

Lipid Nanoparticle-mediated siRNA Transfer Against PCTAIRE1/PCTK1/Cdk16 Inhibits *In Vivo* Cancer Growth

Teruki Yanagi^{1,2}, Kiyoshi Tachikawa³, Rachel Wilkie-Grantham^{1,3}, Asami Hishiki¹, Ko Nagai¹, Ellen Toyonaga¹, Pad Chivukula³ and Shu-ichi Matsuzawa^{1,4}

PCTAIRE1/CDK16/PCTK1 plays critical roles in cancer cell proliferation and antiapoptosis. To advance our previously published *in vitro* results with PCTAIRE1 silencing, we examined the *in vivo* therapeutic potential of this approach by using small interfering RNA (siRNA) encapsulated by lipid nanoparticles. Therapy experiments of PCTAIRE1 siRNA were performed using human HCT116 colorectal cancer cells and human A2058 melanoma cells. A single dose of PCTAIRE1 siRNA-lipid nanoparticles was found to be highly effective in reducing *in vivo* PCTAIRE1 expression for up to 4 days as assayed by immunoblotting. Therapy experiments were started 4 days after subcutaneous injection of cancer cells. Treatment with PCTAIRE1 siRNA-lipid nanoparticles (0.5 mg/kg RNA, twice a week) reduced tumor volume and weight significantly compared with the scramble-control group. Histopathological analysis (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) showed increased apoptosis of tumor cells treated with PCTAIRE1-siRNA. Overall, our results demonstrate that siRNA treatment targeting PCTAIRE1 is effective *in vivo*, suggesting that PCTAIRE1 siRNA-lipid nanoparticles might be a novel therapeutic approach against cancer cells.

Molecular Therapy—Nucleic Acids (2016) 5, e327; doi:10.1038/mtna.2016.40; published online 28 June 2016

Subject Category: siRNAs, shRNAs, and miRNAs; nanoparticles

Introduction

The PCTAIRE family is a branch of kinases related to the Cdk family that includes PCTAIRE1 (also known as PCTK1 and Cyclin-dependent kinase 16 (Cdk16)), PCTAIRE2 and PCTAIRE3.¹ PCTAIRE1 is broadly expressed throughout the body, with highest levels seen in the brain and testes.² PCTAIRE1 has a central kinase domain that shows amino acid sequence similarity to those of Cdk proteins, and this region is flanked by unique N-terminal and C-terminal domains.¹ The mechanisms responsible for PCTAIRE1 activation are unknown, but the finding that deletion of the N-terminal domain abolishes kinase activity *in vitro* suggests that this region is important and that it may bind an unknown cofactor or interact intramolecularly with the central kinase domain to promote active conformations of the catalytic domain.³ We recently discovered that PCTAIRE1 plays an indispensable role in cancer cell proliferation,^{4–6} as well as in antiapoptosis.⁷ We showed that PCTAIRE1-knockdown cancer cells promote late G2-mitotic arrest associated with defects in centrosome dynamics.⁴ Furthermore, PCTAIRE1 phosphorylates p27 at Ser10, which facilitates p27 degradation. PCTAIRE1 is overexpressed by a large number of human tumors, including colon, breast, prostate, and brain cancers, and also malignant melanomas.⁸ Thus, PCTAIRE1 is an attractive target for therapeutic interventions.

Small interfering RNA (siRNA) technology is an intriguing and powerful method of gene down-regulation that has been widely used to study gene function and to target drugs for discovery.^{9,10} Recently, several studies have found highly

efficient methods for the *in vivo* delivery of siRNA, especially by lipid nanoparticles (LNP) technologies.^{11,12}

Based on the known functions of PCTAIRE1 and the promising *in vitro* results of PCTAIRE1 silencing, we investigated the *in vivo* antitumor effects of PCTAIRE1 silencing using siRNA incorporated in LNP.

Results

Knockdown of PCTAIRE1 regulates HCT116 colorectal cancer cell growth and proliferation *in vitro*

To investigate the effect of PCTAIRE1 knockdown in colorectal cancer HCT116 cells, we performed siRNA experiments using two siRNAs that were previously shown to effectively target PCTAIRE1.^{4,5} *In vitro* siRNA experiments were performed with Lipofectamine RNAiMax transfection reagent, since lipid-enabled and unlocked nucleic acid-modified RNA (LUNAR) lipid delivery technology platform was unsuitable for *in vitro* use. Immunoblotting confirmed knockdown of protein levels by both of the PCTAIRE1-targeting siRNAs (see **Supplementary Figure S1a**). Next, human HCT116 colorectal cancer cells were treated with siRNAs targeting PCTAIRE1 versus negative control RNAs, and cell viability was assessed 3 days later. Cultures of PCTAIRE1 knockdown HCT116 cells showed reduced numbers of viable cells compared with control transfected cell cultures using ATP levels (see **Supplementary Figure S1b**). Similar results were obtained by direct cell-counting methods, showing a reduction in the numbers of viable cells in cultures of HCT116 cells within 3 days after PCTAIRE1 siRNA treatment (see **Supplementary Figure S1c**). Based on previous studies,^{4,8} the expression levels of

¹Sanford-Burnham-Prebys Medical Discovery Institute, La Jolla, California, USA; ²Current address: Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; ³Arcturus Therapeutics Inc., San Diego, California, USA; ⁴Current address: Department of Neurology, Kyoto University Graduate School of Medicine Kyoto, Japan. Correspondence: Shu-ichi Matsuzawa, Sanford-Burnham-Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, California 92037, USA. E-mail: smatsuzawa@SBPdiscovery.org

Received 20 February 2016; accepted 5 May 2016; published online 28 June 2016. doi:10.1038/mtna.2016.40

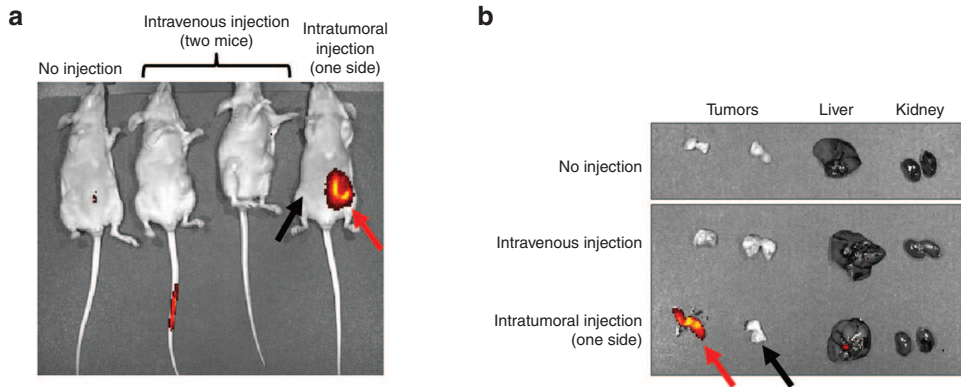


Figure 1 *In vivo* LNP-RNA distribution to tumors and major organs. HCT116 cells were inoculated to both flanks of nu/nu mice. At 2 weeks, siRNAs conjugated with Cy5 (Cy5-siRNAs: 10 μ g) were injected into the mice i.v. or were injected directly into the tumor using LUNAR lipid delivery technology platform. In the intratumoral injection, Cy5-siRNAs were injected into the tumor on one side of the body (red arrow), but not into the tumor on the other side of the body (black arrow). Cy5 signals were photographed by IVIS200, 6 hours after intratumoral or i.v. injection (**a**: whole body, **b**: extracted organs and tumors). LNP, lipid nanoparticles; siRNAs, small interfering RNAs.

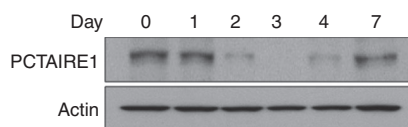


Figure 2 *In vivo* down-regulation of PCTAIRE1 by PCTAIRE1 siRNA. Immunoblot of lysates from tumor samples collected 0, 1, 2, 3, 4, and 7 days after a single administration of PCTAIRE1 siRNA (10 μ g, 0.5 mg/kg) incorporated in lipid nanoparticles (LUNAR lipid delivery technology platform). Representative data of two independent experiments.

c-Myc and tumor suppressor p27 were assessed by immunoblotting, showing that knockdown of PCTAIRE1 leads to the accumulation of p27 and down-regulation of c-Myc (see **Supplementary Figure S1a**).

Delivery of siRNA into xenograft tumor

In this study, LNP-siRNA (size: 40–50 nm) with modification of cationic lipids and polyethylene glycol (PEG) density were used as the delivery vehicle to target xenograft tumors. To examine whether siRNA could be effectively delivered into tumor cells, we used a nonsilencing siRNA tagged with the fluorochrome Cy5 in LNP complexes (LUNAR lipid delivery platform). Nude mice with HCT116 xenograft tumors at 15 days after subcutaneous inoculation of tumor cells were intratumorally or i.v. injected with 10 μ g LNP-conjugated nonsilencing siRNA/Cy5 (0.5 mg/kg). After 6 hours, tumors and major organs were harvested and examined for fluorescence using Xenogen IVIS 200 imaging system imaging. Cy5 signal was detected in tumors but not in major vital organs, suggesting that LNP-RNA was distributed mainly in the xenograft tumors (**Figure 1a, b**).

In vivo down-regulation of PCTAIRE1 by LNP-siRNA

Before initiating therapy experiments, we examined the ability of PCTAIRE1-targeted siRNA incorporated in LNP to down-regulate PCTAIRE1 *in vivo*. Nude mice bearing subcutaneous HCT116 xenograft tumors were injected with a single dose of PCTAIRE1 siRNA (#2)-LNP or scramble-control (0.50 mg/kg), and tumors were harvested 1, 2, 3, 4, and 7 days after

injection for immunoblot analysis to assess the level of PCTAIRE1 expression. The immunoblot analysis revealed a >70% reduction in PCTAIRE1 levels within 2 days, which persisted for at least 4 days (**Figure 2**). PCTAIRE1 expression began to return to basal levels by 7 days after a single treatment. In tumors treated with scramble-control RNA or untreated tumors, the expression levels of PCTAIRE1 were not reduced (see **Supplementary Figure S2**). Therefore, we selected twice-weekly administration of siRNA-LNP as the optimal dosing schedule for subsequent therapy experiments.

In vivo tumor growth was inhibited by LNP-mediated siRNA-transfer against PCTAIRE1

To assess effects from the delivery vehicle, we compared the untreated and LNP-scramble treated tumors. We generated HCT116 cell xenografts by injecting the cells (5×10^6 cells) subcutaneously into nu/nu mice. At day 4, LNP-siRNA intratumoral injections (scramble control RNA, 0.50 mg/kg) were started and were repeated four times (twice weekly). The extracted tumor weights were assessed at day 16, showing that vehicle was not harmful to HCT116 xenograft tumor growth (see **Supplementary Figure S3**). Next, in order to determine the effect of LNP-siRNA against PCTAIRE1 on HCT116 tumor growth *in vivo*, LNP-siRNA intratumoral injections (0.50 mg/kg) were started at day 4 after the cell injections and were repeated four times (twice weekly) as performed above (**Figure 3a**). We continued to measure the tumor volume in each animal every 3 days during the procedure and found that tumor growth was significantly inhibited in the group treated with siRNA targeting PCTAIRE1 compared with the scramble-control groups. In contrast to the large tumors produced by the control groups, the mice treated with siRNA against PCTAIRE1 produced small tumors (**Figure 3c,d,e**). Similar results were obtained in A2058 melanoma cells (see **Supplementary Figure S4**). Total protein lysates were isolated from the tumors of the two groups. The PCTAIRE1 protein was almost undetectable in the siRNA-treated samples (**Figure 3b**), suggesting that the inhibition of tumor growth in the siRNA-treated group was a consequence of PCTAIRE1 depletion in the tumors. Immunohistochemical analysis also showed increased expression of apoptotic markers (TUNEL assay)

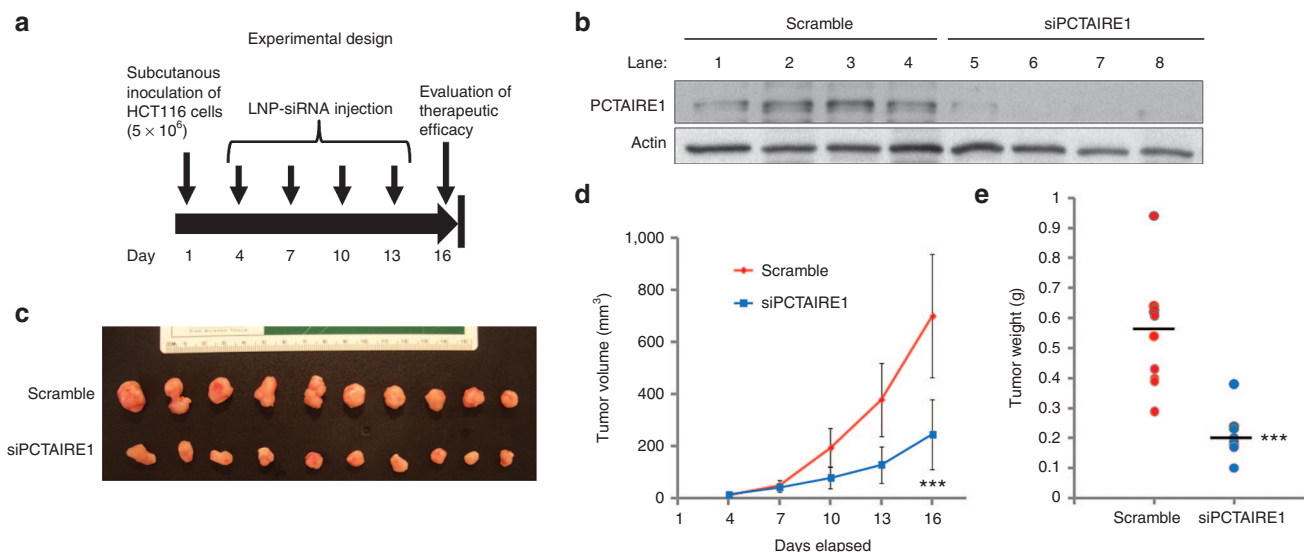


Figure 3 *In vivo* therapeutic efficacy of siRNA-mediated PCTAIRE1 knockdown in HCT116 cells. (a) Experimental design. HCT116 cells (5.0×10^6 cells resuspended in 200 μ l phosphate-buffered saline (PBS)) were subcutaneously injected into the flanks of 7-week-old female nu/nu mice. When tumor volumes reached 10–30 mm³ (Day 4), small interfering RNAs (siRNAs) targeting PCTAIRE1 or scramble-control with lipid nanoparticles (at a dose of 10 μ g siRNAs, 0.5 mg/kg) were injected into tumors twice a week ($n = 10$ mice per group) using LUNAR lipid delivery technology platform. All animals were sacrificed at Day 16 after four siRNA treatments. (b) Immunoblot of lysate from xenograft tumors collected after administration of control siRNA ($n = 4$) or PCTAIRE1-targeting ($n = 4$) siRNA. (c) All the extracted tumors were photographed. (d) Tumor volumes (mm³) were measured every 3 days. *** $P < 0.001$ by *t*-test. (e) Tumors recovered from sacrificed mice were weighed (mean = horizontal line). Differences between siPCTAIRE1 versus scramble-control groups were statistically significant (*** $P < 0.001$, by *t*-test).

in resected PCTAIRE1 knockdown tumor xenografts (Figure 4a–e). These results indicated that siRNA targeting PCTAIRE1 elicited a strong antitumor effect on colorectal cancer *in vivo*.

Discussion

In this study, we report that PCTAIRE1 down-regulation can suppress *in vivo* xenograft tumor growth, which also strongly suggests that this is a promising novel therapeutic approach for cancer cells. We have proposed that PCTAIRE1 may be a therapeutic target for various cancers, especially for malignant lesions where aberrant upregulation of this kinase has been detected.^{4–6} The suppression of growth and increased apoptosis were the most common consequences of PCTAIRE1 knockdown mediated by RNA interference (RNAi) in tumor cells. Interestingly, a recent study reported PCTAIRE1 silencing to be effective in inhibiting *in vitro* growth of medulloblastomas.⁸ Furthermore, we and another group have shown that PCTAIRE1 regulates cell division.¹³ Collectively, the above observations strongly suggest that cancer treatments targeting PCTAIRE1 may be effective against aggressive tumors in which a number of cell divisions take place. Several effects of PCTAIRE1 downregulation, such as the accumulation of tumor suppressor p27 and the down-regulation of c-Myc,⁸ have been observed previously in cancer cells. These phenomena may account for the observed growth suppression or apoptosis of HCT116 and A2058 xenograft tumors. Because of the pivotal role of PCTAIRE1 in many processes associated with cancer progression, interfering with its function may represent novel therapeutic opportunities. Various strategies for targeting PCTAIRE1 have been proposed and small molecule inhibitors that act as competitors for adenosine

triphosphate (ATP) binding at the catalytic site are being developed.⁸ RNAi is a potent and powerful strategy for regulating gene expression, and we and others using siRNA for down-regulating PCTAIRE1 *in vitro* along with the *in vivo* data presented in this article highlight its promise for clinical use.

RNAi delivery is crucial to realizing the potential of RNAi effects, and LNP have been shown to be highly effective in the delivery of siRNAs to the liver.^{12,14} For clinical use of siRNA, Coelho *et al.* reported the safety and efficacy of RNAi for targeting transthyretin amyloidosis, showing that *in vivo* siRNA therapy is effective in humans.¹⁵ Thus, liver-tissue targeted LNP-mediated siRNA therapy is promising for clinical development. Alternative approaches for knockdown in tumors or other organs have also been investigated.¹⁶ Specifically, the targeting of tumors could be enabled through the modulation of LNP (particle sizes or addition of targeting ligands). Targeted receptors for siRNA delivery to tumors include Her2, EGFR, EpCAM, and folate receptors.¹⁶ Reportedly, LNP consisting of antigen or antibody showed successful gene silencing *in vitro*. Furthermore, Kumar *et al.* reported the rational design of LNP-based siRNA delivery systems with an optimal safety and efficacy profile.¹⁷ To date, *in vivo* tumor-targeted siRNA delivery systems have not yet been well established; however, molecules conjugated to LNP could be developed for tumor-targeted systemic siRNA therapy (i.v. or orally) in the near future.

In summary, we have shown that PCTAIRE1 siRNA delivered using a lipid nanoparticle system is highly effective for down-regulating PCTAIRE1 expression *in vivo*. Furthermore, treatment with PCTAIRE1 siRNA was found to be effective in inhibiting cancer cell growth. These findings suggest that PCTAIRE1 siRNA could be a potent therapy in patients with tumors overexpressing PCTAIRE1. Our

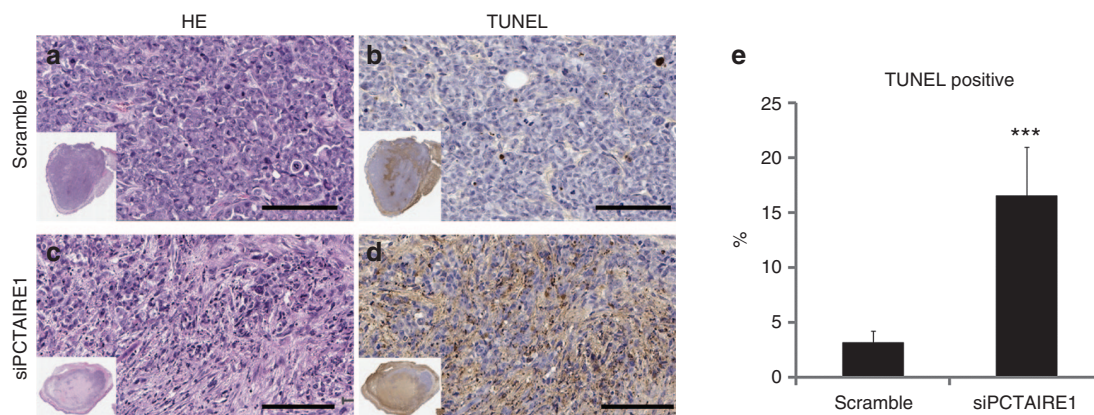


Figure 4 Gene knockdown of PCTAIRE1 leads to apoptosis of xenograft tumors. (a–d) Representative images of hematoxylin-eosin stain (a, c) and immunohistochemistry with TUNEL stain (b, d). (Inset: low magnification; Scale bar = 100 μ m). (e) The number of apoptotic cells (brown) were counted (Columns, mean percentage of TUNEL-positive cells, $n = 12$, determinations based on examination of 100 tumor cells; bars, SD). Four fields per slide and at least three slides per group (all from different animals) were counted. *** $P < 0.001$, by t -test.

previous study shows that PCTAIRE1 knockdown sensitizes cancer cells to tumor necrosis factor- α -family cytokines, including tumor necrosis factor- α -related apoptosis-inducing ligand.⁷ Thus, LNP-mediated *in vivo* PCTAIRE1 gene knockdown may have synergistic effects with tumor necrosis factor- α -family cytokines.

Materials and methods

Cell lines and cell culture. The human HCT116 colorectal cancer cell lines and human A2058 melanoma cells were purchased from the American Type Culture Collection. Cell lines were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. All cells were used within 6 months of continuous passage.

Preparation of siRNA nanoparticles. Using the proprietary LUNAR™ lipid delivery technology platform, Arcturus Therapeutics (San Diego, CA) created test articles containing PCTAIRE1 siRNA (sense: 5'-rG*rGrCrArArArGrCrArArGrCrUrCrArCrArGdT*dT-3'; antisense: 3'-rC*rUrGrUrGrArGrCrUrUrGrCrUrUrUrUrGrCrCdT*dT-5', wherein rN is RNA, dT is 2'-deoxy T, and * is phosphorothioate), nonsilencing control siRNA (sense: 5'-rC*rArArCrArArGrArGrArGrArGrCrArCrAr*dT*dT-3', antisense: 3'-rUrUrG*rGrUrGrCrUrCrUrCrArUrCrUrUrGrUrUrGdT*dT-5', wherein rN is RNA, dT is 2'-deoxy T, and * is phosphorothioate, the sequence is identical to PCTAIRE1-siRNA-2 from Life technologies), which was synthesized by Avetra Bioscience (Mountain View, CA). The LNPs were prepared by mixing appropriate volumes of lipids in ethanol with an aqueous phase containing siRNA duplexes, employing a Nanoassembler™ microfluidic device, followed by downstream processing. For the encapsulation of siRNA, the desired amount of RNA was dissolved in 5 mmol/l citric acid buffer of pH 3.5. Lipids at the desired molar ratio were dissolved in ethanol. The mol% ratio for the constituent lipids is 58% ATX (proprietary ionizable amino lipids), 7% DSPC (1,2-dioctadecanoyl-sn-glycerol-3-phosphocholine) (Avanti Polar Lipids, Alabaster, AL), 33.5% cholesterol (Avanti Polar Lipids), and 1.5% DMG-PEG (1,2-Dimyrystoyl-sn-glycerol, methoxypolyethylene glycol, PEG

chain molecular weight: 2000) (NOF America Corporation, White Plains, NY).^{17–20} At a flow ratio of 1:3 ethanol:aqueous phases, the solutions were combined in the microfluidic device (Precision Nano Systems, Vancouver, Canada) using two HPLC prep pumps (AZURA P 2.1L, Knauer, Berlin, Germany). The total combined flow rate was 12 ml/min per microfluidics chip. Anywhere from 1 to 4 microfluidics chips were utilized, in a custom unit for parallelization (Precision Nano Systems), allowing a variable throughput for different batch sizes. The microfluidics chips employ a herringbone micromixer for extremely quick mixing times, yielding high encapsulation and narrow particle size distribution.^{21,22} The mixed material was then diluted 3 \times with deionized water after leaving the micromixer outlet, reducing the ethanol content to 6.25%. The diluted LNP slurry was concentrated on a tangential flow filtration (TFF) with hollow fiber membranes (mPES Kros membranes, Spectrum Laboratories, Rancho Dominguez, CA), and then diafiltration was performed with modified Dulbecco's phosphate-buffered saline, without magnesium or calcium (HyClone, Logan, UT). A total of 10 diavolumes were exchanged, effectively removing the ethanol. Particle size was determined by dynamic light scattering (ZEN3600, Malvern Instruments, UK). Encapsulation efficiency was calculated by determining unencapsulated siRNA content by measuring the fluorescence upon the addition of RiboGreen (Molecular Probes, Eugene, OR) to the LNP slurry (F_i) and comparing this value to the total siRNA content obtained upon lysis of the LNPs by 1% Triton X-100 (F_t), where % encapsulation = $(F_t - F_i)/F_t \times 100$.

RNAi in vitro. For transient knockdown, cells were transfected with siRNA duplexes by a reverse transfection method using Lipofectamine RNAiMAX according to the manufacturer's instructions (Life Technologies, Carlsbad, CA).

Reagents and antibodies. Predesigned siRNA directed against human PCTAIRE1 (siRNA#1:1472, siRNA#2:1656) and negative scramble control (#1) were purchased from Life Technologies. Antibodies against PCTAIRE1 (rabbit: HPA001366, Sigma, St. Louis, MO), beta-actin (Sigma) and horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Health Care, Chicago, IL) were purchased from the indicated sources.

SDS-PAGE and immunoblotting. Cells were washed twice with phosphate-buffered saline (PBS) and harvested with radioimmunoprecipitation assay (RIPA) buffer composed of 20 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 0.1 mmol/l ethylenediaminetetraacetate (EDTA), 1% Nonidet P-40, 0.1% SDS, 5 mmol/l NaF and an EDTA-free complete protease cocktail tablet (Roche, Basel, Switzerland). Cells were left on ice for 20 minutes before being centrifuged at 14,000g for 10 minutes. The Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) was used to determine protein concentrations. Proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 4–15% gradient gels (Life Technologies) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 hour in Tris-buffered saline with 0.05% Tween-20 and 5% nonfat dry milk, and then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Membranes were rinsed three times in Tris-buffered saline with 0.05% Tween-20 and incubated with secondary HRP-conjugated antibodies for 1 hour at room temperature. An enhanced chemiluminescence method (GE Health Care) was used for detection.

In vivo imaging. After the injection of Cy5-conjugated siRNA, the mice were anesthetized and placed on the imaging stage of the IVIS apparatus in the abdominal (subcutaneous model) position. Images were collected from 1 to 20 minutes using the IVIS Imaging System (Xenogen, PerkinElmer, Waltham, MA). The Cy5 fluorescent signal was detected at a 615–665 nm excitation wavelength and a 695–770 nm emission wavelength using the IVIS 200.

Cell viability assays using ATP measurement. Cell Titer Glo (Promega, Fitchburg, WI) was used for cell viability estimation. Cells were plated at a density of 5,000 cells per well in 100 µl and cultured for 72 hours with or without treatment. The plates were then removed from the incubator and allowed to equilibrate to room temperature for ~10 minutes. Cell Titer Glo solution was added at 100 µl per well, and the plates were kept in the dark for 15 minutes before being assessed for luminescence with a luminometer (Luminoskan Ascent; Thermo Scientific) at a 1 second integration time per sample.

Cell growth assay. To measure cell growth rates, 2.0×10^5 cells were reverse-transfected with siRNAs targeting PCTAIRE1 or the scramble control using Lipofectoamin RNAiMax. After 3 days, the numbers of cells were counted.

Tumor xenograft experiments. All animal experiments were approved by the IACUC of the Sanford-Burnham-Prebys Medical Discovery Institute. Cancer cells (5.0×10^6) resuspended in 200 µl PBS were injected subcutaneously into the flanks of nu/nu mice. Tumor size was measured every 3 days in two dimensions using calipers and tumor volume was calculated using the following formula: (long axis \times short axis²)/2. The endpoint was set at when tumors grow >1000 mm³ (Day 16 in HCT116 xenograft and Day 21 in A2058 xenograft).

Immunohistochemistry. The detection of nuclei with fragmented DNA by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was

accomplished using the ApopTag Peroxidase In situ Apoptosis Detection Kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. To quantify TUNEL-positive cells, the number of brown-positive cells was counted in 12 random fields at $\times 200$ magnification.

Statistical analysis. Means and standard deviation (SD) were calculated statistically from three determinations. The data are expressed as mean \pm SD. The statistical significance of differences between various samples was determined by Student's t-test. $P < 0.05$ was considered significant.

Supplementary material

Figure S1. Knockdown of PCTAIRE1 diminishes HCT116 cell growth *in vitro*.

Figure S2. Scramble RNA does not affect the expression levels of PCTAIRE1 *in vivo*.

Figure S3. LUNAR lipid delivery platform is not harmful to xenograft tumors *in vivo*.

Figure S4. *In vivo* therapeutic efficacy of siRNA-mediated PCTAIRE1 knockdown in A2058 cells.

Acknowledgments T.Y. is supported by a postdoctoral fellowship research abroad from the Japan Society for the Promotion of Science and by research grants from the Kanagawa Foundation for the Promotion of Medical Science and from the Sumitomo Life Social Welfare Services Foundation. This work was supported by funding from Arcturus Therapeutics Inc. (San Diego, CA). Authors are employed by or have received funding from Arcturus Therapeutics.

- Cole, AR (2009). PCTK proteins: the forgotten brain kinases? *Neurosignals* 17: 288–297.
- Le Bouffant, F, Le Minter, P, Traiffort, E, Ruat, M and Sladeczek, F (2000). Multiple subcellular localizations of PCTAIRE-1 in brain. *Mol Cell Neurosci* 16: 388–395.
- Mikolovec, P, Sigl, R, Rauch, V, Hess, MW, Pfaller, K, Barisic, M et al. (2012). Cyclin-dependent kinase 16/PCTAIRE kinase 1 is activated by cyclin Y and is essential for spermatogenesis. *Mol Cell Biol* 32: 868–879.
- Yanagi, T, Krajewska, M, Matsuzawa, S and Reed, JC (2014). PCTAIRE1 phosphorylates p27 and regulates mitosis in cancer cells. *Cancer Res* 74: 5795–5807.
- Yanagi, T, Reed, JC and Matsuzawa, S (2014). PCTAIRE1 regulates p27 stability, apoptosis and tumor growth in malignant melanoma. *Oncoscience* 1: 624–633.
- Yanagi, T and Matsuzawa, S (2015). PCTAIRE1/PCTK1/CDK16: a new oncotarget? *Cell Cycle* 14: 463–464.
- Yanagi, T, Shi, R, Aza-Blanc, P, Reed, JC and Matsuzawa, S (2015). PCTAIRE1-knockdown sensitizes cancer cells to TNF family cytokines. *PLoS One* 10: e0119404.
- Ćwiek, P, Leni, Z, Salm, F, Dimitrova, V, Styp-Rekowska, B, Chiriano, G et al. (2015). RNA interference screening identifies a novel role for PCTK1/CDK16 in medulloblastoma with c-Myc amplification. *Oncotarget* 6: 116–129.
- Taberner, J, Shapiro, GI, LoRusso, PM, Cervantes, A, Schwartz, GK, Weiss, GJ et al. (2013). First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discov* 3: 406–417.
- Zimmermann, TS, Lee, AC, Akinc, A, Bramlage, B, Bumcrot, D, Fedoruk, MN et al. (2006). RNAi-mediated gene silencing in non-human primates. *Nature* 441: 111–114.
- Ozpolat, B, Sood, AK and Lopez-Berestein, G (2010). Nanomedicine based approaches for the delivery of siRNA in cancer. *J Intern Med* 267: 44–53.
- Wittrup, A and Lieberman, J (2015). Knocking down disease: a progress report on siRNA therapeutics. *Nat Rev Genet* 16: 543–552.
- Ivano, S, Satou, A, Matsumura, S, Sugiyama, N, Ishihama, Y and Toyoshima, F (2015). PCTK1 regulates integrin-dependent spindle orientation via protein kinase A regulatory subunit KAP0 and myosin X. *Mol Cell Biol* 35: 1197–1208.
- Judge, AD, Robbins, M, Tavakoli, I, Levi, J, Hu, L, Fronza, A et al. (2009). Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* 119: 661–673.
- Coelho, T, Adams, D, Silva, A, Lozeron, P, Hawkins, PN, Mant, T et al. (2013). Safety and efficacy of RNAi therapy for transthyretin amyloidosis. *N Engl J Med* 369: 819–829.

16. Lorenzer, C, Dirin, M, Winkler, AM, Baumann, V and Winkler, J (2015). Going beyond the liver: progress and challenges of targeted delivery of siRNA therapeutics. *J Control Release* **203**: 1–15.
17. Kumar, V, Qin, J, Jiang, Y, Duncan, RG, Brigham, B, Fishman, S et al. (2014). Shielding of lipid nanoparticles for siRNA delivery: Impact on physicochemical properties, cytokine induction, and efficacy. *Mol Ther Nucleic Acids* **3**: e210.
18. Mui, BL, Tam, YK, Jayaraman, M, Ansell, SM, Du, X, Tam, YY et al. (2013). Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles. *Mol Ther Nucleic Acids* **2**: e139.
19. Akinc, A, Querbes, W, De, S, Qin, J, Frank-Kamenetsky, M, Jayaprakash, KN et al. (2010). Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol Ther* **18**: 1357–1364.
20. Jayaraman, M, Ansell, SM, Mui, BL, Tam, YK, Chen, J, Du, X et al. (2012). Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo. *Angew Chem Int Ed Engl* **51**: 8529–8533.
21. Zhigaltsev, IV, Belliveau, N, Hafez, I, Leung, AK, Huft, J, Hansen, C et al. (2012). Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing. *Langmuir* **28**: 3633–3640.
22. Kastner, E, Kaur, R, Lowry, D, Moghaddam, B, Wilkinson, A and Perrie, Y (2014). High-throughput manufacturing of size-tuned liposomes by a new microfluidics method using enhanced statistical tools for characterization. *Int J Pharm* **477**: 361–368.



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>

© T Yanagi et al. (2016)

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (<http://www.nature.com/mtna>)