~60–100 nt precursor-miRNA (pre-miRNA) with a hairpin-like structure. After pre-miRNAs are transported to the cytoplasm by Exportin-5, they are processed into mature miRNA duplexes by Dicer assembled with transactivating response RNA-binding protein and protein activator of PKR (13,14). Finally, one strand of the mature miRNA duplex, a guide strand, is incorporated into the Argonaut-containing RNA-induced silencing complex, which induces either cleavage or translational repression of targeted mRNAs based on their sequences (Fig. 1). Once the miRNAs are unbalanced or the functions are disordered, they can be involved in the initiation and development of fatal human ailments, including cancer (15). Indeed, many reports have shown that the widespread disruption of miRNAs was correlated with the initiation and progression of human cancer and demonstrated that an injection with synthetic RNA molecules mimics tumor suppressor miRNAs or the inhibitors of oncogenic miRNA (onco-miR) can switch dozens of cancer-related signals on or off (16). In other words, miRNAs are potential therapeutic tools for cancer treatment, representing a superior molecular target approach to the traditional low-molecular compound approach. However, for the realization of RNAi-based therapies using siRNAs, synthetic miRNAs and miRNA inhibitors, more continuous improvements will be required. For example, the technology to avoid unwanted innate immune responses, instability of nucleic acid *in vivo* and off-target side effects strikingly decreases the levels of potency and efficacy of RNAi effector molecules (17–19). Thus, the development of drug delivery systems (DDS) for RNAi therapeutic strategies that are safer, more stable and more effective is a paramount consideration.

Although clinical applications of RNAi-based therapies have not been fully realized, numerous pre-clinical studies in animal models of human disease are providing opportunities for practical use. In this review, we provide an overview of the current clinical and pre-clinical trials of RNAi therapies and discuss strategies toward a pathway of miRNA to practical applications for cancer therapy from the viewpoint of RNAi DDS.

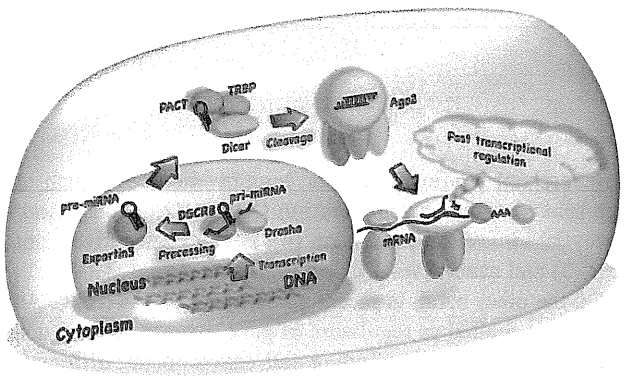

Figure 1. Cellular mechanisms of RNA interference pathway in mammals. First, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and are cleaved by the enzyme Drosha into ~70 nucleotides as precursor-miRNAs (pre-miRNAs). Next, these pre-miRNAs are exported to the cytoplasm with Exportin-5 and are cleaved to double-strand RNAs that do not contain a loop by Dicer. These duplexes are then associated with Argonote2 (Ago2), and one strand is removed. This RNAi-induced silencing complex (RISC) containing the guide strand triggers post-transcriptional regulation of target mRNA depending on the seed sequence of miRNAs.

RNAi THERAPEUTICS DEVELOPMENT PIPELINE

In the development of RNAi technology for therapeutic medication, since the first demonstration of RNAi triggered by siRNA in mammalian cells in 2001 (20,21), some risk-taking biotechnology companies, such as Sirna Therapeutics, Silence Therapeutics and Tekmira, started to establish a platform to develop the new technology using primarily siRNA. At first, some pharmaceutical companies ascribed the RNAi to research for directed gene silencing; however, after the first exploration of *in vivo* gene knockdown (22), major pharmaceutical firms, such as Medtronic, Novartis and Merck, became involved in clinical applications. Observers were surprised by the acquisition by Merck and Roche of Sirna Therapeutics for more than one billion USD. RNAi was considered an exceptional technology for the knockdown of therapeutic target genes, and scientists anticipated that it would significantly shorten the drug development timeline. However, as a consequence of the global economic turmoil that began in 2008 and the slump in development of DDS for RNAi medicine, companies such as Merck, Pfizer, Abbott Labs and Roche were forced to curtail research in these fields. In particular, the fact that Roche halted its development of RNAi technology in 2010 was a shock throughout the industry. The Roche decision resulted in a loss of confidence in the company's ability to innovate, and their withdrawal from RNAi research was followed by other companies. However, the clinical pipeline of RNAi therapies using siRNA has been gradually growing since approximately 2011 as the RNAi technology has matured.

As shown in Table 1, there are many candidates for clinical development in 2012. In particular, there are a number of sites for topical or local administration, such as the skin, retina and airways, which permit safe and efficient delivery without unwanted side effects. For example, according to some recent animal experiments, transtympanic administration of siRNA targeting NOX3 is significantly useful for the attenuation of cisplatin ototoxicity (23). Furthermore, Paller *et al.* (24) at Northwestern University showed that spherical nucleic acid nanoparticle conjugates gold cores surrounded by immobilized siRNA directed against EGFR can be topically delivered more stably into mouse and human skin without undesirable toxicity. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue or cell specifically. Since around 2008, however, the development pipeline has shifted from local to systemic delivery because more advanced delivery vehicles for systemic

Table 1. Current states of clinical candidate pipeline for RNAi therapy

ClinicalTrials.gov identifier	Drug	Route	Delivery	Disease	Target	Phase	States	Company
NCT00499590	Bevasiranib	IVT	Naked siRNA	Wet AMD	VEGF	III	Terminated	Opko Health
NCT00363714, NCT00395057	AGN211745/ Sirna–027	IVT	Naked siRNA	AMD	VEGF-R1	II	Terminated	Allergan/ Sirna
NCT01065935, NCT00658086	ALN-RSV01	Nebulization	Naked siRNA	RSV infection after lung transplantation	RSV Nucleocapsid	II	Completed	Alnylam
NCT00306904	Bevasiranib	IVT	Naked siRNA	DME	VEGF	II	Completed	Opko Health, Inc.
NCT01445899	PF-04523655	IVT	Naked siRNA	DME	RTP801	II	Recruiting	Quark Pharma
NCT01200420	miravirsen	SC	Naked LNA	HCV	miR-122	II	Completed	Santaris Pharma
NCT01551745 NCT01505166	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Ovarian cancer, colon cancer	Bi-shRNA-Furin and GM-CSF	II, II	Recruiting, Recruiting	Gradalis, Inc.
NCT00802347	I5NP	IV	Naked siRNA	DGF in kidney transplantation	P53	I/II	Recruiting	Quark Pharma
NCT01227291	SYL040012	Ophthalmic drops	Naked siRNA in ophthalmic drops	Glaucoma and ocular hypertension	Adrenergic receptor beta-2 siRNA	I/II	Completed	Sylentis
NCT00725686, NCT00713518	PF-04523655	IVT	Naked siRNA	Wet AMD	RTP801	I, II	Completed	Pfizer/Quark
NCT00716014	TD101	Intralesional	Naked siRNA	Pachyonychia congenita	Keratin 6a N171K mutant mRNA	Ib	Completed	TransDerm/ IPCC
NCT00882180, NCT01158079	ALN-VSP02	IV	SNALP	Liver cancer, solid tumors	KSP and VEGF	I, I	Completed	Alnylam
NCT00554359, NCT00683553	I5NP	IV	Naked siRNA	AKI for major cardiovascular surgery	P53	I, I	Completed, terminated	Quark Pharma
NCT01148953	ALN-TTR01	IV	SNALP	TTR-mediated amyloidosis	Transthyretin	I	Completed	Alnylam
NCT00689065	CALAA-01	IV	RONDEL	Solid cancer	RRM2	I	Active	Calando Pharma
NCT00466583	EZN-2968	IV	Naked LNA	Advanced solid tumor, lymphoma	HIF-1a	I	Completed	Santaris Pharma
NCT01120288	EZN-2968	IV	Naked LNA	Liver metastases	HIF-1a	I	Recruiting	NCI
NCT00672542	siRNA in dendritic cells	*Ex vivo*, Intradermal	Electroporation	Metastatic melanoma	Immunoproteasome subunits LMP2, LMP7, MECL1	I	Active	Duke University
NCT01061840	FANG vaccine	*Ex vivo*, Intradermal	Electroporation	Solid tumors	Bi-shRNA-Furin and GM-CSF	I	Recruiting	Gradalis, Inc.
NCT01064505	QPI-1007	IVT	Naked siRNA	Optic atrophy	Caspase-2	I	Active	Quark Pharma
NCT00938574	Atu027	IV	AtuPLEX	Advanced solid cancer	PKN3	I	Completed	Silence Therapeutics
NCT01188785	siG12D LODER	EUS biopsy needle	LODER polymer	Pancreatic ductal adenocarcinoma	KRASG12D	I	Recruiting	Silenseed Ltd
NCT01262235	TKM-080301	IV	SNALP	Cancer	PLK1	I	Recruiting	Tekmira
NCT00927459	PRO-040201	IV	SNALP	Hypercholesterolemia	Apo B	I	Terminated	Tekmira

AKI, acute kidney injury; AMD, age-related macular edema; DGF, delayed graft function; DME, diabetic molecular edema; HCV, Hepatitis C Virus; IV, intravenous; IVT, intravitreal; KSP, Kinesin Spindle Protein; LNA, locked nucleic acids; NCI, National Cancer Institute; PEG, polyethylene glycol; PLK1, Polo-like Kinase I; RRM2, Ribonucleotide Reductase M2; RSV, respiratory syncytial virus; SNALP, stable nucleic acid lipid particle; TF, transferrin; TTR, transthyretin; VEGF, vascular endothelial growth factor; SC, subcutaneous.
*From ClinicalTrials.gov.

application, such as stable nucleic acid lipid particles (SNALPs) and RNAi/oligonucleotide nanoparticle delivery (RONDEL), are available. These technologies have been shown to be effective *in vivo* (25–27), and progress is being achieved in some clinical trials (ALN-VSP02, ALN-TTR01, CALAA-01, TKM-080301, PRO-040201). In cancer treatment, siRNAs targeting polo-like kinase I (PLK1), kinesin spindle protein (KSP) and vascular endothelial growth factor, which are formulated with SNALP or RONDEL, have been developed as candidate pipelines in Phase I (Table 1).

DRUG DELIVERY SYSTEM FOR SYNTHETIC OLIGONUCLEOTIDE

Nucleic acid medicines, including siRNA, miRNA and anti-miRNA, work only after they penetrate hydrophobic cellular membranes. However, it is not easy for them to go through the lipid bilayer membrane without their carrier because synthetic oligonucleotides are negatively charged. In addition to this, RNAs are very easily degraded by RNase *in vivo*. Accordingly, assisting carriers or chemical modifications for the progression of the transmembrane transport and for the inhibition of the degradation by serum RNases are required. Historically, viral and non-viral delivery has been utilized (Table 2). In a viral delivery system, it was reported that an adenovirus carried short hairpin RNA (shRNA) expression vector targeting angiotensin type 1 (AT1) delivered into the brain intracerebroventricularly (ICV) (28) and that the miR-23b expression vector and miR-23b sponge worked in inflammatory autoimmune diseases *via* intra-articular (IA) infection (29). An adeno-associated virus (AAV) was also successful at carrying a miRNA cluster into the muscle and shRNA vectors targeting mutant huntingtin into the brain by topical administration (30,31). Furthermore, miR-34a treatment prevented lung cancer initiation and progression *via* transtracheal infection, and shRNA targeting superoxide dismutase 1 (SOD1) inhibited amyotrophic lateral sclerosis progression by lentiviral-mediated RNAi (32,33). The herpes simplex virus, which commonly causes an eruption of fluid-containing vesicles on the mouth, lips or face, also has potential for cancer treatment and therapeutic pain relief (34,35). Thus, viral-mediated gene silencing is very useful for local infection, particularly at sites that make frequent administration difficult. Although viral delivery has frequently shown higher efficiency than that by non-viral systems, preliminary clinical studies have shown that it triggered strong inflammatory reactions (36), and these delivery vectors have caused the death of several patients in the clinic (37,38). Thus, understanding the details of the inflammatory mechanism and developing safer viral vehicles are important tasks ahead.

On the other hand, the focus has recently been on the non-viral approach because of its advantages over viral vectors, such as non-immunogenicity, low production cost and easy quality control. This approach requires an optimized delivery reagent, such as a cationic lipid, polymers, nanoparticles, carbon nanotubes and atelocollagen (Table 2). In cancer treatment, atelocollagen or cationic liposome- or polymer-mediated transfection reagents have commonly been used to deliver siRNA or miRNA to cells *in vitro* and *in vivo*. In particular, a number of reports have demonstrated a significant anti-cancer effect caused by systemic delivery of siRNA with cationic liposome (39–41). Similarly, a cationic polymer, polyethyleneimine, was commercialized as *in vivo*-jetPEI™, provided by Life Technologies, and was used to successfully deliver siRNAs to cancer cells in animals (42,43). In addition, atelocollagen can be obtained from type I collagen of calf dermis and has also been expected to be a useful carrier because of its low immunogenicity and efficiency (8,44–46). In case of miRNA therapy, a tumor-suppressive miR-16 mimic was successfully delivered by the systemic approach using atelocollagen, and it dramatically inhibited the growth of metastatic prostate cancer (47). Furthermore, chemically functionalized carbon nanotubes also show potential for novel biological applications for the delivery of Caspase-3 siRNA into the brain by topical injection into the cerebral cortex and reduced neurodegeneration without toxic side effects (48).

In a recent study, the focus was on highly stabilized nanoparticles, and these nanoparticles made the systemic delivery system dramatically more efficient (25,49–52). For example, synthetic miR-34a mimic, which was incorporated into cholesterol, and the cationic liposome *N*-[1-(2,3-dioleoyloxy)]-*N,N,N*-trimethyl ammonium propane (DOTAP) (1:1 mol/mol) and polyethylene glycol (PEG)-conjugated CG4-targeting single-chain antibody fragment were efficiently delivered into melanoma and inhibited lung metastasis (53). The nanocarrier 'SNALP' by Tekmira pharmaceuticals is one of the technologies with the most potential in the clinical pipeline. SNALP is a PEG-grafted monolamellar liposome that can easily avoid opsonization and subsequent recognition by the macrophages because the hydrophilic nature of PEG constructs an aqueous coating on its particle surface (54). In the work of Judge *et al.*, SNALP-formulated siRNAs against PLK1 and KSP displayed significant anti-tumor effects in liver tumor model mice (26). Successful results have already been reported in the treatment of transthyretin-mediated amyloidosis, hypercholesterolemia, Ebola virus infection (49) and cancer (50). The clinical trials have been identified as NCT00882180, NCT01158079, NCT01148953, NCT01262235 and NCT00927459 in the ClinicalTrials.gov database (http://clinicaltrials.gov).

The effective systemic delivery of siRNA or miRNA toward target cells or tissues has been enormous challenge for RNAi therapy. Indeed, naked siRNAs are rapidly eliminated by the kidneys, and nanoparticle-formulated siRNAs have a tendency to accumulate in the liver. In particular, their suitability for cancer cells depends on the enhanced permeability and retention effect of nanoparticles. To solve these problems, combined use with orienting molecules, such as a cell-specific ligand, can increase the cell or tumor

Table 2. Technologies for drug delivery systems in RNAi therapy

Delivery	Tissue	Route	RNA	References
Viral vector				
Adenovirus	Articulation	IA	miR-23b	(29)
	Brain	ICV	AT1a, AT1b shRNA	(28)
Adeno-associated virus	Muscles	IM	Anti-VEGF miRNA cluster	(30)
	Brain	Intrastriatal	mHTT shRNA	(31)
Lentivirus	Lung	Transtracheal	miR-34a	(32)
	Spinal cord	Intraspinal	SOD1 shRNA	(33)
Herpes simplex virus	Dorsal root ganglia	Injection into the sciatic nerve	Trpv1 shRNA	(34)
	Glioma	IT	EGFR shRNA	(35)
Non-viral reagent				
Liposome				
Oligofectamine	Colon cancer	IP/IV	B-catenin siRNA	(40)
DOTAP	Liver, spleen	IV	GFP siRNA	(39)
LIC-101	Liver metastasis	IV/SC	BCL-2 siRNA	(41)
PEI	Ovarian canner	IP/SC	HER-2 siRNA	(42)
	Glioblastoma	IP/SC	PTN siRNA	(43)
Nanoparticle				
SNALP	Ebola virus	IP/SC	ZEBOV siRNA	(49)
	Lung cancer	IV	miR-34a/let-7	(50)
RONDEL	Melanoma	IV	RRM2 siRNA	(25)
	Ewing's sarcoma	IV	EWS-FLI 1 siRNA	(51)
AtuPLEX	Prostate/pancreatic cancer	IV	PKN3 siRNA	(52)
DOTAP, cholesterol and PEG	Melanoma	IV	c-Myc/MDM2/VEGF siRNA and miR-34a	(53)
Atelocollagen	Testicular cancer	IT	HST-1/FGF-4 siRNA	(45)
	Osteosarcoma	IV	miR-143	(112)
	Prostate cancer		miR-16	(47)
HDI	Liver	IV	HBV siRNA	(113)
Carbon nanotube	Brain	Into the cerebral cortex	Caspase-3	(48)

ApoB, Apolipoprotein B; AT1, Angiotensin type 1; DDAB, dimethyldioctadecylammonium bromide; DOTAP, (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methylsulfate; HBV, Hepatitis B Virus; HDI, hydrodynamic tail vein injection; HER-2, human epidermal growth factor receptor 2; IA, intra-articular, ICV, intracerebroventricular; IM, intramuscular; IP, intraperitoneal; IT, intratumor; IV, intravenous; mHTT, mutant huntingtin; PBAVE, poly butyl and amino vinyl ethers; PEI, polyethyleneimine; PKN3, Protein Kinase N3; PPARA, peroxisome proliferator-activated receptor alpha; PTN, pleiotrophin; SC, subcutaneously; SLN, solid lipid nanoparticle; SOD1, superoxide dismutase 1; ZEBOV, The Polymerase (L) Gene of the Zaire Species of Ebola Virus.

specificity and delivery efficiency (55–57). Calando's cyclodextrinpolymer-based delivery platform (RONDEL) consists of cyclodextrin-containing polycation, and adamantine-coupled PEG-stabilized some ligands, such as transferrin (TF), and siRNA or miRNA (Fig. 2). This siRNA delivery platform was conceived by Hu-Lieskovan *et al.* in 2005 (51). The TF receptors are known to be upregulated in malignant cells, and TF-stabilized particles are taken up into cancer cells by TF receptor-mediated endocytosis and subsequent release into the cytoplasm in a pH-dependent manner (25). Phase 1b clinical trials of CALAA-01, including the M2 subunit of ribonucleotide reductase (RRM2) targeting siRNA, are being conducted as a novel RNAi therapy for multiple types of solid tumors.

CHEMICAL MODIFICATIONS FOR OLIGONCLEOTIDES

In addition to the nanocarriers mentioned above, others are being sought through chemical modifications. The purpose of such modifications can be permeability into the cells, specificity for specific tissues and stability against nuclease degradation (Fig. 3 and Table 3). For example, as a

permeability-enhancing factor, the covalent conjugation of the lipophilic molecule assists siRNA or miRNA to penetrate into the cellular cytoplasm and trigger gene silencing *in vivo* (58). In particular, high-density lipoprotein (HDL)-conjugated siRNAs are selectivity taken up by the gut, kidney and steroidogenic organs *via* the HDL receptor, scavenger receptor class B, type I (SR-BI) (59–62). In contrast, low-density lipoprotein (LDL)-conjugated siRNAs are efficiently internalized into the hepatocytes after binding to the LDL receptor (59). The Arrowhead Research Corporation demonstrated that the co-injection of cholesterol-siRNA and hepatocyte-targeted endosomolytic polymer achieved high-level target gene knockdown with low doses of cholesterol-siRNA in non-human primates (63). The company is using this strategy and a polymer-based siRNA delivery platform named dynamic polyconjugate polymer in ARC-520, which is a hepatitis B clinical candidate.

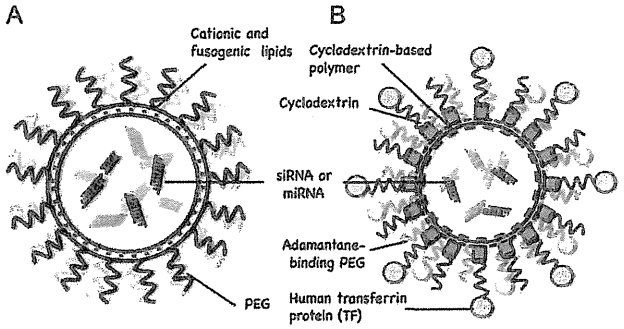

Figure 2. Delivery technology for RNAi therapy. (A) Stable nucleic acid lipid particle (SNALP). The bilayer consists of cationic and neutral lipids and is coated by PEG. The diameter is ~100 nm. (B) RNAi/oligonucleotide nanoparticle delivery (RONDEL). RNAs are protected from degradation in serum by the cyclodextrin-conjugated polymer. The complexes are <100 nm in diameter. In aqueous solution, adamantane easily binds to cyclodextrin as a result of hydrophobic attraction.

In another example, nanoparticles composed of poly (lactic-*co*-glycolic acid) were modified with a cell-penetrating peptide, penetratin, and used for the systemic delivery of the miR-155 inhibitor in the mouse model of lymphoma (64).

On the other hand, cell-specific factors, such as aptamers (65,66), peptide (64,67), antibodies (68,69) and agonists (56), can enhance cell specificity in cases of systemic administration into experimental animals. For example, octaarginine-modified liposomal particles were used to suppress an endogenous gene in the liver at low concentrations of siRNA without any toxicity (67). Usually, targeting proteins were conjugated to cationic bridges, such as polylysine or protamine, which can mediate uptake of nucleic acids, to link targeting proteins to effector oligonucleotide (68,70–72). In contrast, the siRNA-aptamer chimeras have also been of interest because a completely RNA-based approach may have important advantages over other methods for targeted delivery of siRNAs in terms of cost, productivity, safety and flexibility regarding chemical modification. RNA aptamers are single-stranded oligonucleotides and bind with high affinity to specific molecular targets, such as small molecules, proteins and nucleic acids, with their 3D structure (65,66). Here, although antibody-mediated siRNA delivery is required for the biological production of antibodies and antibody-siRNA conjugations by using a linker such as PEG, chimeric aptamer-siRNA can be synthesized as a single unit at once. However, for the utilization of chimeric aptamer-siRNA, more structured RNAs capable of binding with higher affinity and specificity have been required.

Stabilization in serum has been developed for the inhibition of the nuclease activity. Indeed, the backbone linkage introduced phosphorothioate (PS) or the sugar conjugated with protecting groups such as 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP) and 5′-(E)-vinyl-phosphonate (5′-VP)

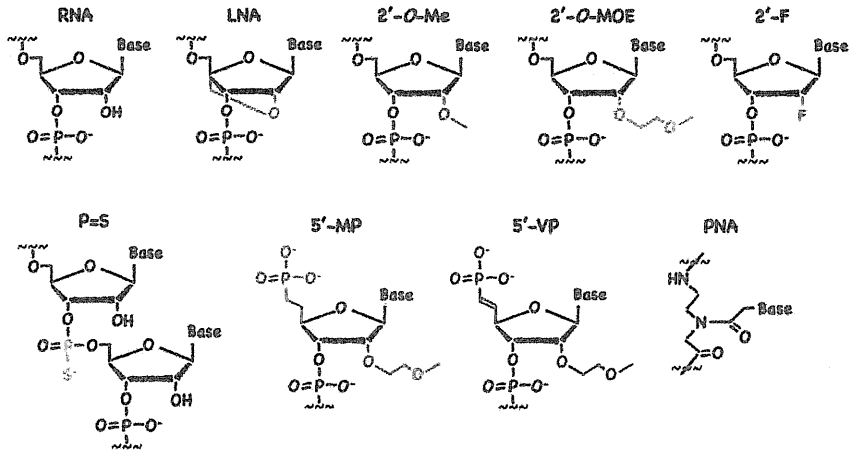

Figure 3. Chemical modifications for stability. Sugar, backbone and base modifications are illustrated. Shown are locked nucleic acid (LNA), phosphorothioate (P = S), 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP), 5′-(*E*)-vinyl-phosphonate (5′-VP) and peptide nucleic acid (PNA).

Table 3. Chemical modifications for permeability and specificity

Chemical modification	Tissue	Factor	Route	RNA	References
PEG, PBAVE and ligand	Liver	NAG	IV	ApoB and PPARA siRNA	(114)
Aptamer	PSMA-positive prostate cancer	Anti-PSMA aptamer	IT	PLK-1/BCL-2 siRNA	(65)
	HIV-infected T cells	Anti-gp120 aptamer	IV	tat/rev siRNA	(66)
Cholesterol	Colon adenocarcinoma	Cholesteryl oligo-D-arginine	IT	VEGF siRNA	(58)
	Liver	HDL/LDL	IV	ApoB siRNA	(59)
Antibody	HIV-infected T cells	Anti-HIV Envelope Fab	IV	gag siRNA	(68)
	Hepatocellular carcinoma	Anti-EGFR Fab	IV	Luciferase siRNA	(69)
Peptide	Liver	Octaarginine	IV	SR-B 1 siRNA	(67)
	Lymphoma	Penetratin	IV/IT	Anti-miR-155-PNA	(64)
Agonist	TLR9 + myeloid cells and B cells	Anti-TLR9 agonist	IV/IT	Stat3	(56)

NAG, *N*-acetylgalactosamine; TLR, toll-like receptor.

enhance the resistance against exonuclease or endonuclease activity (73,74) (Fig. 3). Currently, the most consequential modification is the PS inter-nucleotide linkages that have been developed in the history of anti-sense oligonucleotides and have contributed to remarkable stabilization of double-strand RNA as well as the single-strand oligonucleotide (75,76). However, the influence of chirality in the phosphorus atom on the stability and the activity of duplexes is not entirely understood. Therefore, further investigation of the thermodynamic features and physiological activity with regard to the assignment of the absolute configuration will be required for therapeutic applications.

As reported above, a number of chemical modifications have been produced, which have enhanced the potential of siRNA, miRNA, miRNA inhibitors and anti-sense oligonucleotides. However, it has been required that the optimization of the modifications need to be optimized, as their efficiency depends on the position and combination. In 2012, chemical modifications were optimized for single-stranded siRNAs (ss-siRNAs), and the change was an important advancement for the practical application of RNAi therapeutics. It was shown that ss-siRNA with a number of chemical modifications, such as 5′-phosphonate and 2′-MOE-modified 5′-terminal nucleotide, 2′-F and 2′-*O*-Me motifs with contiguous PS modifications and 2′-MOE-modified adenosine dinucleotide at the 3′ terminus and C16 modification, brought about significant and efficient target gene silencing *in vivo via* the Ago2-mediated RNAi pathway (74). Furthermore, chemically modified ss-siRNAs targeting mutant huntingtin mRNAs have been employed as a novel nucleic acid drug for therapeutic application for Huntington's disease (77). Although single-stranded RNAs (ssRNAs) have been shown to have extremely rapid degradation in serum and poor activities so far (78,79), they have advantages, such as the absence of risk of undesirable off-target effects by passenger strand and the potential of systemic delivery without complex lipid formulations that sometimes trigger the inflammatory toxicities (80). Hence, these stabilized ssRNAs are expected to place RNAi therapy in a prominent class of nucleic acid drugs.

DYSREGULATED MIRNA AS THERAPEUTIC TARGET IN CANCER TREATMENT

The alterations of miRNA expression profiling are significantly related with cancer initiation and progression. To identify dysregulated miRNAs in the physiological and pathological pathway of cancer malignancy is the first step for therapeutic applications. Generally, the widespread disruption of miRNAs is caused by at least three different mechanisms: the loss, amplification or mutation of a fragile cancer-related genomic region; the change of epigenetic control; and the abnormality of miRNA-processing steps. The genetic change has the potential to affect radically the abundance of miRNA, and it was reported that >50% of miRNAs locate on the fragile genomic region in cancer (81–83). For instance, a significant downregulation of miR-15 and miR-16, which is caused by deletion or mutation in chromosome 13q14.3, was observed in 70% of patients with chronic lymphocytic leukemia.

On the other hand, CpG-island hypermethylation and histone modification as good markers for functional miRNA have also been investigated by using 5-aza-2′-deoxycytidine and a histone decetylase inhibitor, such as 4-phenylbutyric acid or trichostatin A (84–86). For example, miR-124a that regulates the expression of cyclin D kinase 6 was located in three chromosome loci, 8p23.1, 8q12.3 and 20q13.33, and these regions were hypermethylated in 75% of patients with primary colorectal tumors (87). In addition to genetic and epigenetic validation, alterations of the protein machinery related to the biogenesis of miRNA might impair global

miRNA expression. Indeed, a copy number change of DICER1 and Ago2 is frequently observed in melanoma, breast and brain cancer (88). Especially, TAR RNA-binding protein 2 (TARBP2), in the DICER-containing complex, showed frameshift mutations and caused a destabilization of DICER1 protein, resulting in global downregulation of mature miRNA in colorectal and gastric tumors (89). According to one estimate, the widespread downregulation of the miRNA expression levels is prevalent in several cancer types (90,91). In contrast, a kind of multi-functional polyphenolic compound, resveratrol, which is present in red wine, induced widespread upregulation of miRNAs and inhibited tumor growth through the acceleration of the expression and activity of Ago2 (92). Thus, the observation and management of the total balance of miRNAs are important for cancer diagnosis and treatment.

INHIBITION OF MIRNA EXPRESSION AND FUNCTIONS

For the therapeutic applications of miRNA, the intracellular expression levels of miRNAs have to be artificially controlled. Although it is relatively easy to upregulate miRNAs, the strategy for the downregulation of miRNAs requires a refined miRNA inhibitor such as a chemically modified antisense oligonucleotide. As the inhibitor against endogenous miRNA, locked nucleic acid (LNA), which has a methylene bridge connecting 2′ and 4′ carbons, is one of the most widely used platforms. LNA nucleotide organizes the phosphate backbone in the *N*-type (C3′-endo) conformation, whereas, in general, the conformations of DNA or RNA duplexes are flexible between *N*-type and S-type (C2′-endo). This conformational change contributes to a more efficient stacking of the nucleobases and functional inhibition of target miRNAs (93). In therapeutic applications, LNA against the liver-expressed miR-122, which is a potential therapeutic target in the hepatitis C virus (HCV), accomplished the long-lasting reduction of mature miR-122 and suppression of HCV viremia (94,95). Furthermore, LNA against hypoxia inducible factor 1α, the primary transcription factor activated by hypoxia that allows glycolysis and angiogenesis to progress, provides significant lowering of the expression of HIF1-α and suppression of tumor growth. Clinical trials of these LNA against miR-122 (SPC3649) and HIF1-α (EZN-2968) have progressed to Phases I and II by Santaris Pharma.

In addition to this, as competitive inhibitors of miRNAs, the miRNA sponge (96), the tough decoy (TuD) RNA (97), antagomirs (98), peptide nucleic acids (PNAs) (99) and anti-miRNA oligonucleotides (AMOs) (100) have also been developed toward medical practice targeting onco-miRNA as well as LNA. Antagomirs composed of 2′-*O*-Me, PS and cholesterol modification were the first miRNA inhibitors that provided a significant reduction in mammals (98,101). However, antagomirs were excluded as clinical candidates because they were less effective than other miRNA inhibitors. PNAs are replaced its sugar-phosphate backbone to

Table 4. Programs of clinical/pre-clinical study in miRNA therapeutics

miRNA	Therapy	Disease	Phase	Company
miR-208/499	Inhibitor	Chronic heart failure	Pre-clinical	MiRagen
miR-15/195	Inhibitor	Post-MI remodeling	Pre-clinical	Therapeutics
miR-451	Inhibitor	Polycythemia vera	Pre-clinical	
miR-122	Inhibitor	HCV	Pre-clinical	
miR-21	Inhibitor	HCC, cancer, fibrosis	Pre-clinical	
miR-10b	Inhibitor	Glioblastoma	Pre-clinical	Regulus
miR-33a/b	Inhibitor	Atherosclerosis	Pre-clinical	Therapeutics
miR-155	Inhibitor	Immuno-inflammatory diseases	Pre-clinical	
miR-122	Inhibitor	HCV	Phase II	Santaris Pharma
miR-29	Mimic	Cardiac fibrosis	Pre-clinical	MiRagen Therapeutics
let-7	Mimic	Lung cancer	Pre-clinical	Mirna Therapeutics
miR-34a	Mimic	Solid tumors	Pre-clinical	
miR-16	Mimic	Cancer	Pre-clinical	
miR-34a	Mimic	Hepatocellular carcinoma	Pre-clinical	Regulus
miR-146a	Mimic	Autoimmunity, cancer	Pre-clinical	Therapeutics

From MiRagen Therapeutics (http://www.miragentherapeutics.com), Regulus Therapeutics (http://www.regulusrx.com), Santaris Pharma (http://www.santaris.com), Mirna Therapeutics (http://www.mirnatherapeutics.com).

N-(2-aminoethyl)glycine units, also have a potential to inhibit miRNA activities. Reports indicate that PNA-DNA chimeras have the potential to inhibit miRNA *in vitro* and *in vivo* (99). On the other hand, unlike chemically modified ASOs, a miRNA decoy can be stably integrated into the chromosomes and degrade miRNA targets. The stable suppression of miR-301a by a miRNA decoy was reported to have inhibited tumor growth by the upregulation of NF-κB-repressing factor in pancreatic cancer (102), and TuD-RNA against miR-122a showed a significant suppression of the HCV replication in liver hepatocytes (103).

PIPELINE OF MIRNA IN CANCER TREATMENT

In a recent study, onco-miRs or tumor-suppressive miRs that work as master regulators in cellular processes have been identified, and a number of pre-clinical trials have been conducted by firms such as MiRagen Therapeutics, Regulus Therapeutics, Santaris Pharma and Mirna Therapeutics (Table 4). For example, miR-34a, which is one of the best-studied tumor-suppressive miRNAs, was a therapeutic target in solid tumor treatment by Mirna Therapeutics and Regulus Therapeutics. miR-34a is commonly downregulated in human cancer, such as prostate, breast, lung, kidney, bladder, ovary and skin cancer (104–106), and was identified as a target of the tumor suppressor gene p53. The reduction of miR-34a by CpG methylation is observed in multiple types of cancer. The restoration of miR-34s has the potential to cause cell cycle arrest, senescence and apoptosis (107). Mirna Therapeutics has also been conducting pre-clinical trials with miR-16 and let-7 mimics, which are potent tumor-suppressive miRNAs (47,108,109). Furthermore, pre-clinical trials of miRNA inhibitors against miR-21 and miR-10b, which are targeted as onco-miRs in hepatocellular carcinoma and glioblastoma, are being conducted. In addition to these developments, a number of non-public candidates for miRNA therapy are being considered by Mirna Therapeutics; they include miR-Rx01, 02, 03, 06 and 07. Thus, miRNA therapeutics using miRNA mimics or inhibitors has been growing in pre-clinical studies and might appear in clinical trials over the next several years.

CONCLUSION

RNAi is one of the most versatile knockdown tools in recent biotechnology, and the potential of RNAi therapeutics using miRNA for cancer treatment has been rapidly expanding. In particular, unlike siRNAs as a tool that specifically impairs the function of a target gene, miRNAs work as key regulators that control target genes and establish balanced cellular organization. Indeed, the disruption of such a balance leads to the possibility of a tumor to become malignant (110,111). To utilize these discoveries of cellular biological basic research for clinical investigation, further innovations in the field of the delivery systemic and chemical modifying strategies are desired. Indeed, although chemically modified ASOs and ss-siRNAs are potentially promising nucleic acid drugs that can efficiently manage RNAi in animals, immeasurable synthesis costs and technical difficulties for bulk production remain. In addition, safer and more effective delivery systems, including a viral approach, are needed. However, the progression of RNAi technology over the past decade has been remarkable, and the hope is that ongoing investigations will result in the use of RNAi therapeutics as a prominent cancer treatment.

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Conflict of interest statement
None declared.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 1998;391:806–11.
2. Mccullough C, Pharmaceuticals A, Pharmaceuticals A, Davidson B. A crucial test. *Nat Med* 2005;11:243–4.
3. McFarland TJ, Zhang Y, Appukuttan B, Stout JT. Gene therapy for proliferative ocular diseases. *Expert Opin Biol Ther* 2004;4:1053–8.
4. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat Med* 2005;11:50–5.
5. Raoul C, Barker SD, Aebischer P. Viral-based modelling and correction of neurodegenerative diseases by RNA interference. *Gene Ther* 2006;13:487–95.
6. Davidson BL, McCray PB. Current prospects for RNA interference-based therapies. *Nat Rev Genet* 2011;12:329–40.

7. Aigner A. Applications of RNA interference: current state and prospects for siRNA-based strategies *in vivo*. *Appl Microbiol Biotechnol* 2007;76:9–21.
8. Takeshita F, Ochiya T. Therapeutic potential of RNA interference against cancer. *Cancer Sci* 2006;97:689–96.
9. Soifer HS, Rossi JJ, Sætrom P. MicroRNAs in disease and potential therapeutic applications. *Mol Ther* 2007;15:2070–9.
10. Harfe BD. MicroRNAs in vertebrate development. *Curr Opin Genet Dev* 2005;15:410–5.
11. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
12. Tomari Y, Zamore PD. Perspective: machines for RNAi. *Genes Dev* 2005;19:517–29.
13. Beezhold KJ, Castranova V, Chen F. Microprocessor of microRNAs: regulation and potential for therapeutic intervention. *Mol Cancer* 2010;9:134.
14. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 2009;11:228–34.
15. Meltzer PS. Small RNAs with big impacts. *Nature* 2005;435:0–1.
16. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. *J Clin Oncol* 2009;27:5848–56.
17. Burchard J, Jackson AL, Malkov V, et al. MicroRNA-like off-target transcript regulation by siRNAs is species specific. *RNA* 2009;15:308–15.
18. de Veer MJ, Sledz CA, Williams BRG. Detection of foreign RNA: implications for RNAi. *Immunol Cell Biol* 2005;83:224–8.
19. Jackson AL, Bartz SR, Schelter J, et al. off-target gene regulation by RNAi. *Nat Biotechnol* 2003;21:635–8.
20. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–8.
21. Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K. Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci* 2001;114:4557–65.
22. McCaffrey AP, Meuse L, Pham T-TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. *Nature* 2002;418:38–9.
23. Rybak LP, Mukherjea D, Jajoo S, Kaur T, Ramkumar V. siRNA-mediated knock-down of NOX3: therapy for hearing loss?. *Cell Mol Life Sci* 2012;69:2429–34.
24. Zheng D, Giljohann DA, Chen DL, et al. Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation. *Proc Natl Acad Sci USA* 2012;109:11975–80.
25. Davis ME, Zuckerman JE, Choi CHJ, et al. Evidence of RNAi in humans from systemically administered siRNA *via* targeted nanoparticles. *Nature* 2010;464:1067–70.
26. Judge AD, Robbins M, Levi J, et al. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* 2009;119:661–73.
27. Zimmermann TS, Lee ACH, Akinc A, et al. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;441:111–4.
28. Chen Y, Chen H, Hoffmann A, et al. Adenovirus-mediated small-interference RNA for *in vivo* silencing of angiotensin AT1a receptors in mouse brain. *Hypertension* 2006;47:230–7.
29. Zhu S, Pan W, Song X, et al. The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-α. *Nat Med* 2012;18:1077–86.
30. Pihlmann M, Askou AL, Aagaard L, et al. Adeno-associated virus-delivered polycistronic microRNA-clusters for knockdown of vascular endothelial growth factor *in vivo*. *J Gene Med* 2012;14:328–38.
31. Rodriguez-Lebron E, Denovan-Wright EM, Nash K, Lewin AS, Mandel RJ. Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol Ther* 2005;12:618–33.
32. Kasinski AL, Slack FJ. miRNA-34 prevents cancer initiation and progression in a therapeutically resistant K-ras and p53-induced mouse model of lung adenocarcinoma. *Cancer Res* 2012;72:5576–87.
33. Raoul C, Abbas-Terki T, Bensadoun J-C, et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med* 2005;11:423–8.
34. Anesti A-M, Peeters PJ, Royaux I, Coffin RS. Efficient delivery of RNA interference to peripheral neurons *in vivo* using herpes simplex virus. *Nucleic Acids Res* 2008;36:e86.
35. Saydam O, Glauser DL, Heid I, et al. Herpes simplex virus 1 amplicon vector-mediated siRNA targeting epidermal growth factor receptor inhibits growth of human glioma cells *in vivo*. *Mol Ther* 2005;12:803–12.
36. Pfeifer A, Verma IM. Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet* 2001;2:177–211.
37. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* 2003;4:346–58.
38. Tomanin R, Scarpa M. Why do we need new gene therapy viral vectors? Characteristics, limitations and future perspectives of viral vector transduction. *Curr Gene Ther* 2004;4:357–72.
39. Sørensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic sirnas in adult mice. *J Mol Biol* 2003;327:761–6.
40. Verma UN, Surabhi RM, Schmaltieg A, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against β-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. *Clin Cancer Res* 2003;9:1291–300.
41. Yano J, Hirabayashi K, Nakagawa S-I, et al. Antitumor activity of small interfering RNA/cationic liposome complex in mouse models of cancer. *Clin Cancer Res* 2004;10:7721–6.
42. Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. *Gene Ther* 2005;12:461–6.
43. Grzelinski M, Urban-Klein B, Martens T, et al. RNA interference-mediated gene silencing of pleiotrophin through polyethylenimine-complexed small interfering RNAs *in vivo* exerts antitumoral effects in glioblastoma xenografts. *Hum Gene Ther* 2006;17:751–66.
44. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. Growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci USA* 2007;104:2–7.
45. Minakuchi Y, Takeshita F, Kosaka N, et al. Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*. *Nucleic Acids Res* 2004;32:e109.
46. Takeshita F, Minakuchi Y, Nagahara S, et al. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen *in vivo*. *Proc Natl Acad Sci USA* 2005;102:12177–82.
47. Takeshita F, Patrawala L, Osaki M, et al. Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors *via* downregulation of multiple cell-cycle genes. *Mol Ther* 2010;18:181–7.
48. Al-jamal KT, Gherardini L, Bardi G, Nunes A, Guo C, Bussy C. Functional motor recovery from brain ischemic insult by carbon nanotube-mediated siRNA silencing. *Proc Natl Acad Sci USA* 2011;108:10952–7.
49. Geisbert TW, Hensley LE, Kagan E, et al. Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J Infect Dis* 2006;193:1650–7.
50. Trang P, Wiggins JF, Daige CL, et al. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther* 2011;19:1116–22.
51. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res* 2005;65:8984–92.
52. Aleku M, Schulz P, Keil O, et al. Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. *Cancer Res* 2008;68:9788–98.
53. Chen Y, Zhu X, Zhang X, Liu B, Huang L. Nanoparticles modified with tumor-targeting scFv deliver siRNA and miRNA for cancer therapy. *Mol Ther* 2010;18:1650–6.
54. Gomes-da-Silva LC, Fonseca NA, Moura V, Pedroso de Lima MC, Simões S, Moreira JN. Lipid-based nanoparticles for siRNA delivery in cancer therapy: paradigms and challenges. *Chem Res* 2012;45:1163–71.
55. Dassie JP, Liu X-Y, Thomas GS, et al. Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. *Nat Biotechnol* 2009;27:839–49.
56. Kortylewski M, Swiderski P, Herrmann A, et al. *In vivo* delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat Biotechnol* 2009;27:925–32.

57. Peer D, Park EJ, Morishita Y, Carman CV, Shimaoka M. Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* 2008;319:627–30.
58. Kim WJ, Christensen LV, Jo S, et al. Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol Ther* 2006;14:343–50.
59. Wolfrum C, Shi S, Jayaprakash KN, et al. Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. *Nat Biotechnol* 2007;25:1149–57.
60. Wu Y, Navarro F, Lal A, et al. Durable protection from Herpes Simplex Virus-2 transmission following intravaginal application of siRNAs targeting both a viral and host gene. *Cell Host Microbe* 2009;5:84–94.
61. Takanashi M, Oikawa K, Sudo K, et al. Therapeutic silencing of an endogenous gene by siRNA cream in an arthritis model mouse. *Gene Ther* 2009;16:982–9.
62. Chen Q, Butler D, Querbes W, et al. Lipophilic siRNAs mediate efficient gene silencing in oligodendrocytes with direct CNS delivery. *J Controlled Release* 2010;144:227–32.
63. Wong SC, Klein JJ, Hamilton HL, et al. Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs *in vivo*. *Nucleic Acid Ther* 2012;22:380–90.
64. Babar IA, Cheng CJ, Booth CJ, et al. Nanoparticle-based therapy in an *in vivo* microRNA-155 (miR-155)-dependent mouse model of lymphoma. *Proc Natl Acad Sci USA* 2012;109:E1695–704.
65. McNamara JO, Andrechek ER, Wang Y, et al. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol* 2006;24:1005–15.
66. Neff CP, Zhou J, Remling L, et al. An Aptamer-siRNA chimera suppresses HIV-1 viral loads and protects from helper CD4+ T cell decline in humanized mice. *Sci Trans Med* 2011;3:1–20.
67. Hayashi Y, Yamauchi J, Khalil IA, Kajimoto K, Akita H, Harashima H. Cell penetrating peptide-mediated systemic siRNA delivery to the liver. *Int J Pharm* 2011;419:308–13.
68. Song E, Zhu P, Lee S-K, et al. Antibody mediated *in vivo* delivery of small interfering RNAs *via* cell-surface receptors. *Nat Biotechnol* 2005;23:709–17.
69. Gao J, Yu Y, Zhang Y, et al. EGFR-specific PEGylated immunoliposomes for active siRNA delivery in hepatocellular carcinoma. *Biomaterials* 2012;33:270–82.
70. Pardridge WM. shRNA and siRNA delivery to the brain. *Adv Drug Del Rev* 2007;59:141–52.
71. Citro G, Perrotti D, Cucco C, et al. Inhibition of leukemia cell proliferation by receptor-mediated uptake of c-myb antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* 1992;89:7031–5.
72. Vornlocher H-P. Antibody-directed cell-type-specific delivery of siRNA. *Trends Mol Med* 2006;12:1–3.
73. Bumcrot D, Manoharan M, Koteliansky V, Sah DWY. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol* 2006;2:711–9.
74. Lima Walt F, Prakash Thazha P, Murray Heather M, et al. Single-stranded sirnas activate RNAi in animals. *Cell* 2012;150:883–94.
75. Choung S, Kim YJ, Kim S, Park H-O, Choi Y-C. Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem Biophys Res Commun* 2006;342:919–27.
76. Kole R, Krainer AR, Altman S. RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat Rev Drug Discov* 2012;11:125–40.
77. Yu D, Pendergraff H, Liu J, et al. Single-stranded RNAs use RNAi to potently and allele-selectively inhibit mutant huntingtin expression. *Cell* 2012;150:895–908.
78. Braasch DA, Jensen S, Liu Y, et al. RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry* 2003;42:7967–75.
79. Wu H, Macleod AR, Lima WF, Crooke ST. Identification and partial purification of human double strand. *J Bio Chem* 1998;273:2532–42.
80. Bennett CF, Swayze EE. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu Rev Pharmacol Toxicol* 2010;50:259–93.
81. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004;101:2999–3004.
82. Rossi S, Sevignani C, Nnadi SC, Siracusa LD, Calin GA. Cancer-associated genomic regions (CAGRs) and noncoding RNAs: bioinformatics and therapeutic implications. *Mamm Genome* 2008;19:526–40.
83. Sevignani C, Calin GA, Nnadi SC, et al. MicroRNA genes are frequently located near mouse cancer susceptibility loci. *Proc Natl Acad Sci USA* 2007;104:8017–22.
84. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 2006;9:435–43.
85. Chen X, Wang J, Shen H, et al. Epigenetics, microRNAs, and carcinogenesis: functional role of microRNA-137 in uveal melanoma. *Invest Ophthalmol Vis Sci* 2011;52:1193–9.
86. Tsai K-W, Wu C-W, Hu L-Y, et al. Epigenetic regulation of miR-34b and miR-129 expression in gastric cancer. *Int J Cancer* 2011;129:2600–10.
87. Lujambio A, Ropero S, Ballestar E, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* 2007;67:1424–9.
88. Zhang L, Huang J, Yang N, et al. microRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci USA* 2006;103:9136–41.
89. Melo SA, Ropero S, Moutinho C, et al. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet* 2009;41:365–70.
90. Ozen M, Creighton CJ, Ozdemir M, Ittmann M. Widespread deregulation of microRNA expression in human prostate cancer. *Oncogene* 2008;27:1788–93.
91. Marton S, Garcia MR, Robello C, et al. Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. *Leukemia* 2008;22:330–8.
92. Hagiwara K, Kosaka N, Yoshioka Y, Takahashi R-U, Takeshita F, Ochiya T. Stilbene derivatives promote Ago2-dependent tumour-suppressive microRNA activity. *Sci Reports* 2012;2:314.
93. Petersen M, Nielsen CB, Nielsen KE, et al. The conformations of locked nucleic acids (LNA). *J Mol Recognit* 2000;13:44–53.
94. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-human primates. *Nature* 2008;452:896–9.
95. Lanford RE, Hildebrandt-Eriksen ES, Petri A, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010;327:198–201.
96. Kluiver J, Slezak-Prochazka I, Smigielska-Czepiel K, Halsema N, Kroesen B-J, van den Berg A. Generation of miRNA sponge constructs. *Methods* 2012;58:113–7.
97. Haraguchi T, Ozaki Y, Iba H. Vectors expressing efficient RNA decoys achieve the long-term suppression of specific microRNA activity in mammalian cells. *Nucleic Acids Res* 2009;37:e43.
98. Krützfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature* 2005;438:685–9.
99. Fabbri E, Brognara E, Borgatti M, et al. miRNA therapeutics: delivery and biological activity of peptide nucleic acids targeting miRNAs. *Epigenomics* 2011;3:733–45.
100. Weiler J, Hunziker J, Hall J. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? *Gene Ther* 2006;13:496–502.
101. Broderick JA, Zamore PD. MicroRNA therapeutics. *Gene Ther* 2011;18:1104–10.
102. Lu Z, Li Y, Takwi A, et al. miR-301a as an NF-κB activator in pancreatic cancer cells. *EMBO J.* 2011;30:57–67.
103. Sakurai F, Furukawa N, Higuchi M, et al. Suppression of hepatitis C virus replicon by adenovirus vector-mediated expression of tough decoy RNA against miR-122a. *Virus Res* 2012;165:214–8.
104. Ji Q, Hao X, Zhang M, et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PloS One* 2009;4:e6816.
105. Wiggins JF, Ruffino L, Kelnar K, et al. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 2010;70:5923–30.
106. Hiroshi Tazawa NT, Izumiya M, Nakagama H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci USA* 2007;104:15472.

107. Lodygin D, Tarasov V, Epanchintsev A, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle* 2008;7:2591–600.
108. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 2002;99:15524–9.
109. Esquela-Kerscher A, Trang P, Wiggins JF, et al. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 2008;7:759–64.
110. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. *FEBS Lett* 2011;585:2087–99.
111. Xu D, Takeshita F, Hino Y, et al. miR-22 represses cancer progression by inducing cellular senescence. *J Cell Biol* 2011;193: 409–24.
112. Osaki M, Takeshita F, Sugimoto Y, et al. MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression. *Mol Ther* 2011;19:1123–30.
113. Morrissey DV, Blanchard K, Shaw L, et al. Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* 2005;41:1349–56.
114. Rozema DB, Lewis DL, Wakefield DH, et al. Dynamic polyconjugates for targeted *in vivo* delivery of siRNA to hepatocytes. *Proc Natl Acad Sci USA* 2007;104:12982–7.

Therapeutic Effects of MicroRNA-582-5p and -3p on the Inhibition of Bladder Cancer Progression

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Many reports have indicated that the abnormal expression of microRNAs (miRNAs) is associated with the progression of disease and have identified miRNAs as attractive targets for therapeutic intervention. However, the bifunctional mechanisms of miRNA guide and passenger strands in RNA interference (RNAi) therapy have not yet been clarified. Here, we show that miRNA (miR)-582-5p and -3p, which are strongly decreased in high-grade bladder cancer clinical samples, regulate tumor progression *in vitro* and *in vivo*. Significantly, the overexpression of miR-582-5p or -3p reduced the proliferation and invasion of UM-UC-3 human bladder cancer cells. Furthermore, transurethral injections of synthetic miR-582 molecule suppressed tumor growth and metastasis in an animal model of bladder cancer. Most interestingly, our study revealed that both strands of miR-582-5p and -3p suppressed the expression of the same set of target genes such as *protein geranylgeranyltransferase type I beta subunit (PGGT1B)*, *leucine-rich repeat kinase 2 (LRRK2)* and *DIX domain containing 1 (DIXDC1)*. Knockdown of these genes using small interfering RNA (siRNA) resulted in the inhibition of cell growth and invasiveness of UM-UC-3. These findings uncover the unique regulatory pathway involving tumor suppression by both strands of a single miRNA that is a potential therapeutic target in the treatment of invasive bladder cancer.

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INTRODUCTION

RNA interference (RNAi), a natural cellular process that regulates gene expression, is the most significant recent contribution to the field of cell biology.[1] Recently, RNAi-based therapies, which are harnessed to control the expression of pathogenic proteins, have been demonstrated in humans and have provided alternative powerful approaches to the traditional small molecule therapies. There has been an increase in the development of RNAi therapies for accessible tissues, such as the skin, retina, liver, and airways, due to their ability to be efficiently and safely delivered without unwanted side effects.[2–4]

Urinary bladder cancer remains one of the most costly cancers with regard to treatment and the monitoring of cytological changes, such as surveillance cystoscopy and periodic imaging.[5] However, despite the existence of appropriate therapies, patients are continually under the threat of ongoing recurrence and progression to muscle.[5,6] Therefore, the development of new treatment strategies to reduce the risk of recurrence and progression based on novel molecular networks is strongly desired. The facts described above provide an insight into an innovative approach that harnesses the power of the RNAi pathway; *i.e.*, the bladder maximizes the effect of RNAi therapy because of its accessibility and closed environment. Although some previous studies showed that the intravesical injection of small interfering RNA (siRNA) has potential as a treatment,[7,8] whether an intravesical strategy can overcome the progression to muscle and metastasis in invasive bladder cancer remains unclear.

In cancer research, the copy number variation of DNA was a focus during the 1990s and the beginning of the 2000s as a result of the development of technologies such as array-based comparative genomic hybridization and microsatellite analysis,[9–11] because copy number gains or losses were believed to be specific markers for functional protein-coding genes. However, if protein-coding genes were not located in these aberrant regions, disease candidate regions were excluded from the functional analysis. On the other hand, recent studies found that microRNAs (miRNAs), which are key post-transcriptional regulators, are the main candidates for cancer-predisposing genes and that approximately half of the miRNAs are located at chromosomal regions that are genetically altered in cancers.[12–14] Indeed, since their discovery, a widespread dysregulation of miRNAs caused by copy number variation is commonly observed in human cancers and has been shown to be involved in diverse physiological/pathological processes.[15–19] In this study, we hypothesized that a novel tumor-suppressive miRNA or onco-miR can be identified more efficiently and more expeditiously by utilizing a vast amount of previously generated information about aberrant chromosomal regions related to bladder cancer.

Cytogenetic studies of bladder cancer have revealed a number of genetic aberrations.[10] For discrimination of miRNAs which denote the abnormal expression in poorly prognostic patients

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on bladder cancer, we focused on the copy number variation in UM-UC-3 cells which is one of the most invasive bladder cancer cell lines. It was reported that a number of genomic regions were deficient or amplified in that cell line.[20] Furthermore, in those genomic regions, 3p11-12, 4q33-34, and 5q12-13 were selected as analysis sets because the correlations between the copy number loss and bladder cancer progression in clinical samples had been reported by at least two groups.[11,21–24] Although these regions include some miRNA genes, such as miR-1305, miR-1324, miR-578, miR-582-5p, and miR-582-3p, the modulation of the miRNA expression profiles and the exact functional mechanism are not thoroughly understood. In the present study, we report that miR-582-5p and miR-582-3p are concurrently downregulated in invasive bladder cancer, which is correlated with a copy number loss of chromosome 5q12. These significant reductions of miR-582-5p and -3p were also observed in clinical samples and strongly correlated with tumor grade. Furthermore, the restorations of miR-582-5p or -3p strongly inhibit cell proliferation and invasion. In the lung metastasis mouse model of human bladder cancer, involving orthotopic transplantation, the injection of the miR-582/cationic liposome complex prevented tumor growth and lung metastasis. This demonstration is the first, to our knowledge, to show inhibition of tumor metastasis in an orthotopic model by intravesical miRNA injection.

Two mature miRNAs can be generated from the same stem-loop pre-miRNA.[25] These 5p and 3p miRNAs, although excised from a single primary transcript, have different sequences and therefore target different mRNAs. Despite nearly a decade of studies on miRNA, the effect of strand-specific mature miRNAs has not yet been fully understood. In the present study, we also provide evidence that the expression of *protein geranylgeranyltransferase type I beta subunit* (*PGGT1B*), *leucine-rich repeat kinase 2* (*LRRK2*), and *DIX domain containing 1* (*DIXDC1*), which are involved in the cell growth and the invasiveness, is bifunctionally regulated by the effects of both strands of miR-582-5p and -3p. These findings suggest that a novel pathway involving miR-582 and cancer-related genes has the potential to be a critical target for the therapeutic treatment of invasive bladder cancer.

RESULTS

Downregulation of miR-582-5p and -3p in bladder cancer cell lines

To identify miRNAs downregulated in bladder cancer cell lines, we performed quantitative reverse transcription-PCR (qRT-PCR) for miR-1305, miR-1324, miR-578, and miR-582-5p and -3p, which are located in aberrant genomic regions correlated with tumor progression (**Supplementary Tables S1 and S2**). The results showed no downregulation of miR-1305 in bladder cancer cells (**Supplementary Figure S1a**), and the expression of miR-1324 and miR-578 was not detected in either bladder cancer cell lines or normal bladder tissue RNA, but we found that miR-582-5p and -3p are strongly downregulated in invasive bladder cancer cell lines (UM-UC-3, 5637, MBT2, J82, TCCSUP) (**Figure 1a**). Furthermore, the copy number of chromosome 5q12, in which the miR-582 gene is located, was lower in UM-UC-3 cells than in HT1376 cells (**Figure 1b**). HT1376 cells were reported to not have a loss in 5q12.[24] However, the degree of downregulation of miR-582 expression in

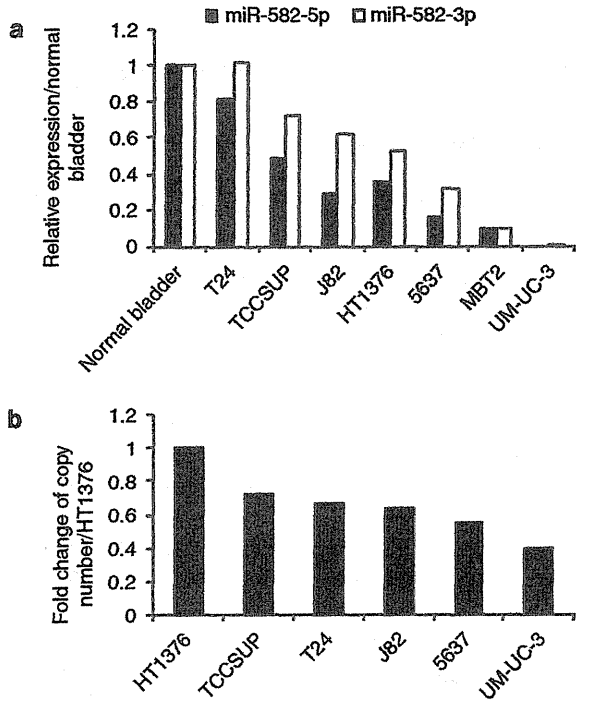

Figure 1 Expression level of miR-582-5p and -3p in human bladder cancer cell lines. (**a**) Expression level of miR-582-5p and -3p in human bladder cancer cell lines and a mouse bladder cancer cell line (MBT2). The relative expression of miR-582 for each of the cell lines was calculated compared with the level in normal bladder tissue RNA. (**b**) Copy number change of the miR-582 loci on chromosome 5q12. The HT1376 cell line was used as the control for this experiment.

UM-UC-3 cells seems too strong compared with the reduction of the copy number. This means that a remarkable reduction of miR-582-5p and -3p expression might be invoked by a combination of copy number loss and other factors. To identify whether the expression of these mature miRNAs are reduced in a transcriptional or biogenic process, we assessed the expression levels of pri-miR-582 and *phosphodiesterase 4D* (*PDE4D*) because miR-582 is located in the intronic region of *PDE4D*. The expression levels of pri-miR-582 and *PDE4D* are strongly reduced in UM-UC-3 and 5637 cells (**Supplementary Figure S1b,c**). These results indicate that the expression of miR-582-5p and -3p is reduced by the combination of copy number loss and transcriptional attenuation.

Downregulation of miR-582-5p and -3p in clinical samples

We examined the expression levels of miR-582-5p and -3p in laser capture-microdissected bladder cancer tissue regions (n = 53) and matched adjacent normal regions (n = 31) derived from 28 patients (**Supplementary Table S3**). Relative to normal regions, the tumor regions showed markedly lower miR-582-5p and -3p expression levels (**Figure 2a**). In particular, we found that these levels tend to decrease more remarkably in invasive tumors (tumor stage ≥pT1) than in superficial tumors (tumor stage pTis and pTa) (**Figure 2b**) (miR-582-5p: superficial tumor P = 0.006, invasive tumor P = 0.003; miR-582-3p: superficial tumor P = 0.035, invasive tumor P < 0.001). Furthermore, a strong correlation was

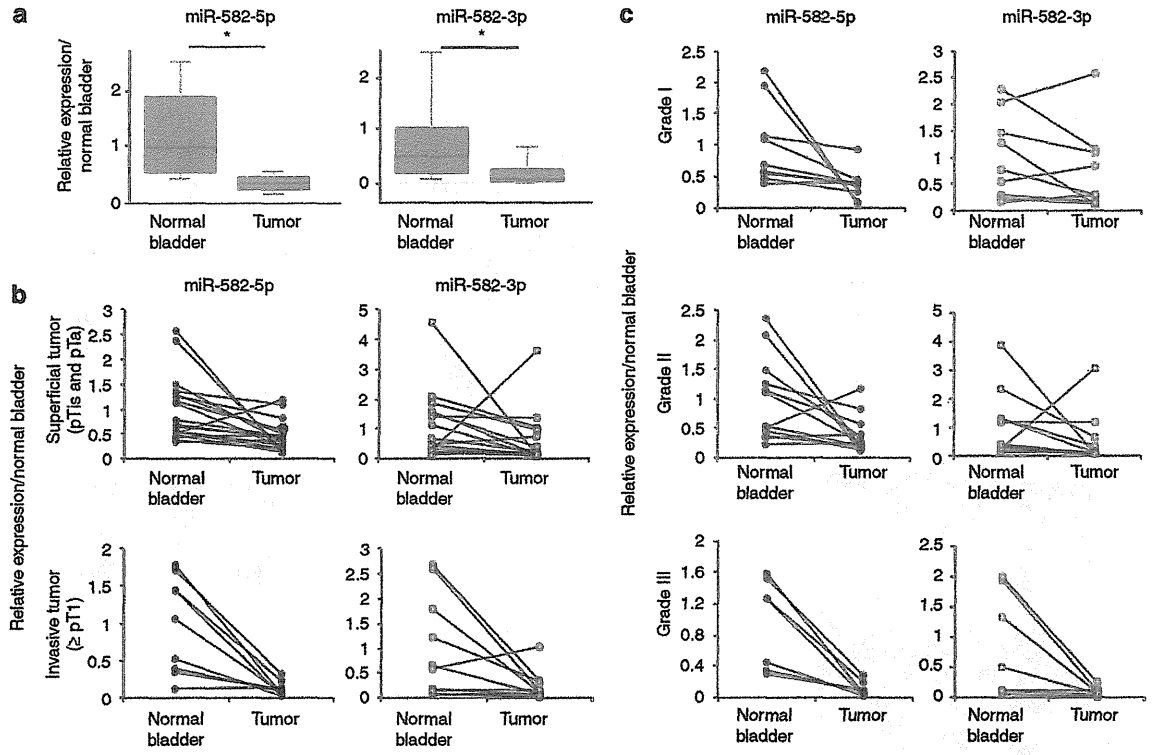

Figure 2 Expression level of miR-582-5p and -3p in bladder cancer tissue samples. (**a**) Expression of miR-582-5p and -3p in clinical samples, which show the downregulation in the tumor region compared with the adjacent normal tissues (tumor: $n = 53$, normal bladder: $n = 31$). The boxes and whiskers range from 25% to 75% and from 10% to 90%, respectively. *$P < 0.01$ versus normal bladder. (**b**) Comparison with the tumor stages in carcinoma cells paired with normal bladder tissue (superficial tumor: $n = 18$, invasive tumor: $n = 10$). (**c**) Comparison with the tumor grades in carcinoma cells paired with normal bladder tissue. All values for the miRNA expression levels were normalized to hsa-miR-103 as an internal control (Grade I: $n = 9$, Grade II: $n = 12$, Grade III: $n = 7$).

Table 1 Downregulation in clinical samples (fold change >2.5)

	Superficial (pTis, pTa)	Invasive (≥pT1)	Grade I	Grade II	Grade III
miR-582-5p (%)	7/18 (38.9)	9/10 (90)	3/9 (33.3)	6/12 (50)	7/7 (100)
miR-582-3p (%)	9/18 (50)	7/10 (70)	2/9 (22.2)	8/12 (66.7)	6/7 (85.7)

also observed between tumor grade and the downregulation of miR-582-5p and -3p (Figure 2c) (miR-582-5p: Grade I $P = 0.042$, Grade II $P = 0.028$, Grade III $P = 0.006$; miR-582-3p: Grade I $P = 0.206$, Grade II $P = 0.270$, Grade III $P = 0.046$). In addition to this, the ratio of samples showing strong downregulation greater than a 2.5-fold change increased as the tumor stage and grade advanced (Table 1). These data suggested that miR-582-5p and -3p levels vary with the malignancy potential and are potential therapeutic markers in the treatment of invasive bladder cancer.

Effect of miR-582-5p and -3p on cell proliferation and invasive ability

To investigate the functional role of miR-582-5p and -3p in bladder cancer, we performed a cell proliferation assay and a cell invasion assay. UM-UC-3, J82, and TCCSUP cells were transfected transiently with miR-582, miR-582-5p, miR-582-3p, and negative control (NC) siRNA. miR-582 is synthesized to mimic endogenous mature miR-582, whereas miR-582-5p and -3p are designed to match perfectly and complementarily to each strand, similar to siRNA. Three days after transfection, the cell viability was decreased from 30% to 80% in the miR-582-, miR-582-5p-, or miR-582-3p-transfected cells compared with the NC cells (Figure 3a). The matrigel invasion assay showed that the cell invasion ability was significantly (>70%) decreased in miR-582-, miR-582-5p-, or miR-582-3p-transfected UM-UC-3, J82, and TCCSUP cells (Figure 3b,c). These results suggest that the cell proliferation and invasive abilities of bladder cancer cells were severely affected only by either strand of miR-582-5p or miR-582-3p. In addition, a reduction of invasiveness was observed in 5637 cells, although T24 cells did not show a significant response to miR-582 in the cell proliferation and cell invasion assays (**Supplementary Figure S2a,b**). The expression and functional data suggest that the tumor-suppressive role of miR-582 is correlated with the reduction of its expression level in bladder cancer cells.

To ensure that the functions of miR-582-5p and -3p are not at a supraphysiological level, we repeated these experiments using a stable miRNA vector that synthesizes mature miRNAs by biological processing. UM-UC-3 cells were transduced with a lentiviral construct stably expressing precursor miR-582, and the expression level was examined (**Supplementary Figure S3a**). Furthermore, the functional analysis revealed that UM-UC-3 cells stably expressing miR-582 (UM-UC-3-miR-582) show a significant repression of cell proliferation and invasion (**Supplementary Figure S3b,c**).

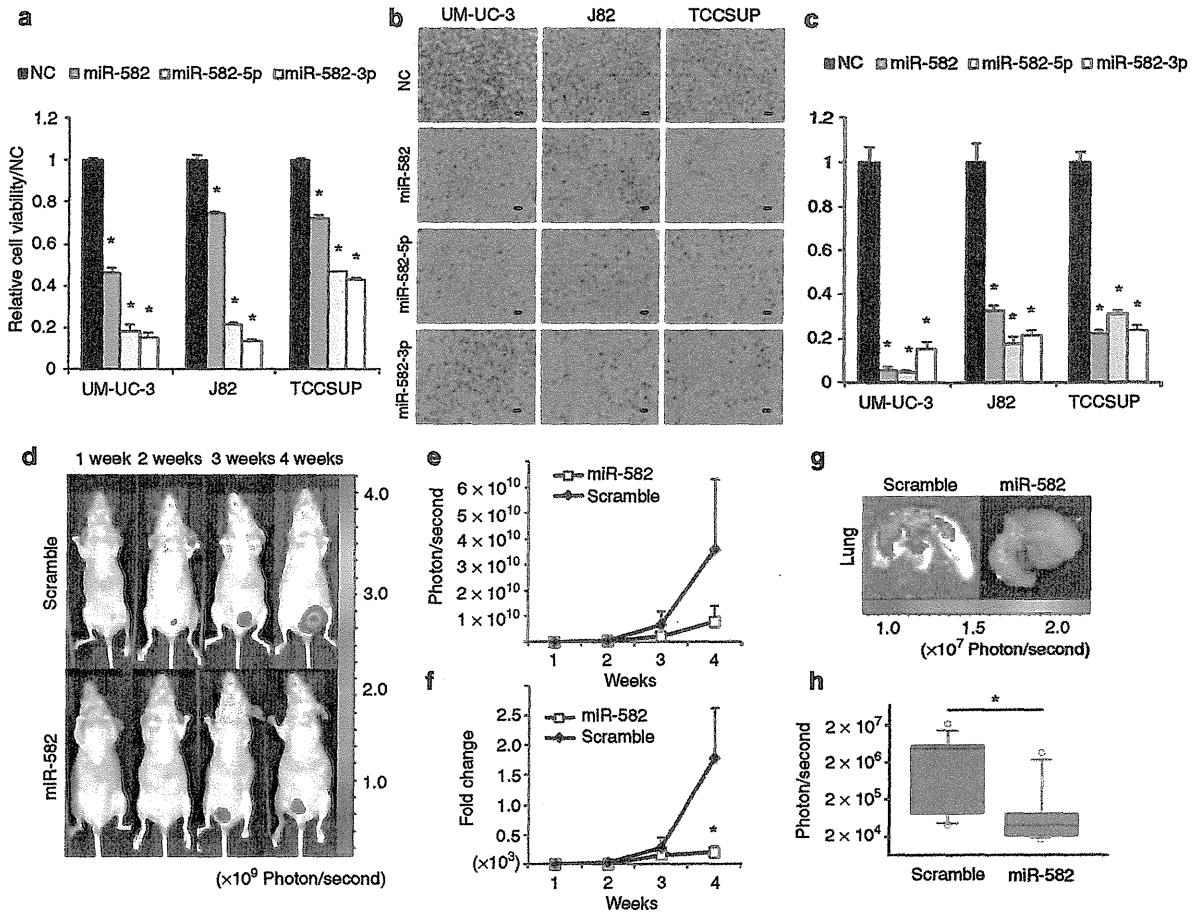

Figure 3 Function of miR-582 in human bladder cancer *in vitro* and *in vivo*. (**a**) Effect of miR-582 on cell proliferation of UM-UC-3, J82 and TCCSUP. The proliferation values were normalized to the values from cells treated with NC ($n = 6$). *$P < 0.01$ versus NC. (**b**,**c**) Effect on cell invasion. The invasive values were normalized to the values from cells treated with NC ($n = 4$). *$P < 0.01$ versus NC (scale bar: 100 μm). (**d**) Inhibition of tumor growth by miR-582 treatment in a murine orthotopic xenograft model of human bladder cancer. The mice were injected with 5×10^6 UM-UC-3-luc cells into the bladder on day 0. miR-582 and miR-582-scramble (10 μg) with cationic liposomes in a 70 μl volume were injected into the bladder on days 4, 6, 8, 11, 13, and 15 after tumor injection. (**e**,**f**) The growth curves of the orthotopically transplanted UM-UC-3-luc cells were measured by IVIS. Filled diamonds: treatment with miR-582-scramble; Open squares: treatment with miR-582 (scramble: $n = 8$, miR-582: $n = 10$). (**g**) Inhibition of lung metastasis by miR-582 treatment in an orthotopic bladder cancer mouse model. The images were obtained by IVIS on day 26 after transplantation. (**h**) Quantitation of bioluminescence emitted from the whole lungs of mice on day 26. The boxes and whiskers range from 25% to 75% and from 10% to 90%, respectively. *$P < 0.05$ versus scramble. NC, negative control.

By contrast, as shown in a loss-of-function assay using the locked nucleic acid–modified anti-miRNA oligonuleotides against each strand of miR-582-5p and -3p, cell proliferation and invasion are promoted in UM-UC-3-miR-582 cells (**Supplementary Figure S3d,e**). These results indicate that the presence of either miR-582-5p or -3p was sufficient to suppress tumor growth and invasiveness.

Inhibition of tumor growth and metastasis *in vivo* with miR-582 treatment

To assess the therapeutic potential of miR-582, we used an orthotopic bladder cancer mouse model featuring UM-UC-3-luc cells, which have the capacity to form tumors in the bladder and spread to the lungs of mice. The cationic liposome, LIC-101 (Nippon Shinyaku, Tsukuba, Japan), was used to deliver miR-582 into the tumor cells in the mouse bladder. The miR-582/LIC-101 or miR-582-scramble/LIC-101 complexes were injected transurethrally into the bladder at 5, 7, 9, 11, 13, and 15 days after tumor transplantation (Figure 3d). These results demonstrated that miR-582 successfully suppressed tumor growth *in vivo*; in particular, we found that miR-582 provided a greater than fourfold reduction of the tumor growth in the fourth week (Figure 3e). Furthermore, there was a significant difference in fold changes relative to the photon counts in the first week (miR-582 = 2,253.5 ± 851.7, scramble = 187.1 ± 108.9, $P = 0.042$) (Figure 3f). At the end of the experiment, 4 weeks after transplantation, the mice treated with miR-582-scramble showed a more frequent presence of tumors in the lung (5 of 8; 63%) than in the miR-582 group (1 of 10; 10%) (Figure 3g). Moreover, there was a significant difference between the two groups regarding the luminescence of the lung (Figure 3h). These results indicated that the intravesical administration of the miR-582/LIC101 complex could be a novel therapeutic strategy for the inhibition of tumor progression and metastasis in bladder cancer.

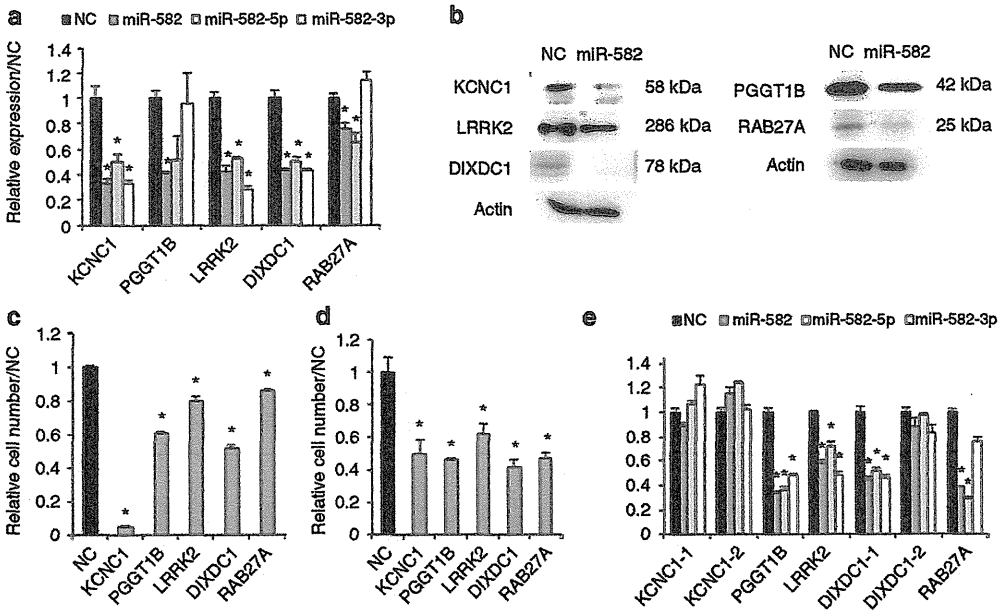

Figure 4 Identification of *PGGT1B, LRRK2, DIXDC1, RAB27A,* and *KCNC1* as miR-582-5p and -3p target genes. Target validations of *KCNC1, PGGT1B, LRRK2, RAB27A,* and *DIXDC1* were confirmed by qRT-PCR, western blot analysis, and cell proliferation, cell invasion, and luciferase reporter assays. (**a**) qRT-PCR was performed in UM-UC-3 cells at 24 hours after transfection with miR-582. The relative expressions of the target mRNAs were calculated compared with the level in NC ($n = 3$). (**b**) Western blot analysis was performed in UM-UC-3 cells at 48 hours after transfection. Actin was used as the loading control. (**c**) Effect of target siRNAs on the proliferation of UM-UC-3. The proliferation values were normalized to values from cells treated with NC ($n = 6$). (**d**) Effect of target siRNAs on the invasion of UM-UC-3. The invasive values were normalized to the values from cells treated with NC ($n = 3$). (**e**) Each luciferase reporter construct containing a target 3′UTR was cotransfected with miR-582, miR-582-5p or -3p, or NC into UM-UC-3 cells ($n = 6$). *$P < 0.05$ versus NC. NC, negative control; qRT-PCR, quantitative reverse transcription-PCR.

Table 2 Summary of the number of target sites for miR-582-5p and -3p

Target 3′UTR		Number of predicted binding site	
		miR-582-5p	miR-582-3p
KCNC1	Site-1	1	0
	Site-2	1	1
PGGT1B		5	1
LRRK2		1	2
RAB27A		4	0
DIXDC1	Site-1	2	2
	Site-2	0	1

Identification of miR-582-5p and -3p target genes

To identify miR-582-5p and -3p target genes, hemagglutinin (HA)-tagged Ago2-immunoprecipitation (Ago2-IP) and mRNA array analysis were performed. The UM-UC-3-Ago2 cells stably expressing HA-tagged-Ago2 were transfected with miR-582, and the Ago2-RNA complexes were precipitated with the anti-HA antibody, which was followed by the microarray analysis. The total RNA from UM-UC-3-Ago2 cells transiently transfected with miR-582 and from UM-UC-3-miR-582 cells was also analyzed (**Supplementary Figure S4a**). As a result, 259 genes were identified as candidates for directly targeted genes, and 1,559 genes were identified as candidates for indirectly targeted genes that are downstream of the direct targets (**Supplementary Tables S4 and S5**). In addition to these experimental approaches, we used two target prediction algorithms (TargetScan: http://www.targetscan.org, miRanda: http://www.microrna.org) as *in silico* approaches. After calculating the fold change and conducting an overlapping prediction analysis, we validated the identified 42 genes by qRT-PCR (**Supplementary Figure S4b**), western blotting analysis, and cell proliferation, cell invasion, and 3′UTR reporter assays. The results of the qRT-PCR and western blotting analyses demonstrated that the expression levels of the mRNA and protein of five genes—*potassium voltage-gated channel subfamily C member 1* (*KCNC1*), *PGGT1B*, *LRRK2*, *DIXDC1*, and *ras-related GTP-binding protein* (*RAB27A*)—were more downregulated by transfection with miR-582, or miR-582-5p, or -3p than the NC siRNA-treated cells (Figure 4a,b). Furthermore, silencing of these target genes by siRNAs showed a remarkable inhibition of cell proliferation and cell invasion in UM-UC-3 cells (Figure 4c,d). These five genes have several putative binding sites for miR-582-5p or -3p in their 3′UTRs (Table 2 and **Supplementary Table S6**). Indeed, the expression of each luciferase reporter gene fused with the 3′UTR of *PGGT1B*, *LRRK2* and *DIXDC1* was suppressed by transfection with miR-582, miR-582-5p, or -3p (Figure 4e). Conversely, the expression of that of *RAB27A* was suppressed by miR-582 and miR-582-5p. There was no significant difference in the expression of that of *KCNC1*. These results indicated that miR-582-5p and -3p regulate the expression of *PGGT1B*, *LRRK2*, and *DIXDC1* by binding their 3′UTRs. One strand, miR-582-5p, can directly bind 3′UTR of *RAB27A* and regulate the expression. In contrast, the downregulation of *KCNC1* by transfection with miR-582-5p and -3p is not a direct consequence of miR-582.

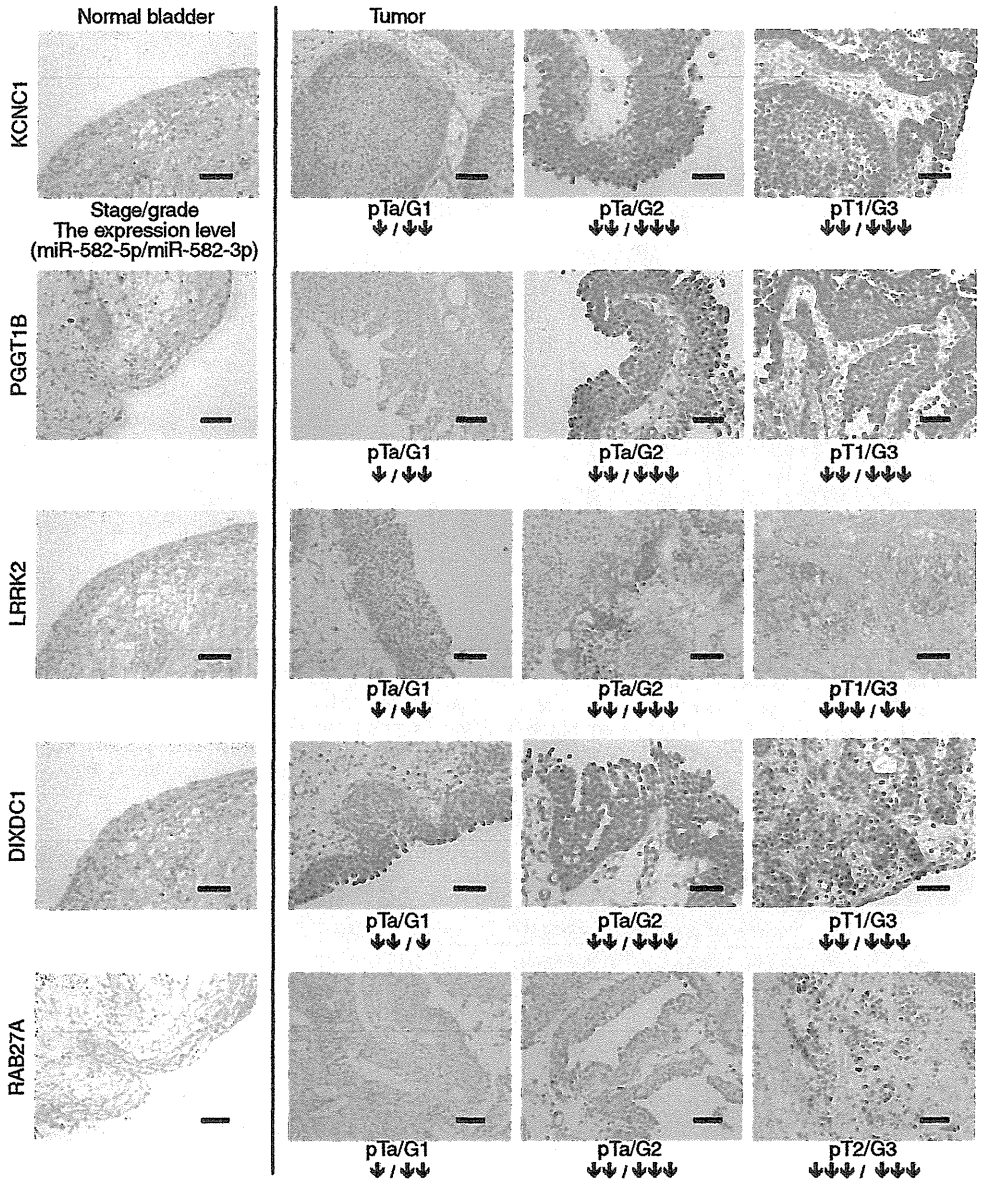

Figure 5 Representative examples of immunohistochemical expression of different gene products in human bladder cancer. Target gene localization in clinical samples by IHC. Left panel: representative normal bladder biopsy (scale bar: 100 μm); Right panel: representative bladder cancer with strong staining observed. A black arrow shows the fold change of the downregulation of miR-582-5p or -3p in tumor tissue compared with the adjacent normal tissues (single black arrow: fold change <2.0, double black arrows: fold change = 2.0 ~ 5.0, triple black arrows: fold change >5.0). Magnification: ×20. IHC, immunohistochemistry.

In addition, we performed an immunohistochemical analysis to observe the expression of these target genes in clinical samples of human bladder cancer. Strong nuclear or cytoplasmic staining was observed in the tumors but not in the adjacent normal bladder tissues (Figure 5). Furthermore, there was a positive correlation between the expression levels of target genes and tumor stage or grade of bladder cancer, and miR-582 was downregulated, whereas, in the low-stage or -grade bladder cancer, a high expression of target genes was not notably observed. These results suggest that the expressions of these cancer-related genes are a clinically relevant biological process, and the levels of the expressions are correlated with malignancy potential in bladder cancer.

DISCUSSION

Genetic changes potentially affect all medical conditions and are associated with thousands of diseases.[26] The amplification of oncogenes or deletion of tumor-suppressor genes can broadly influence tumor initiation and progression.[21,24] Although many previous reports have indicated the correlation between region-specific gain or loss of DNA and tumor progression in bladder cancer, it has not yet been fully elucidated as to which genes, including

noncoding RNAs, are responsible for these different observations. In particular, the losses of chromosomes 3p11-12, 4q32-34, and 5q12-13 loci were reported to be associated with clinicopathological factors in bladder cancer.[11,21–24] Here, we provided evidence on the relationship between the copy number loss of 5q12 and tumor progression via post-transcriptional regulation by miRNA. We demonstrated a novel pathway of paired tumor-suppressive miRNAs, miR-582-5p and -3p located on 5q12, which are specifically attenuated in invasive bladder cancer cells by copy number loss. Furthermore, the expression levels of miR-582-5p and -3p were remarkably lower in bladder cancer tissues and correlated with the tumor grade. These clear correlations suggest an important role for miR-582 on bladder cancer progression.

Despite these considerable dysregulations, there are no reports concerning the expression profiling and functional role of miR-582 in bladder cancer. Thus, focusing on miR-582 as a new candidate tumor-suppressive miRNA in bladder cancer is natural. As expected, the restoration of miR-582-5p or -3p expression inhibited the cell proliferation and invasion in UM-UC-3 cells. Generally, most miRNA duplexes can be functionally asymmetric due to unequal election for assembly into RISC and distinct sequence features. Although previous reports showed that miR199a-5p and -3p or miR-297b-5p and -3p target same mRNAs,[27,28] for this reason, it is extremely rare that each strand of mature miRNA, 5p and 3p, has an identical property in the same physiological/pathological processes. The present experiment indicated that both strands of miR-582-5p and -3p have tumor-suppressive activities based on their sequences.

The number of RNAi-based preclinical and clinical trials has grown over the past several years, and these trials provide opportunities for success.[2,4] These studies were conducted mainly in specific organs, such as eyes, dermis, and lung, which were relatively accessible by topical or local administration. Thus, accessibility is a key requirement for successful RNAi *in vivo* to be delivered tissue specifically or cell specifically. Here, in a closed environment of the bladder, the intravesical injection of miRNA is expected to offer high specificity to a tissue and noninvasive administration. Indeed, a previous study showed that the low-toxic cationic liposome, LIC-101, and the dsRNA complex could be successfully delivered into the bladder tissue and were able to affect tumor growth.[7] However, whether transurethral injection is effective against a severe problem, such as invasion into the muscle and metastasis, remains unknown. In this study, we were successful at providing evidence for this procedure by showing that the instillation of the synthetic miR-582/LIC-101 complex inhibited both lung metastasis and tumor growth. For the first time, our findings have provided a new insight into the availability of a bladder cancer lung metastasis model mouse in RNAi therapeutic trials.

miRNAs are known to have the potential to target thousands of different mRNAs, and each mRNA is also targeted by multiple miRNAs.[29,30] Although target prediction algorithms (TargetScan, miRanda) such as *in silico* approaches based on prior experimental studies can help to predict miRNA-binding targets, computational algorithms are imperfect and insufficient to identify some essential or novel target genes. Hence, in addition to *in silico* analysis, as an optimized and high-throughput biochemical assay, we evaluated the use of Ago2-IP.[31–33] The validation studies revealed that miR-582-5p and -3p have a tumor-suppressive function by both the inhibition of translation and the degradation of *KCNC1*, *PGGT1B*, *LRRK2*, *DIXDC1*, and *RAB27A*. Although miR-582 did not regulate *KCNC1* expression directly, it is very interesting that *KCNC1* has a novel remarkable function in cell proliferation (Figure 4c), because it usually works as a potassium ion transporter. *PGGT1B* is involved in the geranylgeranylation of RhoA and the activation of *MMP-9*, resulting in the disruption of F-actin organization and the progression of cell motility, invasion, and metastasis.[34,35] The kinase domain of *LRRK2* is homologous to B-RAF kinase, which is well known to drive malignant melanoma. Furthermore, *LRRK2* is related to the direct phosphorylation of *Akt1*, resulting in cell survival and the prevention of apoptosis.[36,37] *DIXDC1*, which works as a receptor of the Wnt signaling pathway, is related to the upregulation of *CyclinD1* and the downregulation of p21. The siRNA knockdown of *DIXDC1* was reported to cause G1/S phase arrest in colon cancer.[38,39] *RAB27A*, a member of the RAS oncogene family, is well known to be a driver of melanoma and breast cancer, promoting cell proliferation, invasion, and metastasis potential.[40,41] It is also involved in the regulation of membrane trafficking and exosome formation.[42] This study carries considerable potential to provide novel insights into the pathogenic mechanisms of exosomes regulated by miR-582-5p in invasive bladder cancer.

In conclusion, this study provides two novel findings in bladder cancer. First, we presented evidence of a unique regulatory pathway involving a couple of tumor-suppressive miRNAs, miR-582-5p and -3p, that are downregulated in invasive bladder cancer. The significant dysregulation of the miR-582 network may contribute to the progression of other invasive cancers, and we are actively investigating this hypothesis. Second, an *in vivo* experiment showed the possibility of the application to a therapeutic target against the continued threat of progression to muscle-invasion and metastasis. Importantly, our findings provide an insight into the unique regulatory pathway, which has a coordinated role between miRNA-5p and -3p. These novel findings also provide a potential value of an extreme contribution to the development of the cancer therapy as a prominent class of RNAi therapy.

MATERIALS AND METHODS

Cell culture. The human bladder cancer cell lines UM-UC-3, 5637, J82, TCCSUP, T24, HT1376, and RT4 and the mouse bladder cancer cell line MBT2 were obtained from the American Type Culture Collection (Manassas, VA). The UM-UC-3, J82, TCCSUP, T24, and MBT2 cells were cultured in Modified Eagle's Medium (Sigma-Aldrich, St Louis, MO) containing 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and an antibiotic-antimycotic (Invitrogen) at 37°C in 5% CO_2. The 5637 cells were cultured in RPMI (Invitrogen) containing 10% heat-inactivated fetal bovine serum and an antibiotic-antimycotic at 37°C in 5% CO_2. The T24 and RT4 cells were cultured in McCoy'5A (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum and an antibiotic-antimycotic at 37°C in 5% CO_2.

Patients and human samples. All human bladder tissue samples were derived from the resected bladders of 29 patients who underwent transurethral resection or radical cystectomy at the Department of Urology, St. Marianna University between 2009 and 2011. The samples of tumor and normal epithelium were fixed in formalin, embedded in paraffin, and sectioned for use in microscopic analysis and laser capture microdissection. All materials were obtained with written informed consent, and the study protocols were approved by the Institutional Review Board at the National Cancer Center Research Institute and St. Marianna University.

RNA extraction. Total RNA was extracted from cultured cells using the QIAzol and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Real-time PCR (qRT-PCR). The expression of miRNA was quantified by TaqMan miRNA assays (Applied Biosystems, Foster City, CA). The PCR was performed in 96-well plates using the 7300 Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate. Human-RNU6B or hsa-miR-103 was used as an invariant control.[43]

For the mRNA expression analysis, total RNAs were reverse-transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using a random hexamer primer. The synthesized cDNAs were quantified by TaqMan Gene expression analysis or SYBR Green I qRT-PCR. The β-actin housekeeping gene was used to normalize the variation in the cDNA levels. The primer pairs used for gene amplification are listed in **Supplementary Table S7.**

Quantitative PCR of miR-582 loci on chromosome 5q12. Genomic DNA was extracted from cultured cells using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The qPCR for the miR-582 locus on chromosome 5q12 was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and the primer sequences were 5′-ccacaacaagtcaatctgtgc-3′ and 5′-tattgaaggggttctggtg-3′. The housekeeping gene RNase P was also quantified as a control reference gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) and the TaqMan RNase P Detection Reagents Kit (Applied Biosystems).

Transient miRNA/siRNA transfection. Synthetic hsa-miR-582, hsa-miR-582-5p, hsa-miR-582-3p, and hsa-miR-582-scramble duplexes were obtained from the Bonac (Kurume, Japan). The Allstars Negative Control siRNA was purchased from Qiagen. The siRNAs targeting *KCNC1*, *PGGT1B*, *DIXDC1*, *LRRK2*, and *RAB27A* were purchased from the Bonac. The cells were transfected with 25 nmol/l of either the miRNA or siRNA using DharmaFECT 1 (Thermo Fisher Scientific, San Jose, CA) according to the manufacturer's protocol. The miRNA and siRNA sequences are given in **Supplementary Table S8.**

Cell proliferation assay (MTS assay). For the cell proliferation assay, 24 hours after transfection, 3×10^3 cells were seeded in a 96-well plate. After 3 days of culture, the cell viability was measured using the Tetra Color One assay kit (Seikagaku Kohgyo, Tokyo, Japan) according to the manufacturer's instructions. The absorbance at 450 nm was measured using Envision (PerkinElmer, Norwalk, CT).

Cell invasion assay. The invasive ability of the bladder cancer cells was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm pore size; Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly, the cells were transfected with miRNA or siRNA and, on the following day, 1×10^5 cells were plated in the upper chamber. The lower chamber was supplemented with a medium containing 10% fetal bovine serum. After 24 hours incubation, the cells on the upper surface were scraped off, and the invasive cells attached to the lower surface of the membrane inserts were fixed and stained with Diff-Quik (Sysmex, Kobe, Japan). The invading cells were observed and counted under a microscope in four random fields. All assays were performed in triplicate.

Generation of stable cell lines expressing miR-582 or HA-tagged Ago2 or luciferase. To construct a lentiviral vector for miR-582, pre-miRNA encompassing the stem-loop was amplified from genomic DNA isolated from HT1376 cells by PCR. The PCR product was digested and cloned into the pCDH cDNA cloning lentivector (Cat#CD513B-1; SBI, Mountain View, CA). To construct a lentiviral vector for luciferase, the luc construct was amplified from the pGL4 luciferase reporter vector by PCR. The PCR product was digested and cloned into the pLenti6/V5 Directional TOPO vector (Promega, Madison, WI). The lentiviral vector Lenti-miR-582, Lenti-scramble shRNA (Cat#MZIP000PA-1, SBI), Lenti-HA-Ago2 (RA703B-1, SBI), or Lenti-Luc and the lentiviral packaging plasmids (Invitrogen) were cotransfected into L293T cells. After 48 hours, the lentiviruses in the supernatant were collected and used to infect the UM-UC-3 cells. After antibiotic selection for 2 weeks, stable clones were obtained.

Analysis of miR-582 treatment in a mouse model of bladder cancer. The animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. For the generation of the lung metastasis mouse model, we referred to some previous studies.[7,44–46] Six- to seven-week-old female Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane on day zero and subsequent days. The murine bladder was injected with UM-UC-3-luc cells intravesically at 5×10^6 cells/50 μl/bladder after 15 minutes of trypsin treatment. The development of subsequent tumor growth and metastasis was monitored once a week by *in vivo* imaging. In brief, the mice were injected with 150 mg/kg D-luciferin (Promega) intraperitoneally and imaged 10 minutes later to count the photons from the whole bladder or lung using the IVIS imaging system (Xenogen, Alameda, CA) according to the manufacturer's instructions. The data were analyzed using the LivingImage software (version 2.5; Xenogen). On day 4, the bioluminescence from the implanted cancer cells was measured, and the mice were divided into two treatment groups with equivalent levels of bioluminescence. The transurethral treatment with the miR-582 and LIC101 (Nippon Shinyaku, Kyoto, Japan) complexes at a ratio of 1:16 (w/w) in a volume of 70 μl (10 μg/site) was performed on days 5, 7, 9 11, 13, and 15.

Ago2-IP. The RNA-binding protein immunoprecipitation was performed using an immunoprecipitation kit (RNA-binding protein immunoprecipitation–assay kit for microRNA; MBL, Nagoya, Japan) following the manufacturer's instructions. In brief, UM-UC-3 cells stably expressing HA-Ago2 were transfected with either miR-582 or NC for 48 hours and immunoprecipitated using anti-HA agarose beads (Wako, Osaka, Japan). The Ago2-bound RNA was eluted from beads with the HA peptide (Wako), and the QIAzol reagent was added to extract the total RNA. Ago2-bound total RNA was cleaned further using miRNeasy columns and then subjected to microarray analysis.

Microarray analysis. Total RNA was harvested from UM-UC-3-miR-582, UM-UC-3-shNC, and UM-UC-3-HA-Ago2 cells that were transfected transiently with miR-582 or NC for 48 hours. The Ago2-bound RNA was prepared from the Ago2-IP experiments. The total RNA was labeled with Cy3 using a Low Input Quick Amp Labeling Kit (Agilent, Palo Alto, CA) and hybridized to a SurePrint G3 Human GE 8×60 K array (Agilent) according to the manufacturer's instructions. The data analysis was performed using GeneSpring GX11.5.

Luciferase reporter assay. The 3′-UTRs of human *KCNC1*, *DIXDC1*, *LRRK2*, *PGGT1B*, and *RAB27A* were amplified by PCR from genomic DNA and cloned at the NotI and XhoI sites into pGMT Easy vector (Promega). The PCR primers and oligonucleotide sequences for the constructs are listed in **Supplementary Table S9.** All the constructs were further confirmed by sequencing.

For the luciferase activity analysis, each construct was cotransfected with the miRNA duplex in a 96-well plate using the DharmFECT Duo transfection reagent (Thermo Fisher Scientific) for 24 hours, and the luciferase assays were performed with the Dual-Glo Luciferase assay system (Promega) according to the manufacturer's instructions.

Western blotting. Forty-eight hours after transfection, the cells were homogenized in an M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). The proteins in the total cell lysate or bound to the anti-HA agarose beads were separated by SDS-PAGE gels, which were calibrated using Precision Plus protein standards (Bio-Rad, Richmond, CA). The primary antibodies against KCNC1 (1: 250, ab84823), DIXDC1 (1: 250,

ab67763), LRRK2 (1: 250, ab57329), PGGT1B (1: 250, ab55615), and RAB27A (1: 200, ab55667) were purchased from Abcam (Cambridge, MA), and the primary antibody against ACTIN (1:10,000, MAB1501) was purchased from Millipore (Billerica, MA). The dilution ratio of each antibody is indicated in parentheses. The HRP-linked anti-mouse secondary antibody (GE Healthcare, Buckinghamshire, UK) was used at a dilution of 1:5,000. The bound antibodies were visualized by chemiluminescence using the ECL Plus Western blotting detection system (GE HealthCare), and luminescent images were analyzed using a LuminoImager (LAS-3000; Fuji Film, Inc., Tokyo, Japan).

Immunohistochemistry. All tumors resected from human bladders were fixed with 10% buffered formalin and embedded in paraffin. Sections with a 5-μm thickness were examined using immunohistochemistry. The sections were deparaffinized, the antigens were retrieved by autoclaving in a 10 mmol/l citrate buffer (pH 6.0), and the endogenous peroxidase activity was blocked with the Immuno Pure Peroxidase Suppressor (Pierce, Chester, UK). The primary antibodies used in this study were the same as those used in the western blotting analysis (1:50), followed by incubation with peroxidase-coupled anti-mouse IgG (ImmPRESS Reagent; Vector labs, Burlingame, CA). The immunoreactions were visualized with diaminobenzidine, and the sections were counterstained with hematoxylin.

Statistical analysis. Results are expressed as the mean ± SE. The statistical analyses were conducted using the Bonferroni multiple-comparison test, and the analyses of the luminescence of the lung were conducted using the nonparametric Mann–Whitney–Wilcoxon test. These analyses were performed with the Expert StatView analysis software (version 4; SAS Institute, Cary, NC). $P < 0.05$ was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Figure S1. Expression of miR-1305, pri-miR-582 and PDE4D in human bladder cancer cell lines.
Figure S2. Functions of miR-582 in T24 and 5637 cells.
Figure S3. Functional analysis of UM-UC-3-miR-582 cells.
Figure S4. Identification of miR-582-5p and -3p target genes using HA-Ago2 IP and microarray analysis.
Table S1. Copy number losses detected in UM-UC-3 cell line.
Table S2. Copy number losses correlated with pathological stage in human bladder cancer.
Table S3. Stage and Grade distribution of the patients.
Table S4. A total of 259 genes as candidates for miR-582 direct targets.
Table S5. A total of 1,559 genes as candidates for miR-582 indirect targets.
Table S6. Summary of miR-582-5p and -3p target site.
Table S7. PCR primer sequences and oligonucleotide sequences.
Table S8. *miRNA and siRNA oligonucleotide sequences.*
Table S9. PCR primer sequences for Luciferase assay.

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REFERENCES

1. Fire, A, Xu, S, Montgomery, MK, Kostas, SA, Driver, SE and Mello, CC (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**: 806–811.
2. Davidson, BL and McCray, PB Jr (2011). Current prospects for RNA interference-based therapies. *Nat Rev Genet* **12**: 329–340.
3. Aigner, A (2007). Applications of RNA interference: current state and prospects for siRNA-based strategies *in vivo*. *Appl Microbiol Biotechnol* **76**: 9–21.
4. Takeshita, F and Ochiya, T (2006). Therapeutic potential of RNA interference against cancer. *Cancer Sci* **97**: 689–696.
5. Gingrich, JR (2011). Bladder cancer: chemohyperthermia for bladder cancer–clinically effective? *Nat Rev Urol* **8**: 414–416.
6. Pasin, E, Josephson, DY, Mitra, AP, Cote, RJ and Stein, JP (2008). Superficial bladder cancer: an update on etiology, molecular development, classification, and natural history. *Rev Urol* **10**: 31–43.
7. Nogawa, M, Yuasa, T, Kimura, S, Tanaka, M, Kuroda, J, Sato, K *et al.* (2005). Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. *J Clin Invest* **115**: 978–985.
8. Seth, S, Matsui, Y, Fosnaugh, K, Liu, Y, Vaish, N, Adami, R *et al.* (2011). RNAi-based therapeutics targeting survivin and PLK1 for treatment of bladder cancer. *Mol Ther* **19**: 928–935.
9. Richter, J, Beffa, L, Wagner, U, Schraml, P, Gasser, TC, Moch, H *et al.* (1998). Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. *Am J Pathol* **153**: 1615–1621.
10. Blaveri, E, Brewer, JL, Roydasgupta, R, Fridlyand, J, DeVries, S, Koppie, T *et al.* (2005). Bladder cancer stage and outcome by array-based comparative genomic hybridization. *Clin Cancer Res* **11**(19 Pt 1): 7012–7022.
11. Caballi, MR, Egozcue, J, Gelabert, A, Prat, E and Bernue, M (2001). Detection of chromosomal imbalances in papillary bladder tumors by comparative genomic hybridization. *Urology* **4295**: 5–11.
12. Calin, GA, Sevignani, C, Dumitru, CD, Hyslop, T, Noch, E, Yendamuri, S *et al.* (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* **101**: 2999–3004.
13. Rossi, S, Sevignani, C, Nnadi, SC, Siracusa, LD and Calin, GA (2008). Cancer-associated genomic regions (CAGRs) and noncoding RNAs: bioinformatics and therapeutic implications. *Mamm Genome* **19**: 526–540.
14. Sevignani, C, Calin, GA, Nnadi, SC, Shimizu, M, Davuluri, RV, Hyslop, T *et al.* (2007). MicroRNA genes are frequently located near mouse cancer susceptibility loci. *Proc Natl Acad Sci USA* **104**: 8017–8022.
15. Calin, GA, Dumitru, CD, Shimizu, M, Bichi, R, Zupo, S, Noch, E *et al.* (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* **99**: 15524–15529.
16. Yamada, H, Yanagisawa, K, Tokumaru, S, Taguchi, A, Nimura, Y, Osada, H *et al.* (2008). Detailed characterization of a homozygously deleted region corresponding to a candidate tumor suppressor locus at 21q11-21 in human lung cancer. *Genes Chromosomes Cancer* **47**: 810–818.
17. Agueli, C, Cammarata, G, Salemi, D, Dagnino, L, Nicoletti, R, La Rosa, M *et al.* (2010). 14q32/miRNA clusters loss of heterozygosity in acute lymphoblastic leukemia is associated with up-regulation of BCL11a. *Am J Hematol* **85**: 575–578.
18. Weiss, GJ, Bemis, LT, Nakajima, E, Sugita, M, Birks, DK, Robinson, WA *et al.* (2008). EGFR regulation by microRNA in lung cancer: correlation with clinical response and survival to gefitinib and EGFR expression in cell lines. *Ann Oncol* **19**: 1053–1059.
19. Takeshita, F, Patrawala, L, Osaki, M, Takahashi, RU, Yamamoto, Y, Kosaka, N *et al.* (2010). Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes. *Mol Ther* **18**: 181–187.
20. Hurst, CD, Fiegler, H, Carr, P, Williams, S, Carter, NP and Knowles, MA (2004). High-resolution analysis of genomic copy number alterations in bladder cancer by microarray-based comparative genomic hybridization. *Oncogene* **23**: 2250–2263.
21. Cordon-Cardo, C (1998). Molecular alterations in bladder cancer. *Cancer Surv* **32**: 115–131.
22. Wada, T, Louhelainen, J, Hemminki, K, Adolfsson, J, Wijkström, H, Norming, U *et al.* (2001). The prevalence of loss of heterozygosity in chromosome 3, including FHIT, in bladder cancer, using the fluorescent multiplex polymerase chain reaction. *BJU Int* **87**: 876–881.
23. Polascik, TJ, Cairns, P, Chang, WY, Schoenberg, MP and Sidransky, D (1995). Distinct regions of allelic loss on chromosome 4 in human primary bladder carcinoma. *Cancer Res* **55**: 5396–5399.
24. Richter, J, Jiang, F, Görög, JP, Sartorius, G, Egenter, C, Gasser, TC *et al.* (1997). Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* **57**: 2860–2864.
25. Griffiths-Jones, S, Grocock, RJ, van Dongen, S, Bateman, A and Enright, AJ (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* **34**(Database issue): D140–D144.
26. Almal, SH and Padh, H (2012). Implications of gene copy-number variation in health and diseases. *J Hum Genet* **57**: 6–13.
27. Sakurai, K, Furukawa, C, Haraguchi, T, Inada, K, Shiogama, K, Tagawa, T *et al.* (2011). MicroRNAs miR-199a-5p and -3p target the Brm subunit of SWI/SNF to generate a double-negative feedback loop in a variety of human cancers. *Cancer Res* **71**: 1680–1689.
28. Zhang, T, Luo, Y, Wang, T and Yang, JY (2012). MicroRNA-297b-5p/3p target Mllt3/Af9 to suppress lymphoma cell proliferation, migration and invasion *in vitro* and tumor growth in nude mice. *Leuk Lymphoma* **53**: 2033–2040.
29. Farh, KK, Grimson, A, Jan, C, Lewis, BP, Johnston, WK, Lim, LP *et al.* (2005). The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. *Science* **310**: 1817–1821.
30. Baek, D, Villén, J, Shin, C, Camargo, FD, Gygi, SP and Bartel, DP (2008). The impact of microRNAs on protein output. *Nature* **455**: 64–71.