Novel molecular targets regulated by tumor suppressors *microRNA-1* and *microRNA-133a* in bladder cancer

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Abstract. Our expression signatures of human cancer including bladder cancer (BC) revealed that the expression of *microRNA-1 (miR-1)* and *microRNA-133a (miR-133a)* is significantly reduced in cancer cells. In the human genome, *miR-1* and *miR-133a* are located on the same chromosomal region (*miR-1-2* and *miR-133a-1* on 18q11.2, and *miR-1-1* and *miR-133a-2* on 20q13.33) called cluster. In this study, we identified the novel molecular targets commonly regulated by *miR-1* and *miR-133a* in BC. Genome-wide molecular target search and luciferase reporter assays showed that *prothymosin-α (PTMA*) and *purine nucleoside phosphorylase (PNP)* are directly regulated by *miR-1* and *miR-133a*. Silencing of these two genes significantly inhibited cell proliferation and invasion, and increased apoptosis in BC cells. Immunohistochemistry showed that PTMA expression levels were significantly higher in BC compared to normal bladder epitheliums. *PTMA* and *PNP* were identified as new target genes regulated by the *miR-1* and *miR-133a* cluster in BC. These genes may function as oncogenes contributing to cell proliferation and invasion in BC. Tumor suppressive *miR-1* and *miR-133a*-mediated novel molecular targets may provide new insights into the potential mechanisms of BC oncogenesis.

Introduction

Bladder cancer (BC) is the fourth most common tumor diagnosed and the second most common cancer of the genitourinary tract in the United States (1). Though there have been significant advances in treatment, including surgical technique and adjuvant chemotherapy, approximately 70% of first diagnosed BC recurs after treatment, and 10-15% of recurrent tumors proceed to muscle invasion and metastasis (2). Therefore, it is crucial to find a new strategy for BC diagnosis and treatment based on novel molecular networks in BC oncogenesis.

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules of 20-22 nucleotides which function as negative regulators (cleavage or translational repression) of gene expression by directly bind to specific mRNAs (3). Many studies have shown that miRNAs regulate diverse cellular processes involved in differentiation, apoptosis, cell proliferation, metabolism, immunity, and development (4). Furthermore, recent studies suggest that miRNAs are associated with the development of various cancers: some miRNAs have an oncogenic function, while others have a tumor suppressive function (5).

miR-1 and *miR-133a* are located on the same chromosomal regions, in a so-called 'cluster', and there are two different chromosomal loci that harbor *miR-1* and *miR-133a* clusters (*miR-1-2* and *miR-133a-1* on 18q11.2, and *miR-1-1* and *miR-133a-2* on 20q13.33). Since thousands of miRNAs have been discovered in different species, 247 in human miRNAs have been found to occur in 64 clusters at an inter-miRNA distance of less than 5000 bp (6). In recent studies, *miR-17*/-92 and *miR-221*/*-222* clusters were reported to be over-expressed and to be associated with oncogenic function in several malignancies (7,8). On the other hand, *miR-15*/-16 cluster was reported to be low-expressed and function as tumor suppressor in several malignancies. And *miR-15*/*-16* cluster targets oncogenes such as *BCL2*, *MCL1*, *CCND1*, and *WNT3A*, and induces apoptosis and inhibits cell proliferation (9). It is important to analyze the function of miRNA cluster in oncology.

The expression of *miR-1* and *miR-133a* has been reported to be down-regulated in various cancers, and these miRNAs might have tumor suppressive functions (4,10-13). We previously reported that *miR-1* and *miR-133a* directly regulated oncogenic *LASP1* and *TAGLN2* genes in human BC (14,15). In our previous gene expression profiles in *miR-1*- and *miR-133a*-transfected BC cell lines, *prothymosin-α (PTMA*) and purine *nucleoside phosphorylase (PNP)* were the second and third candidate gene, having these miRNAs as the common target.

In this study, we focused on these two genes (*PTMA* and *PNP*), and hypothesized that *miR-1* and *miR-133a* directly regulate oncogenic *PTMA* and *PNP* in BC. We performed a luciferase reporter assay to determine whether *PTMA* and *PNP* mRNA are actually targeted by *miR-1* and *miR-133a* and a loss of function study using siRNA to elucidate the functional roles

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of PTMA and PNP in BC. We also performed immunohistochemistry to investigate differences in the protein-expression between clinical BC specimens and normal bladder epitheliums (NBEs).

Materials and methods

BC cell lines and cell culture. We used two human BC cell lines: BOY, which was established in our laboratory from an Asian male patient aged 66 and diagnosed with stage III BC with lung metastasis (16); and T24, which was invasive and obtained from the American Type Culture Collection. These cell lines were maintained in a minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Prediction of miRNA candidates targeting PTMA and PNP mRNA. We used the TargetScan (release 5.1, http://www.targetscan.org/) to search for the predicted miRNA candidates targeting *PTMA* and *PNP* mRNA. We also investigated the target sites where the seed regions of these miRNAs bind. The sequences of the predicted mature miRNAs were confirmed by referring to miRBase (release 16.0, Sept 2010; http://microrna.sanger.ac.uk/).

RNA extraction. Total RNA was extracted from frozen fresh tissues using an Isogen™ kit (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's protocol. The integrity of the RNA was checked with an RNA 6000 Nano Assay Kit and a 2100 Bioanalyzer™ (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time RT-PCR. TaqMan probes and primers for *PTMA* (P/N: Hs02339492_g1; Applied Biosystems, Foster City, CA, USA) and *PNP* (P/N: Hs00165367_m1: Applied Biosystems) were assay-on-demand gene expression products. All reactions were performed in duplicate, and a negative-control lacking cDNA was included. We followed the manufacturer's protocol for the PCR conditions. We used *human GUSB* (P/N: Hs99999908_m1; Applied Biosystems) to normalize the data for quantification of *PTMA* and *PNP* mRNA, and we used the $\Delta\Delta C_t$ method to calculate the fold change. As a control RNA, premium total RNA was used from normal human bladder (Clontech, Mountain View, CA, USA).

Mature miRNA and siRNA transfection. As described elsewhere (16), the BC cell lines were transfected with Lipofectamine™ RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM™ (Invitrogen) with 10 nM of mature miRNA molecules. Pre-miR™ and negative-control miRNA (Applied Biosystems) were used in the gain-of-function experiments, whereas *PTMA* and *PNP* siRNA (P/N: s11497; Applied Biosystems, cat no. HSS107263 and HSS181558 Invitrogen) and negative control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA, USA) were used in the loss-of-function experiments. Cells were seeded in a 10-cm dish for protein extraction ($8x10^5$ per dish), a 6-well plate for apoptosis ($1x10^5$ per well), in a 24-well plate for the mRNA extraction, matrigel invasion assay, and luciferase reporter assay ($5x10^4$ per well), and in a 96-well plate for the XTT assay (3,000 per well).

Cell proliferation and invasion assays. Cell proliferation was determined by using an XTT assay (Roche Applied Sciences, Tokyo, Japan) performed according to the manufacturer's instructions. A cell invasion assay was carried out using modified Boyden Chambers consisting of transwell-precoated matrigel membrane filter inserts with 8-mm pores in 24-well tissue culture plates (BD Biosciences, Bedfold, MA). MEM containing 10% foetal bovine serum in the lower chamber served as a chemoattractant, as described previously (10). All experiments were performed in triplicate.

Apoptosis analysis. BC cell lines transiently transfected with transfection reagent si-control, or si-PTMA and si-PNP in 6-well tissue culture plates, as described earlier, were harvested 72 h after transfection by trypsinization and washed in cold PBS. Double staining with FITC-Annexin V and propidium iodide (PI) was carried out using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's recommendations and immediately analyzed within an hour by flow cytometry (FACScan®; BD Biosciences). Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells by using the CellQuest software (BD Biosciences), and then the percentages of early apoptotic and apoptotic cells from each experiment were compared. These experiments were done in triplicate.

Caspase-3/7 activity assays. Caspase-3/7 activity was measured by CellEvent™ Caspase-3/7 Green Detection Reagent (Invitrogen). The BC cell lines were grown in 96-well plates and were transfected with si-RNAs as described above. After 72 h, 5 μM caspase-3/7 reagents were added to each well and incubated for 30 min. Fluorescence was then measured and recorded for each well. For densitometric analysis, the expression level of the sum of fluorescent particles was measured with ImageJ software (http://rsb.info.nih.gov/ij).

Immunohistochemistry in tissue microarray. A tissue microarray of bladder cancer samples was obtained from Biomax, inc. (BL1002; Rockville, MD, USA). Detailed information on all tumor specimens can be found at http://www.biomax.us/index.php. Patient characteristics are summarized in Table II. The tissue microarray was immunostained following the manufacturer's protocol with an UltraVision Detection System (Thermo Scientific). The primary mouse monoclonal antibodies against PTMA (Alexis, San Diego, CA) and the primary rabbit polyclonal antibodies against PNP (Sigma-Aldrich) were diluted 1:300 and 1:450. The slides were treated with biotinylated goat anti-rabbit. Diaminobenzidinehydrogen peroxidase was the chromogen, and the counterstaining was done with 0.5% hematoxylin. The positivity of endothelia and myofibroblasts served as an inner positive control. The immunostaining result was evaluated using the scoring method described previously (17). Each case was scored on the basis of the intensity and area of staining. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, 30-60% stained positive; 3+, >60% stained positive. A combined staining score (intensity + extension) of <2 was low expression, a score between 3 and 4 was moderate expression, and a score between 5 and 6 was high expression. All staining scores were averages of duplicate experiments, and all samples were independently scored by two

Table I. Down-regulated genes in *miR-1*- and *miR-133a*-transfected T24 cell line in gene expression profile.

Entrez Gene ID	Symbol	Fold change (log2 ratio)			Target sites	
		miR-1	*miR-133a*	Average	*miR-1*	*miR-133a*
8407	TAGLN2	-3.33	-2.81	-3.07	+	+
23446	SLC44A1	-3.69	-1.22	-2.46	+	-
80150	ASRGL1	-1.54	-3.01	-2.28	+	-
5757	PTMA	-2.04	-2.50	-2.27	+	+
4860	PNP	-2.54	-1.75	-2.15	+	+
26150	RIBC2	-1.45	-2.72	-2.09	-	-
63827	BCAN	-1.15	-2.97	-2.06	-	-
29956	LASS2	-1.11	-2.85	-1.98	+	+
7170	TPM3	-1.56	-2.35	-1.96	+	+
7095	SEC62	-1.40	-2.41	-1.91	+	-
23531	MMD	-2.08	-1.51	-1.80	+	-
23603	CORO1C	-1.88	-1.67	-1.78	+	+
55276	PGM2	-1.90	-1.64	-1.77	+	-
201895	C4orf34	-1.50	-1.98	-1.74	+	+
11167	FSTL1	-1.23	-2.16	-1.70	+	-
81831	NETO2	-1.90	-1.47	-1.69	+	-
79850	FAM57A	-2.05	-1.28	-1.67	+	+
23607	CD2AP	-1.22	-1.95	-1.59	+	+
55653	BCAS4	-1.57	-1.55	-1.56	-	-
11168	PSIP1	-1.54	-1.51	-1.53	-	-
55179	FAIM	-1.36	-1.52	-1.44	+	+
79841	AGBL2	-1.14	-1.74	-1.44	-	-
25927	CNRIP1	-1.56	-1.22	-1.39	+	-
5819	PVRL2	-1.37	-1.22	-1.30	+	-
57542	KLHDC5	-1.57	-1.02	-1.30	+	+
63929	XPNPEP3	-1.25	-1.29	-1.27	+	-
57171	DOLPP1	-1.15	-1.36	-1.26	+	+
5780	PTPN9	-1.11	-1.31	-1.21	+	-
26999	CYFIP2	-1.04	-1.33	-1.19	+	+
23178	PASK	-1.11	-1.22	-1.17	+	-
11147	HHLA3	-1.15	-1.16	-1.16	+	-
523	ATP6V1A	-1.01	-1.22	-1.12	+	+
64924	SLC30A5	-1.00	-1.04	-1.02	-	+

of the authors (T. Yamasaki and H. Yoshino) who were blinded to the patient status.

Statistical analysis. The relationship between two variables and numerical values was analyzed using the Mann-Whitney U test, and the relationship between three variables and the numerical values was analyzed using the Bonferroni-adjusted Mann-Whitney U test. Expert Stat View analysis software (ver.4; SAS institute Inc., Cary, NC) was used in both analyses. In the comparison of three variables, a non-adjusted statistical level of significance of $P<0.05$ corresponded to the Bonferroni-adjusted level of $P<0.0167$.

Results

Gene expression profile identifying down-regulated genes in miR-1 and miR-133a transfectants. In our previous studies, we performed gene expression analyses using *miR-1* transfectants (BOY and T24) and *miR-133a* transfectants (KK47 and T24) [the gene expression omnibus (GEO) accession No. GSE24782 and GSE19717] (11,15). In this study, we reconstructed a list for common target genes of *miR-1* and *miR-133a* by using the miRNA transfected-T24, because T24 was commonly adapted in these analyses. We found a total of 33 genes were down-regulated <-2.0-fold in both transfectants in comparison with their controls. Among them, *TAGLN2, PTMA* and *PNP* were the top three down-regulated genes that had putative target sites of both *miR-1* and *miR-133a* in their 3'UTR (Table I). We previously reported that *TAGLN2* was targeted by *miR-1* and *miR-133a*. Therefore, we focused on the *PTMA* and *PNP* genes as promising candidates targeted by both *miR-1* and *miR-133a*.

PTMA and PNP mRNA expression in BC cell lines and PTMA and PNP silencing by miR-1 and miR-133a transfection. The

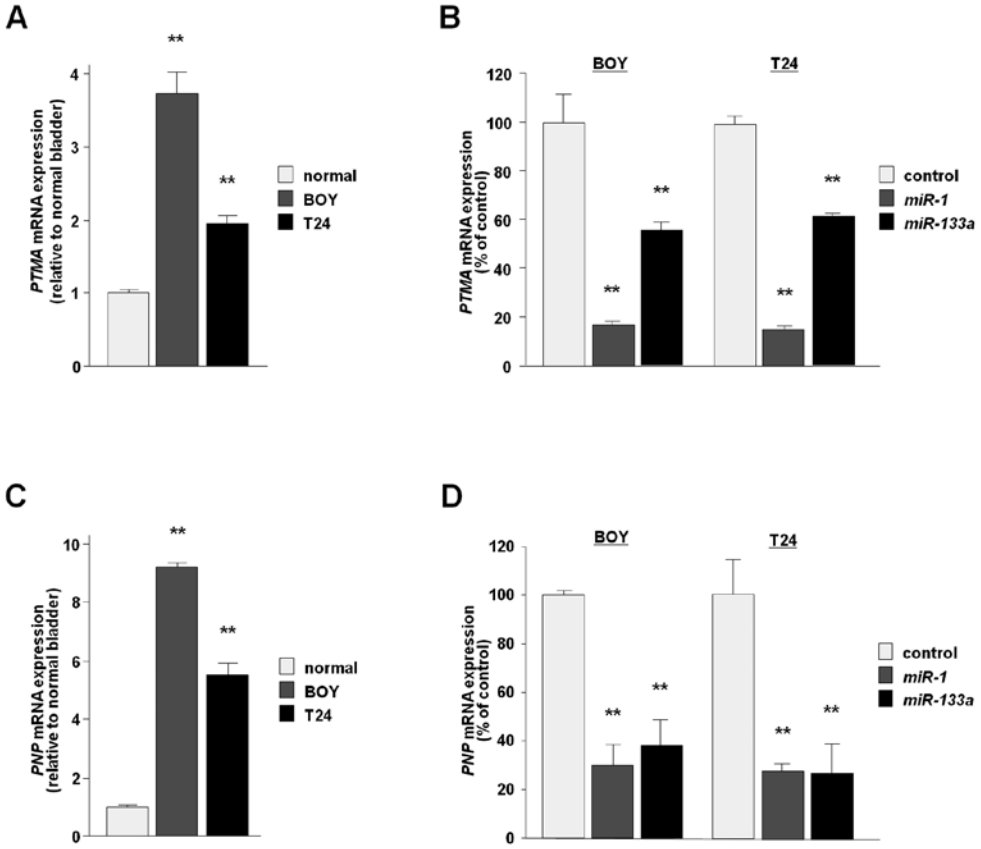

Figure 1. mRNA expression in BC cell lines, and *miR-1* and *miR-133a* transfectants (A) mRNA expression of *PTMA* in the BOY and T24 cell lines and the normal human bladder RNA. (B) *PTMA* mRNA expression after 24-h transfection with 10 nM of the miRNA (*miR-1* and *miR-133a*) in the BOY and T24 cell lines. The mRNA expression level of *PTMA* was repressed in the transfectants. (C) mRNA expression of *PNP* in the BOY and T24 cell lines and the normal human bladder RNA. (D) *PNP* mRNA after 24-h transfection with 10 nM of the miRNA (*miR-1* and *miR-133a*) in the BOY and T24 cell lines. The mRNA expression level of *PNP* was also repressed in the transfectants.

Table II. Patient characteristics.

BC specimens (n=68)	
Median age (range)	54.4 (37-95)
Gender	
Male	54
Female	14
Histologic grade	
G1 + G2	32
G3	33
Unknown	3
Tumor stage	
T1	23
T2	28
T3	15
T4	2

NBEs (urocystitis tissues) (n=17).

quantitative real-time RT-PCR analysis showed that the PTMA mRNA expressions in the BOY and T24 cell lines were respectively higher than that in normal human bladder RNA (P<0.0001, Fig. 1A). The *PTMA* mRNA expression was markedly down-regulated in the *miR-1* and *miR-133a* transfectants compared with the control in the BOY and T24 cell lines (P<0.0001, Fig. 1B). The quantitative real-time RT-PCR analysis showed that the *PNP* mRNA expressions in the BOY and T24 cell lines were respectively higher than in normal human bladder RNA (P<0.0001, Fig. 1C). The *PNP* mRNA expression was markedly down-regulated in the *miR-1* and *miR-133a* transfectants compared with the control in BOY and T24 cell lines (P<0.0001, Fig. 1D).

PTMA and PNP as a target of post-transcriptional repression by miR-1 and miR-133a. We performed a luciferase reporter assay to determine whether *PTMA* and *PNP* mRNA have target sites for *miR-1* and *miR-133a*. We used a vector encoding full-length 3'UTR of *PTMA* and *PNP* mRNA and found that the luminescence intensity was significantly reduced in the *miR-1* and *miR-133a* transfectants, suggesting that these genes have actual binding sites of *miR-1* and *miR-133a* (P<0.0001, Fig. 2).

Effect of PTMA knockdown on cell proliferation and invasion activity, and apoptosis through caspase-3/7 activation in BC cell lines. To examine the functional role of PTMA, we performed loss-of-function studies using si-PTMA-transfected BOY and T24 cell lines. The *PTMA* mRNA expression was

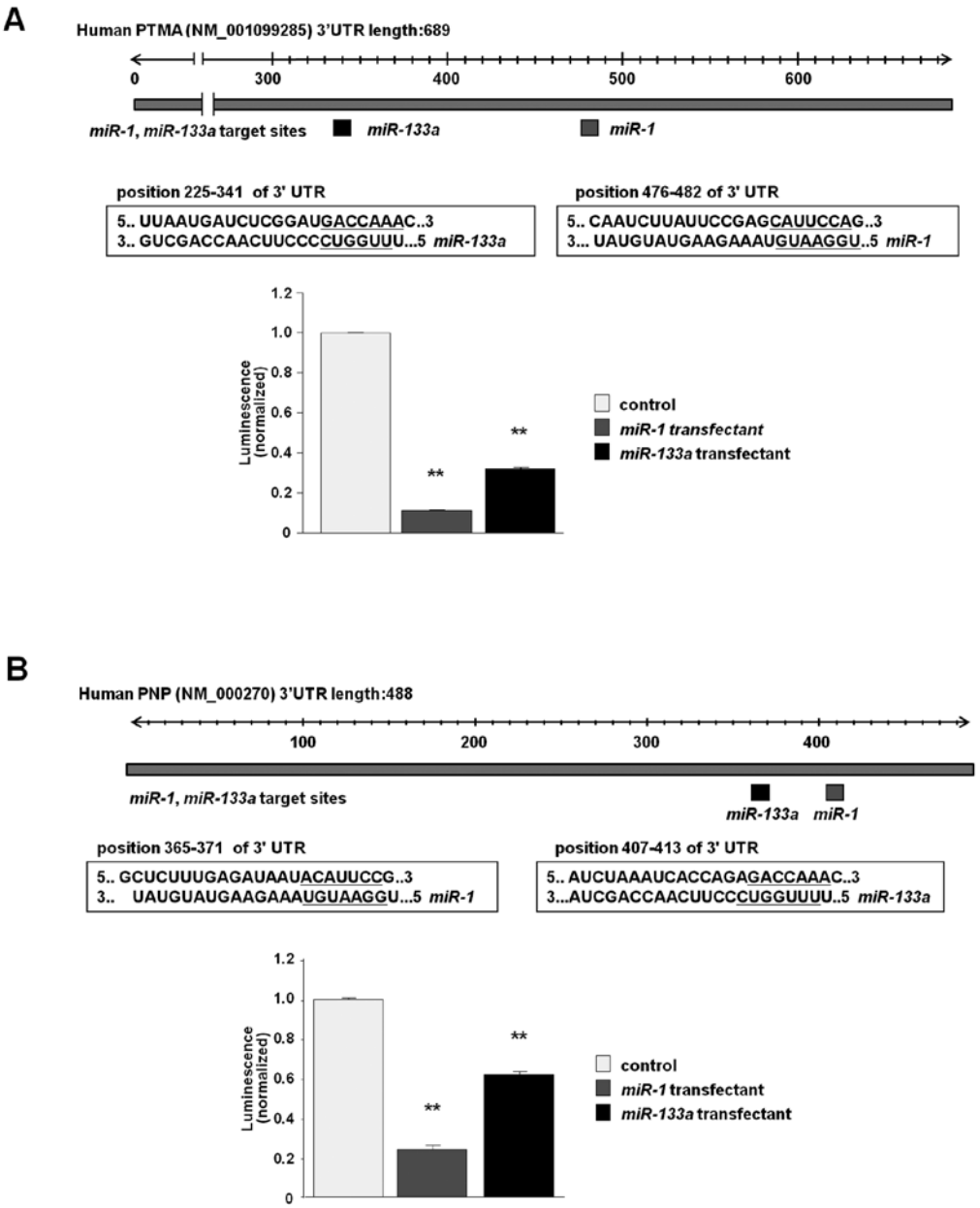

Figure 2. *MiR-1* and *miR-133a* binding sites in 3'-UTR of mRNA and luciferase reporter assay. (A and B, upper) *MiR-1* and *miR-133a* binding sites in 3'-UTR of *PTMA* and *PNP* mRNA. (A and B, lower) luciferase reporter assay using the vector encoding full-length 3'-UTR of *PTMA* and *PNP* mRNA. The Renilla luciferase values were normalized by the firefly luciferase values. **P<0.0001.

markedly repressed by the si-PTMA transfection (P<0.0001, Fig. 3A). The XTT assay revealed significant cell proliferation inhibition in the si-PTMA transfectants in comparison with the si-control transfectants (% of cell viability; BOY, 68.5±6.0 and 100.0±1.3, respectively, P=0.0039; T24, 87.1±7.7 and 100.0±2.9, respectively, P=0.0106; Fig. 3B). The matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in the si-PTMA transfectants compared with the si-control transfectants (% of cell invasion; BOY, 35.7±3.9 and 100.0±10.0, respectively, P<0.0001; T24, 66.2±6.0 and 100.0±5.3, respectively, P=0.0013; Fig. 3C). Cell apoptosis in si-PTMA transfectants was detected using flow cytometry. The apoptotic cell fractions (early apoptotic and late apoptotic, bottom right and upper right quadrants, respectively) were greater in the si-PTMA transfectants than the si-control transfectants 72 h after transfection (relative to control; BOY, 1.86±0.23 and 1.00±0.07, respectively, P=0.0090; T24, 1.86±0.29 and 1.00±0.04, respectively, P=0.0090; Fig. 3D). Caspase-3/7 activity assays showed that fluorescence intensity was also markedly increased in the si-PTMA transfectants compared with the si-control transfectants (relative to control; BOY, 8.47±2.86 and 1.00±0.21, respectively, P=0.0012; T24, 9.56±2.17 and 1.00±0.37, respectively, P=0.0023; Fig. 3E).

Effect of PNP knockdown on cell proliferation, migration and invasion activity in BC cell lines. To examine the functional role of PNP, we performed loss-of-function studies using si-PNP-transfected BOY and T24 cell lines. The *PNP* mRNA expression was markedly repressed by the si-PNP transfection (P<0.0001, Fig. 4A). The XTT assay revealed significant cell proliferation

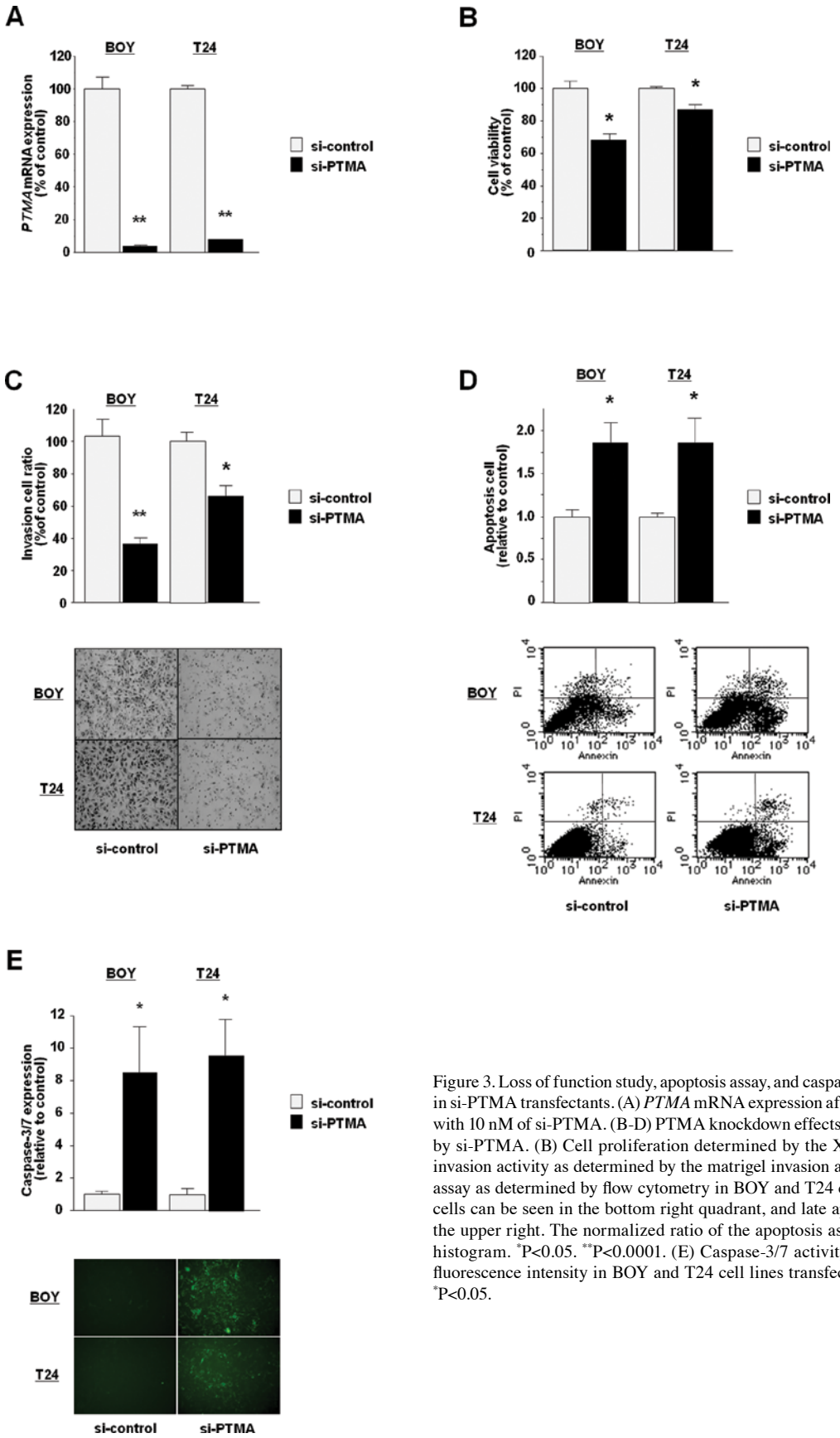

Figure 3. Loss of function study, apoptosis assay, and caspase-3/7 activity assay in si-PTMA transfectants. (A) *PTMA* mRNA expression after 24-h transfection with 10 nM of si-PTMA. (B-D) PTMA knockdown effects on BC cell viability by si-PTMA. (B) Cell proliferation determined by the XTT assay; (C) Cell invasion activity as determined by the matrigel invasion assay; (D) Apoptosis assay as determined by flow cytometry in BOY and T24 cell. Early apoptotic cells can be seen in the bottom right quadrant, and late apoptotic cells are in the upper right. The normalized ratio of the apoptosis assay is shown in the histogram. *P<0.05. **P<0.0001. (E) Caspase-3/7 activity was measured by fluorescence intensity in BOY and T24 cell lines transfected with si-PTMA. *P<0.05.

inhibition in the si-PNP transfectants in comparison with the si-control transfectants (% of cell viability; BOY, 54.4±0.9 and 100.0±2.4, respectively, P=0.0090; T24, 74.6±2.7 and 100.0±2.2, respectively, P=0.0090; Fig. 4B). The matrigel invasion assay demonstrated that the number of invading cells was significantly decreased in the si-PNP transfectants compared with the

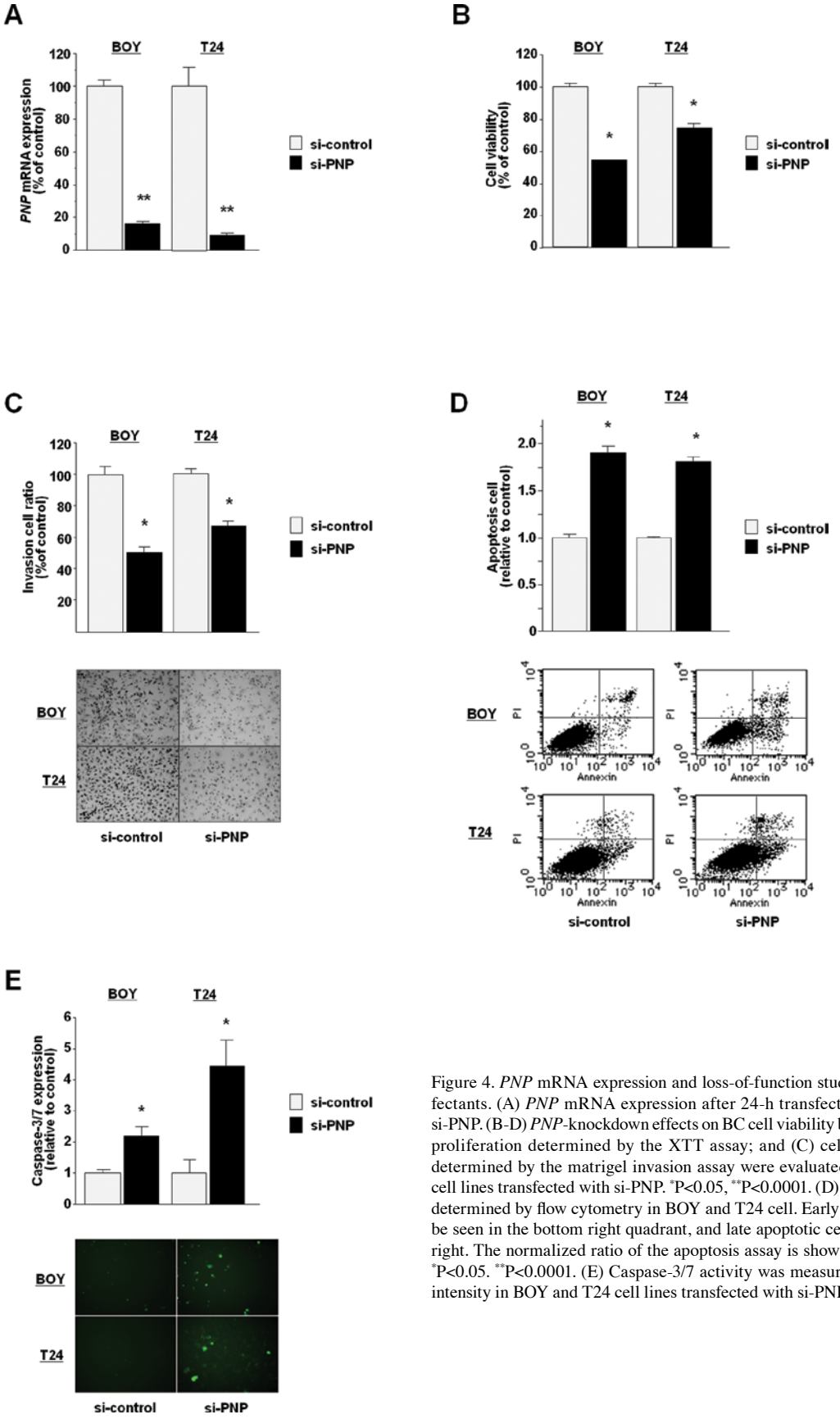

Figure 4. *PNP* mRNA expression and loss-of-function study in si-PNP transfectants. (A) *PNP* mRNA expression after 24-h transfection with 10 nM of si-PNP. (B-D) *PNP*-knockdown effects on BC cell viability by si-RNA. (B) Cell proliferation determined by the XTT assay; and (C) cell invasion activity determined by the matrigel invasion assay were evaluated in BOY and T24 cell lines transfected with si-PNP. *P<0.05, **P<0.0001. (D) Apoptosis assay as determined by flow cytometry in BOY and T24 cell. Early apoptotic cells can be seen in the bottom right quadrant, and late apoptotic cells are in the upper right. The normalized ratio of the apoptosis assay is shown in the histogram. *P<0.05. **P<0.0001. (E) Caspase-3/7 activity was measured by fluorescence intensity in BOY and T24 cell lines transfected with si-PNP. *P<0.05.

si-control transfectants (% of cell invasion; BOY, 50.3±3.3 and 100.0±5.2, respectively, P=0.0080; T24, 65.4±7.2 and 100.0±2.7, respectively, P=0.0016; Fig. 4C). Cell apoptosis in si-PNP transfectants was detected using flow cytometry. The apoptotic cell fractions (early apoptotic and late apoptotic, bottom right and upper right quadrants, respectively) were greater in the si-PTMA

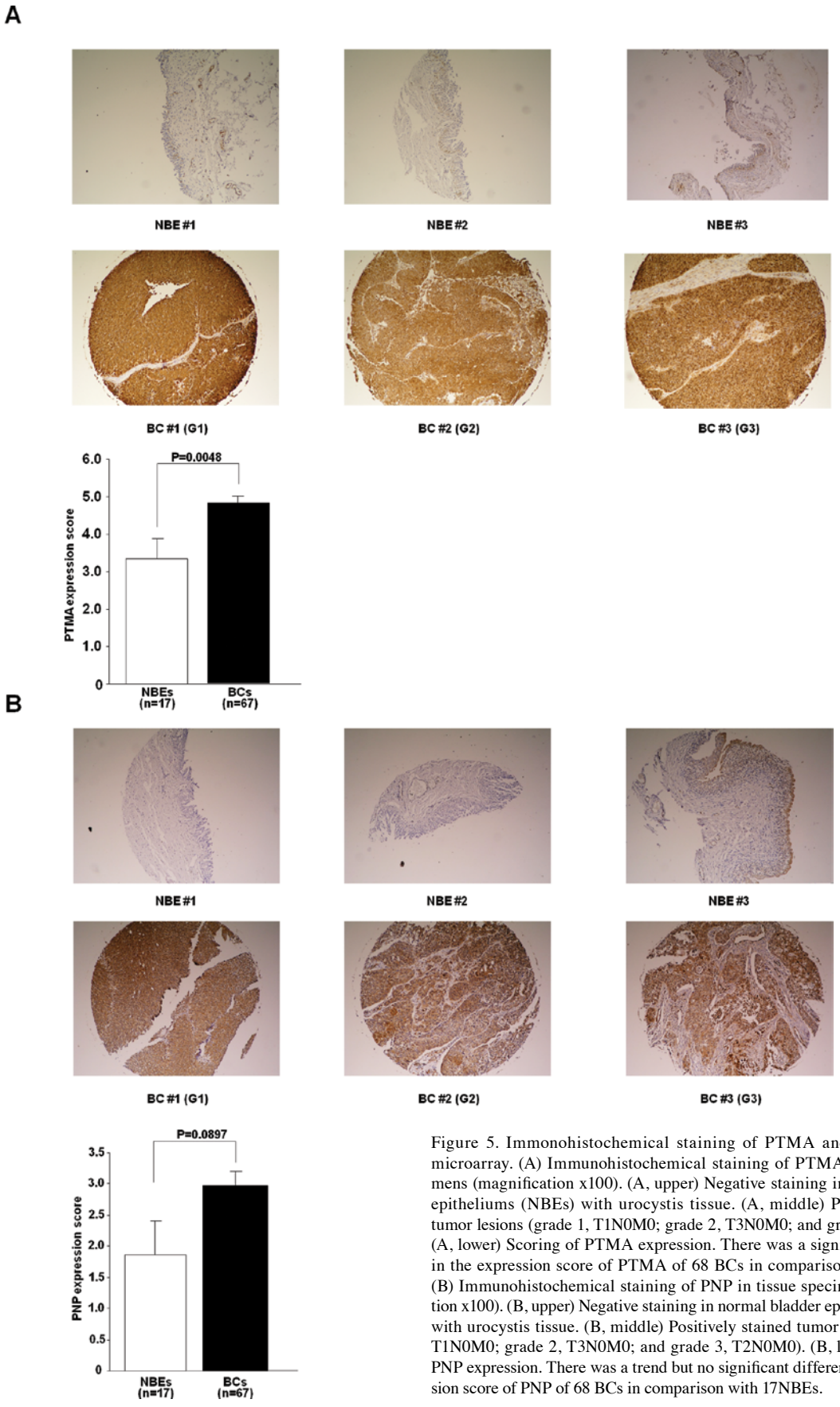

Figure 5. Immonohistochemical staining of PTMA and PNP in tissue microarray. (A) Immunohistochemical staining of PTMA in tissue specimens (magnification x100). (A, upper) Negative staining in normal bladder epitheliums (NBEs) with urocystis tissue. (A, middle) Positively stained tumor lesions (grade 1, T1N0M0; grade 2, T3N0M0; and grade 3, T2N0M0). (A, lower) Scoring of PTMA expression. There was a significant difference in the expression score of PTMA of 68 BCs in comparison with 17 NBEs. (B) Immunohistochemical staining of PNP in tissue specimens (magnification x100). (B, upper) Negative staining in normal bladder epitheliums (NBEs) with urocystis tissue. (B, middle) Positively stained tumor lesions (grade 1, T1N0M0; grade 2, T3N0M0; and grade 3, T2N0M0). (B, lower) Scoring of PNP expression. There was a trend but no significant difference in the expression score of PNP of 68 BCs in comparison with 17NBEs.

transfectants than the si-control transfectants 72 h after transfection (relative to control; BOY, 1.89±0.09 and 1.00±0.03, respectively, P=0.0090; T24, 1.81±0.05 and 1.00±0.01, respectively, P=0.0090; Fig. 4D). Caspase-3/7 activity assays showed that fluorescence intensity was also markedly increased in the si-PNP transfectants compared with the si-control transfectants

(relative to control; BOY, 2.20±0.32 and 1.00±0.13, respectively, P=0.0495; T24, 4.43±0.86 and 1.00±0.43, respectively, P=0.0495; Fig. 4E).

Immunohistochemistry in tissue microarray. Representative immunohistochemistry of PTMA and PNP is shown in Fig. 5. The PTMA was strongly expressed in BCs compared with NBEs (Fig. 5A, upper and middle). The expression score of tumors was significantly higher than that of NBEs and BCs (P=0.0048, Fig. 5A, lower). In terms of PNP, faint to modest staining were found in NBEs, whereas strong staining of cytosol and nuclei were found in BCs (Fig. 5B, upper and middle). Immunohistochemical staining revealed that there was a trend but no significant difference in the expression score of PNP between BC specimens and NBEs (P=0.0897, Fig. 5B, lower). Patient characteristics are summarized in Table II. We found no significant difference between the PTMA and PNP expression score and pathological parameters (tumor stage and grade) of BC patients.

Discussion

Our recent miRNA expression signatures revealed that the expression levels of *miR-1* and *miR-133a* were significantly reduced in various cancer cells (15,18-20). Interestingly, *miR-1-1*/*miR-133a-2* and *miR-1-2*/*miR-133a-1* are clustered on different chromosomal regions in the human genome, 20q13.33 and 18q11.2, respectively. This fact suggests that these miRNAs have an important function in genome. Therefore we performed functional analyses of these miRNAs by using mature miRNA transfection in BC cells. Restoration of *miR-1* or *miR-133a* significantly inhibited cancer cell proliferation, migration and invasion, suggested *miR-1* or *miR-133a* function as tumor suppressors in BC (15). The function analysis of *miR-1* or *miR-133a* in BC cells strongly confirmed our previous data of, head and neck squamous cell carcinoma (HNSCC), renal cell carcinoma (RCC) and prostate cancer (PCa) (18-20).

The unique point of the miRNA biogenesis is that one miRNA regulates many protein coding genes in human genome. However, many databases predict the target genes from miRNA sequences, it is still unknown which genes actually control by miRNAs. That is why the future problem is elucidation of the networks of miRNA that regulates protein coding genes in various cells. Based on this view, we sequentially identified tumor suppressive miRNAs that regulate multiple oncogene networks in various cancer cells (10,11,15,18-20).

When we searched previous reports for *miR-1* regulated genes, recent studies revealed that *miR-1* induced apoptosis through repression of Mcl-1 in lung cancer (4). *MiR-1* also targets *c-Met* in rhabdomyosarcoma (RMS) (13). With regard to *miR-133a*, several reports showed that *miR-133a* was low-expressed in colon cancer, tongue squamous cell carcinoma (SCC), and RMS (21-23). In tongue SCC, *miR-133a* inhibited cell proliferation and induced apoptosis and directly bound to oncogenic *PKM2* (22). Furthermore, *miR-133a* directly regulated multiple oncogenes such as *CAV1* and *GSTP1* and in human SCC (24,25).

From the viewpoint of the *miR-1* and *miR-133a* cluster in human cancers, three profiles of genes regulated by both *miR-1* and *miR-133a* have been reported, BC, PCa and RMS (15,20,23). In RMS, the contributions of two miRNAs were investigated by *miR-1* and *miR-133a* transfectans in RMS cells (27). According to the report, *miR-1* exerts a strong promyogenic influence on these poorly differentiated tumor cells. More recently, we identified both *miR-1* and *miR-133a* regulated genes in BC, PCa, HNSCC and RCC by using the same methods. When we compared the three profiles (BC, PCa, HNSCC), the common genes regulated by both *miR-1* and *miR-133a* were *TAGLN2, PVRL2, C4orf34* and *PNP* (18,20). Among them, *TAGLN2* and *PNP* were directly regulated by *miR-1* and *miR-133a* in BC and HNSCC and PCa, respectively (18,20). Further, TAGLN2 is common in four profiles including the profile of RCC (19).

In this study, *PTMA* and *PNP* were directly down-regulated by both *miR-1* and *miR-133a* transfectants of BC, and contain *miR-1* and *miR-133a* putative binding sites on their 3'UTR regions. Luciferase reporter assay revealed that these genes were directly regulated by both tumor suppressive *miR-1* and *miR-133a*.

PTMA, a small (12.5 kDa) and highly acidic nuclear protein, is highly conserved and widely distributed in mammalian tissues, and it has been implicated in cell cycle progression, cellular and viral transcription, remodeling of chromatin, and anti-apoptosis (26,27). PTMA has been reported to be associated with cancer progression in the liver, lung, prostate, and bladder (28-31). In this study, PTMA knockdown resulted in BC cell viability inhibition and induction of apoptosis. Our results were consistent with previous reports that demonstrated the relationship between PTMA expression and apoptosis (26,30,32). Previous studies revealed that PTMA was a negative regulator of apoptosis, like Bcl-2, and inhibited caspase-3 or induced blocking the apoptosome formation (26,27). Our caspase-3/7 activity assay also showed that fluorescence intensity was markedly increased in the si-PTMA transfectant confirming previous reports.

PNP is an enzyme in the purine salvage pathway and is involved in the phosphorolysis of substrates (i.e., inosine/deoxyinosine, xanthosine/deoxyxanthosine, and guanosine/deoxyguanosine). These conversions result in the inhibition of DNA synthesis and cell death (33). *PNP* expressions have previously been associated with the development of cancers, such as maxillary sinus SCC, PCa and leukemia (18,20,33,34). PNP inhibitors, such as forodesine inhibited cell proliferation by activating the apoptotic pathway in leukemia (33,34), and phase I and II trials using these PNP inhibitors are currently underway for patients with leukemia (33). In this study, PNP knockdown resulted in cell viability inhibition and induction of apoptosis in BC, as forodesine in leukemia. In addition, PNP knockdown increased caspase-3/7 activity and induced apoptosis in BC.

In conclusion, down-regulation of *miR-1* and *miR-133a* was a frequent event in BC and these miRNAs function as tumor suppressors. *PTMA* and *PNP* were directly regulated by *miR-1* and *miR-133a* and oncogenes in BC. Tumor suppressive *miR-1* and *miR-133a* mediate novel cancer pathways providing new insights into the potential mechanisms of BC oncogenesis.

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