and serum lipid level for each strain revealed a correlation between incidence of ACF and TG, but not cholesterol (Fig. 2C). Out of the 18 strains, we selected six strains, readily available and with relatively strong correlations, for more detailed analyses under strict conditions. Specifically, BUF, LEW, F344, and LEA, ACI, NIG-III, were postulated to constitute a tumor- and obesity-prone subgroup and a resistant subgroup, respectively. Both body fat weight and body fat percentage, with the exception of LEW rats, were indeed high in a tumor- and obesity-prone subgroup (Fig. 2D). Similar results were obtained for TG levels in a fasting state, but not cholesterol or glucose levels (Fig. 2E). These results suggested that predisposition to obesity and PhIP-induced colon tumorigenesis in the five strains might be regulated by a common mechanism.

3.3. Correlation between the magnitude of AKT activation and incidence of ACF

To gain insights into the molecular basis for the common predisposition, we set out to determine genes differentially expressed in the colons between F344 and ACI, under LFD and without PhIP. We performed Gene Set Enrichment Analysis (GSEA), which revealed a number of differentially expressed pathways (Table S1). We focused on the PI3K/Akt pathway (Fig. 3A) on the list, because it has been already implicated in both carcinogenesis and metabolism [18]. As many upstream regulators and downstream effectors of the PI3K/Akt pathway are subject to regulation by phosphorylation, we examined the level of ~30 proteins in the pathway for total protein and phosphorylated protein by Western blot analysis. We eventually found that the magnitude of AKT activation had a good correlation with colon tumor susceptibility among the five selected rat strains (Fig. 3B). In line with this observation, FOXOs and Bim, pro-apoptotic molecules inhibited by AKT, were downregulated in the crypts from the strains with higher tumor susceptibility (Fig. 3C).

3.4. Activation of AKT in the colonic cells by PhIP in vivo and in vitro

Having confirmed the static link between the common predisposition and the level of activated AKT in a basal condition, we next investigated whether PhIP and/or HFD could dynamically regulate the magnitude of AKT activation in the colon. To achieve the highest sensitivity in detecting any alterations, we selected BUF rats, which manifested the most pronounced AKT activation (Fig. 3B). Western blotting revealed that AKT was hyper-activated exclusively in colonic crypts from the subgroup treated by PhIP for 2 weeks, followed by LFD for 4 weeks (Fig. 4A). In line with this

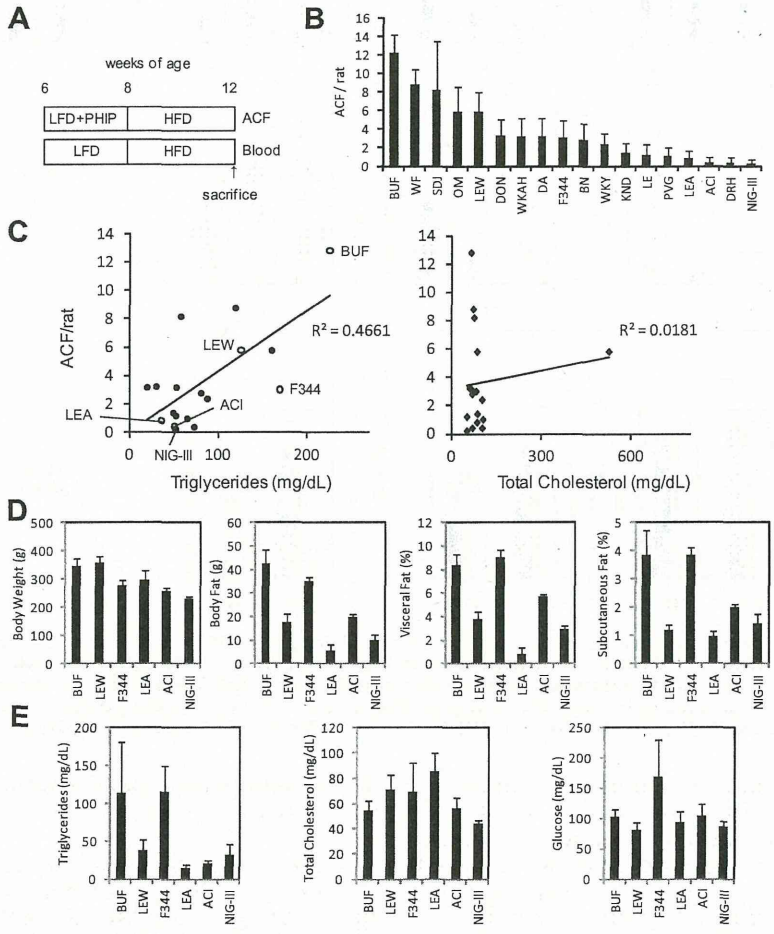

Fig. 2. Correlation between susceptibility to obesity and PhIP-induced colon tumorigenicity. (A) Feeding protocols. (B) The number of PhIP-induced ACF across 18 rat strains ($n = 5$ or 6 each). (C) Correlation between the number of ACF and amount of serum lipid. Triglyceride (*left*), and total cholesterol (*right*). Each circle depicts the mean level of serum lipid taken in a non-fasting condition from 5 individuals of each strain. Open circles labeled by strain name were used in the subsequent analysis D and E. Evaluation of obesity (D) and blood biochemistry (E) in six selected strains. Rats under LFD without PhIP for 2 weeks and subsequently under 4 weeks of HFD were examined ($n = 5$–11 each). BUF and F344, but not LEW, manifested an obesity phenotype, while ACI, LEA, and NIG-III did not (D). Only the level of serum triglyceride exhibited higher in BUF and F344, compared to the others (E).

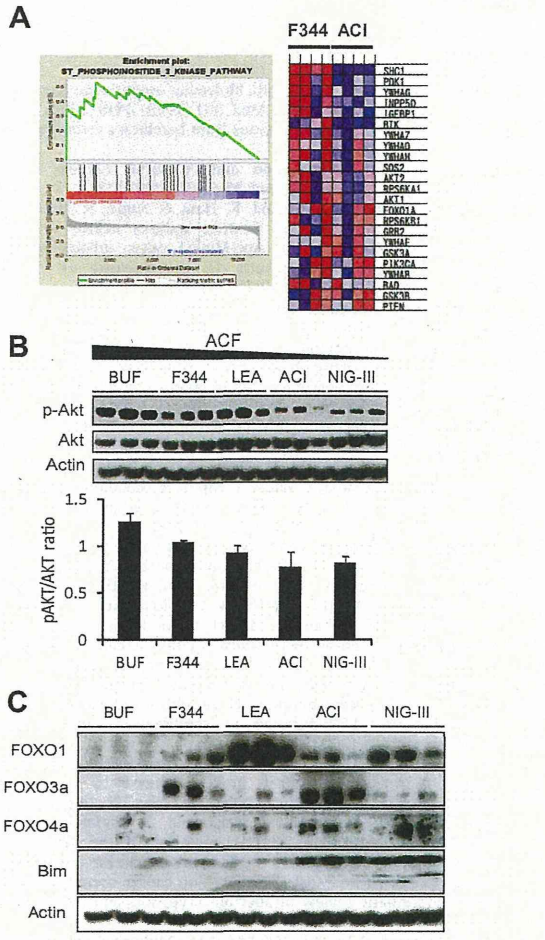

Fig. 3. Basal levels of pAKT in the colon correlate with predisposition to obesity and colon cancer. (A) Gene set enrichment analysis. The colonic crypts from F344 and ACI rats ($n = 4$ each) were subject to microarray analysis. Genes in the PI3K pathway overrepresented in differentially expressed genes (*left*). A heat map showing PI3K pathway genes (*right*). (B) Correlation between magnitude of AKT activation and the incidence of ACF. Western blotting analysis ($n = 3$) of the colonic crypts for total AKT, p-AKT (*upper panel*). Signal intensity ratio of p-AKT to total AKT correlated with the number of PhIP-induced ACF (*lower panel*). (C) Expression of pro-apoptotic molecules downstream of AKT. Western blotting analysis ($n = 3$) revealed FOXO family genes and Bim tended to show lower expression in tumor-prone rat strains. β-actin serves as a loading control.

observation, GSK-3βa key substrate of AKT, was phosphorylated specifically in the same group. The increase of pAKT was marginal without PhIP treatment or under HFD, even after PhIP treatment. To determine if the activation is achieved by a direct effect of PhIP on colonic cells, we treated human normal colon cells FHC with 10 μM of acetoxy-PhIP, a biologically active form of PhIP. Phosphorylation of AKT was indeed observed *in vitro*, albeit at a very early point and in a transient manner (Fig. 4B). These results implied that PhIP directly and promptly activates AKT, which could be sustained *in vivo* only under LFD, by an unknown mechanism.

3.5. PhIP and HFD inhibited apoptosis and activated Wnt pathway by distinct mechanisms

Given that GSK-3β promotes degradation of β-catenin, inactivation of GSK-3β by AKT is supposed to result in β-catenin accumulation leading to Wnt pathway activation. Indeed, PhIP-induced AKT activation increased the amount of total β-catenin, consistent with an earlier study [19], but to a lesser extent compared to HFD

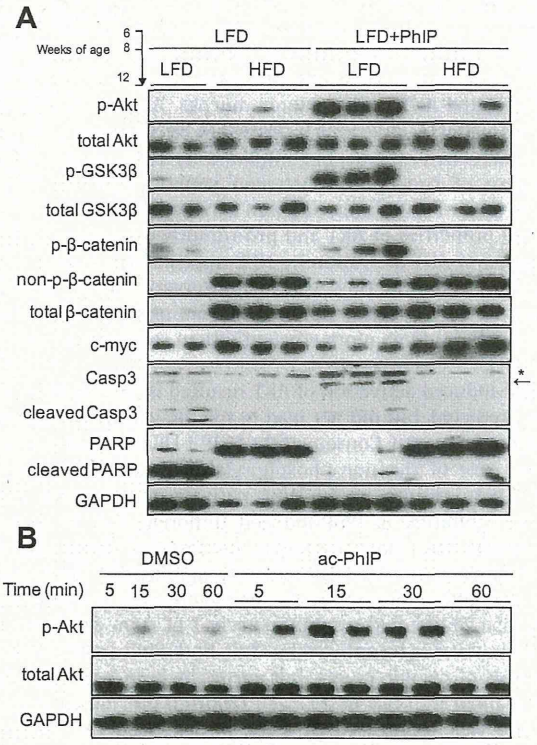

Fig. 4. Wnt pathway activation and inhibition of apoptosis by PhIP and HFD. (A) Characterization of key molecules in the Wnt pathway and apoptosis. Colonic crypts of the BUF rats from 4 subgroups ($n = 3$ each, $n = 2$ for LFD with PhIP) were analyzed by Western blotting analysis. GAPDH serves as a loading control. Non-specific bands (*asterisk*), specific bands (*arrow*) for full-length caspase3. Note that effects by PhIP were sustained even at 4 weeks later under LFD, but not under HFD. (B) Activation of AKT by PhIP in vitro. Normal human colon cells FHC were exposed to 10 μM N-acetoxy-PhIP (ac-PhIP). Negative controls were treated with DMSO ($n = 1$). Cells were collected at 5, 15, 30, and 60 min after PhIP treatment ($n = 2$).

(Fig. 4A). To qualitatively characterize β-catenin, we examined its phosphorylated and non-phosphorylated form, corresponding to an inactive and active form, respectively. It was revealed that HFD exclusively increased the amount of active β-catenin, while PhIP predominantly increased the amount of inactive β-catenin. In line with this observation, the level of c-myc, a major Wnt target gene, was indeed higher under HFD than upon PhIP treatment (Fig. 4A). AKT-induced elevation of inactive β-catenin, however, contradicts with the assumption that inactivation of GSK-3β should result in accumulation of active β-catenin, strongly suggesting that an alternative mechanism might be operating.

We next examined the effects of PhIP and HFD on apoptosis. Caspase3 and poly-ADP ribose polymerase (PARP) were predominantly in cleaved forms in colonic crypts under LFD, indicating massive apoptosis. By contrast, the cleaved forms were not detected under HFD or treated by PhIP, which seems to be achieved via distinct mechanisms. HFD suppressed expression of caspase3, thereby diminishing its cleaved form, while PhIP suppressed cleavage from full-length caspase3. Conversely, PhIP regulated PARP and caspase3 in a reciprocal manner (Fig. 4A). Collectively, HFD and PhIP activated the Wnt pathway and inhibited apoptosis, but through distinct mechanisms in the colon.

4. Discussion

Obesity, a major risk for CRC, has been generally implicated in progression from the initiation step of carcinogenesis. In the

present study, we showed that obesity could be also implicated in the early stages, by sharing a common genetic predisposition with PhIP-induced tumorigenesis. The common genetic predisposition appeared to be conveniently estimated by the level of serum TG and activated AKT in the colonic mucosa. AKT was also dynamically activated by PhIP, which seemed to be promoted by the intestinal microenvironment, but inhibited by HFD, underscoring the relevance of cooperation between genetic and environmental factors toward PhIP-induced colon carcinogenesis. Given the pro-survival properties of AKT and pro-tumorigenic effects of obesity, inhibition of AKT activation by HFD appears paradoxical in terms of tumor promotion. However, this observation might account for the reason why a cycling protocol alternating PhIP with HFD could induce colon tumors more efficiently than continuous exposure to PhIP [11].

PhIP-induced activation of AKT resulted in inactivation of GSK-3β as predicted, but did not lead to full activation of β-catenin for an unknown reason. Consequently, PhIP + LFD induced only a modest increase of the non-phosphorylated β-catenin compared to HFD. These results imply Wnt pathway-independent roles of GSK-3β inhibition in PhIP-induced tumorigenesis. In support of this notion, the colony formation potential of singly dissociated intestinal stem cells in 3D culture is significantly improved by a GSK-3β inhibitor, but not by Wnt3a ligands [20], raising the possibility that PhIP could promote survival of stem cells that might harbor mutations introduced by PhIP. Both PhIP and HFD inhibited apoptosis of colonic crypts, but surprisingly in a completely distinct manner that has never been reported previously. Expression of PARP was suppressed by PhIP, but induced and retained by HFD. As PARP is a component of the TCF4/β-catenin complex and positively regulates its transcriptional activity [21,22], its presence might contribute to a more pronounced activation of the Wnt pathway by HFD than by PhIP.

Considering high serum TG has recently emerged as a high risk factor for CRC in humans [23] [24], consistent with the present study, the findings from this study might have implications on personalized medicine. For instance, those individuals with high serum TG and AKT phosphorylation in the colon might constitute a subgroup with higher risk for CRC, even in the absence of macroscopic colonic lesions. Development of biomarkers for downstream of AKT would be also warranted, which would enable efficient reduction of cancer risk by patient education, early detection of cancer and therapeutic intervention. Taken together, we demonstrated the relevance of AKT in the development of PhIP-induced and obesity-related CRC, providing not only mechanistic insights, but also clinical implications on the diagnosis and prevention of CRC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.059.

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Review Article

RNAi Therapeutics and Applications of MicroRNAs in Cancer Treatment

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RNA interference-based therapies are proving to be powerful tools for combating various diseases, including cancer. Scientists are researching the development of safe and efficient systems for the delivery of small RNA molecules, which are extremely fragile in serum, to target organs and cells in the human body. A dozen pre-clinical and clinical trials have been under way over the past few years involving biodegradable nanoparticles, lipids, chemical modification and conjugation. On the other hand, microRNAs, which control the balance of cellular biological processes, have been studied as attractive therapeutic targets in cancer treatment. In this review, we provide an overview of RNA interference-based therapeutics in clinical trials and discuss the latest technology for the systemic delivery of nucleic acid drugs. Furthermore, we focus on dysregulated microRNAs in human cancer, which have progressed in pre-clinical trials as therapeutic targets, and describe a wide range of strategies to control the expression levels of endogenous microRNAs. Further development of RNA interference technologies and progression of clinical trials will contribute to the achievement of practical applications of nucleic acid drugs.

Key words: RNA interference – microRNA – DDS – Cancer

INTRODUCTION

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants initiated by double-stranded RNA (dsRNA). It is the most significant recent contribution to the field of cell biology, and Fire and Mello who discovered it were awarded the Nobel Prize for Medicine in 2006 (1). The silencing technology to suppress the expression of pathologically or physiologically important genes by using small interfering RNA (siRNA) is applicable to many kinds of research or therapeutics for human diseases caused by specific genes, which are difficult to regulate through traditional approaches. Indeed, as the initial description of RNAi in animals, the development of RNAi-based therapies has provided a powerful new arsenal against various human diseases, such as age-related macular degeneration (AMD) (2,3), respiratory syncytial virus (RSV) infection (4), neurodegenerative disorders (5) and cancers (6–8).

On the other hand, in recent years, microRNAs (miRNAs) have been studied as regulators of gene expression in crucial biological processes, including cell development, differentiation, apoptosis and proliferation (9,10). miRNAs are non-coding small RNAs (~22 nt) which are processed from endogenously expressed transcripts and induce translational suppression and mRNA degradation in animals, plants and viruses (11,12). miRNAs are first transcribed as primary miRNA (pri-miRNA) transcripts by RNA polymerase II and then processed by Drosha in the nucleus to generate

~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.

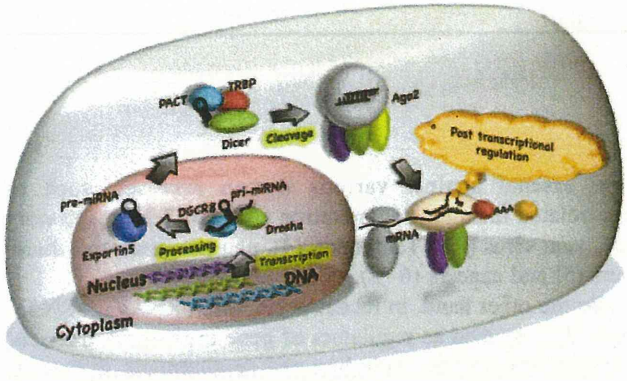

Figure 1. Cellular mechanisms of RNA interference pathway in mammals. First, primary miRNAs (pri-miRNAs) are transcribed by RNA polymerase II and are cleaved by the enzyme Drosha into ~70 nucleotides as precursor-miRNAs (pre-miRNAs). Next, these pre-miRNAs are exported to the cytoplasm with Exportin-5 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.

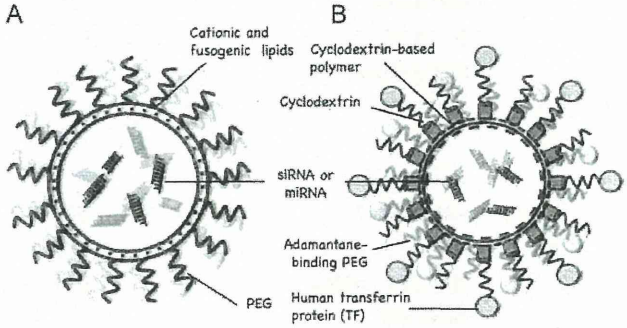

Figure 2. Delivery technology for RNAi therapy. (A) Stable nucleic acid lipid particle (SNALP). The bilayer consists of cationic and neutral lipids and is coated by PEG. The diameter is ~100 nm. (B) RNAi/oligonucleotide nanoparticle delivery (RONDEL). RNAs are protected from degradation in serum by the 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)

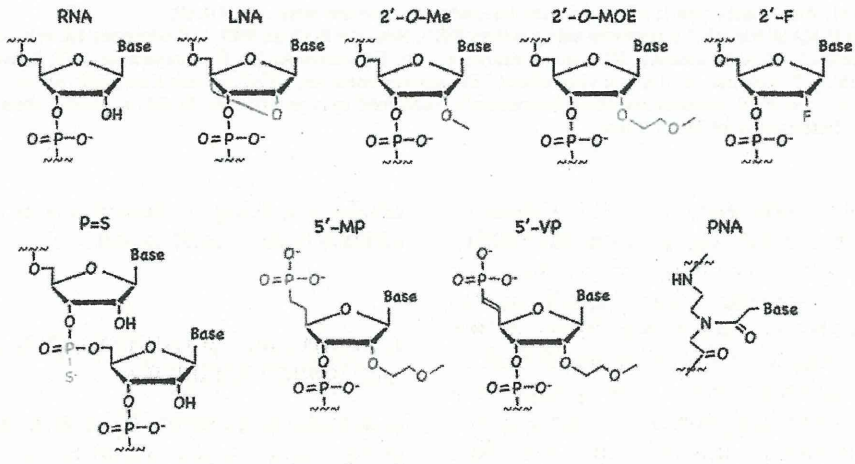

Figure 3. Chemical modifications for stability. Sugar, backbone and base modifications are illustrated. Shown are locked nucleic acid (LNA), phosphorothioate (P = S), 2′-*O*-methyl (2′-*O*-Me), 2′-fluoro (2′-F), 2′-*O*-(2-methoxyethil) (2′-*O*-MOE), 5′-methylenephosphonate (5′-MP), 5′-(*E*)-vinyl-phosphonate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