Targeted p21^{WAF1/CIP1} Activation by RNAa Inhibits Hepatocellular Carcinoma Cells

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RNA activation (RNAa) is a mechanism of gene activation triggered by promoter-targeted small double-stranded RNA (dsRNA), also known as small activating RNA (saRNA). p21^{WAF1/CIP1} (p21) is a putative tumor suppressor gene due to its role as a key negative regulator of the cell cycle and cell proliferation. It is frequently downregulated in cancer including hepatocellular carcinoma (HCC), but is rarely mutated or deleted, making it an ideal target for RNAa-based overexpression to restore its tumor suppressor function. In the present study, we investigated the antigrowth effects of p21 RNAa in HCC cells. Transfection of a p21 saRNA (dsP21-322) into HepG2 and Hep3B cells significantly induced the expression of p21 at both the mRNA and protein levels, and inhibited cell proliferation and survival. Further analysis of dsP21-322 transfected cells revealed that dsP21-322 arrested the cell cycle at the G₀/G₁ phase in HepG2 cells but at G₂/M phase in Hep3B cells which lack functional p53 and Rb genes, and induced both early and late stage apoptosis by activating caspase 3 in both cell lines. These results demonstrated that RNAa of p21 has *in vitro* antigrowth effects on HCC cells via impeding cell cycle progression and inducing apoptotic cell death. This study suggests that targeted activation of p21 by RNAa may be explored as a novel therapy for the treatment of HCC.

Introduction

HEPATOCELLULAR CARCINOMA (HCC), hepatic tumor originated from liver, is the fifth most common cancer worldwide and the third most common cause of cancer mortality (El-Serag and Rudolph, 2007). According to American Cancer Society, 28,720 new cases of liver cancer including intrahepatic bile duct cancer are expected to occur and an estimated 20,550 liver cancer deaths are expected in United States in 2012 (ACS, 2012). The overall prognosis for HCC is poor, with a 5-year survival rate of about 7% despite treatment (Carr, 2004). Early liver cancer can be treated by surgical resection or liver transplantation, but with frequent recurrences. For advanced disease, treatment options are very limited (Carr, 2004; Hwang, 2006).

A number of genes have been identified as tumor suppressor genes for HCC, such as p53, Rb, p21, p16, p15, p27, and RASSF1A (reviewed in Martin and Dufour, 2008). Tumor suppressor genes that are functionally silenced rather than mutated or deleted offer the prospect of reactivation for the exploitation of their natural tumor suppressor function in cancer treatment. Both *in vitro* and *in vivo* studies have shown that enforced expression of these tumor suppressor genes leads to inhibited tumor cell

growth and reversal of tumor phenotypes in HCC cells (Guan et al., 2007; Xue et al., 2007). These studies provide rationale and optimism for the reactivation of tumor suppressor genes in treating cancer.

The p53/p21 pathway is a major fail-safe mechanism to counter uncontrolled cell proliferation. To prevent damaged cells from proliferating or undergoing oncogenic transformation, activation of p53 induces cell growth arrest, cellular senescence, differentiation, DNA repair, or apoptosis (reviewed in Beraza and Trautwein, 2007). p21^{WAF1/CIP1} (p21), located on chromosome 6p21.2, is an immediate downstream effector of p53 (el-Deiry et al., 1994) and acts as a broad-acting cyclin-dependent kinase (CDK) inhibitor. Upon exposed to radiation and other DNA-damaging agents, cells increase their p21 production, predominantly leading to cell cycle arrest at the G₁ checkpoint, which allows time for DNA repair before S phase entry. p21 also interacts with the DNA replication factor proliferating cell nuclear antigen, exerting additional control of cell-cycle progression, DNA replication and repair of damaged DNA (Waga et al., 1994).

p21 generally expresses low in liver tumor tissues compared to their normal counterparts (Furutani et al., 1997; Hui et al., 1997). Ectopic expression of p21 by adenoviral vectors carrying a p21 transgene has been shown to inhibit tumor

growth both *in vitro* and *in vivo* (Eastham et al., 1995; Wu et al., 1998; Teraishi et al., 2003), and to induce apoptosis (Wu et al., 1998; Teraishi et al., 2003). Many chemo-preventive and therapeutic agents exert an antitumor effect via activating p21 such as histone deacetylase (HDAC) inhibitors and phytochemicals (Yamamoto et al., 1998; Liu et al., 2007; Deming et al., 2008; Hou et al., 2008; Kosakowska-Cholody et al., 2008). For example, p21 has been shown to mediate the apoptotic effects of HDAC inhibitor 4-phenylbutyrate and Trichostatin A on hepatocellular carcinoma (Hep3B) cells (Svechnikova et al., 2007). In an animal testing of gene therapy, when p21 gene was delivered with the murine cytokine gene granulocyte-macrophage colony-stimulating factor into hepatoma-bearing mice, the combined administration remarkably inhibited the growth of subcutaneously transplanted hepatoma cells and significantly increased the survival rate of tumor-bearing mice (Liu et al., 2003).

Small RNA molecules that trigger RNA interference (RNAi) are a new class of promising drugs that are under active development due to their high potency and specificity in silencing disease genes, and low toxicity and relative small molecular size (Dykxhoorn et al., 2006). Despite considerable obstacles in delivering small RNA drugs to target tissues in the body, lipid-based formulations, the best developed class of small RNA delivery agents, have been found to be readily taken up by liver cells when administered systemically (Akinc et al., 2009; Vaishnaw et al., 2010; Watts and Corey, 2011). The liver thus represents an ideal target organ for small RNA based therapeutics. However, RNAi therapy can only offer antagonism of specific molecular targets for disease treatment; a method that could provide agonism of specific genes, such as tumor suppressors, may offer similar therapeutic benefits with expanded drugable targets. RNA activation (RNAa), triggered also by small RNAs that target gene promoters, is a gene regulation mechanism that exerts an effect opposite to that of RNAi (Li et al., 2006; Janowski et al., 2007; Chen et al., 2008; Huang et al., 2010; Matsui et al., 2010; Portnoy et al., 2011). Previous studies have shown that RNAa is able to induce robust and prolonged expression of a number of tumor suppressor genes including p21, leading to *in vitro* and *in vivo* anti-growth effects on different tumor cells (Chen et al., 2008; Huang et al., 2010; Junxia et al., 2010; Li et al., 2006; Place et al., 2012; Qin et al., 2012; Wang et al., 2010; Wei et al., 2010; Wu et al., 2011). p21 gene is rarely mutated or deleted in HCC (Furutani et al., 1997; Stein et al., 1998), presenting itself as an ideal target for RNAa-mediated overexpression to restore its tumor suppressor function. In this regard, Wu et al. recently reported that reactivation of the p21 gene by RNAa in several HCC cell lines inhibited cell proliferation/survival and induced apoptotic cell death, providing initial evidence that p21 RNAa may be a new strategy for inhibiting HCC (Wu et al., 2011).

In the present study, we investigated *in vitro* antitumor effects of a p21 small activating RNA (saRNA) we previously identified on two commonly used HCC cell lines including HepG2 and Hep3B and explored the underlying mechanisms. Our results show that p21 saRNA was able to induce p21 expression in HCC cells and result in inhibition of cell proliferation and survival, arrest of the cell cycle, and induction of apoptosis.

Materials and Methods

Double-stranded RNAs

The design and synthesis of double-stranded RNAs (dsRNAs) were carried out as previously described (Li et al., 2006). A 21-nucleotide (nt) dsRNA targeting the p21 promoter at position-322 relative to its transcription start site (dsP21-322) was used to activate p21 expression. Control dsRNAs were similarly designed and synthesized. All dsRNA sequences are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/nat).

Cell Culture and Transfection

Hep3B and HepG2 are HCC lines obtained from American Type Culture Collection. The cells were maintained in Eagle's minimum essential medium and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Before transfection, cells were plated in growth medium without antibiotics at a density of 50%–60% in 6-well plates. dsRNA was transfected at a concentration of 50 nmol/L using Lipofectamine RNAiMax reagent (Life Technologies) and the reverse transcription method according to the manufacture's instruction. The transfected cells were harvested after 72 hours.

RNA Isolation, reverse transcription–polymerase chain reaction

Total cellular RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and 1 µg of the RNA was used for cDNA synthesis using the M-MLV Reverse Transcriptase system (Promega) and oligo(dT) primers. The resulted cDNA was amplified by both regular reverse transcription–polymerase chain reaction (RT-PCR) and real-time RT-PCR using gene-specific primers in conjunction with Power SYBR[®] Green PCR Master Mix (Life Technologies). All primer sequences are listed in Supplementary Table S1.

Western blotting assay

Cells were washed with phosphate buffered saline (PBS) and lysed with radio-immunoprecipitation assay buffer (Thermo Scientific). The cell lysates were centrifuged for 10 minutes at 4°C and supernatants were collected. Protein concentrations were determined by the Bradford assay. The equivalent amounts of proteins were separated by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene fluoride membranes for overnight at 4°C. The resulting blots were blocked in 5% nonfat dry milk and probed with a p21 antibody (Cell Signaling, 1:2,000 dilution for HepG2; 1:1,000 for Hep3B), Caspase-3 (Cell Signaling, 1:1,000 dilution), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling, 1:750 dilution for HepG2; 1:1,000 for Hep3B). The blots were incubated overnight at 4°C for p21 and caspase-3 and 1 hour for PARP. After the primary antibody, blots were incubated with an anti-rabbit horseradish peroxidase-linked immunoglobulin G (IgG) secondary antibody from Jackson ImmunoResearch Lab Inc. (1:2,500 dilution). For protein loading control, blots were probed with α -tubulin (1:15,000) and incubated with an anti-mouse horseradish peroxidase-linked IgG secondary

antibody from Jackson ImmunoResearch Lab Inc. (1:5,000). Then the blots were detected by chemiluminescence using the enhanced chemiluminescence detection system from Thermo Scientific.

Cell proliferation assay

Cell proliferation was detected using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega). Cells were transfected with dsRNA at a final concentration of 50 nM in a 6-well plate using Lipofectamine RNAiMax (Invitrogen) by following the reverse transfection protocol provided with the product. The following day, the cells were seeded in a 96-well plate with cell density of 10,000 cells per well for HepG2 and 5,000 cells per well for Hep3B. The plates were then incubated for 5 days and cell growth was measured at 5 time points from day 1 to day 5 following transfection. At each time point, 10 μ L of CellTiter 96 Aqueous One Solution was added to each well and incubated for 1 hour at 37°C. After 1 hour, absorbance was measured on an ELISA plate reader at 490 nm.

Apoptosis assay

Apoptosis assay was conducted using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen). Both HepG2 and Hep3B cells were trypsinized and centrifuged at 1,000g for 5 minutes at 4°C and washed twice with cold PBS buffer. Then the cells were resuspended in 100 μ L of 1 $\times$ binding buffer from the kit and 5 μ L of PE Annexin V and 5 μ L 7-amino-actinomycin (7-AAD) according to the product's protocol. Samples were analyzed on a FACSCalibur flow cytometer. The experiments were repeated at least 3 times, and each time 10,000 events were sampled for each sample. The data were analyzed using WinMDI (<http://facs.scripps.edu/software.html>).

Flow cytometry

Transfected HepG2 and Hep3B cells were trypsinized and centrifuged at 1,000g for 5 minutes at 4°C and washed with cold PBS buffer. The cells were resuspended with 100 μ L of cold 70% ethanol and incubated overnight at 4°C. The cells were centrifuged at 8,000 rpm for 1 minute and resuspended in 100 μ L Krishan Buffer (0.1% sodium citrate, 0.03% Triton X-100, 0.02 mg/mL RNase A, 0.05 mg/mL propidium iodide) and incubated for 1 hour at 4°C. After 1-hour incubation, 100 μ L of cells were transferred into a 5-mL tube and added 400 μ L of PBS and incubated another 1 hour at 4°C. Then the stained cells were immediately analyzed on a FACSCalibur flow cytometer. The experiments were repeated at least 3 times and, and 10,000 events were analyzed for each sample. The data was analyzed using Modfit LT program (Verity).

Clonogenic survival assay

HepG2 and Hep3B cells were transfected in 6-well plates at 50%–60% density. The following day, the cells were seeded in a 6-well plate at a cell density of 2,500 cells per well and were maintained for 12 days with the culture medium changed every 3 days. At day 12, after removing the medium and washing the cells with PBS, the resulted cell colonies were stained with 0.05% crystal violet for 10 minutes at room temperature and photographed.

Results

p21 saRNA induced p21 expression in HCC cells

To test whether p21 can be activated by RNAa in HCC cells, we used a previously identified p21 saRNA to transfect 2 commonly used HCC cell lines including HepG2 and Hep3B (Fig. 1A, B). The saRNA (dsP21-322) targets the p21 promoter at position –322 relative to its transcription start site. To exclude the possibility that p21 activation is resulted from an off-target effect, we included 3 types of control dsRNA sequences in our transfection experiments: a nonspecific dsRNA which does not have significant homology with any known human sequences, a scrambled dsRNA which was generated by scrambling dsP21-322 (Scrambled), and a mismatched dsRNA which was derived by mutating the last 5 bases of dsP21-322 (Mismatched). dsP21-322 or the control dsRNAs were transfected into HepG2 and Hep3B cells at a concentration of 50 nM and p21 expression levels was evaluated 72 hours later. p21 mRNA expression was significantly induced by dsP21-322 in both cell lines as demonstrated by quantitative RT-PCR analysis (Fig. 1A). dsP21-322 transfection caused a 2.8 ($P < 0.01$) and 2.4-fold ($P < 0.01$) increase in p21 mRNA levels compared to mock transfection in HepG2 and Hep3B cells respectively (Fig. 1B). None of the control dsRNAs caused significant induction of p21 mRNA levels (Fig. 1A). The induction of p21 expression at the protein level by dsP21-322 was verified by western blotting analysis. As shown in Fig. 1B, p21 protein expression was also induced by dsP21-322 (Fig. 1B). Together, the expression

FIG. 1. Induction of p21 expression by p21 saRNA in HCC cells. HepG2 and Hep3B were transfected with 50 nM of the indicated double-stranded RNA (dsRNA) or mock transfected for 72 hours. (A, B) mRNA expression levels of p21 were detected by real-time reverse transcription-polymerase chain reaction and plotted as fold changes relative to mock transfection after normalizing to that of GAPDH. Results are means $\pm$ standard deviation (SD) of 2 independent experiments. *, $P < 0.05$; ** $P < 0.01$ versus mock. (B) Proteins were isolated from the transfected cells and subject to sodium dodecyl sulfate-polyacrylamide gel and western blotting analysis using a p21 antibody. Protein levels of α -tubulin were also detected as a protein loading control.

analyses reveal that dsP21-322 consistently induces sequence-specific p21 gene expression in two HCC cell lines.

p21 saRNA inhibited HCC cell proliferation and survival

Ectopic expression of p21 or induction of endogenous p21 by its saRNA is known to cause cell growth inhibition in different cancer cells including HCC cells (Qin and Ng, 2001;

Wu et al., 2011). Indeed, transfection of dsP21-322 into both HepG2 and Hep3B cells inhibited cell growth as demonstrated by decreased cell density and increased dead cells evident 48 hours later (Fig. 2). To quantitatively measure cell proliferation rates, we performed cell proliferation assays with HepG2 and Hep3B cells. As illustrated in Fig.3A and B, both HepG2 and Hep3B cells transfected with dsP21-322 exhibited progressive retarded growth compared to mock transfection, while cells transfected with control dsRNAs

FIG. 2. p21 small activating RNA (saRNA) induced changes in cell morphology associated with growth inhibition. HepG2 and Hep3B cells were mock transfected or transfected with the indicated dsRNA at 50 nM for 72 hours. Cell images were taken at 100×magnification by phase contrast microscopy.

FIG. 3. p21 saRNA inhibits proliferation and survival of HCC cells. **(A, B)** Cells were transfected with 50 nM of indicated dsRNAs or mock transfected and plated in 96-well plates. Viable cells were measured from day 1 to 5 following transfection using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Results represent the means $\pm$ SD of 3 independent experiments and plotted as percentage relative to mock transfection. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus mock transfection. **(C, D)** Cells were transfected with 10 nM of the indicated saRNA or control dsRNA. The transfected cells were seeded 24 hours following transfection in 6-well plates at a density of 2500 cells per well. Colony formation was analyzed on day 12 by staining the cells with crystal violet.

maintained similar growth as mock transfected cells. By day 5, dsP21-322 transfected HepG2 and Hep3B cells exhibited a 29.4% ($P < 0.01$) and 33.4% ($P < 0.05$) reduction in viable cells respectively compared to mock treatment (Fig. 3A, B).

To further assess the effect of dsP21-322 on HCC cell survival, we performed clonogenic survival assay in dsP21-322 transfected HepG2 and Hep3B cells. As illustrated in Fig. 3C and D, mock and control dsRNA transfected HepG2 and Hep3B cells formed numerous colonies 12 days following transfection, whereas dsP21-322 transfected cells formed colonies significantly lower in number and smaller in size. These results indicate that p21 induction by RNAi

can dramatically inhibit the proliferation and survival of HCC cells.

p21 saRNA induced cell cycle arrest in HCC cells

p21 is a CDK inhibitor known to play an important role in growth arrest after DNA damage and overexpression of p21 can lead to cell cycle arrest at G₀/G₁ or G₂/M phase (Gartel and Tyner, 2002; Niculescu et al., 1998). To determine whether p21 induction by RNAi in HCC cells have an effect on cell cycle progression, we analyzed DNA content in cells transfected for 72 hours by staining the cells with propidium iodide

FIG. 4. p21 saRNA induces cell cycle arrest in HCC cells. **(A, B)** HepG2 **(A)** and Hep3B **(B)** cells were mock transfected or transfected with the indicated dsRNA for 72 hours. The transfected cells were stained with propidium iodide and DNA contents were measured by flow cytometry. The percentage of cells in G₀/G₁, S, and G₂/M phases were calculated using the ModFit program after excluding sub-G₀/G₁ cells. Results represent the means $\pm$ SD of 3 independent experiments and graphed as percentage. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus mock.

followed by flow cytometry to measure DNA content. In HepG2 cells transfected with dsP21-322, a significant increase ($P < 0.001$) in the G_0/G_1 population with corresponding decrease in S and G_2/M phase populations was observed compared to mock transfected cells (Fig. 4A). In contrast, dsP21-322 transfection in Hep3B cells which lacks functional p53 and RB genes (Puisieux et al., 1993; Qin and Ng, 2001) caused a significant increase ($P < 0.001$) in the G_2/M phase with corresponding decrease in G_0/G_1 and S phases (Fig. 4B). These results are consistent with the fact that p21 is a negative regulator of cell cycle progression and can cause cell cycle arrest at G_0/G_1 and/or G_2/M phase (Gartel et al., 1996; Svechnikova et al., 2007) and suggest that p21 activation by its saRNA causes predictable changes in cell cycle profiles.

p21 saRNA induced apoptosis in HCC cells

p21 is known to either induce or antagonize apoptosis depending on genetic makeup of cells (Gartel and Tyner, 2002). To determine the effects of p21 activation on apoptosis in HCC cells, we performed Annexin V staining in conjunction with 7-Amino-Actinomycin (7-AAD) staining followed by flow cytometry to detect apoptotic cells. As shown in Fig. 5A

and B, dsP21-322 transfection caused an increase in both early and late apoptotic cells in both cell lines. This result suggests that p21 activation by saRNA induces apoptosis in HCC cells.

To further confirm apoptosis induced by dsP21-322, we detected procaspase-3 levels in saRNA transfected cells. We observed a decrease in procaspase-3 in both HepG2 and Hep3B cells following dsP21-322 transfection (Fig. 5C), suggesting the conversion of procaspase-3 to active caspase 3, a major executioner protease in apoptotic cell death. In further support, we also detected the cleavage of PARP, a downstream substrate of caspase 3. Cleaved PARP was observed only in HepG2 and Hep3B cells transfected with dsP21-322, but not in any control dsRNA transfected cells (Fig. 5C). These results indicate that p21 activation by dsP21-322 enhances apoptosis due to activation of caspase 3.

Discussion

Promoter-targeted dsRNAs have been shown to be able to alter the transcriptional output of target genes either positively (RNAa) (Janowski et al., 2007; Li et al., 2006) and negatively (transcriptional gene silencing, or TGS) (Jiang et al.,

FIG. 5. p21 saRNA induces apoptosis in HCC cells. **(A)** HepG2 and Hep3B cells were transfected with the indicated dsRNA at a final concentration of 50 nM or mock transfected for 72 hours. The transfected cells were double stained with Annexin V and 7-AAD and analyzed by flow cytometry. Representative log fluorescence dot plots of Annexin V-fluorescein (x-axis) and 7-AAD (y-axis) stained cells are shown in **(A)**. Viable cell population is in the lower left quadrant (Annexin V-/7-AAD-), early apoptotic cells in the lower right quadrant (Annexin V+/7-AAD-), and late apoptotic cells in the upper right quadrant (Annexin V+/7-AAD+). Numbers represent the percentage of cells in each quadrant. **(B)** Histogram plotting of means $\pm$ SD of results **(A)** from 3 independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus mock. **(C)** Cells were transfected as in **(A)**. Protein was isolated from the transfected cells and subject to immunoblotting assay to detect the levels of procaspase 3, pro-poly(ADP-ribose) polymerase (PARP), and cleaved PARP. Protein levels of α -tubulin was also detected as a control for protein loading.

2012; Morris et al., 2004). Despite the exact molecular mechanisms underlying RNAa and TGS remain elusive, such strategies may add to our arsenal against disease including cancer by offering expanded therapeutic targets not drugable by conventional therapies. We previously reported the identification of a p21 saRNA (dsP21-322) that activates p21 expression and inhibits cancer cell growth (Li et al., 2006; Chen et al., 2008; Place et al., 2012). To further explore the therapeutic potential of this saRNA in HCC, we performed *in vitro* assays to characterize its antitumor properties in HCC cells. Our findings reveal that dsP21-322 is a universal p21 activator and a tumor cell growth suppressor. It induces endogenous p21 expression to exert an antigrowth effect through arresting the cell cycle at either the G₀/G₁ or G₂/M phase depending on the genetic makeup of target cells, and inducing apoptotic cell death.

Dual functions for p21 in regulating apoptosis have been observed depending on its subcellular localization and cellular contexts (Gartel and Tyner, 2002; Dong et al., 2005). In certain cell types, p21 may possess anti-apoptotic activity by binding to and degrading caspase 3 to protect cells from apoptotic cell death and to prevent further cell damage (Gorospe et al., 1997; Gartel and Tyner, 2002; Willenbring et al., 2008). On the other hand, p53-dependent or independent proapoptotic function of p21 has been demonstrated in many other cells, although the detailed mechanisms remain not fully understood (Gartel and Tyner, 2002). Some studies have suggested that p21 induces apoptosis through upregulating pro-apoptotic protein Bax (Kang et al., 1999) or activating caspase 3 protein (Lincet et al., 2000). In particular, RNAa of p21 by dsP21-322 has been shown to induce apoptosis by suppressing Bcl-xL in HCC (Wu et al., 2011) and bladder cancer cells (Yang et al., 2008). In consistency with this, we found that dsP21-322 activates caspase 3 and induces both early and late stage apoptosis in both HepG2 and Hep3B cells.

RNAa is able to induce the expression of an endogenous gene resulting in the restoration of a functional protein that causes predictable changes in cell phenotypes and modulation of downstream genes (Wang et al., 2010; Wang et al., 2012). In consistency with this, we observed that in HepG2 cells in which p53 and Rb genes are intact (Puisieux et al., 1993), dsP21-322 mainly caused a cell cycle arrest at the G₀/G₁ phase; whereas in Hep3B cells that do not have functional p53 and Rb, dsP21-322 arrested the cell cycle at the G₂/M phase. This observation is in agreement with a study in which ectopic expression of p21 by a vector in Hep3B cells caused a similar G₂/M phase arrest (Qin and Ng, 2001). Additionally, ectopic expression of p21 also induced apoptotic cell death and inhibition of cell proliferation and survival in Hep3B cells (Qin and Ng, 2001). Therefore, results from RNAa of p21 recapitulated those induced by vector-mediated p21 overexpression, suggesting that RNAa restored a functional p21 protein.

Lipid based formulations are the most promising delivery agents for siRNA and many have advanced into clinical trials. In preclinical studies, it has been demonstrated that systemically delivered siRNA in lipid formulation has the greatest bioavailability in the liver (Akinc et al., 2009; Watts and Corey, 2011). This makes liver disease the most ideal indication for small RNA-based therapies. In a recent study, we reported that lipid nanoparticle formulated and chemically modified dsP21-322 possesses improved pharmaceutical properties and when delivered intratumorally, successfully

activated p21 expression *in vivo* and inhibited tumor growth in a prostate cancer xenograft model (Place et al., 2012). *In vitro* cell transfection studies have revealed that RNAa activity persist longer than RNAi (Li et al., 2006; Place et al., 2010; Wang et al., 2012) and this unique kinetics has been attributed to epigenetic changes triggered by saRNA which are considered inheritable when cells divide (Portnoy et al., 2011). Although it remains to be determined whether RNAa also has prolonged activity *in vivo*, its unique capacity in augmenting gene production, in combination of lipid based delivery platforms, may provide a novel therapeutic option for liver cancer.

Taken together, we demonstrated that the activation of p21 by a promoter-targeted p21 saRNA significantly inhibits HCC cell proliferation and survival. This effect is associated with p21's role of enhancing apoptotic cell death and arresting the cell cycle. Our study suggests that targeted activation of p21 has a potential therapeutic application for HCC disease and warrants further testing in a preclinical setting.

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Author Disclosure Statement

L.C.L. is a named inventor of pending patent applications related to RNAa, which have been filed with the University of California San Francisco and licensed to Alnylam Pharmaceuticals.

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