Tumor-suppressive *microRNA-29s* inhibit cancer cell migration and invasion via targeting *LAMC1* in prostate cancer

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Abstract. Our recent studies of microRNA (miRNA) expression signatures revealed that *microRNA-29s* (*miR-29s*; including *miR-29a/b/c*) were significantly downregulated in prostate cancer (PCa) and was a putative tumor-suppressive miRNA family in PCa. Herein, we aimed to investigate the functional significance of *miR-29* in cancer cells and to identify novel *miR-29s*-mediated cancer pathways and target genes involved in PCa oncogenesis and metastasis. Restoration of *miR-29s* in PC3 and DU145 cell lines revealed significant inhibition of cancer cell migration and invasion. To identify *miR-29s*-mediated molecular pathways and targets, we used gene expression data and *in silico* database analysis. Our analysis demonstrated that *miR-29s* modulated the focal adhesion pathway. Moreover, the laminin γ1 *(LAMC1)* gene was a candidate target of *miR-29s* regulation. Luciferase reporter assays showed that *miR-29s* directly regulated *LAMC1*. Silencing of *LAMC1* significantly inhibited cell migration and invasion in cancer cells, and *LAMC1* was upregulated in PCa. *miR-29s* acted as tumor suppressors, contributing to cancer cell migration and invasion and directly targeting laminin signaling. Recognition of tumor-suppressive miRNA-mediated cancer pathways provides new insights into the potential mechanisms of PCa oncogenesis and metastasis, and suggests novel therapeutic strategies for treating this disease.

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); however, PCa can eventually become resistant to ADT and progress to castration-resistant prostate cancer (CRPC). Currently, CRPC is difficult to treat, and most clinical trials for advanced PCa have shown limited benefits, with disease progression and metastasis to the bone or other sites (2,3). Therefore, understanding the molecular mechanisms of CRPC and the metastatic pathways underlying PCa using currently available genomic approaches would help to elucidate for this disease.

The discovery of non-coding RNAs (ncRNAs) in the human genome was an important conceptual breakthrough in the post-genome sequencing era (4). Further improving our 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 protein-coding gene expression by repressing translation or cleaving RNA transcripts in a sequence-specific manner (5). Numerous studies have shown that miRNAs are aberrantly expressed in many human cancers and that they play significant roles in the initiation, development and metastasis of those cancers (6-9). Moreover, normal regulatory mechanisms can be disrupted by the aberrant expression of tumor-suppressive or oncogenic miRNAs in cancer cells (10,11). Therefore, identification of aberrantly expressed miRNAs is an important first step toward elucidating miRNA-mediated oncogenic pathways.

Based on these data, we have sought to elucidate the miRNA expression signatures of PCa clinical specimens and have investigated the specific roles of miRNAs in PCa oncogenesis using differentially expressed miRNAs (12). Recently, we demonstrated that several miRNAs are downregulated in PCa and that *miR-1/133a* and *miR-143/145* clusters function as tumor suppressors, targeting several oncogenic genes (13,14). Data from our analysis of the miRNA signature of PCa showed that *miR-29s* (including *miR-29a/b/c*) are significantly downregulated in cancer tissues, suggesting that miR-29s may act as tumor suppressors.

The aim of the present study was to investigate the functional significance of *miR-29s* and to identify the molecular pathways and targets regulated by these miRNAs in PCa cells.

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Our data demonstrated that restoration of mature *miR-29s* inhibited cancer cell proliferation. Moreover, gene expression data and *in silico* database analysis showed that laminin γ1 *(LAMC1)*, which is involved in the 'focal adhesion' pathway, was a potential target of *miR-29*-mediated regulation. Silencing of the *LAMC1* gene significantly inhibited cell migration and invasion in cancer cells. Thus, our study revealed that tumor-suppressive *miR-29s* regulated cancer pathways and cancer-related target molecules, providing new insights into the potential mechanisms of PCa oncogenesis and metastasis.

Materials and methods

Clinical prostate specimens. Clinical specimens were obtained from patients at the Teikyo University Chiba Medical Center from 2012 to 2013. All patients had elevated levels of prostate-specific antigen (PSA) and had undergone transrectal prostate needle biopsy. Non-cancerous prostate tissue (n=33) was obtained from patients who were negative for malignancy without indurations on the prostate. Prostate cancer tissues (n=37) contained 90-100% malignant cells in the biopsy cores.

The patient backgrounds and clinicopathological characteristics are summarized in Table 1A and B. 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 Teikyo University.

Cell culture and RNA extraction. Human prostate cancer cells (PC3 and DU145) were obtained from the American Type Culture Collection (Manassas, VA, USA) and 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 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). The procedure for PCR quantification was carried out as previously described (12-14). TaqMan probes and primers for LAMC1 (P/N: Hs00267056_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-29a* (Assay ID: 002112), *miR-29b* (Assay ID: 000413) and *miR-29c* (Assay ID: 000587) were analyzed by TaqMan quantitative real-time PCR (TaqMan MicroRNA Assay; Applied Biosystems) and normalized to the expression of *RNU48* (Assay ID: 001006). All reactions were performed in triplicate, and each assay included negative control reactions that lacked cDNA.

Transfection with mature miRNA and small-interfering RNA (siRNA). The following mature miRNA species were used in this study: mirVana miRNA mimic for *hsa-miR-29a-3p* (product ID: MC12499; Applied Biosystems), *hsa-miR-29b-3p* (product ID: MC10103), and *hsa-miR-29c-3p* (product ID: MC10518). The following si-RNAs were used: Stealth Select RNAi si-RNA, *si-LAMC1* (P/N: HSS105959, HSS180591; Invitrogen), and negative control miRNA/siRNA (P/N: AM17111; Applied Biosystems). RNAs were incubated with OPTI-MEM (Invitrogen) and Lipofectamine RNAiMax reagent (Invitrogen). 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 (15).

Cell proliferation, migration and invasion assays. To investigate the functional significance of *miR-29s*, we performed cell proliferation, migration and invasion assays using PC3 and DU145 cells. The experimental procedures were performed as described in our previous studies (12-16).

Western blotting. Cells were harvested 72 h after transfection, and lysates were prepared. Fifty micrograms of protein from each lysates was separated on Mini-Protean TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. Immunoblotting was performed with mouse anti-LAMC1 antibodies (1:250; HSA001909; Sigma-Aldrich, St. Louis, MO, USA), and anti-GAPDH antibodies (1:1000; ab8245, Abcam, Cambridge, UK) were used as an internal loading control.

Genome-wide gene expression and in silico analysis for the identification of genes regulated by miR-29s. To identify target genes of *miR-29s*, we used *in silico* analysis and genome-wide gene expression analysis. First, we screened genes using TargetScan release 6.2 (http://www.targetscan.org/). These genes were then categorized into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways using GeneCodis analysis (http://genecodis.cnb.csic.es/). To identify upregulated genes in PCa, we analyzed a publicly available gene expression data set in GEO (accession no.: GSE29079).

Plasmid construction and dual-luciferase reporter assay. Partial sequences of the *LAMC1* 3'-untranslated region (UTR) of putative *miR-29s* binding sites (180 bp) were inserted between the *Xho*I-*Pme*I restriction sites in the 3'-UTR of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). PC3 cells were transfected with 5 ng of the vector and 10 nM *miR-29s* 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 as previously described (17).

Statistical analysis. The relationships between 2 groups and the numerical values obtained by real-time RT-PCR were analyzed using paired t-tests. The relationships among 3 variables and numerical values were 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

Expression levels of miR-29s (miR-29a/b/c) in PCa specimens. The chromosomal locations of *miR-29s* in the human

Table I. Patient characteristics.

A, Patient characteristics used for *miR-29s* expression

No.	Age (yrs.)	PSA (ng/ml)	Gleason score	TNM classification		
				T	N	M
PCa						
1	67	244	4+4	4	1	1
2	70	395	4+4	3a	1	1
3	83	49.9	4+5	3a	0	0
4	68	212	4+4	3b	1	0
5	80	589	4+4	3b	1	0
6	72	2530	4+5	3b	0	1
7	76	12.5	4+5	3b	1	1
8	67	153	4+4	4	1	1
9	82	808.8	4+4	4	1	1
10	88	50.5	4+4	3a	0	0
11	69	3.45	4+3	3a	0	0
12	64	486	4+5	4	1	1
13	74	60.8	5+5	4	1	1
14	63	49.6	4+4	3b	1	0
15	69	1060	4+5	4	1	1
16	67	34.9	4+4	3b	1	1
17	64	7.23	4+4	3b	1	0
18	79	3750	4+4	3a	0	1
19	78	1400	4+4	3a	0	1
20	77	2640	4+4	4	1	1
21	69	554	4+4	3b	1	1
22	82	59.5	4+5	3b	0	0
23	70	1030	4+4	3b	1	1
24	78	892	4+5	4	1	1
25	86	24.2	4+4	3b	0	0
26	67	64.6	4+5	3b	0	1
27	71	63.8	4+3	3a	0	0
Non-PCa						
28	69	12.2				
29	66	11.9				
30	85	10.1				
31	55	11.2				
32	67	22				
33	66	7.33				
34	69	7.92				
35	68	7.24				
36	53	4.33				
37	62	5.14				
38	63	10				
39	62	5.11				
40	74	8.3				
41	65	4.3				
42	72	5.43				
43	66	5.35				
44	67	5				
45	66	19.5				
46	63	12.9				
47	53	6.69				
48	63	5.3				
49	66	7.73				
50	57	7.3				

Table I. Continued.

B, Patient characteristics used for *LAMC1* expression

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

genome are shown in Fig. 1. These miRNAs were clustered at 2 different human genomic loci; *miR-29b-1* and *miR-29a* were located at 7q32, and *miR-29b-2* and *miR-29c* were located at 1q32.

First, we evaluated the expression levels of *miR-29s (miR-29a/b/c)* in normal prostate tissues (n=23) and PCa tissues (n=27). In patients from whom normal prostate tissues were collected, the median PSA level was 7.3 ng/ml (range, 4.3-22 ng/ml). In contrast, in patients from whom PCa tissues were collected, PSA levels were quite high, with a median of 212 ng/ml (range, 3.5-3750 ng/ml). Twenty-one PCa patients had progressive disease with N1 or M1 according to TNM classification (Table IA).

To validate our previous miRNA profiling results in PCa, we evaluated expression of *miR-29s* in clinical PCa specimens. The expression levels of *miR-29a, miR-29b* and *miR-29c* were significantly lower in tumor tissues than in corresponding non-cancerous tissues (P<0.0001, P=0.0111 and P=0.0004, respectively; Fig. 2A). Spearman's rank test showed a positive correlation between the expression of *miR-29a* and that of *miR-29b* (R=0.752 and P<0.0001, Fig. 2B). The expression of *miR-29a* was positively correlated with that of *miR-29c* (R=0.804 and P<0.0001, Fig. 2B). Similarly, the expression of *miR-29b* was positively correlated with that of *miR-29c* (R=0.753 and P<0.0001, Fig. 2B).

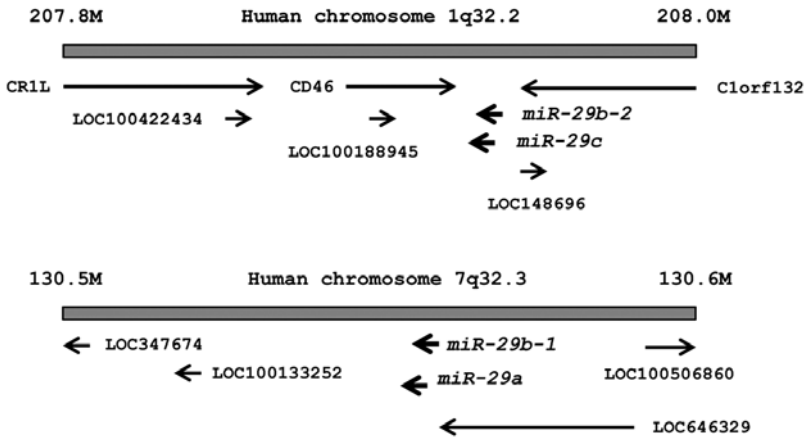

Figure 1. Chromosomal locations of *miR-29* family members. Chromosomal locations of the *miR-29b-2* and *miR-29c* cluster (human chromosome 1q32.2) and the *miR-29a* and *miR-29b-1* cluster (human chromosome 7q32.3) are shown.

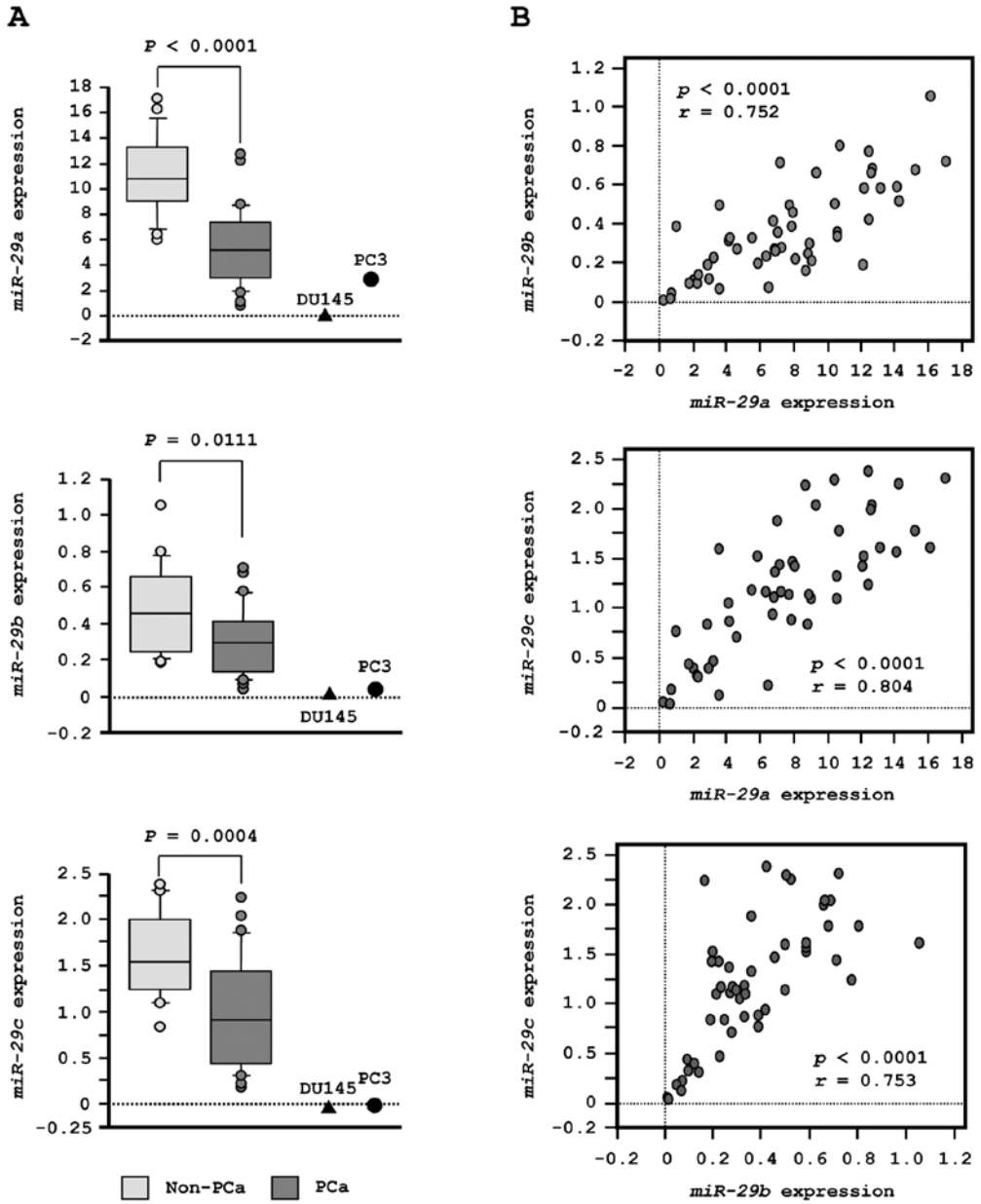

Figure 2. The expression levels of *miR-29a*, *miR-29b*, *miR-29c* in clinical specimens and PCa cell lines (PC3 and DU145). (A) Real-time PCR showing changes in expression levels of *miR-29s* in PCa tissues and cell lines as compared to normal prostate tissues. RNU48 was used as an internal control. (B) Correlations between *miR-29a*/*miR-29b*, *miR-29a*/*miR-29c* and *miR-29b*/*miR-29c*.

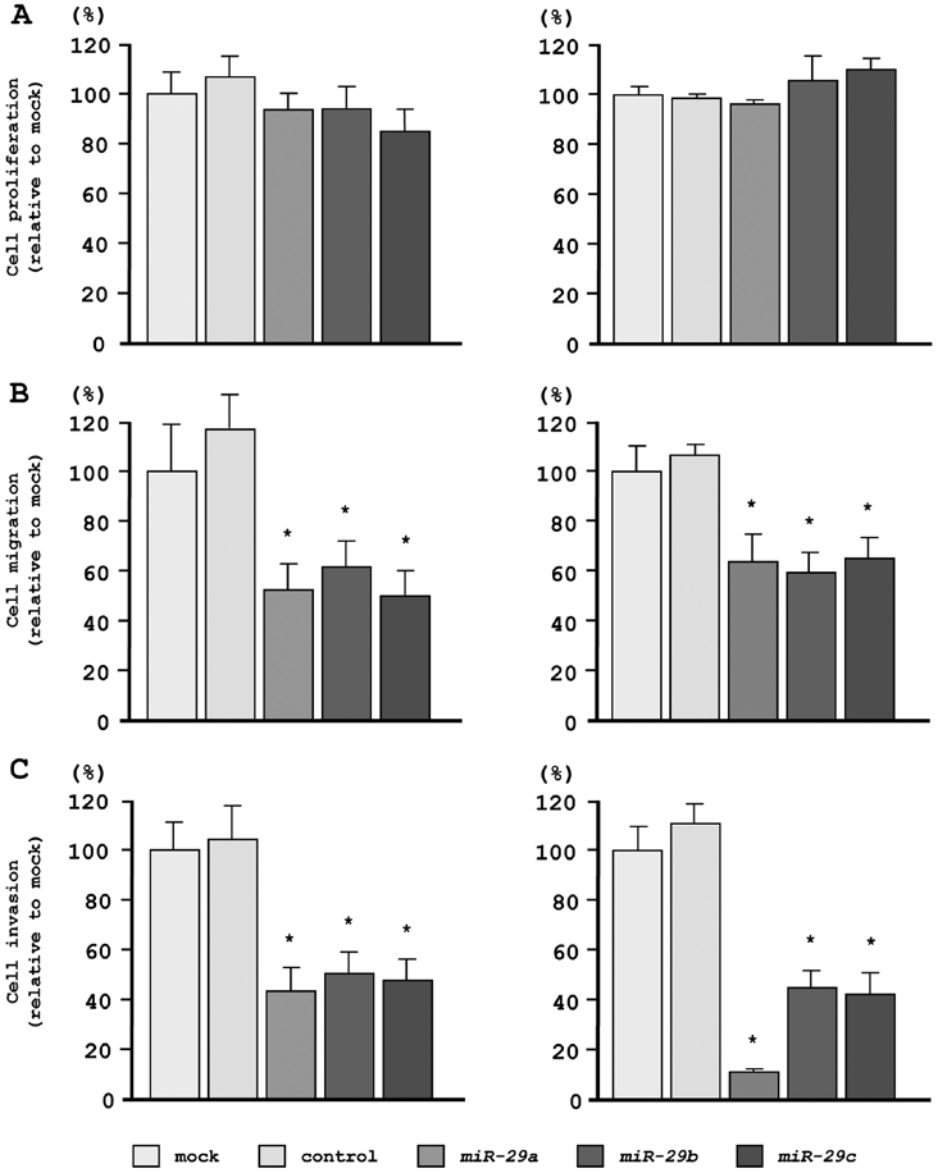

Figure 3. Effects of *miR-29s* transfection in PCa cell lines (PC3 and DU145). (A) Cell proliferation was determined with XTT assays 72 h after transfection with 10 nM *miR-29s* (10 nM) or miR-control or after mock transfection. (B) Cell migration activity was determined using wound healing assays. (C) Cell invasion activity was determined using Matrigel invasion assays. *P<0.001.

Table II. Significantly enriched pathways among predicted *miR-29s* target genes (top 10 pathways).

No. of genes	P-value	Annotations
77	8.47E-18	(KEGG) 05200: Pathways in cancer
58	1.13E-17	(KEGG) 04510: Focal adhesion
34	3.41E-15	(KEGG) 05222: Small cell lung cancer
30	9.11E-12	(KEGG) 04512: ECM-receptor interaction
46	6.31E-11	(KEGG) 04144: Endocytosis
32	6.86E-11	(KEGG) 05146: Amoebiasis
33	2.52E-09	(KEGG) 05145: Toxoplasmosis
25	6.83E-09	(KEGG) 04974: Protein digestion and absorption
30	4.35E-07	(KEGG) 04360: Axon guidance
29	7.07E-07	(KEGG) 04722: Neurotrophin signaling pathway

Effects of restoring miR-29s expression levels on cell proliferation, migration, and invasion in PC3 and DU145 PCa cells. To investigate the functional effects of *miR-29s*, we performed gain-of-function studies using miRNA transfection in PC3 and DU145 cell lines.

As observed using XTT assays, cell proliferation was not inhibited in *miR-29s* transfectants, as compared with mock- or miR-control-transfected cells (Fig. 3A). However, cell migration activity was significantly inhibited in *miR-29s* transfectants, as compared with mock- or miR-control-transfected cells (Fig. 3B). Moreover, in Matrigel invasion assays, transfection with *miR-29s* significantly inhibited cell invasion as compared with mock- or miR-control-transfected cells (Fig. 3C).

Identification of candidate genes targeted by miR-29s. To identify genes targeted by *miR-29s*, we analyzed a combination of *in silico* and gene expression data in PCa. First, we screened *miR-29s*-targeted genes using the TargetScan database and

Table III. Candidate target genes for *miR-29s* in focal adhesion pathway.

Entrez gene ID	Gene symbol	Gene name	Location	GEO expression data		Conserved sites	Poorly conserved sites
				Fold change	Log FC		
1277	COL1A1	Collagen, type I, α1	17q21.33	2.35	1.23	3	0
1278	COL1A2	Collagen, type I, α2	7q22.1	1.74	0.80	2	0
3480	IGF1R	Insulin-like growth factor 1 receptor	15q26.3	1.69	0.71	0	1
1281	COL3A1	Collagen, type III, α1	2q31	1.67	0.74	2	0
3915	LAMC1	Laminin, γ1 (formerly LAMB2)	1q31	1.63	0.71	1	0
7058	THBS2	Thrombospondin 2	6q27	1.54	0.62	0	1
7057	THBS1	Thrombospondin 1	15q15	1.53	0.61	0	1
5159	PDGFRB	Platelet-derived growth factor receptor, β polypeptide	5q33.1	1.30	0.38	1	0
8503	PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3 (γ)	1p34.1	1.28	0.36	1	0
1282	COL4A1	collagen, type IV, α1	13q34	1.26	0.33	2	0
2889	RAPGEF1	Rap guanine nucleotide exchange factor (GEF) 1	9q34.3	1.26	0.33	0	1
1293	COL6A3	Collagen, type VI, α3	2q37	1.25	0.32	1	0
1290	COL5A2	Collagen, type V, α2	2q14-q32	1.19	0.25	2	0
22801	ITGA11	Integrin, α11	15q23	1.19	0.25	1	0
208	AKT2	v-akt murine thymoma viral oncogene	19q13.1-q13.2	1.15	0.21	0	4
1284	COL4A2	homolog 2 collagen, type IV, α2	13q34	1.15	0.21	1	0
1399	CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	22q11.21	1.11	0.15	0	1

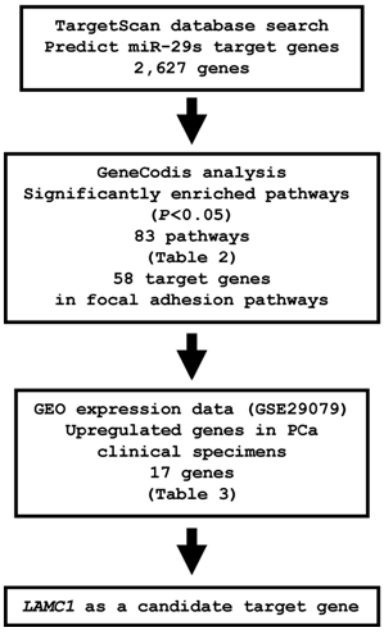

Figure 4. Workflow for selection of candidate target genes regulated by *miR-29s*. To identify *miR-29s*-target genes, we screened putative targets using the TargetScan database. These genes were then categorized into KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways using GeneCodis analysis. Among the identified pathways, we focused on the focal adhesion pathway, and 58 genes were identified. The gene set was then analyzed using a publicly available gene expression data set in GEO (accession no.: GSE29079), and genes upregulated in PCa were chosen.

identified 2,627 genes, which were then categorized into KEGG pathways using GeneCodis analysis, yielding 83 pathways identified as significantly enriched (Table II). Among these pathways, we focused on the focal adhesion pathway because this pathway has been implicated in cancer cell migration and invasion. A total of 58 genes were identified in this pathway. The gene set was then analyzed with available gene expression data from the GEO (accession no.: GSE29079), and genes upregulated in PCa were chosen (Fig. 4).

From this selection, 17 candidate genes were identified as *miR-29s* targets (Table III). Among these candidates, we focused on the *LAMC1* gene and examined *LAMC1* function and characteristics in further analyses.

LAMC1 is a direct target of miR-29s in PCa cells. We performed quantitative real-time RT-PCR and western blotting in PC3 and DU145 cells to investigate whether restoration of *miR-29s* altered *LAMC1* gene and LAMC1 protein expression. The mRNA and protein expression levels of *LAMC1*/LAMC1 were significantly repressed in *miR-29s* transfectants as compared with mock- or miR-control transfected cells (Fig. 5A and B).

Therefore, we next performed luciferase reporter assays in PC3 cells to determine whether *LAMC1* mRNA had target sites for *miR-29s*. The TargetScan database predicted that the putative *miR-29s* binding site existed in the 3'-untranslated

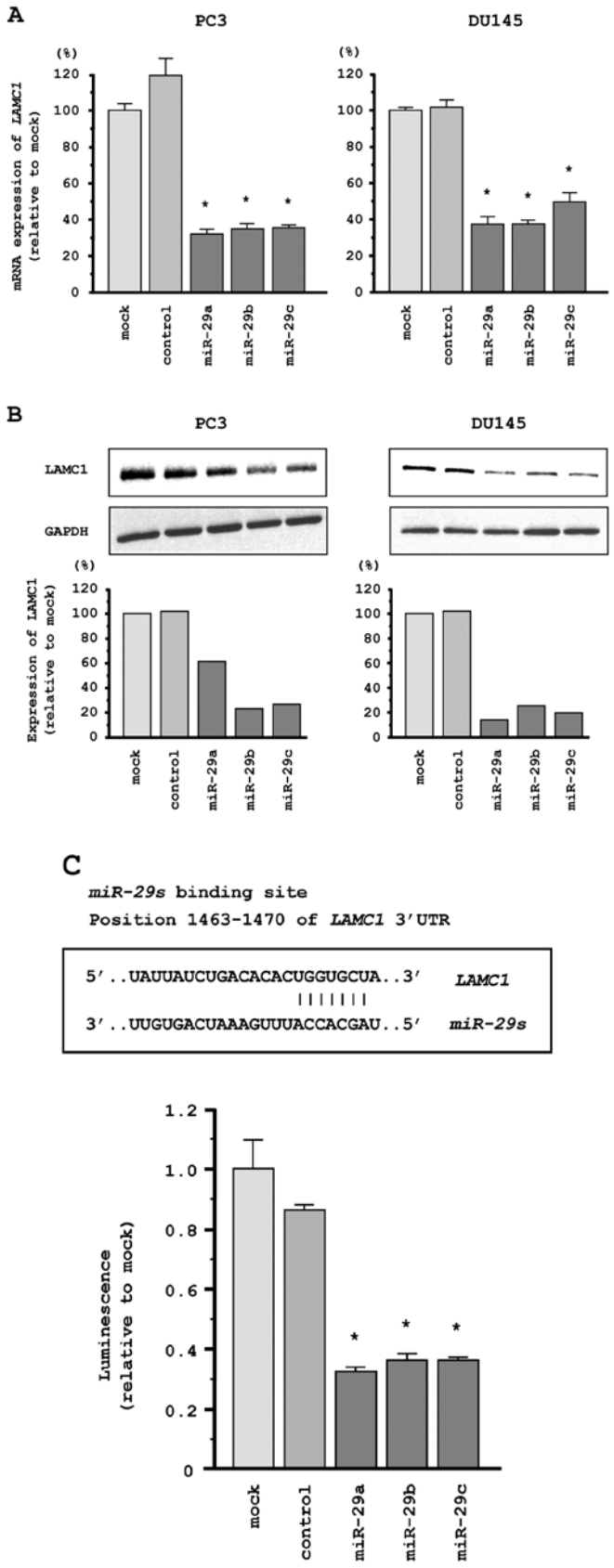

Figure 5. *LAMC1* expression was suppressed by transfection of PCa cells with *miR-29s*. (A) *LAMC1* mRNA expression 72 h after transfection with *miR-29s*. *GUSB* expression was used for normalization. (B) LAMC1 protein expression 72 h after transfection with *miR-29s*. GAPDH was used as a loading control. (C) *miR-29s* binding sites in the 3'-UTR of *LAMC1* mRNA. Luciferase reporter assays using the vector encoding putative *miR-29s* target site at 1463-1470. *Renilla* luciferase values were normalized to firefly luciferase values. *P<0.001.

region (UTR) of *LAMC1* (position 1463-1470). We used vectors encoding a partial wild-type sequence of the 3'-UTR of *LAMC1* mRNA, including predicted *miR-29s* target sites. We found that the luminescence intensity was significantly reduced by transfection of *miR-29s* with the vector carrying the wild-type 3'-UTR of *LAMC1* (Fig. 5C).

Effects of downregulating LAMC1 on cell proliferation, migration and invasion in PCa cell lines. To investigate the functional role of *LAMC1* in PCa cells, we performed loss-of-function studies using *si-LAMC1* transfectants. First, we evaluated the knockdown efficiency of *si-LAMC1* transfection in PC3 and DU145 cells. Quantitative real-time RT-PCR and western blotting indicated that the siRNA effectively downregulated *LAMC1*/LAMC1 expression in both cell lines (Fig. 6A and B).

In our functional analyses, XTT assays demonstrated that cell proliferation was not inhibited in *si-LAMC1* transfectants, as compared with mock- or miR-control-transfected cells (Fig. 7A). In contrast, transfection with *si-LAMC1* inhibited both cell migration and invasion, as compared with mock- or miR-control-transfected cells (Fig. 7B and C), similar to the results observed for restoration of *miR-29s*.

LAMC1 expression in PCa specimens. Finally, we evaluated the mRNA expression levels of *LAMC1* in normal prostate tissues (n=10) and PCa tissues (n=10). The expression of *LAMC1* was significantly higher in PCa tissues compared with normal tissues (P=0.0041), as demonstrated by RT-PCR (Fig. 8). In this analysis, an independent set of clinical specimens was used (Table IB).

Discussion

Emerging evidence has demonstrated that aberrantly expressed miRNAs upset the tightly regulated miRNA/protein-coding gene networks and cause initiation, progression and metastasis of human cancers (11). Therefore, identification of aberrantly expressed miRNAs is an important initial step in elucidating miRNA-mediated oncogenic pathways. Based on this strategy, we analyzed the miRNA expression signature of PCa and identified tumor-suppressive miRNAs and their associated PCa oncogenic pathways (12-14,16). The past studies supported the legitimacy of our strategy for miRNA analysis and contributed to the discovery of novel tumor-suppressive miRNAs in PCa.

In this study, we focused on the *miR-29s* (*miR-29a, miR-29b* and *miR-29c*) because we observed downregulation of these miRNAs in our PCa expression signature. Our data confirmed that all members of the *miR-29* family were significantly downregulated in PCa tissues. Recently, we also observed that *miR-29s* were downregulated in head and neck and cervical squamous cell carcinomas (HNCSSs) (17,18). Moreover, downregulation of *miR-29*-family miRNAs has been described by other groups in several types of cancers (19); these studies are all consistent with our results. Although the molecular mechanisms through which *miR-29s* are silenced in PCa are still unknown, recent data have suggested that transforming growth factor (TGF)-β1 inhibits the expression of *miR-29s* and promotes the expression of extracellular matrix (ECM) components (20-22). The ECM functions as a

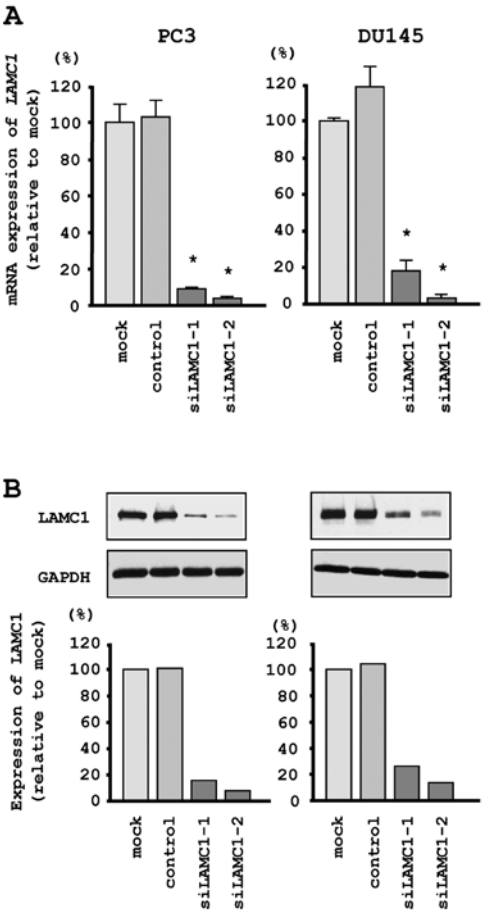

Figure 6. *LAMC1* mRNA and LAMC1 protein expression levels were suppressed by transfection of DU145 and PC3 cells with *si-LAMC1*. (A) *LAMC1* mRNA expression 72 h after transfection with *si-LAMC1*. *GUSB* expression was used for normalization. (B) LAMC1 protein expression 72 h after transfection with *si-LAMC1*. GAPDH was used as a loading control. The ratio of LAMC1/GAPDH expression was evaluated using ImageJ software (ver. 1,43; http://rsbweb.nih.gov/ij/index.htmL).

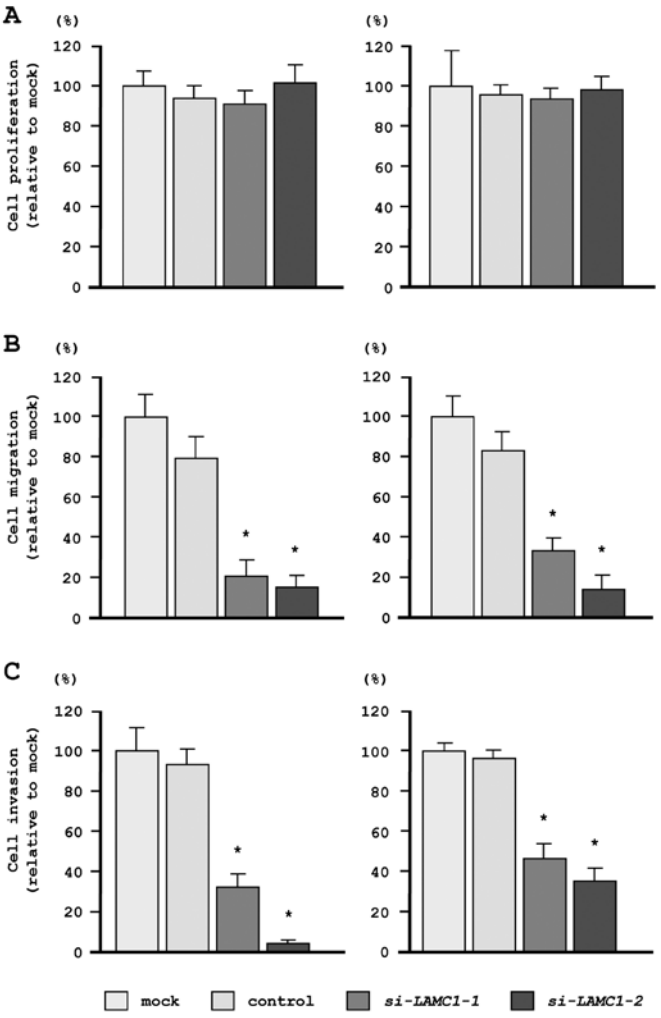

Figure 7. Effects of *LAMC1* downregulation by *si-LAMC1* in PCa cells (PC3 and DU145). (A) Cell proliferation was determined with XTT assays. (B) Cell migration activity was determined using wound healing assays. (C) Cell invasion activity was determined using Matrigel invasion assays. *P<0.001.

critical source for growth, survival, motility and angiogenic factors that significantly affect tumor biology and progression (23-25). Additionally, TGF-β1 signaling is known to contribute to the epithelial-to-mesenchymal transition (EMT), an important step in cancer progression and metastasis (26). Our data demonstrated that restoration of *miR-29s* significantly inhibited cancer cell migration and invasion. Therefore, upregulation of ECM proteins caused by TGF-β-dependent silencing of *miR-29s* is an important step for metastasis in PCa cells.

Full understanding of the targets and signaling pathways in PCa that are regulated by the *miR-29s* family may contribute to our knowledge on PCa metastasis. We categorized *miR-29s*-target genes into known pathways using KEGG pathways (17,18). From our data in this study, we focused on the 'focal adhesion' pathway because restoration of *miR-29s* inhibited cancer cell migration and invasion in PCa cell lines. Furthermore, we combined the gene expression data of upregulated genes in PCa, generating 17 candidate target genes for *miR-29s* in focal adhesion pathways. The upregulation of collagen genes in primary tumors with metastatic potential was consistent with recent observations that epithelial-mesenchymal interactions are critical determinants of tumor cell behavior (27,28). High levels of type 1 collagen in metastatic lesions and in the serum of individuals with metastatic disease have also been reported (29,30).

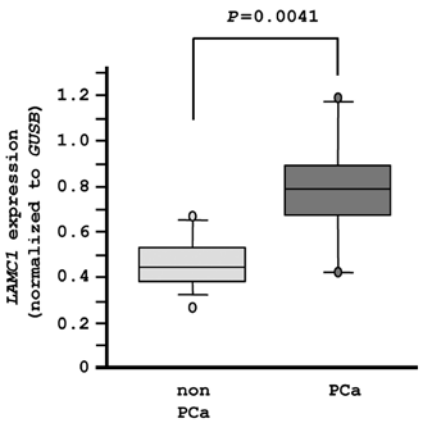

Figure 8. The mRNA expression levels of *LAMC1* in clinical specimens. The expression of *LAMC1* was analyzed in PCa specimens and normal prostate tissues. *P=0.0041.

In this study, we selected *LAMC1*, a member of the laminin super family as a target gene that contributes to cancer cell migration and invasion in PCa cells. Laminins are trimeric proteins that contain α-, β-, and γ-chains and are important ECM regulators that are and biologically active part of the basal lamina, influencing cell differentiation, migration and adhesion, as well as phenotype and survival (31). During tumor progression, several members of the laminin family have been shown to be upregulated in cancer cells and involved in cancer cell migration and invasion (32,33). Our recent studies in HNSCC have demonstrated that laminin-integrin signaling promotes cancer cell migration and invasion and that these signal pathways are regulated by tumor-suppressive *miR-218* and *miR-29s* (17,34).

This is the first study demonstrating that *miR-29s* directly regulated *LAMC1* in cancer cells and that silencing of *LAMC1* inhibited cancer cell migration and invasion. The LAMC1 chain is the most widely expressed laminin chain, found predominantly in the basement membrane (35,36). Upregulation of LAMC1 in meningiomas correlates with shorter times to tumor recurrence and decreased progression-free survival (37). Furthermore, treatment with the specific LAMC1 peptide enhances pulmonary metastasis of B16 melanoma cells and induces the production of matrix metalloproteinase (MMP)-9 from B16 cells (38). Some studies have suggested that LAMC1 functions to promote metastasis and might be a novel therapeutic target in the treatment of human cancer. Further studies are needed to determine whether LAMC1-mediated molecular cascades contribute to PCa metastasis. A complete understanding of tumor-suppressive *miR-29s* and their regulation of *LAMC1* signaling should shed light on the mechanisms of PCa metastasis and facilitate the development of more effective strategies for treating PCa.

Our data showed that all members of the *miR-29*-family were frequently downregulated in PCa cells. Moreover, these miRNAs functioned as tumor suppressors and inhibited cancer cell migration and invasion through regulation of focal adhesion pathways, especially via *LAMC1*. Elucidation of cancer pathways regulated by tumor-suppressive *miR-29s* should shed light on PCa metastasis and facilitate the development of more effective strategies for future therapeutic interventions for this disease.

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