

ORIGINAL ARTICLE

Regulation of metastasis-promoting LOXL2 gene expression by antitumor microRNAs in prostate cancer

Mayuko Kato^{1,2}, Akira Kurozumi^{1,2}, Yusuke Goto^{1,2}, Ryosuke Matsushita³, Atsushi Okato^{1,2}, Rika Nishikawa^{1,2}, Ichiro Fukumoto¹, Keiichi Koshizuka¹, Tomohiko Ichikawa² and Naohiko Seki¹

Our recent studies of microRNA (miRNA) expression signatures of prostate cancer (PCa) showed that six miRNAs (specifically, *miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c* and *miR-218*) were markedly reduced in cancer tissues. Moreover, ectopic expression of these miRNAs suppressed PCa cell aggressiveness, indicating that these miRNAs acted in concert to regulate genes that promoted metastasis. Genome-wide gene expression analysis and *in silico* database analysis identified a total of 35 candidate genes that promoted metastasis and were targeted by these 6 miRNAs. Using luciferase reporter assays, we showed that the lysyl oxidase-like 2 (*LOXL2*) gene was directly controlled by these tumor-suppressive miRNAs in PCa cells. Overexpression of *LOXL2* was confirmed in PCa tissues and knockdown of the *LOXL2* gene markedly inhibited the migration and invasion of PCa cells. Aberrant expression of *LOXL2* enhanced migration and invasion of PCa cells. Downregulation of antitumor miRNAs might disrupt the tightly controlled RNA networks found in normal cells. New insights into the novel molecular mechanisms of PCa pathogenesis was revealed by antitumor miRNA-regulated RNA networks.

Journal of Human Genetics (2017) 62, 123–132; doi:10.1038/jhg.2016.68; published online 9 June 2016

INTRODUCTION

The current 5-year survival rate of patients with localized prostate cancer (PCa) is almost 100%, which is attributed to efficacy of treatments of early-stage disease.¹ Although most patients with PCa are initially responsive to androgen-deprivation therapy, their cancers eventually become resistant to this mode of treatment, and they progress to castration-resistant PCa.^{1,2} Unfortunately, most clinical trials for advanced PCa have shown limited benefits, eventually resulting in disease progression and metastasis.^{1,2} Distant metastasis of advanced PCa is common and leads to significant morbidity and mortality. Therefore, we have proposed that current therapies for advanced PCa could be improved by using new genomic approaches to better understand the molecular mechanisms of metastasis and disease progression.

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules (19–22 bases in length) that regulate the expression of protein-coding or protein non-coding genes in a sequence-specific manner.^{3,4} There is substantial evidence that miRNAs can be oncogenic or act to suppress tumors. Dysregulated expression of oncogenic miRNAs can disrupt the normally controlled RNA networks present in normal cells and enhance the development of cancer.^{5–8} Identification of aberrantly expressed miRNAs in cancer cells provides important new information regarding the initiation, progression and metastasis of cancer cells.

We have identified antitumor miRNAs and miRNA-mediated oncogenic genes in PCa cells by using our PCa miRNA expression

signatures.^{9,10} Our recent studies showed that six miRNAs (*miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c* and *miR-218*) were down-regulated in PCa tissues and restoration of these miRNAs markedly suppressed cancer cell aggressiveness.^{11–13} Our data suggested that these six miRNAs act as antimetastatic miRNAs in PCa cells. Therefore, we hypothesized that the genes regulated by these miRNAs significantly contributed to PCa metastasis. The aim of this study was to identify the genes targeted by these six antitumor miRNAs and to investigate their functional roles in migration and invasion in PCa cells.

Present study identified that a total of 35 putative metastasis-promoting genes were targeted by the 6 abovementioned miRNAs. Here we focused on the lysyl oxidase-like 2 (*LOXL2*) gene, a member of the lysyl oxidase (LOX) family.¹⁴ The function of *LOXL2* is to promote crosslinking of collagen and elastin in the extracellular matrix (ECM).^{15–17} Past studies showed that *LOXL2* contributed to the regulation of extracellular and intracellular cell signaling pathways.^{15–17} Our recent studies demonstrated that *LOXL2* was controlled by several tumor-suppressive miRNAs and promoted cancer cell aggressiveness in renal cell carcinoma, head and neck cancer and lung cancer.^{18–20} Furthermore, several studies demonstrated that upregulation of *LOXL2* occurred in many types of cancers and its expression contributed to cancer cell metastasis.^{21–23} Our present data show that insights into the molecular mechanisms of PCa pathogenesis can be revealed by identification of antitumor miRNA-regulated RNA networks.

¹Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba, Japan; ²Department of Urology, Chiba University Graduate School of Medicine, Chiba, Japan and ³Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan
Correspondence: Dr N Seki, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan.
E-mail: naoseki@faculty.chiba-u.jp

Received 2 April 2016; revised 29 April 2016; accepted 11 May 2016; published online 9 June 2016

MATERIALS AND METHODS

PCa cell lines and RNA extraction

Two human PCa cell lines (PC3 and PC3M) was obtained from ATCC (Manassas, VA, USA). The maintenance of PCa cells and RNA extraction procedures were as described previously.^{11,24}

Quantitative real-time reverse transcription-PCR (qRT-PCR)

TaqMan probes and primers of *LOXL2* (P/N: Hs00158757_m1; Applied Biosystems, Foster City, CA, USA) and *GAPDH* (P/N: Hs02758991_g1; Applied Biosystems) was used as an internal control. PCR quantification method was as described previously.^{13,25,26}

Transfection with miRNA mimic and small-interfering RNA (siRNA)

The following miRNA mimics were used in the present analysis: Ambion (Austin, TX, USA) Pre-miR miRNA precursor for *hsa-miR-26a-5p* (product ID: PM10249), *hsa-miR-26b-5p* (product ID: PM12899), *hsa-miR-29a-3p* (product ID: MC12499), *hsa-miR-29b-3p* (product ID: MC10103), *hsa-miR-29c-3p* (product ID: MC10518) and *hsa-miR-218* (product ID: AM17100). Following siRNAs were used in this study: *si-LOXL2* (P/N: HSS180848, HSS106125; Invitrogen, Carlsbad, CA, USA) and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). The procedures of transfection of miRNAs and siRNAs were as described previously.^{12,13}

Selection of putative miR-26a/b-, miR-29a/b/c- and miR-218-regulated targets in PCa cells

To identify putative targets of these miRNAs, we carried out a combination of *in silico* database analysis and comprehensive gene expression analysis, as described in previous studies.^{12,13,25} Our strategy for identification of putative targets is shown in Figure 1.

Immunohistochemistry and western blotting

A tissue microarray containing PCa, prostatic intraepithelial neoplasias (PINs) and prostatic hyperplastic tissues was obtained from Provitro (Berlin, Germany) (Cat. no. 401 2209, Lot no. 146.1 P020212, 26–46). The information about these tissues can be found at <http://www.provitro.com/fileadmin/provitro-data/TMA/4012209.pdf>. Tissue immunostaining of LOXL2 and scoring method were as described previously.^{24,25}

The procedures of LOXL2 western blotting were as described previously.^{12,13,25}

Cell proliferation, migration and invasion assays in PCa cells

To know the functional significance of *LOXL2* in PCa cells, we investigated the knockdown effects of *LOXL2* on cell proliferation, migration and invasion assays using *si-LOXL2*-transfected PC3 and PC3M cells. These assays were as described previously.^{12,13,25}

Plasmid construction and dual-luciferase reporter assays

Partial wild-type sequences of the *LOXL2* 3' untranslated region (3'-UTR) or those with deleted sequences of miRNAs (*miR-26a/b-*, *miR-29a/b/c-* and *miR-218*-binding sites) were inserted in the hRluc gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). The procedure for the dual-luciferase reporter assay was as described previously.^{12,13,25}

Statistical analysis

Relationships between two or three variables and numerical values were investigated using the Mann-Whitney *U*-test or Bonferroni adjusted Mann-Whitney *U*-test. Expert StatView software, version 4 (SAS Institute Inc., Cary, NC, USA), was used in these analyses.

RESULTS

Identification of candidate targets regulated by antitumor miRNAs in PCa cells

To identify target genes of the six miRNAs, we carried out a combination of *in silico* database analysis and genome-wide gene expression analysis. Seed sequences of *miR-26a* and *miR-26b* are

identical, and seed sequences of *miR-29a*, *miR-29b* and *miR-29c* are identical (Figure 1). First, we screened putative target genes using the TargetScan database Release 6.2 (<http://www.targetscan.org/>) and selected 334 genes that had putative binding sequences for these miRNAs in their 3'-UTRs. Next we narrowed down the list of genes by oligomicroarray analysis using PC3 cells and found that 35 genes were downregulated (\log_2 ratio < 0) following transfection with *miR-26a* or *miR-29a* or *miR-218* as compared with expression levels in miR-control-transfected cells (Table 1). Expression data were deposited in the Gene Expression Omnibus database (accession numbers, GSE56243 and GSE77790).

We investigated the regulation of hyaluronan synthase 3 (*HAS3*), *LOXL2* and LIM and SH3 protein 1 (*LASP1*) by these antitumor miRNAs. The data showed that *miR-26a* and *miR-26b* slightly suppressed the expression of *HAS3* and *LASP1* in PCa cells (data not shown). Therefore, we excluded *HAS3* and *LASP1* genes in this analysis. In this study, we focused on *LOXL2* because many reports showed that this gene had a pivotal role in cancer metastasis. Furthermore, we previously reported that *miR-29a/b/c* acted as antitumor miRNAs via targeting of *LOXL2* in lung squamous cell carcinoma and renal cell carcinoma.^{18,20}

Direct regulation of LOXL2 by antitumor miRNAs in PCa cells

First, qRT-PCR and western blotting were carried out in PC3 and PC3M cells transfected with *miR-26a*, *miR-26b*, *miR-29a*,

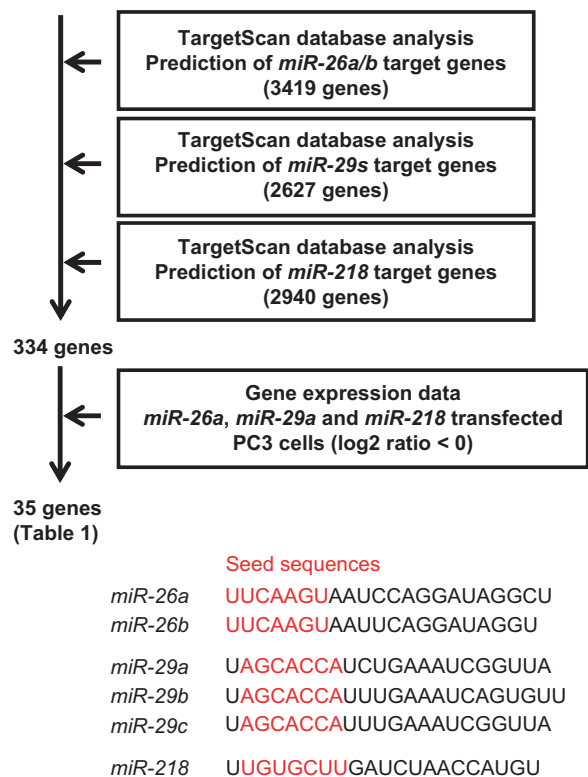


Figure 1 Workflow of the strategy for selection of putative target genes regulated by tumor-suppressive miRNAs (*miR-26a/b*, *miR-29s* and *miR-218*) in PCa. We screened candidate targets using the TargetScan database. In total, 334 genes were identified as putative targets containing binding sites for *miR-26a/b*, *miR-29s* and *miR-218*. Among these, 35 genes were downregulated in *miR-26a*-, *miR-29a*- and *miR-218*-transfected PC3 cells (\log_2 ratio < 0). The sequences of *miR-26a/b*, *miR-29* family and *miR-218* miRNAs. Seed sequences are shown by red letters.

Table 1 Putative target genes regulated by miR-26a, miR-29a and miR-218 in PCa cells

Gene symbol	Representative transcript	Gene name	miR-26a		miR-29a		miR-218		miR-26ab			miR-29abc			miR-218		
			transfection	transfection	transfection	transfection	Conserved	Poorly	Conserved	Poorly	Conserved	Poorly	Conserved	Poorly	Conserved	Poorly	
HAS3	NM_001199280	Hyaluronan synthase 3	-1.56	-2.10	-3.25	1	0	3	0	0	0	1	0	0	1		
LOXL2	NM_002318	Lysyl oxidase-like 2	-2.09	-2.18	-2.12	2	0	1	1	0	0	1	0	0	1		
LASP1	NM_006148	LIM and SH3 protein 1	-0.51	-0.49	-1.96	1	0	2	0	1	0	1	0	1	2		
SLC4A7	NM_003615	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	-0.30	-1.35	-1.95	0	1	0	1	0	0	1	0	0	1		
LIF	NM_002309	Leukemia inhibitory factor (cholinergic differentiation factor)	-1.02	-0.13	-1.91	1	0	1	0	0	0	0	0	0	1		
VAMP7	NM_001145149	Vesicle-associated membrane protein 7	-0.43	-1.24	-1.86	0	2	1	0	2	1	0	2	1			
TRAK2	NM_015049	Trafficking protein, kinesin binding 2	-0.18	-0.66	-1.67	0	1	0	1	0	0	1	0	1			
RBBP9	NM_006606	Retinoblastoma-binding protein 9	-0.80	-0.32	-1.57	0	2	0	1	0	0	3	0	3			
EMP2	NM_001424	Epithelial membrane protein 2	-0.61	-0.32	-1.42	0	1	1	0	0	0	1	0	1			
PTP4A1	NM_003463	Protein tyrosine phosphatase type IVA, member 1	-0.73	-0.12	-1.05	1	0	1	0	2	0	0	2	0			
ZHX3	NM_015035	Zinc fingers and homeobox 3	-0.04	-0.88	-0.95	0	1	1	0	0	0	3	0	3			
EPB41L1	NM_012156	Erythrocyte membrane protein band 4.1-like 1	-0.57	-0.64	-0.73	0	1	0	2	1	0	1	0	0			
SPI100	NM_001080391	SP100 nuclear antigen	-0.30	-0.31	-0.72	0	1	0	1	0	0	1	0	1			
LARP4B	NM_015155	La ribonucleoprotein domain family, member 4B	-0.54	-0.22	-0.69	2	0	1	0	1	0	1	0	0			
VPS13D	NM_015378	Vacuolar protein sorting 13 homolog D (<i>S. cerevisiae</i>)	-0.26	-1.31	-0.55	0	1	0	1	0	0	1	0	1			
PBX2	NM_002586	Pre-B-cell leukemia homeobox 2	-0.48	-0.53	-0.54	0	1	0	1	0	1	1	0	0			
NRAS	NM_002524	Neuroblastoma RAS viral (<i>v-ras</i>) oncogene homolog	-0.01	-0.71	-0.52	1	2	1	0	0	0	0	0	4			
ZNF592	NM_014630	Zinc finger protein 592	-0.19	-0.79	-0.47	0	1	0	1	0	0	1	0	1			
MYO5A	NM_000259	Myosin VA (heavy chain 12, myosin)	-0.09	-0.34	-0.44	0	1	0	2	0	0	1	0	1			
ACER3	NM_018367	Alkalin ceramidase 3	-0.64	-0.02	-0.42	1	2	0	0	1	0	1	0	1			
WDR33	NM_018383	WD repeat domain 33	-0.52	-0.24	-0.42	1	1	0	2	1	0	2	1	0			
HDAC4	NM_006037	Histone deacetylase 4	-0.69	-0.50	-0.37	0	1	1	0	0	0	1	0	1			
PIKFYVE	NM_015040	Phosphoinositide kinase, FYVE finger containing	-0.47	0.00	-0.27	0	3	0	1	0	0	0	0	1			
DTWD2	NM_173666	DTW domain containing 2	-0.18	-0.71	-0.27	0	1	1	0	0	0	1	0	1			
SERP1	NM_001018067	SERPINE1 mRNA-binding protein 1	-1.25	-0.73	-0.27	2	1	1	0	1	0	1	0	0			
RBM33	NM_053043	RNA-binding motif protein 33	-0.34	-0.72	-0.27	0	1	0	1	0	1	0	0	1			
REPS2	NM_001080975	RALBP1-associated Eps domain containing 2	-0.76	-0.76	-0.24	1	1	1	0	0	2	0	2	2			
GTF2H5	NM_207118	General transcription factor IIH, polypeptide 5	-0.10	-0.02	-0.21	0	1	0	1	0	0	1	0	1			
CACNA1C	NM_000719	Calcium channel, voltage-dependent, L type, alpha 1C subunit	-0.15	-0.02	-0.20	1	1	0	2	0	0	2	0	1			
EXD2	NM_001193360	Exonuclease 3'-5' domain containing 2	-1.16	-0.03	-0.15	0	1	0	1	0	1	1	0	0			
USP31	NM_020718	Ubiquitin-specific peptidase 31	-0.88	-0.56	-0.14	0	1	1	0	1	0	1	1	0			
TDP1	NM_001008744	Tyrosyl-DNA phosphodiesterase 1	-0.41	-0.08	-0.14	0	1	0	1	0	1	0	1	1			
YPEL1	NM_013313	Yippee-like 1 (<i>Drosophila</i>)	-0.24	-0.37	-0.14	1	3	0	1	0	1	0	0	1			
IQCJ	NM_001042706	IQ motif containing J	-0.13	-0.11	-0.14	0	1	0	1	0	1	0	1	1			
LOX	NM_001178102	Lysyl oxidase	-0.25	-1.06	-0.03	0	1	3	0	1	0	1	0	1			

Abbreviation: PCa, prostate cancer.

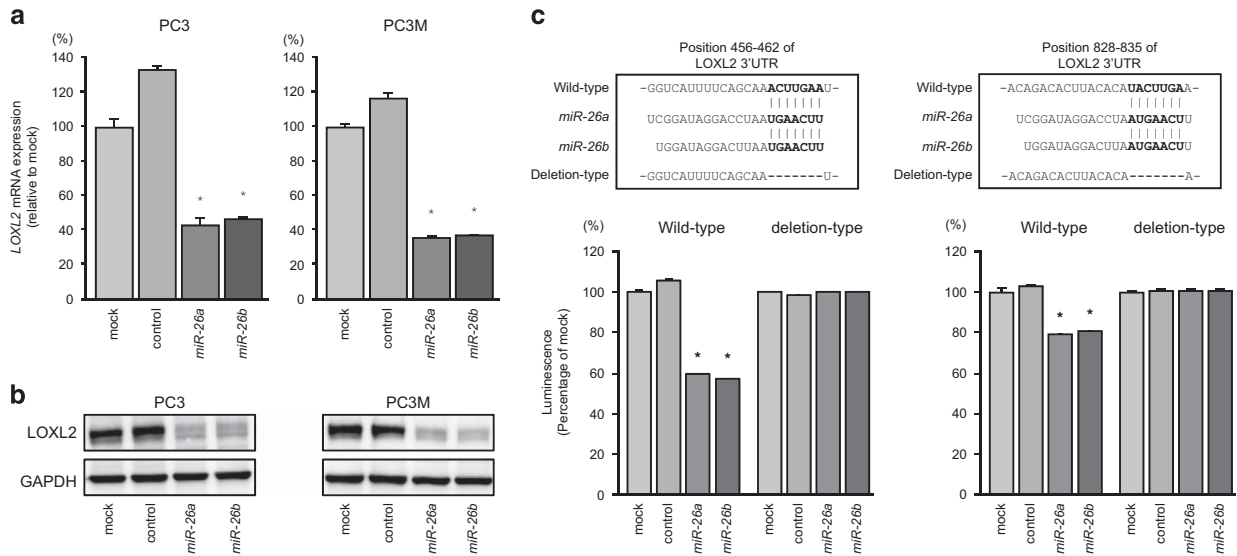


Figure 2 Direct regulation of *LOXL2* by *miR-26a* and *miR-26b* in PCa cell lines. (a) *LOXL2* mRNA expression 72 h after transfection with *miR-26a* or *miR-26b*. *GAPDH* expression was used for normalization. * $P < 0.0001$. (b) *LOXL2* protein expression 72 h after transfection with *miR-26a/b*. *GAPDH* was used as a loading control. (c) The *miR-26a*- and *miR-26b*-binding site in the 3'-UTR of *LOXL2* mRNA. Luciferase reporter assays were performed using vectors that included (WT) or lacked (DEL) the wild-type sequences of the putative *miR-26a* and *miR-26b* target site. * $P < 0.0001$.

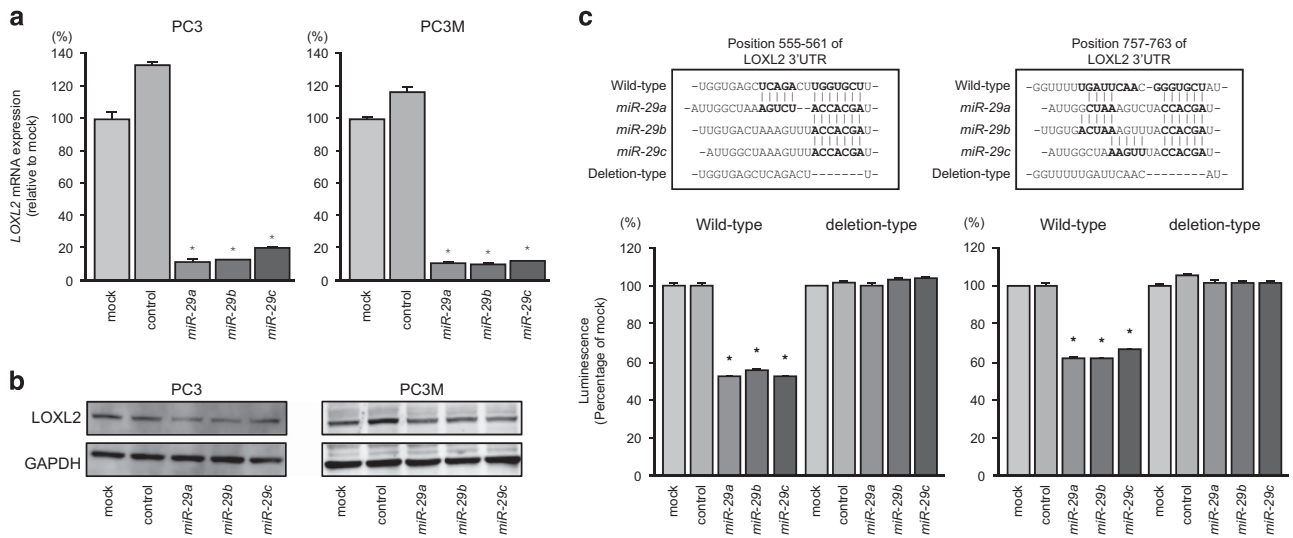


Figure 3 Direct regulation of *LOXL2* by *miR-29a*, *miR-29b* and *miR-29c* in PCa cell lines. (a) *LOXL2* mRNA expression 72 h after transfection with *miR-29s*. *GAPDH* expression was used for normalization. * $P < 0.0001$. (b) *LOXL2* protein expression 72 h after transfection with *miR-29s*. *GAPDH* was used as a loading control. (c) The *miR-29s*-binding site in the 3'-UTR of *LOXL2* mRNA. Luciferase reporter assays were performed using vectors that included (WT) or lacked (DEL) the wild-type sequences of the putative *miR-29s* target site. * $P < 0.0001$.

miR-29b, *miR-29c* or *miR-218* to investigate whether *LOXL2* expression was downregulated by restoration of these miRNAs. The expression levels of *LOXL2* mRNA and protein were markedly suppressed in transfected cells with these miRNAs (Figures 2a,b, 3a,b and 4a,b). Next we performed luciferase reporter assays in PC3 cells to determine whether *LOXL2* mRNA had actual target sites for these miRNAs. We used vectors encoding either a partial wild-type sequence or a sequence in which the miRNA-binding sequence was deleted from *LOXL2* mRNA. The luminescence intensity was reduced by co-transfection with *miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c*, *miR-218* and the vector carrying the wild-type *LOXL2* 3'-UTR in PC3 cells (Figures 2c, 3c and 4c).

Effects of knockdown of *LOXL2* on cell proliferation, migration and invasion in PCa cell lines

To investigate the cancer cell-promoting role of *LOXL2*, we performed loss-of-function assays using *si-LOXL2* transfectants. Knockdown efficiency of *si-LOXL2* was evaluated in PC3 and PC3M transfectant cells. Both mRNA and protein expression levels of *LOXL2* were downregulated in PC3 and PC3M cells (Figures 5a and b). XTT cell proliferation, wound-healing and invasion activities demonstrated that cell proliferation, migration and invasion activities were significantly suppressed in *si-LOXL2*-transfectant cells (Figures 5c–e).

Immunostaining of *LOXL2* in PCa tissues

We validated the expression levels of *LOXL2* in PCa tissues using immunostaining. We used a tissue microarray containing 51 PCa, 10

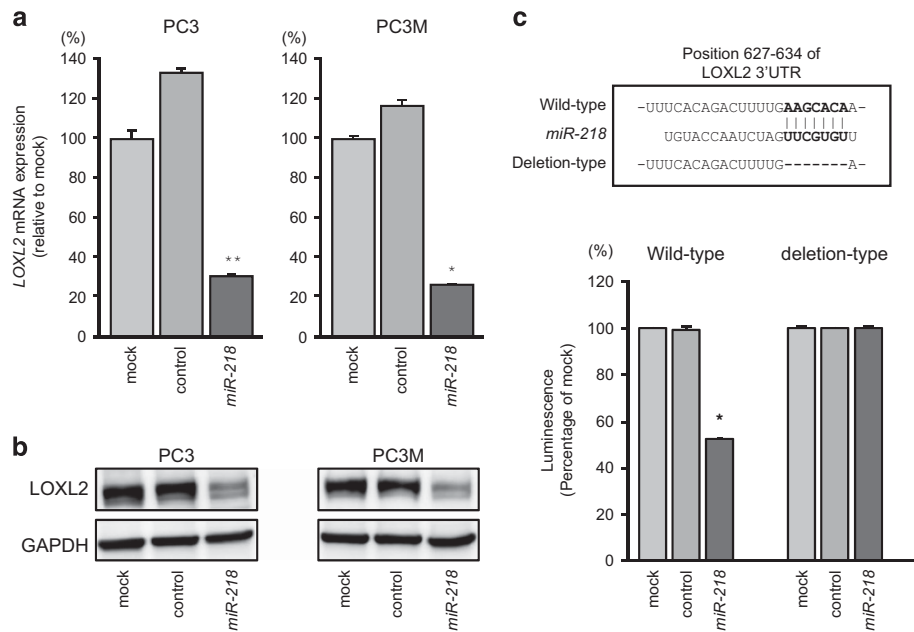


Figure 4 Direct regulation of *LOXL2* by *miR-218* in PCa cell lines. **(a)** *LOXL2* mRNA expression 72 h after transfection with *miR-218*. *GAPDH* expression was used for normalization. * $P < 0.0001$. ** $P = 0.0002$. **(b)** *LOXL2* protein expression 48 h after transfection with *miR-218*. *GAPDH* was used as a loading control. **(c)** The *miR-218*-binding site in the 3'-UTR of *LOXL2* mRNA. Luciferase reporter assays were performed using vectors that included (WT) or lacked (DEL) the wild-type sequences of the putative *miR-218* target site. *Renilla* luciferase assays were normalized to firefly luciferase values. * $P < 0.0001$.

PINs and 10 prostatic hyperplastic specimens (Table 2). Upregulation of *LOXL2* protein was confirmed in the PCa tissues compared with noncancerous tissues (Figure 6).

DISCUSSION

Aberrantly expressed miRNAs might disturb normally regulated RNA networks and contribute to cancer cell pathology. Strategies to identify aberrant expression of miRNA-mediated RNA networks are being developed as a new direction in cancer research in the postgenome sequencing era. Based on the miRNA expression signature of PCa cells, we have continued the identification of tumor-suppressive miRNAs and their regulated novel PCa metastatic pathways.^{10–13,24–27} Our recent studies showed that restoration of antitumor miRNAs (*miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c* and *miR-218*) markedly suppressed PCa cell aggressiveness via targeting of La-related protein 1 (*LARPI*), laminin γ 1 (*LAMC1*) and *LASPI*, respectively.^{11–13} Moreover, these miRNA-targeted genes were overexpressed in PCa tissues and expression of these genes enhanced cancer cell migration and invasion.^{11–13} *LASPI* encodes a LIM motif at its N-terminus and an src homology 3 (SH3) domain at its C-terminus. In *LASPI*, SH3 domain functions in protein–protein interactions^{28,29} and *LASPI* acts as an actin-binding protein and overexpression of *LASPI* enhances cancer cell proliferation and invasion.^{13,28,30} Our present data showed that *LASPI* is a putative candidate of these antitumor miRNA regulation, suggesting that *LASPI* is a key molecule for cancer cell aggressiveness.

The *miR-26* family is comprised of three subtypes in the human genome: *miR-26a-1* (chromosome 3p22.2), *miR-26a-2* (12q14.1), and *miR-26b* (2q35). The seed sequences of these miRNAs are identical, suggesting that the *miR-26* family might regulate the same genes in human cells (miRBase: release 21; <http://www.mirbase.org/>). Downregulation of the *miR-26* family and its antitumor effects have been reported in several cancers, such as bladder cancer, breast cancer, hepatocellular carcinoma, oral cancer and PCa.^{31–34} Our past study

showed that downregulation of these miRNAs enhanced cancer cell migration and invasion in oral cancer through direct regulation of *TMEM184B*.³⁵ In PCa, *EZH2*, a histone-lysine *N*-methyltransferase enzyme, was directly regulated by *miR-26a* and *miR-26b*.³⁶ Overexpression of *EZH2* is observed in several cancers and contributes to cancer aggressiveness.^{36,37}

The *miR-29* family consists of four miRNAs: *miR-29a*, *miR-29b-1*, *miR-29b-2*, and *miR-29c*. These miRNAs form cluster miRNA on two human chromosome loci: *miR-29b-1* and *miR-29a* in 7q32 and *miR-29b-2* and *miR-29c* in 1q32 (miRBase: release 21; <http://www.mirbase.org/>). Our miRNA expression signatures showed that all member of *miR-29* family were downregulated in several cancers and that restoration of these miRNAs markedly inhibited cancer cell aggressiveness via targeting of ECM–integrin pathways.^{12,38} Antitumor roles of the *miR-29* family were demonstrated in several cancers.^{12,18,20,38,39} Interestingly, recent studies demonstrated molecular mechanisms of silencing of *miR-29* family expression in cancer cells.⁴⁰ The genome structure of *miR-29b-1/miR-29a* promoter region contains two E-box (MYC-binding) sites and four nuclear factor- κ B-binding sites such that overexpressed Myc and nuclear factor- κ B inhibited the expression of *miR-29b-1/miR-29a* at the transcriptional level.⁴⁰ Overexpression of Myc was frequently observed in advanced PCa,⁴¹ and this phenomenon might enhance PCa cell progression and metastasis.

The miRNA database indicates that the *miR-218* family is distributed between two human chromosomal loci: *miR-218-1* at 4p15.31 and *miR-218-2* at 5q35.1. Similar to *miR-26a*, *miR-26b* and *miR-29* family, antitumor function of *miR-218* have been described in many types of cancers.^{13,42–44} Our past studies demonstrated that loss of *miR-218* enhanced cancer cell migration and invasion through dysregulation of genes involved in the focal adhesion pathway, including *CAV2*, *LAMA3* and *LASPI*.^{13,42,44} Two miRNAs, *miR-218-1* and *miR-218-2*, are located on the introns of *SLIT2* and *SLIT3* genes, respectively, and control of the expression depends on the same

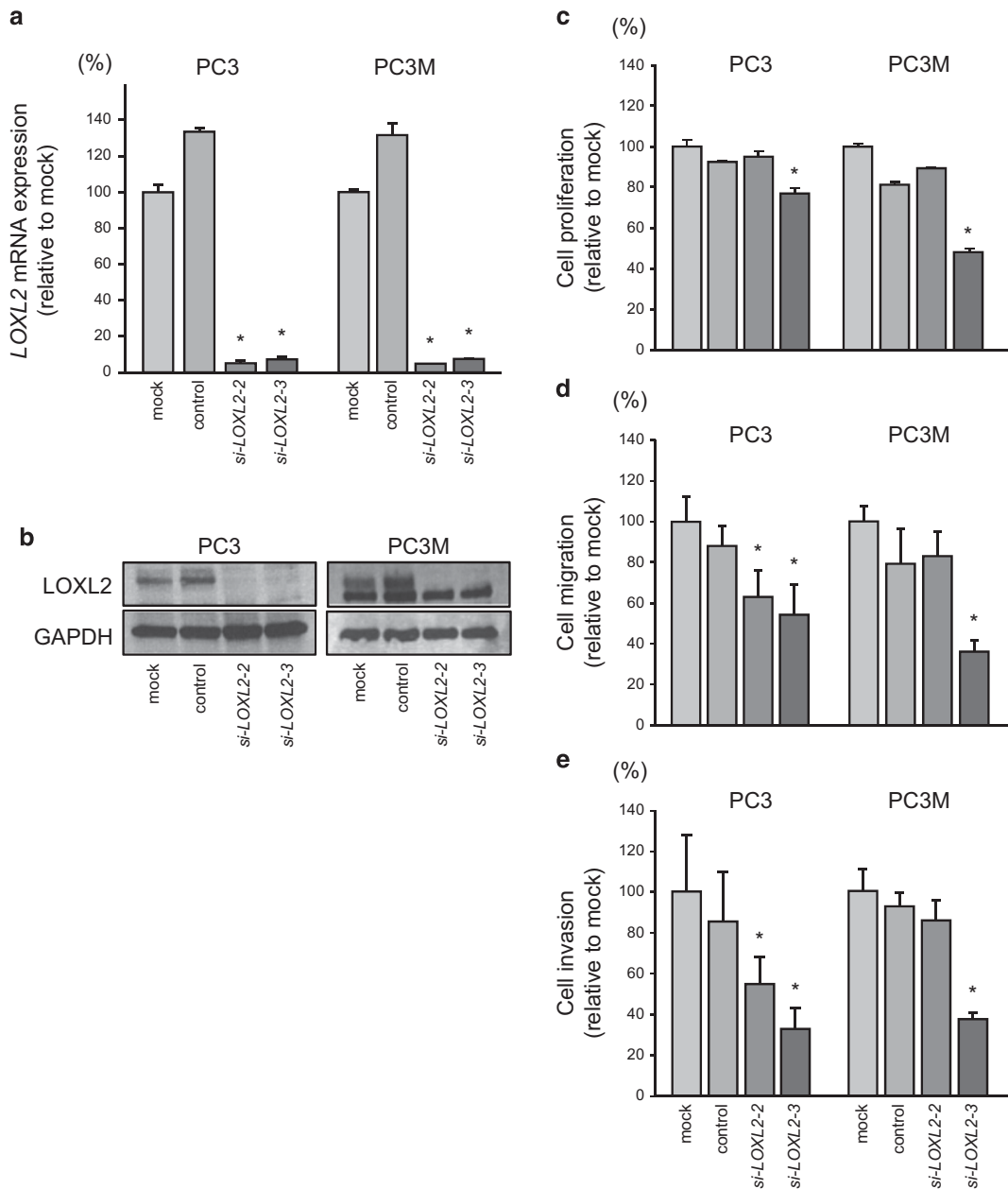


Figure 5 Effects of *si-LOXL2* transfection on cell proliferation, migration and invasion in PCa cell lines. (a) *LOXL2* mRNA expression levels were measured by qRT-PCR 72 h after transfection with 10 nM *si-LOXL2*. *GAPDH* was used for normalization. * $P < 0.0001$. (b) *LOXL2* protein expression 72 h after transfection with 10 nM *si-LOXL2*. *GAPDH* was used as a loading control. (c) Cell proliferation was determined by XTT assay 72 h after transfection with 10 nM *si-LOXL2*. * $P < 0.0001$. (d) Cell migration activity was determined by migration assay 48 h after transfection with 10 nM *si-LOXL2*. * $P < 0.0001$. (e) Cell invasion activity was determined by Matrigel invasion assay 48 h after transfection with 10 nM *si-LOXL2*. * $P < 0.0001$.

promoter in their host genes.⁴⁵ Several reports showed that the promoter regions of these genes were frequently methylated in cancer cell lines and clinical specimens.^{45,46} Thus hypermethylation of their promoter regions caused silencing of *miR-218-1* and *miR-218-2* expression in cancer cells.

The highly invasive properties of PCa cells cause distant metastasis in patients with PCa, and metastasis is the primary reason for the high mortality of advanced PCa.^{1,2} Studying the non-coding RNA networks could reveal the molecular mechanisms underlying metastatic pathways and facilitate the development of novel therapies to block progression of the disease. In this study, we hypothesized that several antitumor miRNAs (the *miR-26a/b*, the *miR-29* family and

miR-218) coordinately regulate genes that have key roles in PCa metastasis.

Here we focused on the *LOXL2* gene as a putative regulatory target of these antitumor miRNAs in PCa cells. Recently, we showed that *LOXL2* was directly regulated by *miR-29a*, *miR-29b* and *miR-29c* and its expression promoted cancer cell aggressiveness in renal cell carcinoma, non-small cell lung cancer and head and neck squamous cell carcinoma.^{18–20} Present data demonstrated that six antitumor miRNAs (*miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c* and *miR-218*) directly regulated *LOXL2* in PCa cells. Moreover, over-expression of *LOXL2* was confirmed in PCa tissues and knockdown of *LOXL2* markedly impaired cancer cell aggressiveness. Notably,

Table 2 Clinical characteristics and IHC score of LOXL2 in tissue microarray

No.	Diagnosis	Age	Gleason score	Stage pT	Stage pN	IHC score of LOXL2
1	PCa	64	4+3	3b	0	5
2	PCa	67	3+4	2b	0	5
3	PCa	58	3+4	2b	0	5
4	PCa	63	7	3b	0	6
5	PCa	65	3+3	2b	0	5
6	PCa	61	4+4	3b	×	5
7	PCa	62	3+4	2b	×	4
8	PCa	66	4+4	2b	×	4
9	PCa	61	3+4	3a	×	4
10	PCa	74	4+3	2b	×	5
11	PCa	54	3+4	2c	×	5
12	PCa	68	3+4	3a	0	5
13	PCa	58	3+4	3a	0	5
14	PCa	65	3+3	2a	0	5
15	PCa	77	3+4	4	0	5
16	PCa	58	3+4	3a	0	5
17	PCa	50	4+3	2b	0	5
18	PCa	53	3+3	2b	0	5
19	PCa	59	4+5	3a	0	5
20	PCa	70	2+3	2b	0	5
21	PCa	65	5+4	3a	0	4
22	PCa	57	3+5	2b	0	4
23	PCa	68	4+4	2b	0	5
24	PCa	58	3+3	2b	0	5
25	PCa	63	3+4	2b	0	5
26	PCa	56	3+4	2b	0	5
27	PCa	63	5+3	3a	0	3
28	PCa	64	3+5	3a	0	5
29	PCa	60	3+4	2b	0	5
30	PCa	60	3+3	3a	0	4
31	PCa	57	3+2	2b	0	4
32	PCa	50	3+3	2a	0	5
33	PCa	68	3+3	3a	0	4
34	PCa	65	3+4	3b	1	3
35	PCa	69	5+5	3a	1	3
36	PCa	51	2+3	2b	0	4
37	PCa	62	3+3	3a	0	5
38	PCa	61	3+4	3a	0	4
39	PCa	53	4+4	3b	1	3
40	PCa	56	4+3	2b	0	4
41	PCa	59	2+3	2b	0	3
42	PCa	61	3+4	2b	0	4
43	PCa	62	3+4	3b	1	3
44	PCa	66	3+3	3a	0	5
45	PCa	62	3+3	2b	0	5
46	PCa	56	3+3	2b	0	5
47	PCa	58	3+3	3a	0	5
48	PCa	66	5+4	3a	0	4
49	PCa	55	3+4	3a	0	4
50	PCa	67	2+3	2b	0	4
51	PCa	61	3+5	2b	0	4
52	PIN	59	—	—	—	0
53	PIN	58	—	—	—	2
54	PIN	62	—	—	—	0
55	PIN	51	—	—	—	2
56	PIN	58	—	—	—	3
57	PIN	68	—	—	—	3
58	PIN	64	—	—	—	4
59	PIN	56	—	—	—	5
60	PIN	61	—	—	—	3

Table 2 (Continued)

No.	Diagnosis	Age	Gleason score	Stage pT	Stage pN	IHC score of LOXL2
61	PIN	51	—	—	—	4
62	Non-PCa	70	—	—	—	4
63	Non-PCa	63	—	—	—	0
64	Non-PCa	62	—	—	—	2
65	Non-PCa	81	—	—	—	0
66	Non-PCa	67	—	—	—	2
67	Non-PCa	76	—	—	—	0
68	Non-PCa	66	—	—	—	3
69	Non-PCa	69	—	—	—	3
70	Non-PCa	63	—	—	—	3
71	Non-PCa	71	—	—	—	4

Abbreviations: IHC, immunohistochemical; LOXL2, lysyl oxidase-like 2; PCa, prostate cancer; PIN, prostatic intraepithelial neoplasia. Immunohistochemical status and characteristics of PCa, PIN and normal prostate cases.

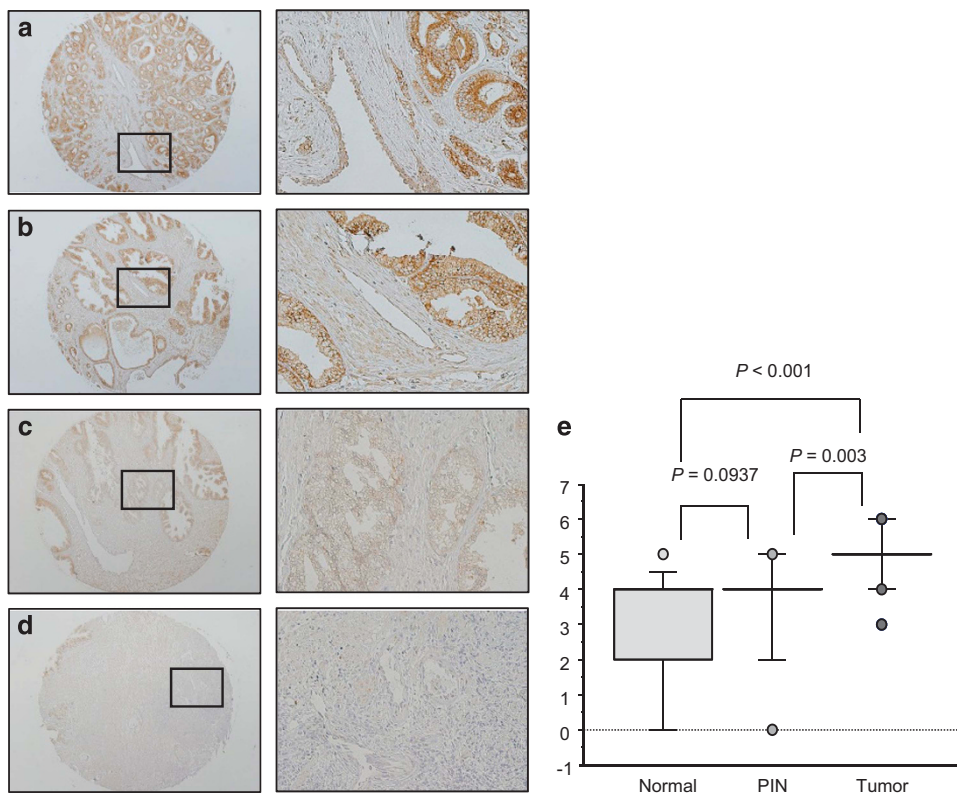


Figure 6 Immunohistochemical staining of LOXL2 in PCa clinical specimens. LOXL2 was expressed more strongly in several cancer lesions, weakly stained in PIN lesions than in normal tissues. (a) Prostate cancer, pT4N0, Grade3a, Gleason score 3+4; (b) Prostate cancer, pT2bN0, Grade3a, Gleason score 4+3; (c) PIN; (d) normal prostate tissue. (e) Quantification of LOXL2 expression. Expression of LOXL2 was upregulated in PCa and PIN specimens compared with normal prostate tissues.

we showed that LOXL2 regulation by these miRNAs was also observed in head and neck squamous cell carcinoma cells.²⁰ To identify regulation of *LOXL2* by these miRNAs is needed to elucidate the metastatic roles of antitumor miRNAs in cancer.

The LOX protein family is comprised of five proteins (LOX and LOXL1–LOXL4). Their primary functions appear to be covalent crosslinking of collagen to elastin in the ECM.^{14–17} Overexpression of the LOX family was observed in several cancers,^{18–20} indicating that dysregulated expression of the LOX family enhances ECM deposition and subsequent tissue stiffness. Overexpression of ECM component

proteins is frequently observed in many types of cancer tissues. This aberration promotes cancer cell aggressiveness by dysregulation of cell adhesion and ECM remodeling.¹⁴ Thus aberrant expression of the LOX family is a trigger of malignant transformation of cancer cells through ECM dysregulation. Several studies have indicated that high expression of LOXL2 was correlated with poor prognosis in patients with gastric, breast, lung and laryngeal cancers.^{19–22} To investigate the clinical features of PCa patients and *LOXL2* expression is an important theme. A large scale of cohort study will be necessary in future. Interestingly, transcriptional control of *LOXL2* was regulated by

hypoxia-inducible factor-1. LOXL2 directly interacts with transcriptional factor SNAIL1 in the nucleus and repressed the expression of E-cadherin.^{47,48} Thus hypoxic conditions induced LOXL2 expression, and the accumulating LOXL2 enhanced the aberrant activation of epithelial-mesenchymal transition signaling in cancer cells.

In conclusion, direct regulation of *LOXL2* by antitumor miRNAs (*miR-26a*, *miR-26b*, *miR-29a*, *miR-29b*, *miR-29c* and *miR-218*) was observed in PCa cells. Overexpression of LOXL2 was validated in PCa tissues and aberrantly expressed LOXL2 enhanced PCa cell aggressiveness. Understanding of novel RNA networks regulated by the antitumor miRNAs may lead to a better understanding of PCa metastasis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by KAKENHI, grant numbers (C) 15K10801, (C) 15K20070, (C) 15K20071 and (B) 25293333.

- Chi, K. N., Bjartell, A., Dearnaley, D., Saad, F., Schroder, F. H., Sternberg, C. et al. Castration-resistant prostate cancer: from new pathophysiology to new treatment targets. *Eur. Urol.* **56**, 594–605 (2009).
- Sturge, J., Caley, M. P. & Waxman, J. Bone metastasis in prostate cancer: emerging therapeutic strategies. *Nat. Rev. Clin. Oncol.* **8**, 357–368 (2011).
- Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
- Carthew, R. W. & Sontheimer, E. J. Origins and mechanisms of miRNAs and siRNAs. *Cell* **136**, 642–655 (2009).
- Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* **9**, 102–114 (2008).
- Hobert, O. Gene regulation by transcription factors and microRNAs. *Science* **319**, 1785–1786 (2008).
- Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
- Iorio, M. V. & Croce, C. M. MicroRNAs in cancer: small molecules with a huge impact. *J. Clin. Oncol.* **27**, 5848–5856 (2009).
- Goto, Y., Kurozumi, A., Enokida, H., Ichikawa, T. & Seki, N. Functional significance of aberrantly expressed microRNAs in prostate cancer. *Int. J. Urol.* **22**, 242–252 (2015).
- Goto, Y., Kojima, S., Nishikawa, R., Kurozumi, A., Kato, M., Enokida, H. et al. MicroRNA expression signature of castration-resistant prostate cancer: the microRNA-221/222 cluster functions as a tumour suppressor and disease progression marker. *Br. J. Cancer* **113**, 1055–1065 (2015).
- Kato, M., Goto, Y., Matsushita, R., Kurozumi, A., Fukumoto, I., Nishikawa, R. et al. MicroRNA-26a/b directly regulate La-related protein 1 and inhibit cancer cell invasion in prostate cancer. *Int. J. Oncol.* **47**, 710–718 (2015).
- Nishikawa, R., Goto, Y., Kojima, S., Enokida, H., Chiyomaru, T., Kinoshita, T. et al. Tumor-suppressive microRNA-29s inhibit cancer cell migration and invasion via targeting LAMC1 in prostate cancer. *Int. J. Oncol.* **45**, 401–410 (2014).
- Nishikawa, R., Goto, Y., Sakamoto, S., Chiyomaru, T., Enokida, H., Kojima, S. et al. Tumor-suppressive microRNA-218 inhibits cancer cell migration and invasion via targeting of LASP1 in prostate cancer. *Cancer Sci.* **105**, 802–811 (2014).
- Barker, H. E., Cox, T. R. & Erler, J. T. The rationale for targeting the LOX family in cancer. *Nat. Rev. Cancer* **12**, 540–552 (2012).
- Kagan, H. M. & Li, W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J. Cell. Biochem.* **88**, 660–672 (2003).
- Vadasz, Z., Kessler, O., Akiri, G., Gengrinovitch, S., Kagan, H. M., Baruch, Y. et al. Abnormal deposition of collagen around hepatocytes in Wilson's disease is associated with hepatocyte specific expression of lysyl oxidase and lysyl oxidase like protein-2. *J. Hepatol.* **43**, 499–507 (2005).
- Kim, Y. M., Kim, E. C. & Kim, Y. The human lysyl oxidase-like 2 protein functions as an amine oxidase toward collagen and elastin. *Mol. Biol. Rep.* **38**, 145–149 (2011).
- Nishikawa, R., Chiyomaru, T., Enokida, H., Inoguchi, S., Ishihara, T., Matsushita, R. et al. Tumour-suppressive microRNA-29s directly regulate LOXL2 expression and inhibit cancer cell migration and invasion in renal cell carcinoma. *FEBS Lett.* **589**, 2136–2145 (2015).
- Fukumoto, I., Kikkawa, N., Matsushita, R., Kato, M., Kurozumi, A., Nishikawa, R. et al. Tumor-suppressive microRNAs (miR-26a/b, miR-29a/b/c and miR-218) concertedly suppressed metastasis-promoting LOXL2 in head and neck squamous cell carcinoma. *J. Hum. Genet.* **61**, 109–118 (2016).
- Mizuno, K., Seki, N., Mataka, H., Matsushita, R., Kamikawaji, K., Kumamoto, T. et al. Tumor-suppressive microRNA-29 family inhibits cancer cell migration and invasion directly targeting LOXL2 in lung squamous cell carcinoma. *Int. J. Oncol.* **48**, 450–460 (2016).
- Kasashima, H., Yashiro, M., Kinoshita, H., Fukuoka, T., Morisaki, T., Masuda, G. et al. Lysyl oxidase-like 2 (LOXL2) from stromal fibroblasts stimulates the progression of gastric cancer. *Cancer Lett.* **354**, 438–446 (2014).
- Ahn, S. G., Dong, S. M., Oshima, A., Kim, W. H., Lee, H. M., Lee, S. A. et al. LOXL2 expression is associated with invasiveness and negatively influences survival in breast cancer patients. *Breast Cancer Res. Treat.* **141**, 89–99 (2013).
- Peinado, H., Moreno-Bueno, G., Hardisson, D., Perez-Gomez, E., Santos, V., Mendiola, M. et al. Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. *Cancer Res.* **68**, 4541–4550 (2008).
- Kurozumi, A., Goto, Y., Matsushita, R., Fukumoto, I., Kato, M., Nishikawa, R. et al. Tumor-suppressive microRNA-223 inhibits cancer cell migration and invasion by targeting ITGA3/ITGB1 signaling in prostate cancer. *Cancer Sci.* **107**, 84–94 (2016).
- Kojima, S., Chiyomaru, T., Kawakami, K., Yoshino, H., Enokida, H., Nohata, N. et al. Tumour suppressors miR-1 and miR-133a target the oncogenic function of purine nucleoside phosphorylase (PNP) in prostate cancer. *Br. J. Cancer* **106**, 405–413 (2012).
- Goto, Y., Nishikawa, R., Kojima, S., Chiyomaru, T., Enokida, H., Inoguchi, S. et al. Tumour-suppressive microRNA-224 inhibits cancer cell migration and invasion via targeting oncogenic TP52 in prostate cancer. *FEBS Lett.* **588**, 1973–1982 (2014).
- Nishikawa, R., Goto, Y., Kurozumi, A., Matsushita, R., Enokida, H., Kojima, S. et al. MicroRNA-205 inhibits cancer cell migration and invasion via modulation of centromere protein F regulating pathways in prostate cancer. *Int. J. Urol.* **22**, 867–877 (2015).
- Orth, M. F., Cazes, A., Butt, E. & Grunewald, T. G. An update on the LIM and SH3 domain protein 1 (LASP1): a versatile structural, signaling, and biomarker protein. *Oncotarget* **6**, 26–42 (2015).
- Grunewald, T. G. & Butt, E. The LIM and SH3 domain protein family: structural proteins or signal transducers or both? *Mol. Cancer* **7**, 31 (2008).
- Pappas, C. T., Bliss, K. T., Zieseniss, A. & Gregorio, C. C. The Nebulin family: an actin support group. *Trends Cell Biol.* **21**, 29–37 (2011).
- Lin, Y., Chen, H., Hu, Z., Mao, Y., Xu, X., Zhu, Y. et al. miR-26a inhibits proliferation and motility in bladder cancer by targeting HMGAL1. *FEBS Lett.* **587**, 2467–2473 (2013).
- Zhang, B., Liu, X. X., He, J. R., Zhou, C. X., Guo, M., He, M. et al. Pathologically decreased miR-26a antagonizes apoptosis and facilitates carcinogenesis by targeting MTDH and EZH2 in breast cancer. *Carcinogenesis* **32**, 2–9 (2011).
- Zhu, Y., Lu, Y., Zhang, Q., Liu, J. J., Li, T. J., Yang, J. R. et al. MicroRNA-26a/b and their host genes cooperate to inhibit the G1/S transition by activating the pRb protein. *Nucleic Acids Res.* **40**, 4615–4625 (2012).
- Jia, L. F., Wei, S. B., Gan, Y. H., Guo, Y., Gong, K., Mitchelson, K. et al. Expression, regulation and roles of miR-26a and MEG3 in tongue squamous cell carcinoma. *Int. J. Cancer* **135**, 2282–2293 (2014).
- Fukumoto, I., Hanazawa, T., Kinoshita, T., Kikkawa, N., Koshizuka, K., Goto, Y. et al. MicroRNA expression signature of oral squamous cell carcinoma: functional role of microRNA-26a/b in the modulation of novel cancer pathways. *Br. J. Cancer* **112**, 891–900 (2015).
- Koh, C. M., Iwata, T., Zheng, Q., Bethel, C., Yegnasubramanian, S. & De Marzo, A. M. Myc enforces overexpression of EZH2 in early prostatic neoplasia via transcriptional and post-transcriptional mechanisms. *Oncotarget* **2**, 669–683 (2011).
- Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G. et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624–629 (2002).
- Kinoshita, T., Nohata, N., Hanazawa, T., Kikkawa, N., Yamamoto, N., Yoshino, H. et al. Tumor-suppressive microRNA-29s inhibit cancer cell migration and invasion by targeting laminin-integrin signalling in head and neck squamous cell carcinoma. *Br. J. Cancer* **109**, 2636–2645 (2013).
- Yamamoto, N., Kinoshita, T., Nohata, N., Yoshino, H., Itesako, T., Fujimura, L. et al. Tumor-suppressive microRNA-29a inhibits cancer cell migration and invasion via targeting HSP47 in cervical squamous cell carcinoma. *Int. J. Oncol.* **43**, 1855–1863 (2013).
- Mott, J. L., Kurita, S., Cazanave, S. C., Bronk, S. F., Werneburg, N. W. & Fernandez-Zapico, M. E. Transcriptional suppression of miR-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB. *J. Cell. Biochem.* **110**, 1155–1164 (2010).
- Koh, C. M., Bieberich, C. J., Dang, C. V., Nelson, W. G., Yegnasubramanian, S. & De Marzo, A. M. MYC and prostate cancer. *Genes Cancer* **1**, 617–628 (2010).
- Kinoshita, T., Hanazawa, T., Nohata, N., Kikkawa, N., Enokida, H., Yoshino, H. et al. Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma. *Oncotarget* **3**, 1386–1400 (2012).
- Yamamoto, N., Kinoshita, T., Nohata, N., Itesako, T., Yoshino, H., Enokida, H. et al. Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion by targeting focal adhesion pathways in cervical squamous cell carcinoma. *Int. J. Oncol.* **42**, 1523–1532 (2013).
- Yamasaki, T., Seki, N., Yoshino, H., Itesako, T., Hidaka, H., Yamada, Y. et al. MicroRNA-218 inhibits cancer cell migration and invasion in renal cell carcinoma through targeting caveolin-2 involved in focal adhesion pathway. *J. Urol.* **190**, 1059–1068 (2013).
- Alajez, N. M., Lenarduzzi, M., Ito, E., Hui, A. B., Shi, W., Bruce, J. et al. MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res.* **71**, 2381–2391 (2011).

- 46 Uesugi, A., Kozaki, K., Tsuruta, T., Furuta, M., Morita, K., Imoto, I. *et al*. The tumor suppressive microRNA miR-218 targets the mTOR component Rictor and inhibits AKT phosphorylation in oral cancer. *Cancer Res.* **71**, 5765–5778 (2011).
- 47 Schietke, R., Warnecke, C., Wacker, I., Schodel, J., Mole, D. R., Campean, V. *et al*. The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. *J. Biol. Chem.* **285**, 6658–6669 (2010).
- 48 Moon, H. J., Finney, J., Xu, L., Moore, D., Welch, D. R. & Mure, M. MCF-7 cells expressing nuclear associated lysyl oxidase-like 2 (LOXL2) exhibit an epithelial-to-mesenchymal transition (EMT) phenotype and are highly invasive in vitro. *J. Biol. Chem.* **288**, 30000–30008 (2013).