Dual regulation of receptor tyrosine kinase genes *EGFR* and *c-Met* by the tumor-suppressive *microRNA-23b/27b* cluster in bladder cancer

TAKESHI CHIYOMARU[1], NAOHIKO SEKI[3], SATORU INOGUCHI[1], TOMOAKI ISHIHARA[1], HIROKO MATAKI[2], RYOSUKE MATSUSHITA[1], YUSUKE GOTO[3], RIKA NISHIKAWA[3], SHUICHI TATARANO[1], TOSHIHIKO ITESAKO[1], MASAYUKI NAKAGAWA[1] and HIDEKI ENOKIDA[1]

Departments of [1]Urology and [2]Pulmonary Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8520; [3]Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan

Abstract. Recent clinical trials of chemotherapeutics for advanced bladder cancer (BC) have shown limited benefits. Therefore, new prognostic markers and more effective treatment strategies are required. One approach to achieve these goals is through the analysis of RNA networks. Our recent studies of microRNA (miRNA) expression signatures revealed that the *microRNA-23b/27b (miR-23b/27b)* cluster is frequently downregulated in various types of human cancers. However, the functional role of the *miR-23b/27b* cluster in BC cells is still unknown. Thus, the aim of the present study was to investigate the functional significance of the *miR-23b/27b* cluster and its regulated molecular targets, with an emphasis on its contributions to BC oncogenesis and metastasis. The expression levels of the *miR-23b/27b* cluster were significantly reduced in BC clinical specimens. Restoration of mature *miR-23b* or *miR-27b* miRNAs significantly inhibited cancer cell migration and invasion, suggesting that these clustered miRNAs function as tumor suppressors. Gene expression data and *in silico* analysis demonstrated that the genes coding for the epidermal growth factor receptor (*EGFR*) and hepatocyte growth factor receptor (*c-Met*) were potential targets of the *miR-23b/27b* cluster. Luciferase reporter assays and western blotting demonstrated that *EGFR* and *c-Met* receptor trypsine kinases were directly regulated by these clustered miRNAs. We conclude that the decreased expression of the tumor-suppressive *miR-23b/27b* cluster enhanced cancer cell proliferation, migration and invasion in BC through direct regulation of *EGFR* and *c-Met* signaling pathways. Our data on RNA networks regulated by tumor-suppressive *miR-23b/27b* provide new insights into the potential mechanisms of BC oncogenesis and metastasis.

Introduction

In developed contries, bladder cancer (BC) is the fifth most commonly diagnosed tumor and the second most common cause of death in patients with genitourinary tract malignancies (1). BCs can be classified into two categories: non-muscle-invasive tumors and muscle-invasive tumors. The 5-year survival frequency for patients with non-muscle-invasive BC is close to 90%, whereas patients with muscle-invasive tumors have 5-year survival frequencies of ~60% (2). Patients with non-muscle-invasive BC tend to have a high rate of recurrence. Moreover, some patients are found to have muscle-invasive BC at recurrence (3). Recent clinical trials of chemotherapeutics for advanced BC have shown limited benefits. Therefore, new prognostic markers and more effective treatment strategies are required. One approach to achieve these goals is through the analysis of RNA networks.

Recent studies have demonstrated the importance of non-coding RNAs (ncRNAs). Participation of these RNAs in human diseases, including cancer, is now apparent (4). For example, microRNAs (miRNAs) are 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 recent studies have reported that miRNAs are aberrantly expressed in many human cancers. In fact, miRNAs play significant roles in the initiation, development and metastasis of human cancers (6).

Important new information has been gained through the analysis of the cancer-related miRNA networks. We have used our miRNA expression signatures to investigate several tumor-suppressive miRNAs and their regulated cancer pathways (7-11). Notably, some miRNAs are located in close proximity in the human genome; these are termed clustered miRNAs. We previously reported that *miR-1/133a*, *miR-29s*,

miR-143/145 and *miR-195/497* formed clusters and that these clusters function as tumor suppressors, targeting several oncogenic genes in human cancers, including BC (7,12-21).

Previously, our miRNA expression signatures revealed that *miR-23b/27b* clustered miRNAs were significantly reduced in several cancer tissues (8,9). Our deep sequencing miRNA signature of BC also annotated downregulation of *miR-23b* in cancer tissues (7). In contrast, Jin *et al* showed that the expression of *miR-23b* and *miR-27b* was highly upregulated in human breast cancer, and knockdown of these miRNAs substantially repressed breast cancer growth (22). Thus, the expression status of the *miR-23b/27b* cluster is not consistent among different types of cancers. Importantly, the functional roles of the *miR-23b/27b* cluster have not been fully investigated in BC.

The aim of the present study was to investigate the functional significance of *miR-23b/27b* clustered miRNAs and to identify the molecular targets regulated by these miRNAs in BC cells. We found that restoration of *miR-23b* or *miR-27b* mature miRNAs significantly inhibited cancer cell migration and invasion. Gene expression data and *in silico* analysis demonstrated that epidermal growth factor receptor (*EGFR*) and hepatocyte growth factor receptor (*c-Met*) were potential targets of the *miR-23b/27b* cluster. Elucidation of the cancer pathways and targets regulated by tumour-suppressive *miR-23b/27b* cluster will provide new insights into the potential mechanisms of oncogenesis and metastasis of BC.

Materials and methods

Clinical specimens. A total of 58 BC and 25 normal bladder specimens were collected from patients who underwent cystectomy or transurethral resection of bladder tumors (TUR-BT) at the Kagoshima University Hospital. The 25 normal bladder specimens were derived from patients without BC. Samples were processed and stored in RNAlater® (Qiagen, Valencia, CA, USA) at -20°C until RNA extraction. The samples were staged in accordance with the tumor-node-metastasis classification system of the American Joint Committee on Cancer/Union Internationale Contre le Cancer (UICC), and they were histologically graded. Written informed consent was obtained from all patients and the present study was approved by the Bioethics Committee of Kagoshima University. The patients' backgrounds and clinicopathological characteristics are summarized in Table I.

Cell culture and RNA extraction. We used the human BC cell lines BOY and T24. BOY was established in our laboratory from a male Asian patient, 66-years old, who was diagnosed with stage III BC with lung metastasis. T24 was obtained from the ATCC. The cell lines were incubated in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and maintained in a humidified incubator (5% CO_2) at 37°C. Total RNA was extracted as previously described (23).

Quantitative real-time RT-PCR. Stem-loop RT-PCR for *miR-23b* (P/N 000400; Applied Biosystems, Foster City, CA, USA) and *miR-27b* (P/N 000409; Applied Biosystems) were used to quantitate miRNAs according to previously published

Table I. Patient characteristics.

Characteristics	Data
Bladder cancer	
Total number	58
Median age (range) (years)	71 (47-91)
Gender	
Male	45 (78%)
Female	13 (22%)
Pathological tumor stage	
pTis	2 (3%)
pTa	7 (12%)
pT1	10 (17%)
pT2	15 (26%)
pT3	7 (12%)
pT4	5 (9%)
Unknown	12 (21%)
Grade	
G1	2 (3%)
G2	29 (50%)
G3	21 (36%)
Unknown	6 (1%)
Operation	
Cystectomy	23 (40%)
TUR-BT	35 (60%)
Normal bladder epithelium	25

conditions (11). To normalize data for quantifying the miRNAs, we used *RNU48* (P/N 001006; Applied Biosystems). The δ-δ threshold count method was used to calculate the fold-change.

Mature miRNA transfection. As previously described (11), the BC cell lines were transfected with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) with 10 nM mature miRNA molecules. As the negative control, Pre-miR miRNA precursor (P/N AM17111; Applied Biosystems) was used in gain-of-function experiments.

Cell proliferation, migration and invasion assays. Cell proliferation was determined using an XTT assay performed according to the manufacturer's instructions. Cell migration activity was evaluated with a wound healing assay and cell invasion assays were done using modified Boyden chambers as previously described (23). All experiments were performed in triplicate.

Putative miR-23b and miR-27b target gene pathway analysis and expression. To obtain putative miR-23b- and *miR-27b*-regulated genes, we used the TargetScan database (Release 6.2, http://www.targetscan.org/). To identify signaling pathways regulated by the *miR-23b/27b* cluster, *in silico* and gene expression data were analyzed in the Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway
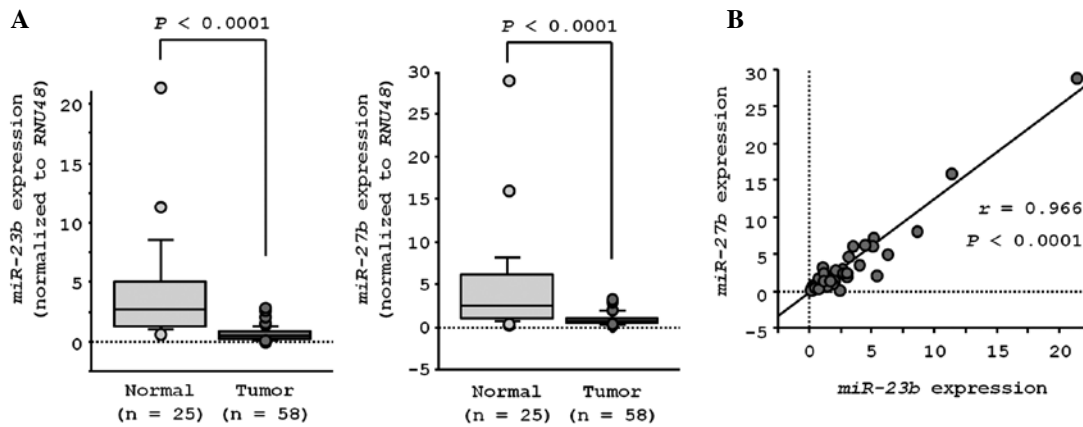
Figure 1. Expression levels of *miR-23b/27b* in clinical bladder specimens. (A) *miR-23b/27b* expression levels were significantly lower in 58 BC clinical specimens than in 25 normal bladder specimens. (B) The expression of *miR-23b* and *miR-27b* was positively correlated.

categories using the GeneCodis program. We performed gene expression analyses for all candidate genes involved in each of the pathways identified by GeneCodis3 software analysis using microarray expression data. The data were approved by the Gene Expression Omnibus (GEO) and were assigned GEO accession numbers (GSE11783 and GSE31684). For microarray expression data, we examined 90 BCs and 6 normal bladder epithelium collected from patients, none of whom had been exposed to chemotherapy before surgery. The data were normalized and analyzed with GeneSpring (Agilent Technologies, Santa Clara, CA, USA). Statistical analyses were conducted using the Mann-Whitney U test.

Western blotting. Three days after transfection, protein lysates (20 μg) were separated in NuPAGE on 4-12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes as previously described (23). Antibodies against EGFR and c-Met were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against GAPDH were purchased from Chemicon (Temecula, CA, USA). Specific complexes were visualized with an echochemiluminescence (ECL) detection system (GE Healthcare, Little Chalfont, UK).

Plasmid construction and dual-luciferase reporter assays. miRNA target sequences were inserted between the *Xho*I-*Pme*I restriction sites in the 3'-untranslated region (UTR) of the *hRluc* gene in the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). miRNA target sequences targeted by *miR-23b* and *miR-27b* are summarized in Table II.

T24 cells were transfected with 50 ng of the vector and 10 nM miRNA 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.

Statistical analysis. Relationships between 2 or 3 variables and numerical values were analyzed using the Mann-Whitney U test or Bonferroni adjusted Mann-Whitney U test. Spearman's rank test was used to evaluate the correlation between the expressions of *miR-23b* and *miR-27b*. Expert StatView, version 4 was used in these analyses.

Results

Expression levels of miR-23b/27b cluster in BC clinical specimens. We evaluated the expression levels of *miR-23b* and *miR-27b* in BC tissues (n=58) and normal bladder specimens (n=25). The expression levels of *miR-23b* and *miR-27b* were significantly lower in tumor tissues than in corresponding non-cancerous tissues (both P<0.0001; Fig. 1A). Spearman's rank test showed a positive correlation between the expression of *miR-23b* and that of *miR-27b* (r=0.966 and P<0.0001; Fig. 1B). These results suggested that *miR-23b* and *miR-27b* were significantly downregulated in BC and could represent putative tumor suppressors in BC.

Effects of restoring miR-23b and miR-27b expression on cell proliferation, migration and invasion activities in cancer cell lines. To examine the functional roles of *miR-23b* and *miR-27b*, we performed gain-of-function studies using miRNA transfection into BOY and T24 cells. XTT assays revealed significant inhibition of cell proliferation in BOY and T24 cells transfected with *miR-23b* and *miR-27b* in comparison with mock-transfected cells and control transfectants (BOY: P=0.0011 and P<0.0001, respectively; T24: P=0.0035 and P<0.0001, respectively) (Fig. 2A).

Moreover, wound healing assays demonstrated significant inhibition of cell migration was observed in BOY and T24 cells transfected with *miR-23b* and *miR-27b* (BOY: P<0.0001 and P=0.0001, respectively; T24: both P<0.0001) (Fig. 2B).

Similarly, *Matrigel* invasion assays revealed that transfection with these miRNAs reduced cell invasion. Indeed, the number of invading cells was significantly decreased in BOY and T24 cells transfected with *miR-23b* and *miR-27b* (BOY: P<0.0024 and P<0.0001, respectively; T24: P<0.0075 and P<0.0001, respectively) (Fig. 2C).

Identification of targets pathways and genes regulated by the miR-23b/27b cluster in BC cells. To gain further insight into the molecular mechanisms and pathways regulated by the tumor-suppressive *miR-23b/27b* cluster in BC, we performed a combination of gene expression and *in silico* analyses. The strategy for selecting *miR-23b/27b* cluster-regulated pathways is shown in Fig. 3.

Table II. Insert 3'-UTR sequence of *EGFR* and *c-Met*.

| Gene	Target sites	3'-UTR position		Sequence (5'-3')
EGFR	miR-27b	200-207	Wild	gccaggaagtacttccacctcgggcacatttgggaagttgcattccttgtcttcaa**acttgtgaag**catttacagaaacgcatccagcaag aatattgtcccttgagcagaaatttatcttcaaagagggtatatttgaaaaaaaaaaaagtatatgtgaggattttattgattgg
			Mutation	gccaggaagtacttccacctcgggcacatttgggaagttgcattccttgtcttcat**gacactt**gcatttacagaaacgcatccagcaag aatattgtcccttgagcagaaatttatcttcaaagagggtatatttgaaaaaaaaaaaagtatatgtgaggattttattgattgg
		430-436	Wild	ggatcttggagtttttcattgtcgctattgatttttacttcaatgggctcttccaacaaggaagaagcttgtcgtgtagcacttgctaccctg agttcatccaggcccaa**ctgtga**gcaaggagcacaagccacaagtcttccagagagtgctgttcagtggttctgcttcaaggctt
			Mutation	ggatcttggagtttttcattgtcgctatttgatttttacttcaatgggctcttccaacaaggaagaagcttgtcgtgtagcacttgctaccctg agttcatccaggcccaat**gacactt**gcaaggagcacaagccacaagtcttccagagagtgctgttcagtggttctgcttcaaggctt
c-Met	miR-23b	1019-1026	Wild	cattaagaaaatttgtatgaaataattagtcatcatgaaatatttagttgtcatataaaaccacctgtttgagaatgatgctactctgat ctaat**gaatgtga**acatgtagatgtttgtgttgtatttttaaatgaaaactcaaaataagacaagtaatttgttgataaatatttt
			Mutation	cattaagaaaatttgtatgaaataattagtcatcatgaaatatttagttgtcatataaaaccacctgtttgagaatgatgctactctgat ctaat**gttacact**tcatgtagatgtttgtgttgtatttttaaatgaaaactcaaaataagacaagtaatttgttgataaatatttt
		2065-2072	Wild	gaactcggggaaacatcccatcaacaggactacacactgtatatacattcttgagacaactgc**aatgtga**aaatcacgtttgctatttata aacttgtccttagattaatgtgtctggacagattgtgggagttaagtaagtgattcttcaagatacttgtcactgcctatacctgc
			Mutation	gaactcggggaaacatcccatcaacaggactacacactgtatatacattcttgagacaactgc**ttacact**taatcacgtttgctatttata aacttgtccttagattaatgtgtctggacagattgtgggagttaagtaagtgattcttcaagatacttgtcactgcctatacctgc
EGFR	miR-27b	1564-1571	Wild	taactggtttgtcgacgtaaacatttaaagtgtatattttttataaaaagtttattttataaatgtgagaaaaatttgttaggccac aaaaacactgc**actgtgaac**atttagaaaaggtatgtcgacgtgggattaatgacagcatgatttcaatgactgtaaattgcgata
			Mutation	taactggtttgtcgacgtaaacatttaaagtgtatattttttataaaaagtttattttataaatgtgagaaaaatttgttaggccac aaaaacactgc**tgacactt**catttagaaaaggtatgtcgacgtaaacatttcaatgactgtaaattgcgata
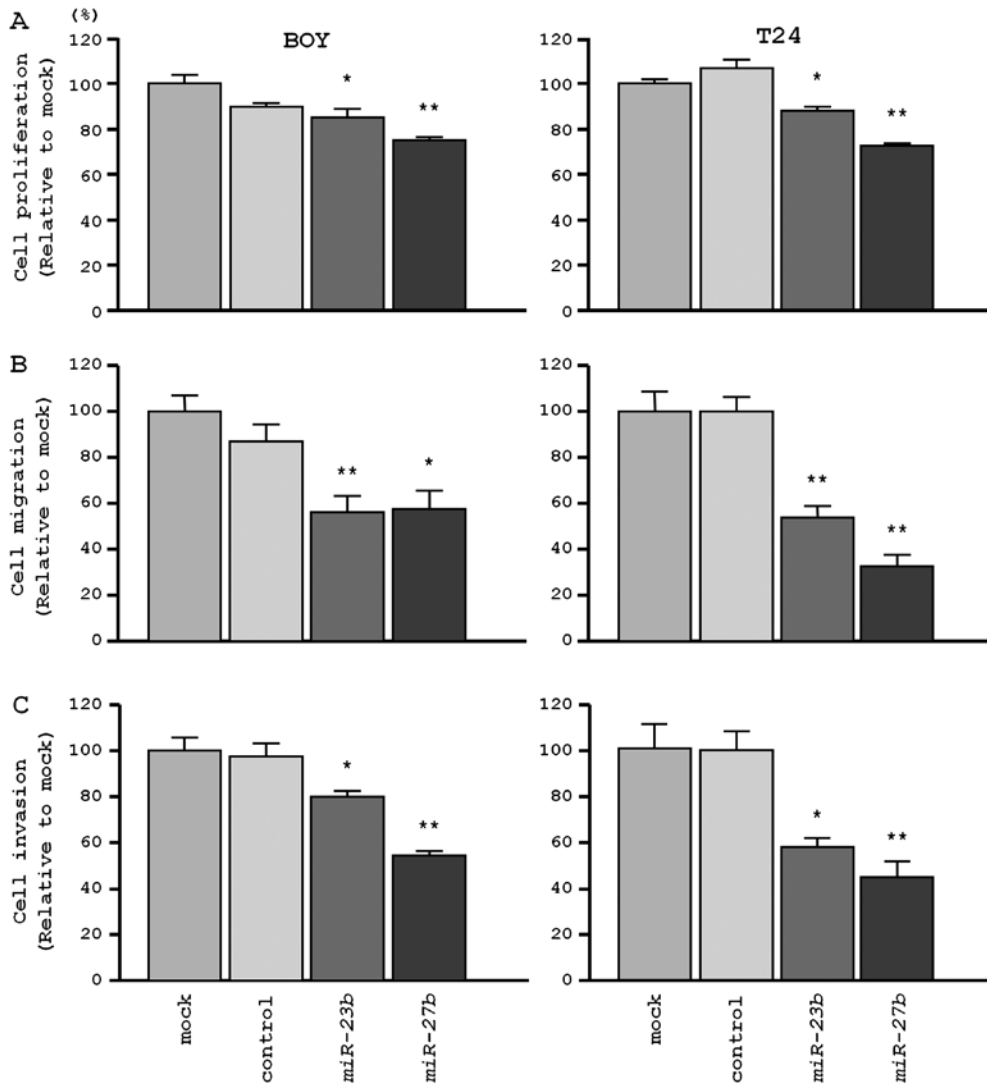
Figure 2. Effects of *miR-23b/27b* transfection into BC cell lines BOY and T24. (A) Cell proliferation determined by XTT assay. $^{*}P<0.005$. $^{**}P<0.0001$. (B) Cell migration activity determined with the wound healing assay. $^{*}P<0.0005$. $^{**}P<0.0001$. (C) Cell invasion activity determined with the Matrigel invasion assay. $^{*}P<0.01$. $^{**}P<0.0001$.
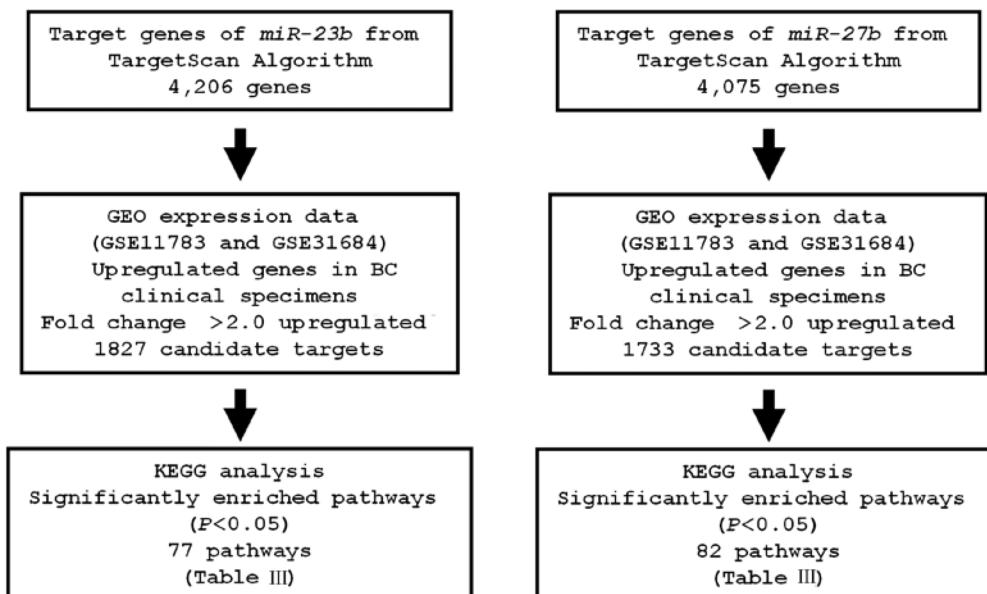

Figure 3. The strategy for selecting target pathways regulated by the *miR-23b/27b* cluster.

Table III. Top 10 enriched pathways in *miR-23b* and *miR-27b*.

Annotations	No. of genes	P-value
miR-23b		
Pathways in cancer	50	5.09E-15
Neuroactive ligand-receptor interaction	39	5.52E-11
MAPK signaling pathway	24	4.60E-04
Cytokine-cytokine receptor interaction	23	8.97E-04
Endocytosis	22	6.98E-05
Focal adhesion	21	2.03E-04
Regulation of actin cytoskeleton	21	3.72E-04
Calcium signaling pathway	20	1.35E-04
Glutamatergic synapse	19	6.90E-06
Chemokine signaling pathway	19	5.79E-04
miR-27b		
Pathways in cancer	42	2.14E-10
MAPK signaling pathway	30	1.30E-06
Neuroactive ligand-receptor interaction	29	4.67E-06
Calcium signaling pathway	24	1.23E-06
Axon guidance	23	2.23E-08
Regulation of actin cytoskeleton	22	1.42E-04
Endocytosis	20	3.85E-04
Glutamatergic synapse	19	3.35E-06
Chemokine signaling pathway	18	1.34E-03
Cytokine-cytokine receptor interaction	18	2.10E-02

The TargetScan program showed that 4206 and 4075 genes had putative target sites for *miR-23b* and *miR-27b-*, respectively, in their 3'-UTR regions. To confirm the expression levels of these genes in clinical BC tissues, GEO database (GEO accession number: GSE11783 and GSE31684) analysis was performed. The data showed that 1827 and 1733 genes were 2.0-fold or more upregulated in BC tissues compared to normal tissues for *miR-23b* and *miR-27b* target genes, respectively. KEGG analysis revealed that the top 'pathway' (greatest number of genes) was 'Pathways in cancer' (Table III). Several common putative target genes were included in this pathway (Table IV). We focused on *EGFR*, *RET* and *c-Met* genes that coded for tyrosine kinase receptors.

EGFR and c-Met were regulated by miR-23b and miR-27b. We performed western blot analysis of BOY and T24 cells to investigate whether EGFR, RET and c-Met expression were downregulated by restoration of *miR-23b* and *miR-27b*. Expression of EGFR protein was significantly repressed in *miR-27b* transfectants in comparison with mock or miR-control transfectants (Fig. 4). The protein expression level of c-Met was significantly repressed in *miR-23b* and *miR-27b* transfectants (Fig. 4). RET protein expression was not repressed in either *miR-23b* or *miR-27b* transfectants (Fig. 4).

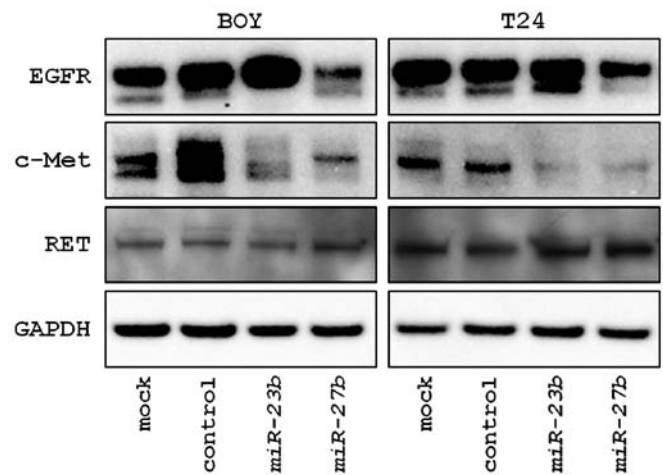*EGFR and c-Met were directly targeted by miR-23b and miR-27b.* We performed a luciferase reporter assay in T24

Figure 4. EGFR and c-Met protein expression levels were suppressed by *miR-23b/27b* transfection in BOY and T24 cells. Expression of EGFR, c-Met and RET protein as revealed by western blot analysis. GAPDH was used as a loading control.

to determine whether *EGFR* and *c-Met* had target sites for *miR-23b* and *miR-27b*. The TargetScan database predicted that two putative *miR-27b* binding sites existed in the 3'-UTR of *EGFR* (positions 200-207 and 430-436; Fig. 5A). The database showed that one putative *miR-23b* binding site existed in the 3'-UTR of *EGFR*. However, EGFR protein expression was not repressed in *miR-23b* transfectants (Fig. 4). Therefore, we performed a luciferase reporter assay to determine whether *EGFR* had target sites for *miR-27b*. The database also predicted that two putative *miR-23b* binding sites and one putative *miR-27b* binding site existed in the *c-Met* 3'-UTR (positions 1019-1026, 2065-2072 and 1564-1571, respectively; Fig. 5B). We used wild-type and mutant vectors encoding either the partial sequence of the 3'-UTRs of *EGFR* and *c-Met*, including the predicted *miR-23b* and *miR-27b* target sites.

We found that the luminescence intensity was significantly reduced by transfection of *miR-27b* with the wild-type vector carrying the 3'-UTR of *EGFR* (position 200-207: P<0.0001; position 430-436: P<0.0001; Fig. 5B), whereas transfection with a mutant vector showed no decrease in luminescence.

With regards to *c-Met*, the luminescence intensity was significantly reduced by transfection of *miR-23b* with vectors carrying a portion of the 3'-UTR of *c-Met* (position 1019-1026: P<0.0001; position 2065-2072: P<0.0001; Fig. 5B) and the luminescence intensity was also reduced by transfection of *miR-27b* with the vector carrying the 3'-UTR of *c-Met* (position 1564-1571: P<0.0001; Fig. 5B), whereas transfection with a mutant vector failed to decrease luminescence.

Discussion

Aberrant expression of the *miR-23b/27b* cluster has been reported in several types of human cancers; however, the expression status varies according to the cancer type. Decreased expression of the *miR-23b/27b* cluster has been observed in castration-resistant prostate cancer (24) and drug-resistant Ehrlich ascites tumor (25). In contrast, upregulation of the *miR-23b/27b* cluster was reported in breast cancer (22)

Table IV. *miR-23b/27b* common target genes highly expressed in bladder cancer.

Clinical BCs				
Change	P-value	Entrez gene ID	Symbol	Description
19.55	4.67E-05	7849	PAX8	Paired box 8
6.79	1.14E-04	1021	CDK6	Cyclin-dependent kinase 6
5.98	1.64E-04	1956	EGFR	Epidermal growth factor receptor
5.00	2.65E-04	2034	EPAS1	Endothelial PAS domain protein 1
4.74	1.21E-04	208	AKT2	v-akt murine thymoma viral oncogene homolog 2
4.60	5.91E-04	2246	FGF1	Fibroblast growth factor 1 (acidic)
4.20	8.23E-04	861	RUNX1	Runt-related transcription factor 1
3.96	3.16E-03	2250	FGF5	Fibroblast growth factor 5
3.91	3.75E-04	7170	TPM3	Tropomyosin 3
3.75	4.46E-04	4089	SMAD4	SMAD family member 4
3.75	5.59E-04	3918	LAMC2	Laminin, γ 2
3.42	7.80E-05	862	RUNX1T1	Runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
3.31	6.45E-05	5979	RET	Ret proto-oncogene
3.09	1.75E-02	4286	MITF	Microphthalmia-associated transcription factor
2.87	8.69E-04	4233	c-Met	Met proto-oncogene
2.83	1.15E-02	2259	FGF14	Fibroblast growth factor 14
2.83	1.14E-04	3845	KRAS	Kirsten rat sarcoma viral oncogene homolog
2.68	2.32E-02	7188	TRAF5	TNF receptor-associated factor 5
2.63	2.73E-03	2932	GSK3B	Glycogen synthase kinase 3 β
2.60	1.82E-03	5594	MAPK1	Mitogen-activated protein kinase 1
2.40	1.48E-03	4193	MDM2	MDM2 oncogene, E3 ubiquitin protein ligase
2.37	1.40E-03	5579	PRKCB	Protein kinase C, β

and chemoresistant ovarian cancer cells (26). In the present study, our data demonstrated that *miR-23b* and *miR-27b* expression was significantly downregulated in BC clinical specimens. Functional analysis demonstrated that restoration of *miR-23b* and *miR-27b* in BC cells inhibited cancer cell proliferation, migration and invasion. Those results suggest that the *miR-23b/27b* cluster functions as a tumor suppressor and may contribute to metastasis in BC. Of particular interest, our recent study showed that *miR-24-1*, which is located close to the *miR-23b/27b* cluster, was downregulated in BC tissues and functioned as a tumor suppressor targeting FOXM1 (27). Thus, our data suggest that the *miR-23b/27b* cluster, including *miR-24-1*, functions as a tumor suppressor and significantly contributes to BC oncogenesis and metastasis.

Next, we investigated the pathways/targets that were regulated by the tumor-suppressive *miR-23b/27b* cluster in BC cells. To identify tumor-suppressive, miRNA-regulated molecular pathways, we used a combination of expression data and *in silico* database analysis. Using this strategy, we have identified molecular targets and pathways in several types of cancer that are regulated by tumor-suppressive miRNAs, including BC (7-11). In the present study, 'pathways in cancer', the 'MAPK signaling pathway' and 'cytokine-cytokine receptor interaction' were significantly selected as candidate pathways regulated by the *miR-23b/27b* cluster in BC cells. Among these pathways, we focused attention on 'pathways in cancer' and searched for putative targets of *miR-23b/27b* regulation. We focused on tyrosine kinase receptors such as *EGFR*, *RET*

and *c-Met* genes because molecularly targeted therapies aimed at inhibiting their activities have been developed recently for several types of cancer (28). Our results showed that *EGFR* was directly regulated by *miR-27b* and that *c-Met* was directly regulated by both *miR-23b* and *miR-27b*. Unfortunately, *RET* was not controlled by either miRNA.

EGFR is the cell-surface receptor for members of the epidermal growth factor family of extracellular protein ligands (29). Receptor activation initiates several signal transduction cascades, including the MAPK, Akt and JNK pathways, leading to DNA synthesis and cell proliferation (30). Previous studies showed that overexpression of EGFR occurred in BC and the expression level correlated with tumor grade, stage and survival (31-33).

Another receptor, *c-Met* (hepatocyte growth factor receptor) activates multiple signal transduction pathways such as those involving RAS, PI3K-Akt, STAT and β-catenin (34-37). The expression of c-Met and phosphorylated c-Met are positively correlated with tumor grade, stage, tumor size and survival of several types of cancers. It is likely that c-Met could be a promising therapeutic target in disease (38,39). The amplification frequency of c-Met is approximately 20% in patients who have acquired resistance to EGFR tyrosine kinase inhibitors (40). Therefore, inhibition of EGFR and c-Met and their associated signaling pathways could be a potent strategy for cancer therapy. Studies of dual tyrosine kinase inhibitors are underway (41). Several laboratories have shown that miRNAs directly inhibit EGFR or/and c-Met
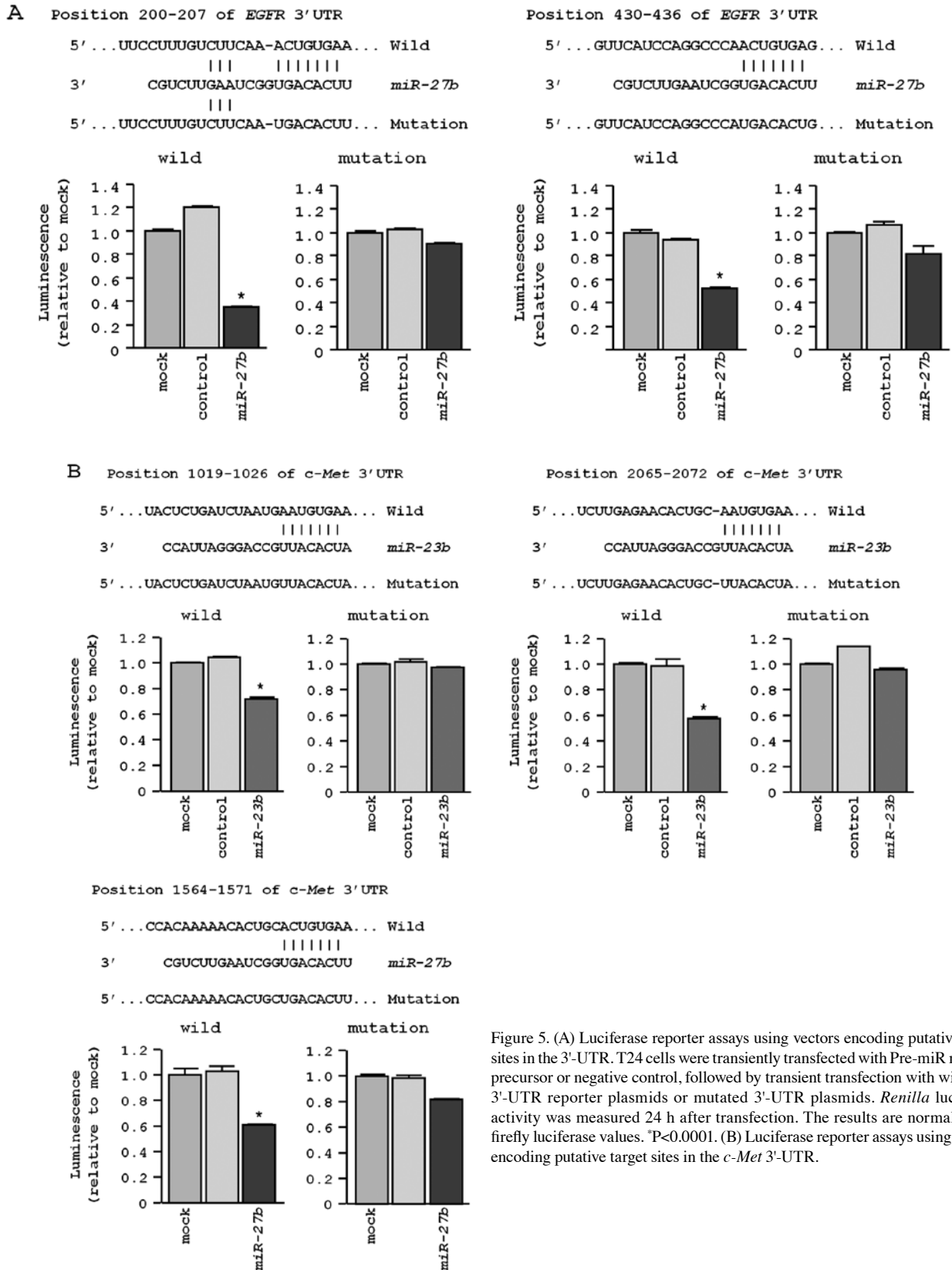
Figure 5. (A) Luciferase reporter assays using vectors encoding putative target sites in the 3'-UTR. T24 cells were transiently transfected with Pre-miR miRNA precursor or negative control, followed by transient transfection with wild-type 3'-UTR reporter plasmids or mutated 3'-UTR plasmids. *Renilla* luciferase activity was measured 24 h after transfection. The results are normalized to firefly luciferase values. *P<0.0001. (B) Luciferase reporter assays using vectors encoding putative target sites in the *c-Met* 3'-UTR.

expression, such as *miR-7*, *miR-146a*, *miR-574-3p*, *miR-34a*, *miR-130a* and *miR-1/206* (42-47). In addition, a recent study demonstrated that *miR-27a* regulated both EGFR and c-Met in non-small cell lung cancer (48). In the present study, the *miR-23b/27b* cluster regulated EGFR and c-Met in BC cells. Therefore, inhibition of two separate tyrosine kinases via the

tumor-suppressive *miR-23b/27b* cluster represents an attractive possibility in the development of new treatment options in cancer.

In conclusion, downregulation of the *miR-23b/27b* cluster is a frequent event in BC. Moreover, tumor-suppressive *miR-23b* and *miR-27b* directly regulated tyrosine kinase receptor genes

EGFR and *c-Met*. Identification of molecular targets regulated by tumor-suppressive miRNAs will provide insights into the potential mechanisms of BC oncogenesis and metastasis, facilitating the development of novel therapeutic strategies for the treatment of BC.

Acknowledgements

References

1. Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin 63: 11-30, 2013.
2. Luke C, Tracey E, Stapleton A and Roder D: Exploring contrary trends in bladder cancer incidence, mortality and survival: implications for research and cancer control. Intern Med J 40: 357-362, 2010.
3. Zuiverloon TC, Nieuweboer AJ, Vekony H, Kirkels WJ, Bangma CH and Zwarthoff EC: Markers predicting response to bacillus Calmette-Guerin immunotherapy in high-risk bladder cancer patients: a systematic review. Eur Urol 61: 128-145, 2012.
4. Mattick JS: RNA regulation: a new genetics? Nat Rev Genet 5: 316-323, 2004.
5. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
6. Esquela-Kerscher A and Slack FJ: Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
7. Itesako T, Seki N, Yoshino H, *et al*: The microRNA expression signature of bladder cancer by deep sequencing: the functional significance of the miR-195/497 cluster. PLoS One 9: e84311, 2014.
8. Fuse M, Kojima S, Enokida H, *et al*: Tumor suppressive microRNAs (miR-222 and miR-31) regulate molecular pathways based on microRNA expression signature in prostate cancer. J Hum Genet 57: 691-699, 2012.
9. Hidaka H, Seki N, Yoshino H, *et al*: Tumor suppressive microRNA-1285 regulates novel molecular targets: aberrant expression and functional significance in renal cell carcinoma. Oncotarget 3: 44-57, 2012.
10. Nohata N, Hanazawa T, Kikkawa N, *et al*: Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. Br J Cancer 105: 833-841, 2011.
11. Ichimi T, Enokida H, Okuno Y, *et al*: Identification of novel microRNA targets based on microRNA signatures in bladder cancer. Int J Cancer 125: 345-352, 2009.
12. Yoshino H, Chiyomaru T, Enokida H, *et al*: The tumour-suppressive function of *miR-1* and *miR-133a* targeting TAGLN2 in bladder cancer. Br J Cancer 104: 808-818, 2011.
13. Nishikawa R, Goto Y, Kojima S, *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.
14. Kojima S, Enokida H, Yoshino H, *et al*: The tumor-suppressive microRNA-143/145 cluster inhibits cell migration and invasion by targeting GOLM1 in prostate cancer. J Hum Genet 59: 78-87, 2014.
15. Kinoshita T, Nohata N, Hanazawa T, *et al*: Tumour-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.
16. Yoshino H, Enokida H, Itesako T, *et al*: Tumor-suppressive microRNA-143/145 cluster targets hexokinase-2 in renal cell carcinoma. Cancer Sci 104: 1567-1574, 2013.
17. Yamasaki T, Yoshino H, Enokida H, *et al*: Novel molecular targets regulated by tumor suppressors microRNA-1 and microRNA-133a in bladder cancer. Int J Oncol 40: 1821-1830, 2012.
18. Nohata N, Hanazawa T, Enokida H and Seki N: microRNA-1/133a and microRNA-206/133b clusters: dysregulation and functional roles in human cancers. Oncotarget 3: 9-21, 2012.
19. Kojima S, Chiyomaru T, Kawakami K, *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.
20. Kawakami K, Enokida H, Chiyomaru T, *et al*: The functional significance of miR-1 and miR-133a in renal cell carcinoma. Eur J Cancer 48: 827-836, 2012.
21. Nohata N, Hanazawa T, Kikkawa N, *et al*: Identification of novel molecular targets regulated by tumor suppressive miR-1/miR-133a in maxillary sinus squamous cell carcinoma. Int J Oncol 39: 1099-1107, 2011.
22. Jin L, Wessely O, Marcusson EG, Ivan C, Calin GA and Alahari SK: Prooncogenic factors miR-23b and miR-27b are regulated by Her2/Neu, EGF, and TNF-alpha in breast cancer. Cancer Res 73: 2884-2896, 2013.
23. Chiyomaru T, Enokida H, Tatarano S, *et al*: *miR-145* and *miR-133a* function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer 102: 883-891, 2010.
24. Ishteiwy RA, Ward TM, Dykxhoorn DM and Burnstein KL: The microRNA-23b/-27b cluster suppresses the metastatic phenotype of castration-resistant prostate cancer cells. PLoS One 7: e52106, 2012.
25. Husted S, Sokilde R, Rask L, *et al*: MicroRNA expression profiles associated with development of drug resistance in Ehrlich ascites tumor cells. Mol Pharm 8: 2055-2062, 2011.
26. Park YT, Jeong JY, Lee MJ, *et al*: MicroRNAs overexpressed in ovarian ALDH1-positive cells are associated with chemoresistance. J Ovarian Res 6: 18, 2013.
27. Inoguchi S, Seki N, Chiyomaru T, *et al*: Tumour-suppressive *microRNA-24-1* inhibits cancer cell proliferation through targeting *FOXM1* in bladder cancer. FEBS Lett 588: 3170-3179, 2014.
28. Shaw AT, Hsu PP, Awad MM and Engelman JA: Tyrosine kinase gene rearrangements in epithelial malignancies. Nat Rev Cancer 13: 772-787, 2013.
29. Herbst RS: Review of epidermal growth factor receptor biology. Int J Radiat Oncol Biol Phys 59: 21-26, 2004.
30. Oda K, Matsuoka Y, Funahashi A and Kitano H: A comprehensive pathway map of epidermal growth factor receptor signaling. Mol Syst Biol 1: 2005.0010, 2005.
31. Chow NH, Liu HS, Lee EI, *et al*: Significance of urinary epidermal growth factor and its receptor expression in human bladder cancer. Anticancer Res 17: 1293-1296, 1997.
32. Mellon K, Wright C, Kelly P, Horne CH and Neal DE: Long-term outcome related to epidermal growth factor receptor status in bladder cancer. J Urol 153: 919-925, 1995.
33. Bue P, Wester K, Sjostrom A, *et al*: Expression of epidermal growth factor receptor in urinary bladder cancer metastases. Int J Cancer 76: 189-193, 1998.
34. O'Brien LE, Tang K, Kats ES, Schutz-Geschwender A, Lipschutz JH and Mostov KE: ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. Dev Cell 7: 21-32, 2004.
35. Graziani A, Gramaglia D, Cantley LC and Comoglio PM: The tyrosine-phosphorylated hepatocyte growth factor/scatter factor receptor associates with phosphatidylinositol 3-kinase. J Biol Chem 266: 22087-22090, 1991.
36. Boccaccio C, Ando M, Tamagnone L, *et al*: Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature 391: 285-288, 1998.
37. Monga SP, Mars WM, Pediaditakis P, *et al*: Hepatocyte growth factor induces Wnt-independent nuclear translocation of beta-catenin after Met-beta-catenin dissociation in hepatocytes. Cancer Res 62: 2064-2071, 2002.
38. Cheng HL, Trink B, Tzai TS, *et al*: Overexpression of c-met as a prognostic indicator for transitional cell carcinoma of the urinary bladder: a comparison with p53 nuclear accumulation. J Clin Oncol 20: 1544-1550, 2002.
39. Miyata Y, Sagara Y, Kanda S, Hayashi T and Kanetake H: Phosphorylated hepatocyte growth factor receptor/c-Met is associated with tumor growth and prognosis in patients with bladder cancer: correlation with matrix metalloproteinase-2 and -7 and E-cadherin. Hum Pathol 40: 496-504, 2009.
40. Engelman JA, Zejnullahu K, Mitsudomi T, *et al*: MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316: 1039-1043, 2007.
41. Goldman JW, Laux I, Chai F, *et al*: Phase 1 dose-escalation trial evaluating the combination of the selective MET (mesenchymal-epithelial transition factor) inhibitor tivantinib (ARQ 197) plus erlotinib. Cancer 118: 5903-5911, 2012.

42. Kefas B, Godlewski J, Comeau L, *et al*: microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res 68: 3566-3572, 2008.

43. Li Y, Vandenboom TG II, Wang Z, *et al*: miR-146a suppresses invasion of pancreatic cancer cells. Cancer Res 70: 1486-1495, 2010.

44. Chiyomaru T, Yamamura S, Fukuhara S, *et al*: Genistein up-regulates tumor suppressor microRNA-574-3p in prostate cancer. PLoS One 8: e58929, 2013.

45. Acunzo M, Visone R, Romano G, *et al*: miR-130a targets MET and induces TRAIL-sensitivity in NSCLC by downregulating miR-221 and 222. Oncogene 31: 634-642, 2012.

46. Yan D, Dong Xda E, Chen X, *et al*: MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. J Biol Chem 284: 29596-29604, 2009.

47. Taulli R, Bersani F, Foglizzo V, *et al*: The muscle-specific microRNA miR-206 blocks human rhabdomyosarcoma growth in xenotransplanted mice by promoting myogenic differentiation. J Clin Invest 119: 2366-2378, 2009.

48. Acunzo M, Romano G, Palmieri D, *et al*: Cross-talk between MET and EGFR in non-small cell lung cancer involves miR-27a and Sprouty2. Proc Natl Acad Sci USA 110: 8573-8578, 2013.