). We focused on 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.[2] 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. To elucidate the molecular mechanisms underlying PCa, we have examined tumour-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 its functioned as a tumour 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. Human genome database indicates that *miR-218* is located 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 the *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 provided that promoter hypermethylation of *SLIT2* and *SLIT3* regions drove PCa progression and derived 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 (VASP), 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 this 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.[31,32] 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.[33,34] 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[16], 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 tumor-suppressive miRNAs such as *miR-1*, *miR-133a* and *miR-218* regulated LASP1.[16] Recent studies demonstrated that LASP1 was regulated by *miR-203* in in breast cancer and esophageal cancer.[35-37] Immunohistchemical 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 of primary PCa tissues and cell lines might be suggesting that downregulation of *miR-218* caused to upregulation of LASP1 in PCa cells. Improved understanding of tumour-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.

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Disclosure Statement

The authors declare no conflicts of interest.

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Figure Legends

Figure 1

The expression levels of *miR-218* in clinical specimens and PCa cell lines (PC3 and DU145).

Real-time PCR showed that the expression levels of *miR-218* were significantly lower in PCa tissues and cell lines than in normal prostate tissues. *RNU48* was used as an internal control.

Figure 2

Effects of *miR-218* transfection on prostate cancer (PCa) cell lines (PC3 and DU145).

(A) Cell proliferation was determined with XTT assays 72 h after transfection with ten nM *miR-218*, miR-control, or mock transfection. (B) Cell invasion activity was determined with the Matrigel invasion assay. (C) Cell migration activity determined with the wound healing assay. $*P < 0.001$.

Figure 3

***LASP1* expression was suppressed by *miR-218* transfection of prostate cancer (PCa) cells lines.**

(A) *LASP1* mRNA expression 72 h after transfection with *miR-218. GUSB* expression was used for normalization. (B) LASP1 protein expression 72 h after transfection with *miR-218.* GAPDH was used as a loading control. (C) *miR-218* binding sites in the 3'-UTR of *LASP1* mRNA. Luciferase reporter assays using three vectors encoding putative *miR-218 t*arget sites at positions 686-692, 1587-1593 and 2080-2087 for both wild-type (WT) and deletion (DEL). *Renilla* luciferase values were normalized to firefly luciferase values. $*P < 0.001$.

Figure 4

***LASP1* mRNA and LASP1 protein expression levels were suppressed by si-LASP1 transfection of DU145 and PC3 cells.**

(A) *LASP1* mRNA expression 72h after transfection with *si-LASP1. GUSB* expression was used for normalization. (B) LASP1 protein expression 72h 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).

Figure 5

Effects of *LASP1* downregulation by *si-LASP1* on prostate cancer (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. $*P < 0.001$.

Figure 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, 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 X50; right panel, original magnification X100).

Supporting information

Supplemental Figure 1

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

Supplemental Figure 2

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

Supplemental Figure 3

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

Supplemental Figure 4

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

Figure 1

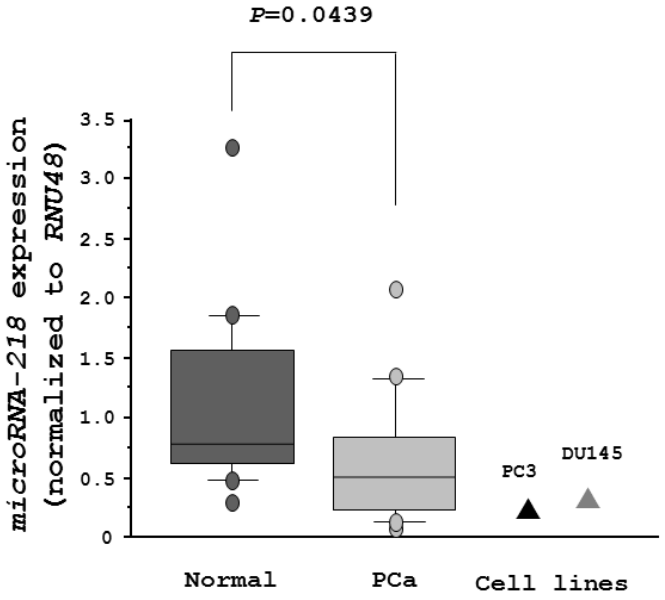


Figure 2

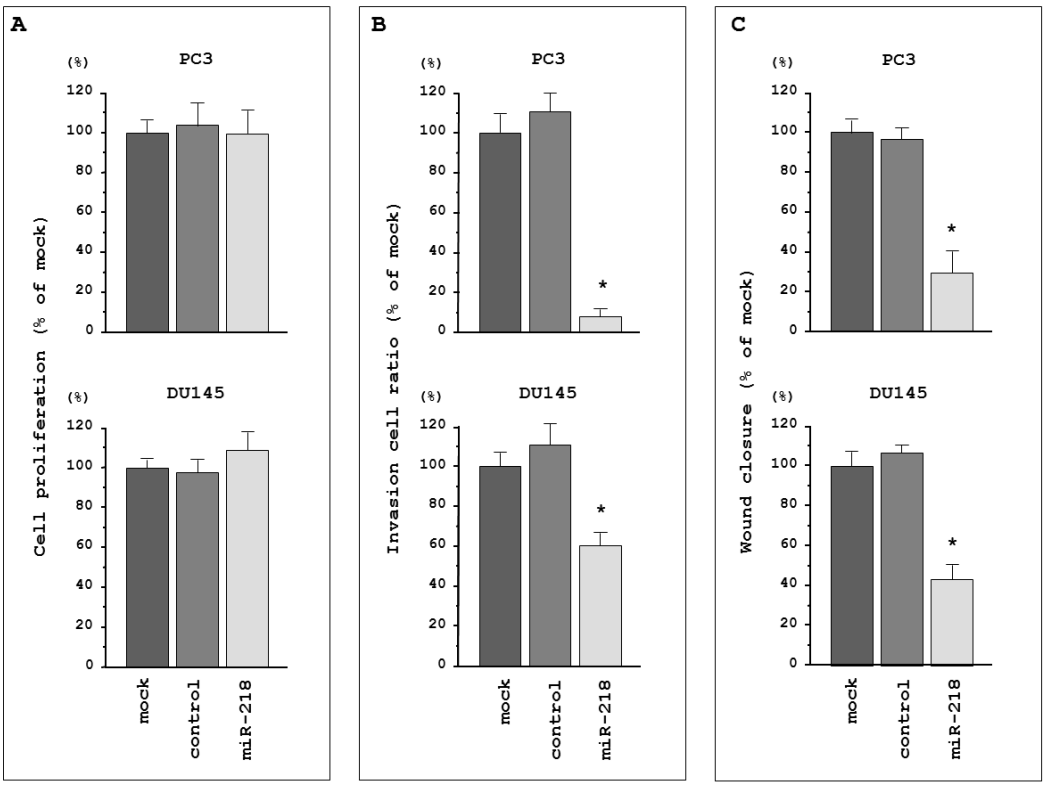


Figure 3

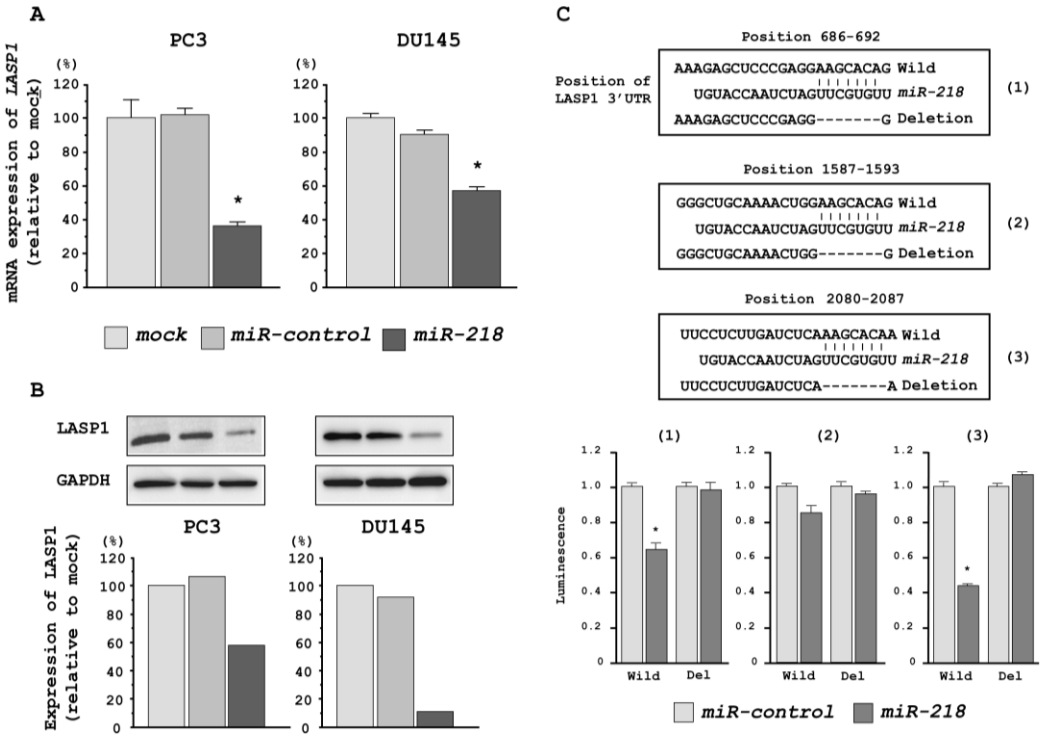


Figure 4

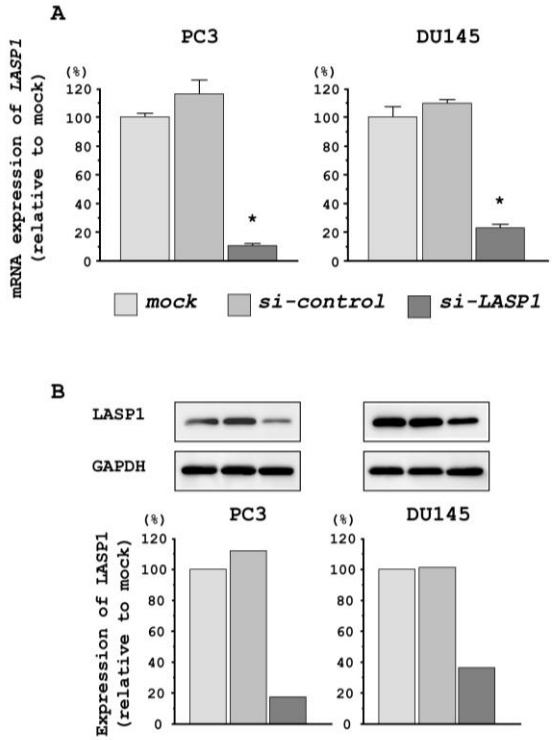


Figure 5

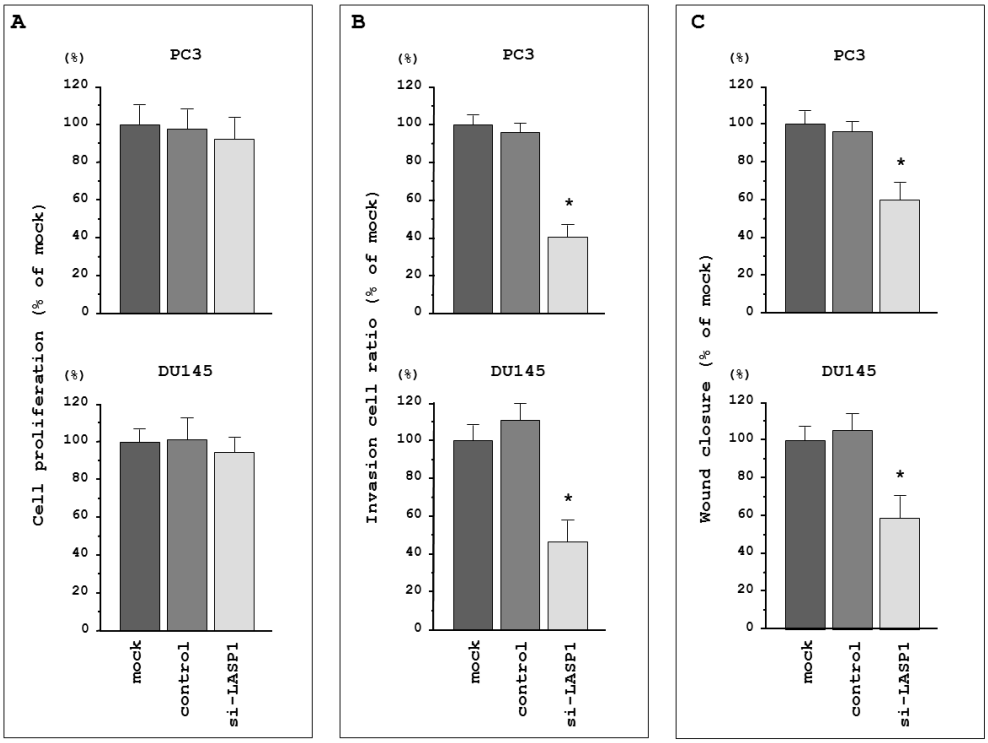
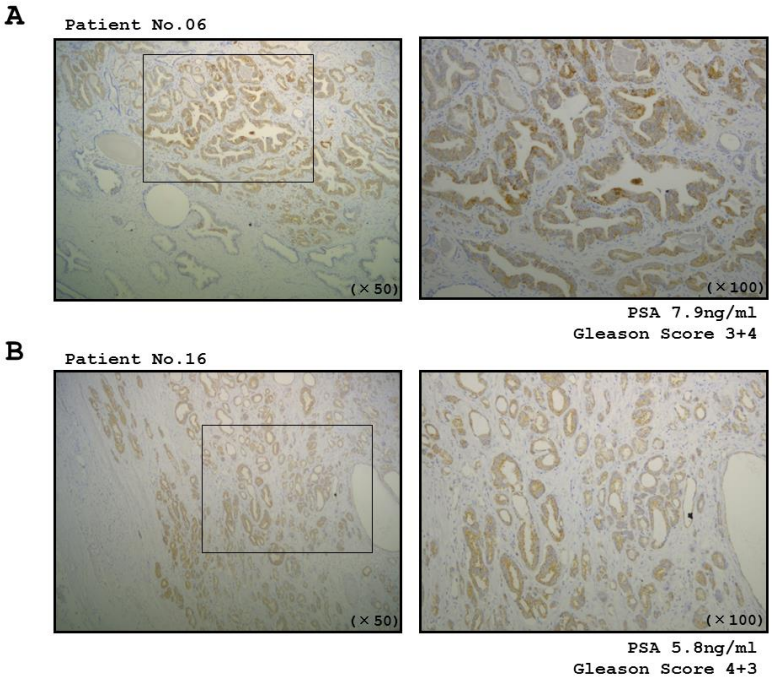
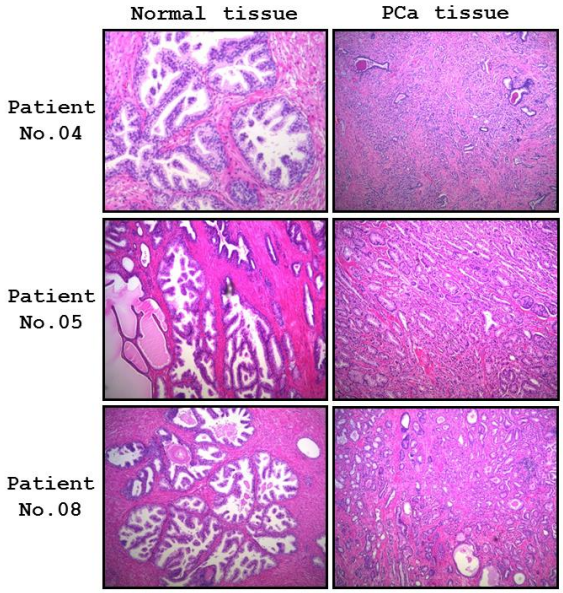


Figure 6

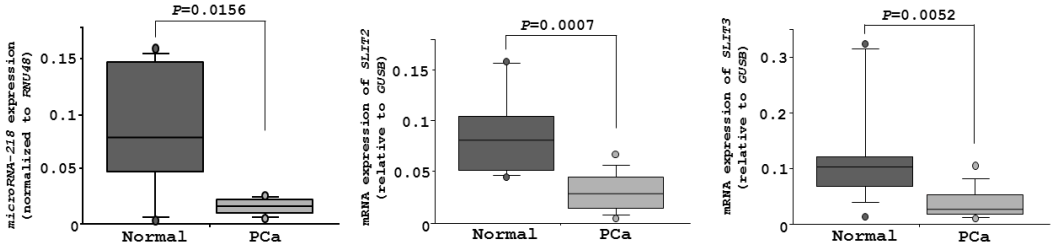


Supplemental Figure 1



Supplemental Figure 1
HE staining of the FFPE prostate specimens.
FFPE samples used for expression analysis showed massive infiltration of high grade PCa cells and normal prostate tissues from the same patients (patient numbers 4, 5 and 8).

Supplemental Figure 2

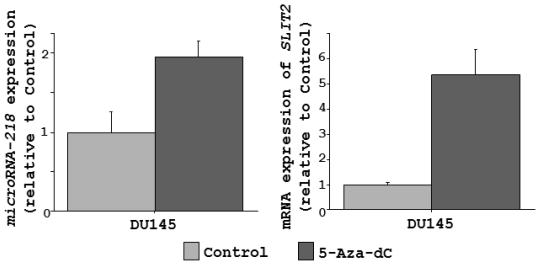


Patients' characteristics

PCa Patients							non-PCa patients		
				TNM classification					
No.	Age (yrs.)	PSA (ng/ml)	Gleason score	T	N	M	No.	Age (yrs.)	PSA (ng/ml)
1	78	989	4+4	4	1	1	11	60	5.6
2	69	26	3+4	3b	0	0	12	56	8.4
3	78	19.3	4+4	3a	0	0	13	61	8.6
4	73	478	4+3	3b	0	1	14	62	35.5
5	72	102	4+4	3a	0	0	15	73	6.0
6	65	212	4+4	4	1	1	16	57	5.2
7	81	11.4	4+4	2	0	0	17	64	4.4
8	78	121	4+5	3b	1	0	18	60	5.7
9	79	633	4+5	3b	1	0	19	63	11.4
10	58	482	4+4	3b	1	0	20	65	13.2

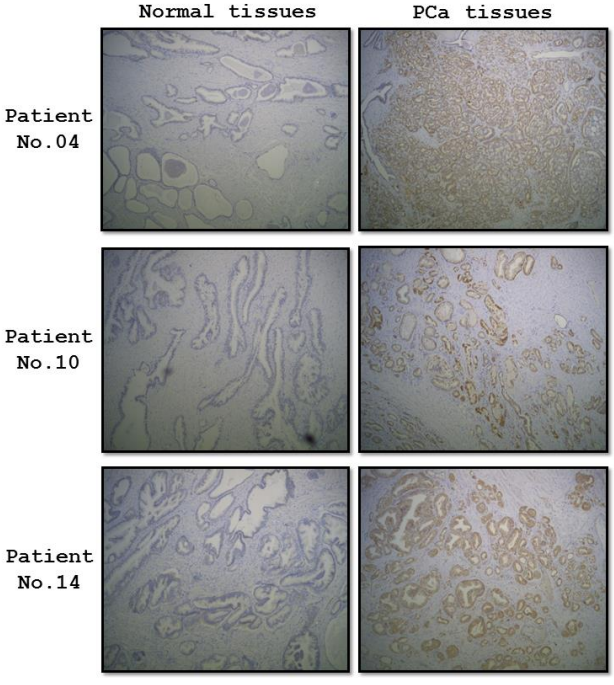
Supplemental Figure 2
Expression levels of *SLIT2*, *SLIT3* and *miR-218* in PCa tissues.
Preparing clinical specimens (needle biopsy specimens) independently from Table 1, and measured the expression of *SLIT2*, *SLIT3* and *miR-218* by TaqMan quantitative real-time PCR methods. *GUSB* and *RNU48* were used for normalization. Patients' characteristics were summarized in the lower. TaqMan probes and primers for *SLIT2* (P/N: Hs00191193_m1) and *SLIT3* (P/N: Hs00171524_m1) were obtained from Applied Bisystems (Assay-On-Demand Gene Expression Products).

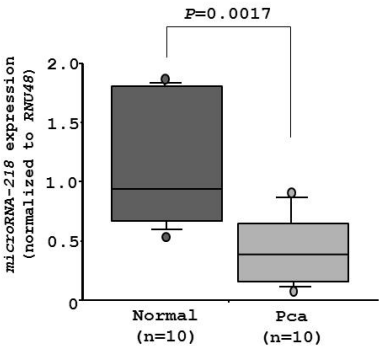
Supplemental Figure 3



Supplemental Figure 3
Effects of 5-aza-2'-deoxycytidine (5-aza-dc) treatment of PCa cells.
The effects of the demethylation agent treatment (5-aza-dc, Sigma-Aldrich, St Louis, MO, USA) on PCa cell line, DU145. Cells were treated with 5μM of the agent, and cells were harvested for 72 h treatment. Expression levels of *SLIT2*, *SLIT3* and *miR-218* were measured by TaqMan quantitative real-time PCR methods. *GUSB* and *RNU48* were used for normalization. *SLIT3* was not detected in DU145 cells.

Supplemental Figure 4





Supplemental Figure 4
Expression levels of *miR-218* in PCa tissues.
LASP1 positive cancer tissues (right slides) and corresponding LASP1 negative normal tissues (left slides) were sorted by the degree of the LASP1 staining in Table 1. A total of 10 radical prostatectomy specimens were measured for *miR-218* expression, total RNAs were extracted from FFPE tissues and expression levels of *miR-218* were measured by TaqMan quantitative real-time PCR methods. *RNU48* was used for normalization.

Table1

Patients' characteristics

						TNM classification		
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	C	3b	0	0
4	PCa	69	25.8	4+5	B	2a	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
1	non-PCa							
2	non-PCa							
3	non-PCa							
4	non-PCa							
5	non-PCa							
6	non-PCa							
7	non-PCa							
8	non-PCa							
9	non-PCa							
10	non-PCa							
11	non-PCa							
12	non-PCa							
13	non-PCa							
14	non-PCa							
15	non-PCa							
16	non-PCa							
17	non-PCa							

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
10983	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 protein, 29kDa	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	6p24.3	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	FAM20B	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	12q24	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	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, 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 accumbens 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 HMGIC 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 immunoglobulin 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

Table 3

Downregulated genes both *si-LASP1* and *miR-218*- transfects in PC3 cells

			Expression (log2 ratio)	
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 PZP-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	ABI3BP	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-LASP* 1 in PC3 cells

Number of genes	*P* value	Annotations
17	8E-08	(KEGG) 04512: ECM-receptor interaction
16	6.7E-05	(KEGG) 04142: Lysosome
18	0.00013	(KEGG) 05010: Alzheimer's disease
15	0.00054	(KEGG) 04910: Insulin signaling pathway
11	0.00062	(KEGG) 04146: Peroxisome
16	0.00079	(KEGG) 04141: Protein processing in endoplasmic reticulum
18	0.0008	(KEGG) 04510: Focal adhesion
8	0.00105	(KEGG) 00280: Valine, leucine and isoleucine degradation
15	0.00184	(KEGG) 00230: Purine metabolism
9	0.00462	(KEGG) 03320: PPAR signaling pathway
15	0.00537	(KEGG) 05016: Huntington's disease
22	0.00548	(KEGG) 05200: Pathways in cancer
6	0.00593	(KEGG) 00640: Propanoate metabolism
10	0.01371	(KEGG) 05146: Amoebiasis
6	0.02207	(KEGG) 00480: Glutathione metabolism
11	0.02289	(KEGG) 00190: Oxidative phosphorylation
11	0.02296	(KEGG) 05012: Parkinson's disease
13	0.02655	(KEGG) 04020: Calcium signaling pathway
8	0.03817	(KEGG) 05222: Small cell lung cancer
8	0.04822	(KEGG) 05322: Systemic lupus erythematosus
8	0.04917	(KEGG) 05414: Dilated cardiomyopathy

Table 5

Downregulated genes involved in Focal adhesion pathway by *si-LASP1* transfectant in PC3 cells

			Expression (log2 ratio)	
Entrez Gene ID	Gene symbol	Gene name	miR-218 transfectant	siLASP1 transfectant
7791	ZYX	zyxin	-0.64	-0.51
1288	COL4A6	collagen, type IV, alpha 6	-0.9	-0.86
3913	LAMB2	laminin, beta 2 (laminin S)	-0.53	-0.85
2064	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	-0.63	-0.91
3675	ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	-0.7	-0.79
1287	COL4A5	collagen, type IV, alpha 5	-0.45	-0.7
7148	TNXB	tenascin XB	-1.18	-1.71
1290	COL5A2	collagen, type V, alpha 2	1.57	-0.82
25759	SHC2	SHC (Src homology 2 domain containing) transforming protein 2	0.72	-0.85
207	AKT1	v-akt murine thymoma viral oncogene homolog 1	-0.52	-0.53
3910	LAMA4	laminin, alpha 4	0.49	-1.07
5881	RAC3	ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	-0.07	-0.58
1292	COL6A2	collagen, type VI, alpha 2	0.19	-1.3
1289	COL5A1	collagen, type V, alpha 1	1.34	-0.84
3691	ITGB4	integrin, beta 4	-0.82	-1.17
3912	LAMB1	laminin, beta 1	0.2	-0.58
7059	THBS3	thrombospondin 3	-1.2	-1.09
5500	PPP1CB	protein phosphatase 1, catalytic subunit, beta isozyme	-2.1	-0.5

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