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The RNA Activator ds-p21 Potentiates the Cytotoxicity Induced by Fludarabine in Dohh2 Cells

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Recently, it has been reported that, in several tumor cell lines, short double-stranded RNAs tailored for promoter regions of specific genes are able to activate their transcription. Such molecules (named RNA activators) act opposite to other double-stranded RNA molecules (named RNA inhibitors) in that the overexpression instead of underexpression of a given gene is triggered. In Dohh2 non-Hodgkin lymphoma cells, the transcriptional repressor BCL6, which negatively controls both p53 and p21, is overexpressed, so that the cells can escape the check point governed by p53 and proliferate. The aim of this work was to investigate whether the RNA activator p21 can represent a tool to circumvent the transcriptional control of BCL6 and induce the blockage of cell proliferation in Dohh2 non-Hodgkin lymphoma cells. For that, Dohh2 cells were transfected with either a control RNA activator (ds-NC) or an RNA activator specific for human p21 promoter (ds-p21). At various time points after transfection, the cells were collected and p21 was measured. Dohh2 cells transfected with ds-p21 showed a slight but significant overexpression of p21 at both mRNA and protein levels. Nonetheless, cell proliferation, cell cycle, and apoptosis were not significantly modified. In contrast, the exposure of Dohh2 cells transfected with ds-p21 to fludarabine potentiates the cytotoxicity of the drug, suggesting the RNA activator p21 complements the fludarabine-dependent cell death pathways.

Introduction

Tumor cells have a high proliferation rate mostly because of cell cycle deregulation. Cell cycle is controlled by a complex network of cyclins, and cyclin-dependent kinases are the core of this network. P21 being an inhibitor of cyclin-dependent kinases is a negative regulator of cell cycle (Harper et al., 1993) and as such acts as a potential tumor suppressor gene. In normal cells, p21 is under p53 control. In the presence of DNA damage, the axis p53-p21 is activated and cells are blocked at G1/S to be repaired or addressed toward cell death. In tumor cells, p53 is often mutated, so that cells escape the G1 check point and proliferate. Dohh2 lymphoma cells have a functional p53 negatively controlled by BCL6, which also inhibits p21 transcription. These positive and negative feedbacks of p21 expression suggest that a tightly controlled expression of the tumor suppressor gene p21 is functional to Dohh2 cell proliferation. Hypothetically, the upregulation of p21 uncoupled from BCL6 and p53 is expected to restore the tumor suppressor activity in Dohh2 cells.

Recently, it has been shown that the transfection of a double-stranded RNA (ds-RNA) of 21 nt specific for the promoter region of p21 is able to induce the overexpression of p21 at transcriptional and translational levels in several tumor cell lines (Janowski et al., 2006b; Chen et al., 2008; Yang et al., 2008; Whitson et al., 2009). This process, named RNA activation (RNAa), acts opposite to RNA interference (RNAi) (Poliseno et al., 2004) in that the overexpression instead of underexpression of a given gene is triggered. The existence of RNA activators derives from several observations. A short RNA isolated in neuronal staminal cells is able to induce neural differentiation in adult staminal cells by targeting NRSE/RE1 (neuronal restrictive elements) (Kuwabara et al., 2004). A microRNA specific for hepatocytes (miR-122) has been demonstrated to increase viral replication targeting a noncoding region at the 5′ of viral genome (Jopling et al., 2005); moreover, another microRNA (miR-373) was demonstrated to induce expression of genes with complementary promoter sequence (Place et al., 2008). Recently, RNAa was shown to be a mechanism conserved in mammalian cells (Huang et al., 2010). The main differences between RNA activators and RNA inhibitors are the following: first of all, RNA activator recognizes the promoter of the gene transcriptionally activated, whereas RNAi recognizes mRNA regions such as the 3′UTR. Second, RNA inhibitors act very rapidly after transfection, whereas RNA activators act at later times and

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their effect is persistent. Third, RNA activators require the presence of a 21-nt ds-RNA perfectly complementary at the 5′ region (seed region) to DNA target (Janowski et al., 2006b). Finally, the proteins necessary for functioning of RNAa and RNAi are different: AGO1, AGO3, and AGO4 are slightly involved, whereas AGO2 is fundamental for RNAa (Janowski et al., 2006b; Place et al., 2008), even if controversial data on the role of AGO2 in RNAa also exist (Janowski et al., 2006a).

In this work, we investigated whether the RNA activator ds-p21 is able to induce the transcription of p21 in Dohh2 cells and modify cell proliferation and apoptosis, the 2 main responses to p21 overexpression. We report that the expression of p21 was transiently enhanced by ds-p21 at the mRNA and protein levels and that cell proliferation and apoptosis were accordingly but not significantly modified. In contrast, ds-p21 coupled to fludarabine treatment potentiated the death of Dohh2 cells.

Materials and Methods

Double-stranded RNAs

The following ds-RNAs were used: ds-NC (ACUACU GAGUGACAGUAGA[dT][dT], UCUACUGUCACUCAGU AGU [dT][dT]) and ds-p21 (CCAACUCAUUCUCCAAGU A[dT][dT], UACUUGGAGAAUGAGUUG G[dT][dT]) correspond to sequences already used (Li et al., 2006); si-NC (UUCUCCGAACGUGUCACGUTT, ACGUGACACGUUC GGAGAATT); si-Bcl6 (GUCGAGACA UCUUGACUGAUU, UCAGUCAAGAUGUCUCGACUU) corresponds to the se-

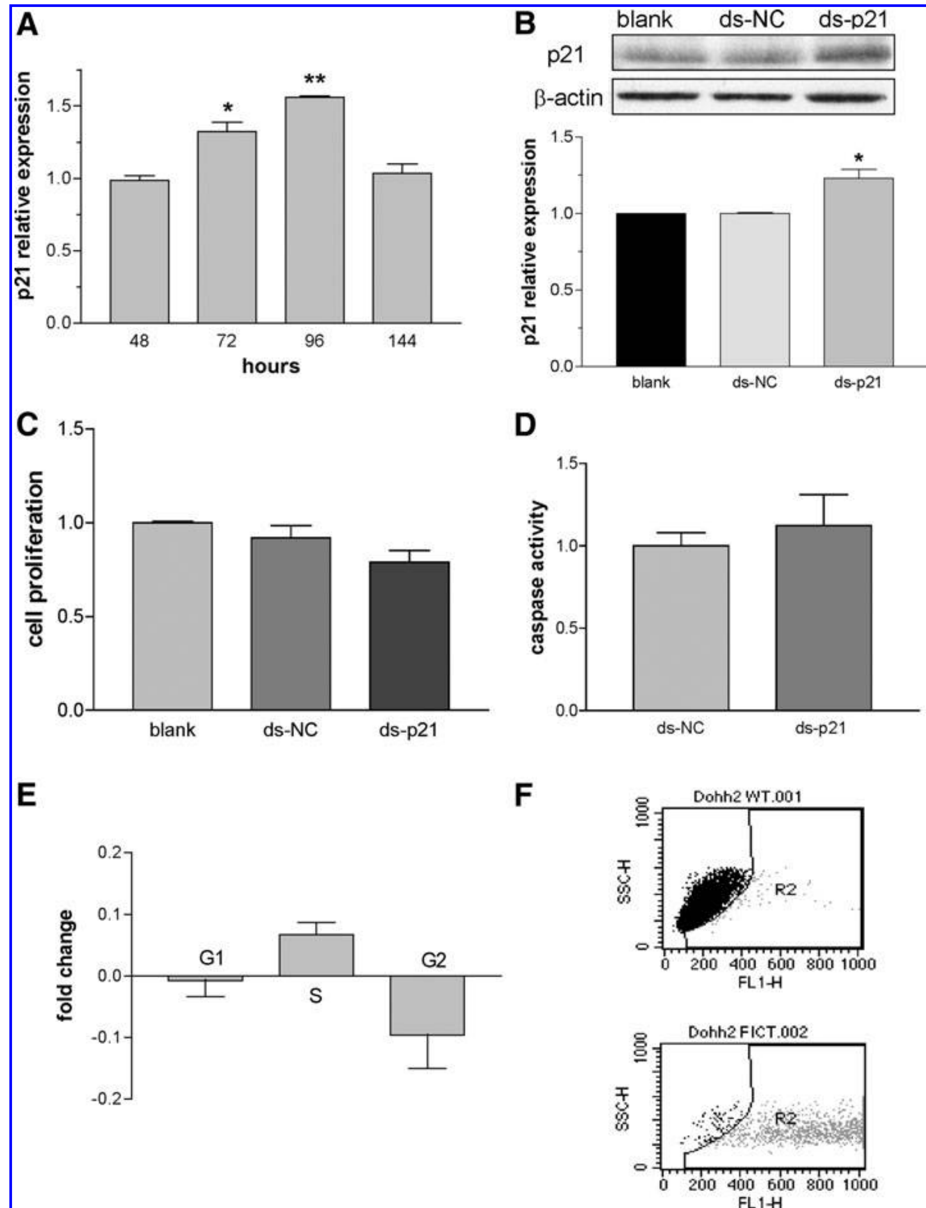

FIG. 1. (**A**) p21 transcription. Total RNA was extracted and qRT-PCR was performed at specified time points after transfection to quantify p21 transcripts. Each point represents the expression of p21 in ds-p21– versus ds-NC–transfected cells. Data are shown as SEM from 3 independent experiments (*$P < 0.05$; **$P < 0.01$, unpaired *t*-test). (**B**) Expression of p21 protein. ds-NC cells showed a p21 expression similar to that of untransfected cells, whereas ds-p21 cells showed an enhanced p21 expression. p21 was detected by western blot (*upper panel*) and quantified by densitometry at 72 hours after double-stranded RNA (ds-RNA) transfection (*lower panel*). Data are shown as SEM from 3 independent experiments (*$P < 0.05$, unpaired *t*-test). Signals were normalized to that of β-actin. (**C**) Cell proliferation. In comparison to untransfected cells, ds-NC– and ds-p21–transfected cells showed no significant reduction of cell proliferation. Data are shown as SEM from 3 independent experiments. (**D**) Caspase activity. Dohh2 transfected cells were distributed in a 96-well plate and caspase 3/7 activity was measured using Caspase-Glo® 3/7 Assay. The luminiscent signal of ds-p21–transfected cells was no significantly higher than that of ds-NC–transfected cells at 3 days after transfection. Data are shown as SEM from 3 independent experiments. (**E**) Cell cycle analysis. Bars indicate fold changes (negative and positive) of cells in G1, S, and G2 at 3 days after transfection. Each value was obtained by dividing the percentages of ds-p21–transfected cells versus the percentage of ds-NC–transfected cells in each phase. (**F**) Scattergram of FACS analysis of Dohh2 untransfected (*upper panel*) and transfected with oligo FITC (*lower panel*). Transfection efficiency was ~98%.

quence already used to silence BCL6 (Phan and Dalla-Favera, 2004). All the ds-RNAs were synthesized by GenePharma.

Cell culture

Dohh2 cells and HeLa cells were grown, respectively, in Roswell Park Memorial Institute 1640 (RPMI 1640) medium and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin and streptomycin 1% in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection

Transfection of ds-RNAs was carried out using Amaxa Nucleofector® Device and Nucleofector® Solution (Lonza) in Dohh2 cells. After nucleofection, the cells were distributed in two 75-cm flasks with 15 mL complete medium. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen).

Cell cycle

At specific times, cells were stained with propidium iodide and the DNA content profile was determined using FACScalibur cytofluorimeter (BD Biosciences).

Caspase activity

Dohh2 cells were distributed in a 96-well plate and caspase 3/7 activity was measured using Caspase-Glo® 3/7 Assay (Promega); the luminescent signal was read using GloMax-Multi Detection System (Promega).

RNA extraction

Total RNA was extracted using Trizol Reagent (Invitrogen).

qRT-PCR

Total mRNA was reverse transcribed using QuantiTect Reverse Trascription Kit (Qiagen). The resulting cDNA samples were amplified by quantitative Real-Time PCR (qRT-PCR) using SYBR Green (MesaGreen; Eurogentec) and LightCycler® 480 (Roche). We used specific primers for p21 (forward: TCACTGTCTTGTACCCTTGTGC; reverse: GGCG TTTGGAGTGGTAGAAA), p53 (forward: AGGCCTTGG AACTCAAGGAT; reverse: CCCTTTTTGGACTTCAGGTG), BCL6 (forward: TTCCGCTACAAGGGCAAC; reverse: TGC AACGATAGGGTTTCTCA), and GAPDH (forward: AGCC ACATCGCTCAGACA; reverse: GCCCAATACGACCAAA TCC); amplification of GAPDH served as a loading control.

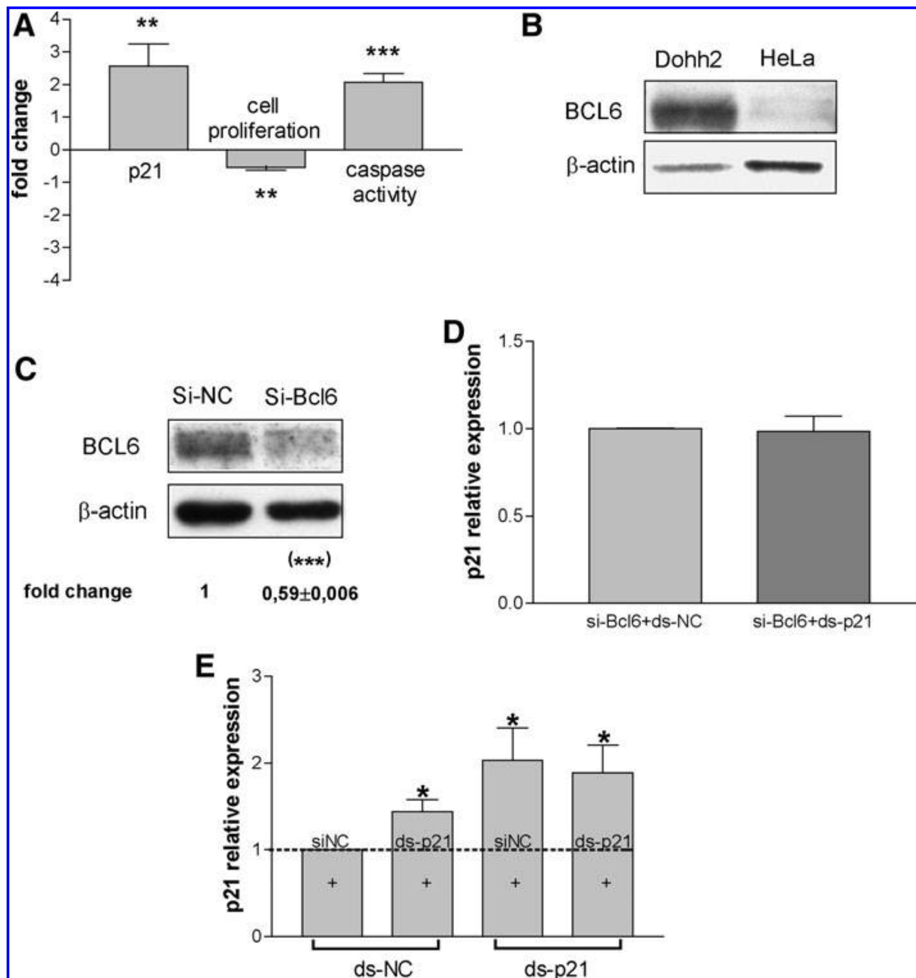

FIG. 2. (**A**) Effects of ds-p21 transfection in HeLa cells. Seventy-two hours posttransfection of either ds-NC or ds-p21, cells were collected and used to determine the expression of p21, cell proliferation, and caspase activity. The fold changes are calculated by taking as reference the corresponding values of ds-NC–transfected cells. Data are shown as SEM from 3 independent experiments (**$P < 0.01$; ***$P < 0.001$, unpaired t-test). (**B**) Expression of BCL6 protein. Dohh2 cells highly express the transcriptional repressor BCL6, compared with HeLa cells. BCL6 was detected by western blot. (**C**) Effects of ds-p21 in BCL6-silenced cells. Dohh2 cells were prior transfected with an siRNA against BCL6 and, 48 hours later, with either ds-NC or ds-p21. The protein BCL6 was significantly reduced in Dohh2 cells transfected with si-BCL6, whereas neither ds-NC nor ds-p21 modified the transcription of p21 in BCL6-silenced Dohh2 cells (**D**). Effects of ds-p21 in HeLa cells. Dohh2 cells were prior transfected with either ds-NC or ds-p21 and, 48 hours later, with either si-NC or ds-p21. In comparison to ds-NC+si-NC cells, the expression of p21 was significantly enhanced in ds-NC+ds-p21–, ds-p21+si-NC–, and ds-p21+ds-p21–transfected cells. Data are shown as SEM from 3 independent experiments (*$P < 0.05$, unpaired t-test). Dashed line shows the reference value (**E**).

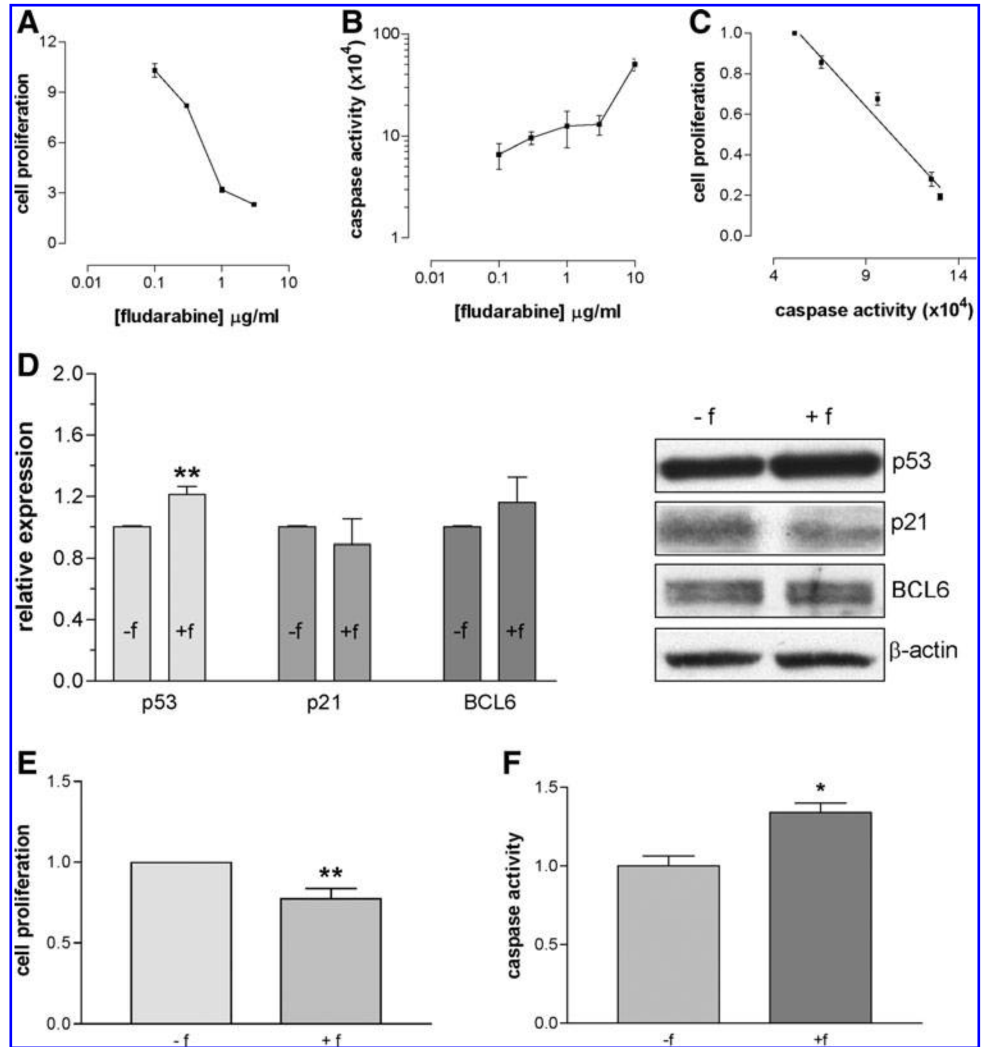

FIG. 3. (**A**) Dose–response curve of Dohh2 cells exposed to increasing concentrations of fludarabine. After 72 hours of treatment, cells were counted and cell proliferation reduction was calculated. (**B**) Caspase activity in fludarabine-treated cells. The caspase 3/7 activity of cells exposed to specified fludarabine concentrations was measured using Caspase-Glo® 3/7 Assay. (**C**) Dot plot of cell proliferation reduction versus caspase activity. Effects of 0.3 μg/mL fludarabine on (**D**) Quantification (left panel) of p53, p21 and BCL6 protein (right panel); (**E**) cell proliferation; and (**F**) caspase activity. Data are shown as SEM from 3 independent experiments (* $P < 0.05$; ** $P < 0.01$, unpaired *t*-test).

We performed a relative quantification using fit point method.

Western blot

Equivalent amounts of protein were resolved on 5%–12% SDS-PAGE gels and transferred to Hybond-C extra membranes (Amersham Biosciences) by electroblotting. The resulting blots were blocked with 5% BSA solution. We used anti-ACTB, anti-p21, anti-p53, and BCL6 primary antibodies (SantaCruz Biotechnologies); immunoblotting secondary antibodies were HRP conjugates (SantaCruz Biotechnologies). Specific proteins were detected by enhanced chemiluminescence (ECL Plus Western Blot Detection Reagent; Amersham Bioscience).

Results and Discussion

Exponentially growing Dohh2 cells were transfected with either ds-NC (nick named as ds-NC cells) or ds-p21 specific for human p21 promoter (nick named as ds-p21 cells). After transfection, cell suspensions were distributed in a series of flasks and cultured up to 6 days. After 3 days, cells were subcultured to restore the optimal cell growth conditions. At specified time points, cells were collected and used for detection of molecular and cellular endpoints. In comparison to ds-NC cells, ds-p21 cells showed a significant increase of p21 transcription between 72 and 96 hours after transfection, which thereafter decreases (Fig. 1A). In accordance with the enhanced transcription, p21 protein was more expressed in ds-p21 cells than in ds-NC cells ($P < 0.05$) (Fig. 1B). At cellular level, we found that ds-p21 cells proliferate no significantly less than ds-NC cells (Fig. 1C). In parallel, we measured apoptotis (Fig. 1D) and analyzed cell cycle (Fig. 1E), which were both no significantly modified. In this respect, the RNA activator ds-p21 is biologically irrelevant. There may be many reasons why the transcription of p21 was not so efficiently induced by ds-p21 in Dohh2 cells. First of all, we investigated whether the reduced transcription of p21 was due to a defective transfection. We transfected Dohh2 cells using Amaxa apparatus and the transfection efficiency was determined using an oligo FITC. FACS analysis showed that ~98% of cells were FITC positive (Fig. 1F), and therefore, we can reasonably exclude that neither ds-p21 nor ds-NC was efficiently transfected. Another possibility is that the ds-p21 used was not biologically active in Dohh2 cells. To address this point, either ds-NC or ds-p21 was transfected in HeLa cells. We found >3-fold increase of the transcription of p21, 2-fold de-

crease of cell proliferation, and 3-fold increase of apoptosis in HeLa cells transfected with ds-p21 in comparison to HeLa transfected with ds-NC (Fig. 2A). Together these data indicate that ds-p21 was active in promoting p21 expression at molecular and cellular levels in HeLa cells and suggest that Dohh2 cells are refractory to the RNA activator ds-p21. Many explanations have been so far evoked to explain RNAa. One of them is that the transcriptional activity is due to chromatin remodeling. The chromatin associated with genes susceptible to RNAa has been shown to have demethylated (Janowski et al., 2006b) or methylated (Janowski et al., 2006a) histone H3 lysines. The demethylation of CpG islands seems also to favor RNAa (Janowski et al., 2006b). Finally, the position of ds-RNA is fundamental (Janowski et al., 2006a): the shift of a single base of the positioning of the ds-RNA could lead to activation or inhibition. This indicates that optimal position sites exist, but whether they are common or not to each gene is still unknown. As the observed increase of p21 in Dohh2 cells is much less and less persistent than that found in other tumor cell lines (Li et al., 2006), it seems plausible that the epigenetic context of Dohh2 cells is at the basis of the slight RNAa. In HeLa cells, RNAa has been reported to be methylation dependent (Li et al., 2006). Nevertheless, in HeLa cells and follicular lymphomas, the p21 promoter is not hypermethylated (Ying et al., 2004). No data are available on the hypermethylation of p21 promoter in Dohh2 cells. However, epigenetic mechanisms of p21 gene regulation involve also histone methylation and deacetylation (Duan et al., 2005; Ocker and Schneider-Stock, 2007; Kim et al., 2009). As histone deacetylase inhibitors trichostatin A, phenylbutyrate, and subercylanilide hydroxamic acid can also activate *p21* expression, the possibility of epigenetic repression of this gene cannot be ruled out (Richon et al., 2000; Sakajiri et al., 2005).

Dohh2 cells express high level of BCL6 (Fig. 2B). BCL6 is a bifunctional protein consisting of 2 functional domains, a N-terminal POZ (Poxvirus and zinc finger) domain and a C-terminal kruppel-type (C2H2) zinc finger domain. The C-terminal zinc fingers mediate specific DNA recognition and binding, whereas the N-terminal POZ domain recruits histone deacetylase repressing transcription (Pasqualucci et al., 2003). BCL6 is able to repress both p53 (Phan and Dalla-Favera, 2004) and p21 (Phan et al., 2005). To circumvent the epigenetic control of BCL6, we transfected Dohh2 cells prior with a siRNA specific for BCL6 to reduce the endogenous BCL6 and then with either ds-NC or ds-p21 to test whether the transcription of p21 takes advantage from BCL6 reduction. As expected, we observed a significant reduction of BCL6 in si-BCL6–transfected cells in comparison to si-NC–transfected cells (Fig. 2C). Nonetheless, despite this reduction, the expression of p21 in ds-NC– and ds-p21–transfected cells was similar (Fig. 2D), suggesting that the residual endogenous BCL6 is still sufficient to repress the transcription of p21. However, it cannot be excluded that the lack of ds-p21 effect in si-BCL6–transfected cells might be due to a reduced availability of the RNAi apparatus. To address the point, we transfected HeLa cells, which have a negligible expression of BCL6 (Fig. 2B), with ds-NC and, 48 hours later, with either si-NC or ds-p21. Cells were collected at 48 hours after the second transfection. The expression of p21 was enhanced in ds-NC+ds-p21 cells (column 2) in comparison to ds-NC+si-NC cells (column 1), suggesting ds-p21 is not competed by ds-NC. By transfecting HeLa cells prior with ds-p21 and then with either si-NC or ds-p21, we found that the expression of p21 was enhanced in both ds-p21+siNC (column 3) and ds-p21+ds-p21 (column 4), suggesting that ds-p21 is not competed by si-NC. These data suggest that the RNAi apparatus is not a limiting step in the activation of ds-p21 and so it is plausible that the poor responsiveness of Dohh2 cells to the RNA activator ds-p21 is attributable to the epigenetic context established by the BCL6 overexpression in these cells.

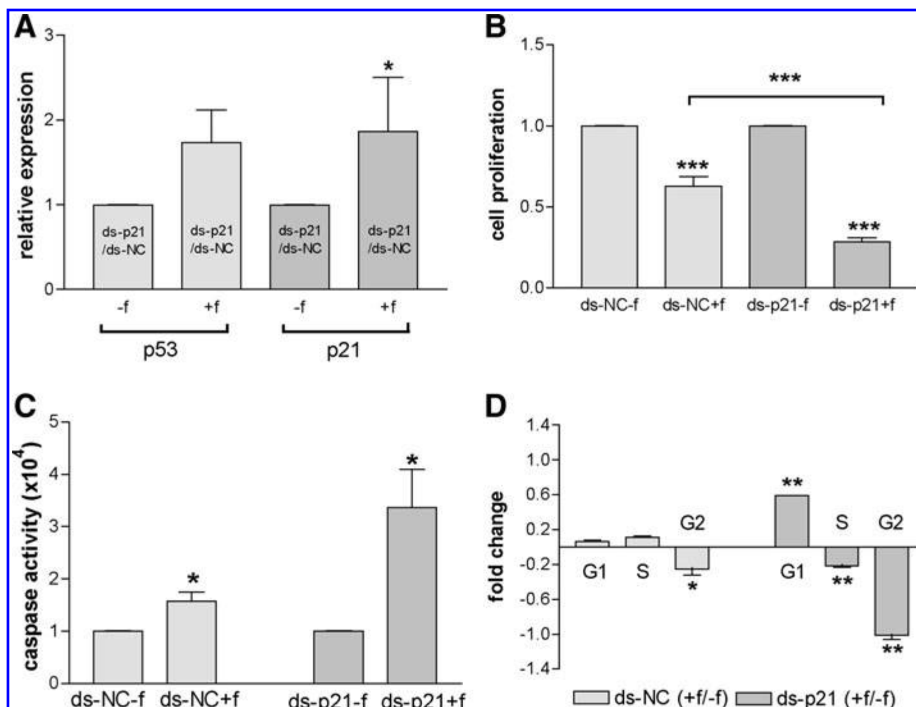

FIG. 4. Dohh2 cells transfected with either ds-NC or ds-p21 were exposed or not to 0.3 μg/mL fludarabine. After 72 hours, the expression of p53 and p21 proteins (**A**), cell proliferation (**B**), caspase activity (**C**), and cell cycle (**D**) were measured. The ratio (ds-p21/ds-NC) of unexposed cells was used to normalize the ratio (ds-p21/ds-NC) of exposed cells. Data are shown as SEM from 3 independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, unpaired *t*-test).

The finding that the modifications of cell proliferation, cell cycle, and apoptosis albeit coherent to the enhanced expression of p21 were not significantly modified (Fig. 1C–E) does not exclude that the RNA activator ds-p21 could have a biological role. To give support to this interpretation, we challenged Dohh2 cells with fludarabine, a drug of prominent use in hematopoietic malignancies and exhibiting indolent growth profiles. The choice of this drug was due to the fact that modulating agents to potentiate fludarabine cytotoxicity or circumvent fludarabine resistance have been yet considered (Alas et al., 2000; Anderson and Perry, 2007). To address whether the RNA activator ds-p21 can act as a modulating agent of fludarabine cytotoxicity, first of all we determined the sensitivity of Dohh2 cells to the drug. The dose–response curve showed that at increasing concentrations of fludarabine, cell proliferation decreases (Fig. 3A) and caspase activity increases (Fig. 3B). The dot plot of cell proliferation reduction versus caspase activity showed that the reduction of cell numbers correlates well with the increase of caspase activity ($r^2 = 0.9736$) (Fig. 3C). Then we measured the expression of p53, p21, and BCL6 in Dohh2 cells at increasing concentrations of fludarabine and selected 0.3 μg/mL, because at this concentration p53 was significantly enhanced, whereas p21 and BCL6 were stably expressed (Fig. 3D). The cellular outcomes of the exposure to 0.3 μg/mL fludarabine were a significant reduction of cell proliferation (Fig. 3E) and an increase of apoptosis (Fig. 3F), suggesting that both the effects are attributable to the overexpression of p53 without an apparent contribution of p21 because fludarabine *per se* is not able to induce p21 expression. Then, we tested whether ds-p21 can act as a modulating agent of fludarabine cytotoxicity. For that, Dohh2 cells, transfected with either ds-p21 or ds-NC, were grown for 3 days in the presence or absence of 0.3 μg/mL fludarabine. In comparison to ds-NC (+f) cells, ds-p21 (+f) cells showed both p53 and p21 transcriptional increases (Fig. 4A) and a potentiated reduction of cell proliferation (Fig. 4B) accompanied by a further potentiation of apoptosis (Fig. 4C). Moreover, we observed that ds-p21 (+f) cells more than ds-NC (+f) cells showed a marked exit from G2 followed by an accumulation in G1, a state that renders cells particularly prone to apoptosis (Fig. 4D). The 2-fold reduction of cell proliferation accompanied by an enhanced caspase activity, on one hand, highlights a role of the RNA activator p21 as a modulating agent of fludarabine cytotoxicity and, on the other hand, indicates that this occurs via the potentiation of the apoptotic pathways. This means that ds-p21 and fludarabine work cooperatively and that ds-p21 is able to enhance the cytotoxic effect of fludarabine in Dohh2 cells, even if it has weak effects in these cells when tested alone.

In conclusion, we demonstrated that (1) Dohh2 cells are poorly responsive to the RNA activator ds-p21, as the enhanced expression of p21 was unable to affect cell proliferation and apoptosis, the 2 main responses to p21 overexpression; and (2) the RNA activator ds-p21 potentiates the responsiveness of Dohh2 cells to fludarabine, suggesting that ds-p21 molecule could be used as a modulating agent of fludarabine cytotoxicity.

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Author Disclosure Statement

No competing financial interests exist.

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