Biochemical and Biophysical Research Communications 376 (2008) 169–173

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Modulating endogenous gene expression of mammalian cells via RNA–small molecule interaction

Nazgul Tuleuova [a,b], Chung-Il An [a], Erlan Ramanculov [b], Alexander Revzin [a,*], Yohei Yokobayashi [a,*]

[a] Department of Biomedical Engineering, University of California-Davis, 451 Health Sciences Drive, Davis, CA 95616, USA
[b] National Center for Biotechnology, Astana, Kazakhstan

ARTICLE INFO

Article history:
Received 18 August 2008
Available online 31 August 2008

Keywords:
RNA interference
Aptamer
Gene silencing
Theophylline

ABSTRACT

RNA interference (RNAi) has emerged as a powerful technology to silence arbitrary genes by designing small RNA constructs based on the targeted messenger RNA sequences. We recently developed a small molecule-controlled RNAi gene switch that combined the molecular recognition by *in vitro* selected RNA aptamers with versatile gene silencing by small interfering RNAs, and demonstrated for the first time, posttranscriptional modulation of RNAi through direct RNA–small molecule interaction. In this report, we describe the first application of this technology to regulate an endogenous gene in mammalian cells. As a proof-of-concept demonstration we chose to modulate expression of albumin—serum protein produced by the liver. We designed and constructed a theophylline aptamer-fused short hairpin RNA (shRNA) expression vector targeting albumin mRNA in hepatic (HepG2) cells. Transfection of HepG2 cells with the aptamer–shRNA expression vector allowed to control albumin gene expression by adding theophylline into the culture media in dose dependent fashion.

The emergence of RNA interference (RNAi) technology transformed many aspects of mammalian genetics research. Introduction of a short (~21 bp) duplex RNA with complementary sequence to the targeted messenger RNA (mRNA) into mammalian cells results in site specific digestion of the mRNA and efficient silencing of gene expression [1,2]. Recent advance in predictive algorithms to identify efficiently targeted sequences within mRNAs [3–5] and deeper understanding of the molecular mechanisms of the RNAi pathway have allowed many researchers to use RNAi to study gene functions and discover new genes.

RNAi can be induced by several methods. Direct transfection of synthetic double-stranded small interfering RNA (siRNA) or short hairpin RNA (shRNA) is effective for gene silencing in cultured mammalian cells. Alternatively, plasmid or viral DNA vectors can be used to transfect or transduce mammalian cells to express siRNA precursors in the cells.

Further regulation of RNAi could be useful for studying temporal and dose-dependent effects of gene expression. A number of engineered transcription factors that respond to small molecule inducers have been adapted to control transcription of shRNAs expressed from appropriately modified promoters [6]. These methods require introduction of large transcription factors and engineered promoters which may not be compatible with some applications such as gene therapy. The choice of inducer molecules is also inherently limited by the availability of the transcription factors. In another approach, several groups developed chemically modified siRNA derivatives that are activated by light [7–10]. These photocaged siRNAs are induced irreversibly and often to suboptimal levels. The released photoprotective groups may also exhibit undesired cytotoxicity.

As an alternative strategy, we recently developed a method to chemically regulate RNAi via RNA–small molecule interaction [11]. An RNA aptamer, selected *in vitro* for binding to theophylline [12,13], was inserted in the loop region of an shRNA targeting enhanced green fluorescent protein (EGFP). This aptamer–shRNA construct was delivered using a plasmid vector, resulting in silencing of EGFP expression. However, the embedded aptamer allowed inhibition of the observed gene silencing (enhanced EGFP expression) by added theophylline in a dose-dependent manner. Modulation of RNAi by theophylline was attributed to inhibition of the posttranscriptional processing of the aptamer-fused shRNA into siRNA by Dicer (Fig. 1). The spatial overlap of the aptamer–theophylline binding site with the Dicer cleavage site was determined to be critical for the observed effect [11].

In this study, we report an important advance in our aptamer-fused shRNA technology that demonstrates modulation of an endogenous gene in mammalian cells. Specifically, we targeted albumin expression in hepatic (HepG2) cells using an aptamer-fused shRNA. Albumin synthesis of hepatic cells transfected with aptamer–RNA construct was decreased to 20% compared to untransfected controls. However, adding theophylline allowed to rescue albumin expression in a dose dependent fashion with

* Corresponding authors. Fax: +1 530 754 5739.
E-mail addresses: arevzin@ucdavis.edu (A. Revzin), yoko@ucdavis.edu (Y. Yokobayashi).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.08.112

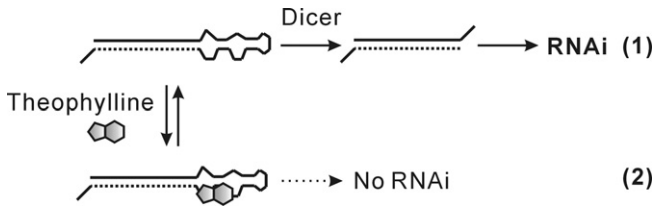

Fig. 1. Schematic illustration of the mechanism of RNAi modulation by aptamer-fused shRNA. Aptamer-fused shRNA is normally processed by Dicer to yield siRNA duplex to induce gene silencing (1). In the presence of the aptamer ligand, Dicer processing of the aptamer-fused shRNA is inhibited, resulting in inhibition of RNAi (11).

2 mM concentration corresponding to 80% of normal albumin synthesis. To our knowledge, this is the first example of using RNA–small molecule interaction to regulate an endogenous gene in mammalian cells, and sets the stage for further applications of the technology.

Materials and methods

Plasmid construction. pSilencer 2.1-U6 hygro (Ambion) was used for constructing all RNAi vectors. pAlb191, pAlb192, and pAlb193 (Alb19 stands for *19* bp of shRNA encoding an *Alb*umin target sequence) and pAlb192T (T stands for *t*heophylline aptamer inserted in the loop region of shRNA) were produced by ligating phosphorylated and annealed oligonucleotides with pSilencer 2.1-U6 hygro. Oligonucleotides used were as follows (a pair of annealed oligonucleotides is indicated by "&"): pAlb191: 5′-GATCCGCCAGAAGA CATCCTTACTTTCAAG & 5′-TTCTCTTGAAAGTAAGGATGACTTCTGG CG and 5′-AGAAGTAAGGATGTCTTCTGGCTTTTTGGA & 5′-AGCTT CCAAAAAGCCAGAAGACATCCTTAC; pAlb192: 5′-GATCCGGAAGAG CCTCAGAATTTATTCAAG & 5′-ATCTCTTGAATAAATTCTGAGGCTCTT CCG, and 5′-AGATAAATTCTGAGGCTCTTCCTTTTTGGA & 5′-AGCTT CCAAAAAGGAAGAGCCTCAGAATTT; pAlb193: 5′-GATCCGCAGATA TATGCACACTTTATCAAG & 5′-TACTCTTGATAAAGTGTGCATATATCT GCG and 5′-AGTAAAGTGTGCATATATCTGCTTTTTGGA & 5′-AGCTT CCAAAAAGCAGATATATGCACACTT; pAlb19T: 5′-GATCCGGAAGAG CCTCAGAATTTAATACCAGCCGAAAG & 5′-GGGCCTTTCGGCTGGTAT TAAATTCTGAGGCTCTTCCG, and 5′-GCCCTTGGCAGTAAATTCTGAG GCTCTTCCTTTTTGGA & 5′-AGCTTCCAAAAAGGAAGAGCCTCAGA ATTTACTGCCAA. The target sequences within human albumin mRNA were chosen according to standard criteria described elsewhere [3,4].

Cell culture and transfection. Hepatic cells (HepG2 human hepatoma cell line) were maintained in a 5% CO_2 humidified incubator at 37 °C in MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). On the day of transfection HepG2 cells were trypsinized and diluted with fresh medium without antibiotics to a final concentration of 5×10^5 cells/ml, and transferred to 96-well plate (100 μl per well). Transfection of shRNA expression vectors was carried out using FuGeneHD (Roche) according to the manufacturer's instructions with modifications. Specifically, a ratio of 2:10 DNA:FuGENE reagent complex was used with 10 μl of complex per well. Cells were incubated in a 5% CO_2 humidified incubator at 37 °C in the HepG2 medium supplemented either with or without the indicated concentrations of theophylline 72 h before albumin secretion measurements.

Quantifying albumin silencing induced by aptamer-fused shRNA. RNAi activity in transfected cells was quantified using enzyme-linked immunosorbent assay (ELISA) for albumin and another liver specific protein, α1-AT, that served as a control in albumin silencing experiments. HepG2 cells containing aptamer–shRNA construct were cultured for 72 h in media supplemented with 0–2 mM concentration of theophylline. After incubation, media samples were collected from individual wells of a 96-well plate, diluted 1:100 with fresh MEM and analyzed for secreted albumin and α1-AT using standard ELISA [14]. Measurements were performed on MultiScan Ascent plate reader (Thermo) in triplicate for each sample. Total protein concentration in each sample was also measured using standard Bradford technique using Coomassie (Bradford) Protein Assay Kit (Pierce) according to the manufacturer's instructions using Safire^2 microplate reader (Tecan). Production of liver specific protein determined by ELISA was normalized by total protein concentration, and the average values and standard deviations of albumin/total protein and α1-AT/total protein from triplicate samples were calculated. These average values and the standard deviations were then normalized by liver protein/total protein values from untransfected cells or cells transfected with an shRNA expression vector which has a scrambled target sequence with no significant homology to the human genome (pSilencer 2.1-U6 hygro Negative Control, Ambion, which expresses an shRNA with the following sequence: 5′-ACUACCGUUGUUAUAGGUGUUCAAGA GACACCUAUAACAACGGUAGUU-3′; double-stranded stem underlined).

Transcript level measurements by quantitative real-time RT-PCR. Cells transiently transfected with shRNA expression vectors were exposed to varying concentrations of theophylline and harvested after 72 h. RNA isolation and cDNA synthesis were performed using SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers for human albumin gene detection were designed using Primer3 software (available online http://frodo.wi.mit.edu/) while primers for human α1-AT and beta actin (housekeeping gene) were selected from a database http://medgen.ugent.be/rtprimerdb). Primer (Sigma Genosys) concentrations were optimized before use. SYBR Green Master Mix (1×) was used with the appropriate concentrations of forward and reverse primers (Table 1) in a total volume of 12 μl that also included 1 μl cDNA. All PCR reactions were done in duplicate. PCR amplification was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 58 °C for 10 s and 68 °C for 1 min on Mastercycler ep Realplex (Eppendorf). The comparative Ct value method using beta actin housekeeping gene as internal standard was employed to determine relative levels of albumin and α1-AT gene expression.

Table 1
Primer sequences used in quantitative real-time RT-PCR

Gene	Accession No.	Primer	Sequence 5′–3′	Conc. (μM)
β-actin	NM_001101	F	CTGGAACGGTGAAGGTGACA	0.5
		R	AAGGGACTTCCTGTAACAATGCA	0.5
Albumin	NM_000477	F	CCTCTTGTGGGAAGAGCCTCA	1
		R	TACCCCAAGTGTCAACTCCA	1
α1-antitrypsin	NM_001002235	F	GTCAAGGACACCGAGGAAGA	1
		R	TATTTCATCACAGCAGCACCCA	1

Results

Identification of RNAi target sequence in human albumin mRNA

We constructed human U6 promoter-driven plasmid vectors (pAlb191, pAlb192, pAlb193, Fig. 2A) that transcribe shRNAs targeting different sequences within the human albumin mRNA. The target sequences were identified based on the published algorithms [3,5,4]. Transient transfection of HepG2 cells with several variants of shRNAs resulted in 50–75% reduction in levels of secreted albumin compared to cells transfected with negative control vector. The most efficient gene silencing was observed by pAlb192 (75%). Considering that the maximal transfection efficiency as estimated using an EGFP expression vector was approximately 85% (data not shown), the albumin mRNA is efficiently targeted by pAlb192 (Fig. 2B). Consequently, we based our aptamer-fused shRNA design on this construct.

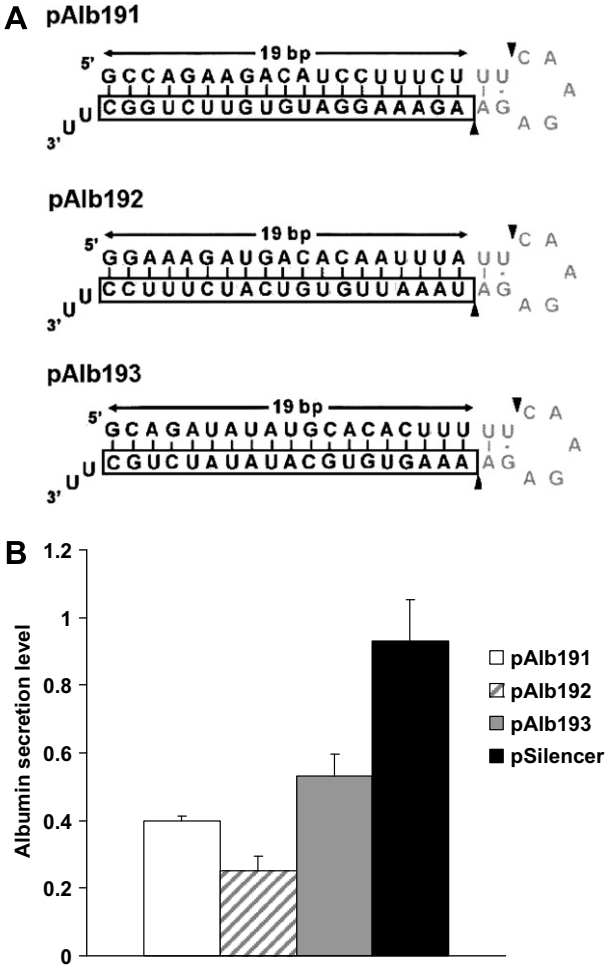

Fig. 2. Design of shRNAs targeting albumin mRNA. (A) Secondary structures of putative transcripts from pAlb191, pAlb192, and pAlb193. Boxed nucleotides indicate the antisense strand targeting different regions in albumin mRNA, gray nucleotides indicate the loop sequence derived from the pSilencer vector (Ambion). (B) Gene silencing of albumin induced by the shRNAs. HepG2 cells were transiently transfected with pAlb191, pAlb192, pAlb193, or pSilencer (pSilencer 2.1-U6 hygro Negative Control, see Materials and methods) in a 96-well microplate. Albumin secretion level measurements were performed 72 h after transfection and the ratios of albumin and total protein amount were calculated. The ratios were then normalized to untransfected cells. The data are averages of triplicate transfection experiments and error bars represent standard deviations.

Design of theophylline aptamer-fused shRNA targeting human albumin

In vitro selection from a random sequence library was previously used to identify an RNA aptamer that tightly binds to a small molecule drug theophylline, while simultaneously discriminating against the structurally similar molecules [12]. We recently showed that engineered shRNAs in which the loop region is replaced with the theophylline aptamer retain gene silencing activity, but addition of theophylline results in dose-dependent inhibition of RNAi [11]. We attributed the observation to the inhibition of Dicer mediated processing of the shRNA due to spatial overlap of the theophylline binding site and the Dicer cleavage site in the aptamer-fused shRNA. It was also found that the spacing between the RNAi-inducing duplex stem and the aptamer is critical for theophylline-modulated gene silencing.

Based on the best albumin-targeting shRNA expression vector pAlb192 and the sequence requirements reported by us previously [11], we designed and constructed an shRNA expression vector with a theophylline aptamer embedded in the loop region denoted as pAlb192T (Fig. 3A).

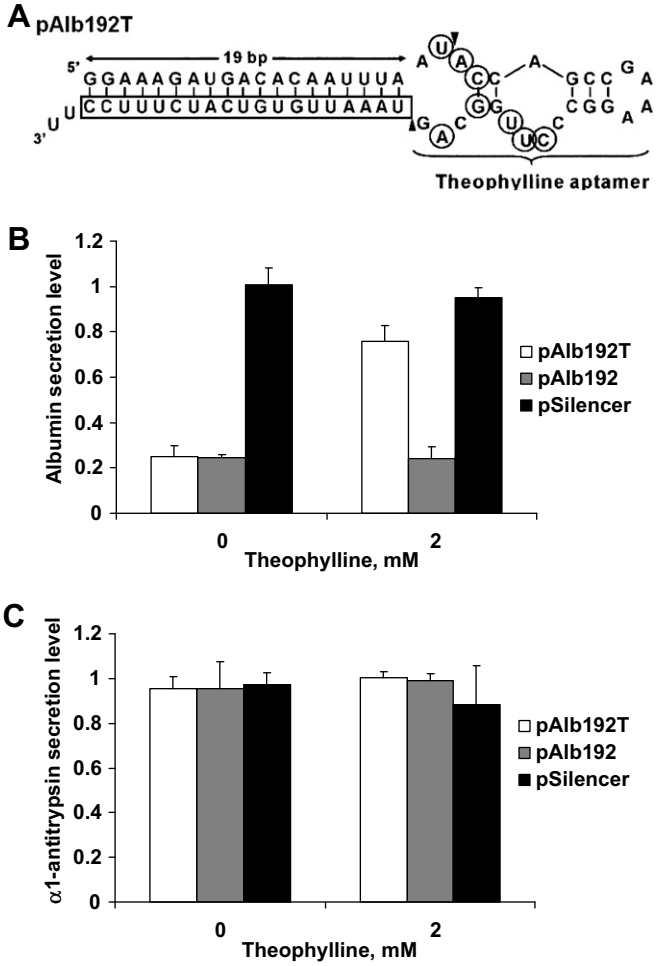

Fig. 3. Design of the aptamer-fused shRNA targeting the albumin mRNA and inhibition of RNAi by theophylline. (A) Secondary structure of the shRNA expressed from pAlb192T. Circled nucleotides indicate bases that interact with theophylline [13]. (B) Inhibition of RNAi against the albumin gene by theophylline. Albumin gene silencing was induced by pAlb192 (without aptamer) or pAlb192T (with aptamer) in the presence or absence of theophylline. (C) Expression of α1-AT was measured as an untargeted control to show the absence of nonspecific RNAi. The data shown are averages of triplicate transfection experiments normalized to untransfected cells, with error bars representing standard deviations.

Inhibition of RNAi by theophylline

Secreted albumin by the cultured HepG2 cells transiently transfected with the shRNA expression vectors were measured by ELISA. The reduction of secreted albumin by cells transfected with pAlb192T was virtually identical to that of the cells transfected with pAlb192 lacking the aptamer. Furthermore, the albumin level of pAlb192T-transfected cells increased significantly in the presence of 2 mM theophylline while that of pAlb192-transfected cells remained constant (Fig. 3B). Another liver protein produced by HepG2, alpha1-antitrypsin (α1-AT), was assayed as a negative control to demonstrate the specificity of gene silencing. As expected, α1-AT expression was unaffected by the shRNAs and theophylline (Fig. 3C).

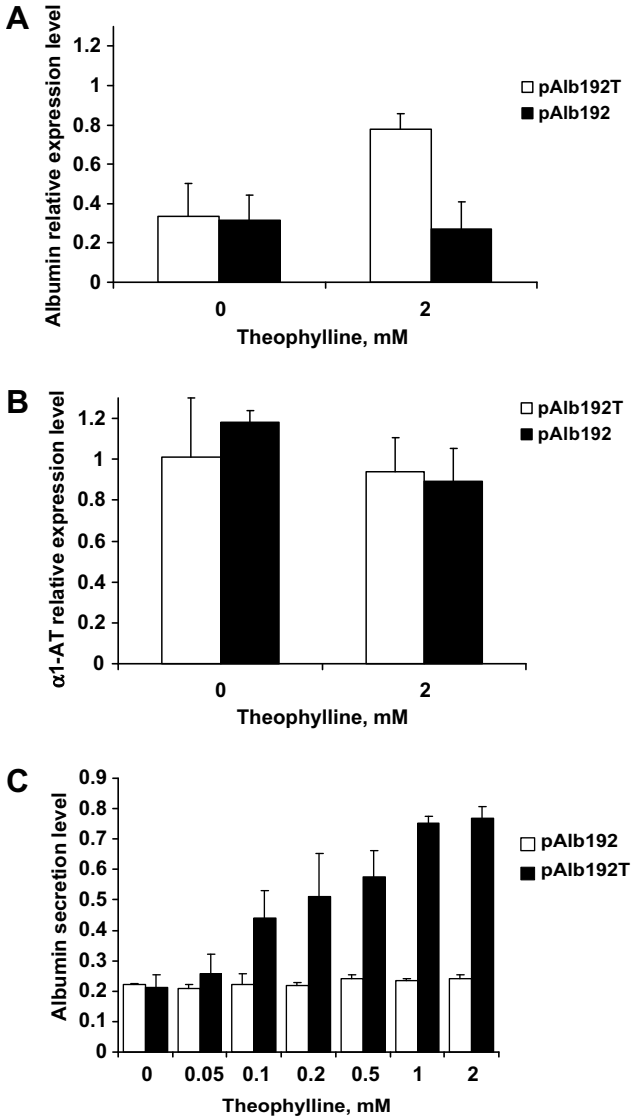

Fig. 4. Modulation of gene expression by the aptamer–shRNA construct. (A) Quantification of mRNA level of albumin by quantitative real-time RT-PCR. The expression levels were normalized to a house keeping gene (beta actin). The data were then normalized to those from cells transfected with pSilencer (pSilencer 2.1-U6 hygro Negative Control, see Materials and methods). The data are averages of triplicate transfections and error bars represent standard deviations. (B) Quantification of mRNA level of α1-AT as described for albumin. (C) Dose-dependent inhibition of albumin synthesis in hepatic cells transfected with aptamer–shRNA constructs. HepG2 cells were transfected with pAlb192T (with aptamer) and pAlb192 (without aptamer) in a 96-well microplate. Secreted albumin was analyzed by ELISA 72 h after transfection and the ratios of albumin to total protein were calculated. The ratios were then normalized to those from cells transfected with pSilencer (pSilencer 2.1-U6 hygro Negative Control, see Materials and methods). The data are averages of triplicate transfections and error bars represent standard deviations.

In addition to monitoring protein synthesis with ELISA, we employed quantitative real-time RT-PCR to quantitate the mRNA levels for albumin (target), α1-AT (untargeted control) and beta actin (house keeping gene). To achieve adequate results, the primers used to detect albumin gene were designed to span the expected siRNA-directed cleavage site in the mRNA, and all the primer pairs were designed to span an exon–exon boundary to avoid any amplification of genomic DNA contaminant. The results show decreased albumin mRNA levels in cells expressing the shRNAs. However, addition of theophylline results in recovery of the mRNA level in pAlb192T (aptamer containing)-transfected cells, but not in pAlb192-transfected cells (Fig. 4A). The untargeted control α1-AT mRNA showed no changes under similar conditions (Fig. 4B).

The observed selective modulation of albumin expression was dose-dependent up to 2 mM theophylline (Fig. 4C). Importantly, hepatocytes remained viable and retained normal morphology of the cells of hepatic lineage after 72 h incubation in 2 mM theophylline (data not shown). From these results, we concluded that shRNA expressed by pAlb192T selectively knocks down human albumin expression by RNAi in the absence of theophylline, but theophylline restores albumin expression by inhibiting shRNA processing.

Discussion

Our first demonstration of posttranscriptional regulation of RNAi by RNA–small molecule interaction used exogenous reporter genes as the target gene [11]. The present work significantly extends the applicability of the strategy to modulate gene expression of a highly expressed endogenous gene, human albumin, and sets the stage for investigations of other endogenous genes that influence various biological functions such as differentiation, cell division, and cell death. Modulation of endogenous genes by aptamer-fused shRNA complements the existing inducible RNAi systems based on transcriptional regulation of shRNAs [6]. Unlike the transcriptional regulation of shRNAs, aptamer-fused shRNAs do not require large exogenous protein factors, providing a genetically compact package, decreasing the risk of immunogenic complications, and thus, making aptamer–shRNA constructs advantageous for applications in gene therapy and viral transduction.

RNA aptamer–small molecule interactions have been exploited to control gene expression in a variety of cell types [15–17]. However, very few aptamer-mediated gene regulations in mammalian cells have been reported. Werstuck and Green first demonstrated translation inhibition by aptamer–small molecule interaction within 5′ untranslated region (UTR) of mRNA in CHO cells [18]. More recently, Kim et al. engineered the theophylline aptamer to regulate splicing of pre-mRNA in HeLa cells [19]. However, both of these methods, as well as most other aptamer-based gene switches in other cell types, are designed to regulate genes in *cis*, allowing control of only exogenous genes. The notable exception is the yeast RNA antiswitches developed by Bayer and Smolke that function in *trans* [20]. However, targeting of endogenous genes by antiswitches has not been reported. To our knowledge, our work represents the first aptamer-based RNA gene switch to control an endogenous gene in any cell type.

In this study, we have shown that RNA–small molecule interaction can be used to modulate an endogenous gene expression via RNAi in mammalian cells. Molecular recognition capacity afforded by aptamers combined with the ability of RNAi to target arbitrary genes may lead to new biomedical applications of

RNAi. For example, RNA aptamers that bind to cell surface antigens have been fused to siRNAs to target siRNAs to specific cell types [21,22]. The aptamer-based RNA gene switches should also provide promising interface with other RNA-based decision-making circuits that operate in mammalian cells [23] to build intelligent gene therapeutic systems or engineered mammalian cellular factories.

Acknowledgments

This work was supported in part by National Institutes of Health grant (DK073901) awarded to A.R. and National Science Foundation grant (CBET 0755053) awarded to Y.Y. N.T. was supported through a Biotechnology Fellowship from the National Center for Biotechnology, Republic of Kazakhstan.

References

[1] G.J. Hannon, J.J. Rossi, Unlocking the potential of the human genome with RNA interference, Nature 431 (2004) 371–378.
[2] G. Meister, T. Tuschl, Mechanisms of gene silencing by double-stranded RNA, Nature 431 (2004) 343–349.
[3] R. Kumar, D.S. Conklin, V. Mittal, High-throughput selection of effective RNAi probes for gene silencing, Genome Res. 13 (2003) 2333–2340.
[4] A. Reynolds, D. Leake, Q. Boese, S. Scaringe, W.S. Marshall, A. Khvorova, Rational siRNA design for RNA interference, Nat. Biotechnol. 22 (2004) 326–330.
[5] K. Yoshinari, M. Miyagishi, K. Taira, Effects on RNAi of the tight structure, sequence and position of the targeted region, Nucleic Acids Res. 32 (2004) 691–699.
[6] M. Wiznerowicz, J. Szulc, D. Trono, Tuning silence: conditional systems for RNA interference, Nat. Methods 3 (2006) 682–688.
[7] V. Mikat, A. Heckel, Light-dependent RNA interference with nucleobase-caged siRNAs, RNA 13 (2007) 2341–2347.
[8] S. Shah, S. Rangarajan, S.H. Friedman, Light activated RNA interference, Angew. Chem. Int. Ed. 44 (2005) 1328–1332.
[9] R.A. Blidner, K.R. Svoboda, R.P. Hammer, W.T. Monroe, Photoinduced RNA interference using DMNPE-caged 2′-deoxy-2′-fluoro substituted nucleic acids in vitro and in vivo, Mol. Biosyst. 4 (2008) 431–440.
[10] Q.N. Nguyen, R.V. Chavli, J.T. Marques, P.G. Conrad, D. Wang, W.H. He, B.E. Belisle, A.G. Zhang, L.M. Pastor, F.R. Witney, M. Morris, F. Heitz, G. Divita, B.R.G. Williams, G.K. McMaster, Light controllable siRNAs regulate gene suppression and phenotypes in cells, Biochim. Biophys. Acta Biomembr. 1758 (2006) 394–403.
[11] C.-I. An, V.B. Trinh, Y. Yokobayashi, Artificial control of gene expression in mammalian cells by modulating RNA interference through aptamer–small molecule interaction, RNA 12 (2006) 710–716.
[12] R.D. Jenison, S.C. Gill, A. Pardi, B. Polisky, High-resolution molecular discrimination by RNA, Science 263 (1994) 1425–1429.
[13] G.R. Zimmermann, R.D. Jenison, C.L. Wick, J.P. Simorre, A. Pardi, Interlocking structural motifs mediate molecular discrimination by a theophylline-binding RNA, Nat. Struct. Biol. 4 (1997) 644–649.
[14] J.C.Y. Dunn, M.L. Yarmush, H.G. Koebe, R.G. Tompkins, Hepatocyte function and extracellular matrix geometry—long-term culture in a sandwich configuration, FASEB J. 3 (1989) 174–177.
[15] J.P. Gallivan, Toward reprogramming bacteria with small molecules and RNA, Curr. Opin. Chem. Biol. 11 (2007) 612–619.
[16] B. Suess, J.E. Weigand, Engineered riboswitches—overview, problems and trends, RNA Biol. 5 (2008) 1–6.
[17] M.N. Win, C.D. Smolke, RNA as a versatile and powerful platform for engineering genetic regulatory tools, Biotechnol. Genet. Eng. Rev. 24 (2007) 311–346.
[18] G. Werstuck, M.R. Green, Controlling gene expression in living cells through small molecule–RNA interactions, Science 282 (1998) 296–298.
[19] D.S. Kim, V. Gusti, K.J. Dery, R.K. Gaur, Ligand-induced sequestering of branch point sequence allows conditional control of splicing, BMC Mol. Biol. 9 (2008) 23.
[20] T.S. Bayer, C.D. Smolke, Programmable ligand-controlled riboregulators of eukaryotic gene expression, Nat. Biotechnol. 23 (2005) 337–343.
[21] T.C. Chu, K.Y. Twu, A.D. Ellington, M. Levy, Aptamer mediated siRNA delivery, Nucleic Acids Res. 34 (2006) e73.
[22] J.O. McNamara II, E.R. Andrechek, Y. Wang, K.D. Viles, R.E. Rempel, E. Gilboa, B.A. Sullenger, P.H. Giangrande, Cell type-specific delivery of siRNAs with aptamer–siRNA chimeras, Nat. Biotechnol. 24 (2006) 1005–1015.
[23] K. Rinaudo, L. Bleris, R. Maddamsetti, S. Subramanian, R. Weiss, Y. Benenson, A universal RNAi-based logic evaluator that operates in mammalian cells, Nat. Biotechnol. 25 (2007) 795–801.