RESEARCH ARTICLE

Potent subunit-specific effects on cell growth and drug sensitivity from optimised siRNA-mediated silencing of ribonucleotide reductase

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ABSTRACT

Ribonucleotide reductase (RR) has an essential role in DNA synthesis and repair and is a therapeutic target in a number of different cancers. Previous studies have shown that RNAi-mediated knockdown of either the RRM1 or RRM2 subunit sensitizes cells to the cytotoxic effects of the nucleoside analogs and more recently it has been shown that RRM2 knockdown itself has a growth inhibitory effect. Here we compare the effects of siRNA-mediated knockdown of both RRM1 and RRM2 subunits of RR in A549 and HCT-116 cells using an optimized transfection protocol. Growth of A549 cells was strongly inhibited by efficient siRNA-mediated silencing of either RRM1 or RRM2, and knockdown of each subunit led to long-term growth inhibition and cell-cycle arrest. Knockdown with sub growth inhibitory siRNA concentrations sensitized A549 and HCT-116 cells to gemcitabine when RRM1 was targeted, whereas RRM2 knockdown led to hydroxyurea sensitization. These results suggest that the inhibition of cell growth, rather than drug sensitization, is the major effect of RRM1 and RRM2 knockdown. In an A549 xenograft model, cells transfected with RRM1-specific siRNA failed to form tumors in 6 out of 8 CD1 nude mice, whereas those transfected with RRM2-specific siRNA grew but at a reduced rate. Taken together, these data demonstrate that siRNA-mediated knockdown of the RRM1 subunit is more effective than knockdown of RRM2 in inhibiting the growth of cancer cell lines and suggest that RRM1 is a potential target for nucleic acid-based cancer therapies, either alone or in combination with gemcitabine.

KEYWORDS: Ribonucleotide reductase, RNAi, gene silencing, gemcitabine

INTRODUCTION

In order for DNA replication and repair to proceed, cells must synthesize sufficient quantities of 2'-deoxyribonucleotide precursors. Their generation by reduction of the corresponding ribonucleotides, the rate-limiting step in DNA synthesis, is the preserve of ribonucleotide reductase (RR). RR is a tightly regulated tetrameric enzyme consisting of homodimers of the RRM1 and RRM2 subunits, both of which are required for the formation of the active site (Nordlund and Reichard, 2006). RRM1 is constitutively expressed, whereas RRM2 is expressed in a cell cycle-dependent manner, with expression beginning in S-phase and peaking in mitosis, after which it is rapidly degraded.

An RRM2 paralog, p53R2 (or RRM2B), is activated by p53 following genotoxic stress and is involved in DNA repair (Xue et al, 2003).

RR has long been targeted with anti-cancer therapies, including hydroxyurea and the nucleoside analog gemcitabine. These drugs have distinct mechanisms of action on RR. Hydroxyurea is a specific reversible inhibitor that interferes with radical formation essential to the catalytic activity of the enzyme. In contrast, gemcitabine is a substrate analog which competitively inhibits *de novo* DNA synthesis. RRM1 (Davidson et al, 2004; Jordheim et al, 2005) and RRM2 (Zhou et al, 1995; Goan et al, 1999) were found to be upregulated in cell lines resistant to gemcitabine and hydroxyurea, respectively. In addition, RRM1 was found upregulated following *in vivo* selection with gemcitabine (Bergman et al, 2005).

Despite the central role of RR in cancer cell growth, there are conflicting reports as to its contribution to tumorigenesis. Expression of RRM1 and RRM2 is suppressed by the retinoblastoma tumor suppressor (pRB) and loss of pRB in tumor cells is associated with increased dNTP levels and resistance to 5-fluorouracil (5-FU) and hydroxyurea (Angus et al, 2002). Other reports suggest that RRM1 has a tumor suppressor role and inhibits growth when expressed at high levels (Cao et al, 2003).

Previous studies in which RRM1 or RRM2 expression was inhibited using antisense oligodeoxynucleotides (AS-ODN) (Chen et al, 2000; Lee et al, 2003; Lee et al, 2006) or RNAi (Duxbury et al, 2004a; Duxbury et al, 2004b; Lin et al, 2004; Bepler et al, 2006; Oguri et al, 2006) showed varying levels of growth inhibition, and RNAi-mediated knockdown of RRM1 or RRM2 also sensitized cells to gemcitabine or hydroxyurea, respectively (Bepler et al, 2006; Lin et al, 2007). Similarly, inhibiting the expression of the p53R2 subunit was shown to enhance 5-FU toxicity in oral cancer cell lines (Yanamoto et al, 2005). The promise of molecular inhibition of RR has led to AS-ODN against each subunit reaching clinical trial for non-small cell lung cancer (Desai et al, 2005; Juhasz et al, 2006).

Considering the apparent non-redundant central role of RRM1 in DNA synthesis, it is surprising that previous reports of siRNA-mediated reduction in RRM1 expression showed only a modest growth inhibitory effect on tumor cells (Nakahira et al, 2007). Initial studies on the silencing of RRM2 reported similar effects; more recently, however, RRM2 silencing has been shown to inhibit tumor cell growth both *in vitro* and *in vivo* (Avolio et al, 2007; Heidel et al, 2007). We have used synthetic siRNAs to further investigate the role of RRM1 on the growth and chemoresistance of cancer cell lines, compared with knockdown of RRM2. By using optimized transfection conditions, we found that inhibiting the expression of either RRM1 or RRM2 with nanomolar concentrations of siRNA had a strong inhibitory effect on the growth of cancer cell lines derived from a range of tumors; at low picomolar concentrations the siRNAs no longer inhibited growth but caused subunit-specific effects on sensitization to hydroxyurea and gemcitabine. These observations suggest that, with appropriate tumor-selective delivery, a nucleic acid-based strategy targeting RRM1 could form a viable therapeutic strategy against a variety of tumor types.

MATERIALS AND METHODS

Cell lines and chemicals

The A549 and HCT-116 cell lines used in this study were obtained from ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (both from Invitrogen Corporation, Carlsbad, CA), at 37ºC in humidified air with 5% (v/v) CO^2. Hydroxyurea was purchased from Sigma (St Louis, MO) and gemcitabine was obtained from Eli Lilly (Indianapolis, IN). SYBR® Green I and Hoechst 33342 were from Invitrogen Corporation. All other chemicals were purchased from Sigma.

siRNAs and transfection

All siRNAs and transfection reagents were purchased from Invitrogen. The siRNAs used were blunt-ended 25 nucleotide duplexes with sense strands modified by Invitrogen's proprietary Stealth™ technology. Three independent siRNAs targeting RRM1 and RRM2 were used. See below for guide strand sequences (5' → 3').

siRNAs targeting RRM1:
RRM1-1: AUUCAAAGAUGUCUAAAUGCCAAGG
RRM1-2: AAGAUCUGCUUAUUCAGUAACUGGG
RRM1-3: UAGAAGUGCAUACUAGUGAGUUUGC

siRNAs targeting RRM2:
RRM2-1: UGUACCAGGUGUUUGAACAUCAGGC
RRM2-2: AAUUCAUCCCAAUGAGCUUCACAGG
RRM2-3: UUAUACAUCUGCCAGAUAUCAUGGU

Non-targeting controls:
81-ctrl: CCACACGAGUCUUACCAAGUUGCUU
809-ctrl: AAAUCAAUGGGAGGAGACAUUUCCC
RRM1-REV: CCCAGUUACUGAAUAAGCAGAUCUU

Transfection of each cell line was first optimized by measuring the level of FITC-labeled Block-iT™ duplex introduced with the transfection reagent Lipofectamine™ 2000 (L2K), using flow cytometry (see below). Once optimal conditions were determined, Lipofectamine™ RNAiMax, a reagent optimized for siRNA transfection but incompatible with FITC fluorescence measurements, was used in all subsequent experiments to introduce Stealth™ siRNAs into cells via reverse transfection. Briefly, siRNAs and Lipofectamine were diluted in RPMI without serum, and incubated for 5-10 min at room temperature. After optimization, a Lipofectamine concentration of 0.8 µl/ml was used for transfections with all cell lines. The Lipofectamine mix was added drop-wise to the siRNA, and lipoplex formation was allowed to proceed for 20-30 min at room temperature. Lipoplexes were then transferred to multi-well tissue culture plates and, unless otherwise noted, overlaid with $5x10^3$ cells/cm^2. Following overnight incubation, the transfection medium was replaced with RPMI containing 10% (v/v) FBS, and cells were analyzed at indicated time points. Where required, cells were further treated with chemotherapeutic drugs as described in the figure legends.

Real-time PCR

RNA was purified from cells using PureLink™ 96 RNA Purification kit as per the manufacturer's protocol (Invitrogen). First-strand cDNA was generated using the SuperScript® VILO™ cDNA Synthesis kit (Invitrogen), from approximately 140ng purified RNA. For semi-quantitative RT-PCR, the cDNA was diluted 1:4 in ultraPURE™ RNase-free water, and real-time PCR carried out on a LightCycler® 480 II (Roche) using LightCycler® 480 SYBR Green I Master Mix (Roche) and gene- specific primers at 180 nM. Levels of RRM1 and RRM2 mRNA were normalized to the housekeeping gene LMNA, and change in mRNA following transfection with specific siRNAs compared to control siRNAs was calculated. The following primers were used:

RRM1 forward primer:
5' GGCAAACTCACTAGTATGCACTTC

RRM1 reverse primer:
5' AAATAATACATCCCAGTCTTCAAACC

RRM2 forward primer:
5' CAGCAAGCGATGGCATAGT

RRM2 reverse primer:
5' AGCGGGCTTCTGTAATCTGA

LMNA forward primer:
5' TGAGGCCAAGAAGCAACTTCA

LMNA reverse primer:
5' CTCATGACGGCGCTTGGT.

Western blotting

Cells were harvested 48 or 72 hr post-transfection with RRM1- or RRM2-specific or control siRNAs, and lysates were prepared in lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 7.4, 1%, v/v NP40) supplemented with a protease inhibitor cocktail (Roche). Following quantification using the BioRad's Protein Assay kit 10 µg of total protein/lane was loaded and separated by electrophoresis on a pre-cast 10% polyacrylamide gel (Invitrogen) and blotted onto an Immobilon™-P PVDF membrane (Millipore). After blocking for 1 hr with 5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20, membranes were probed with a goat anti-human polyclonal antibodies specific for RRM1 (T-16, Santa Cruz), RRM2 (N-18, Santa Cruz) (both used at 0.3 µg/ml) and GAPDH (V-18, Santa Cruz, 0.1 mg/ml) in PBS containing 2.5% (w/v) BSA and 0.1% (v/v) Tween-20. HRP-conjugated donkey anti-goat IgG (Santa Cruz) was used as a secondary antibody at 80 ng/ml, and the signal was detected with ECL-Plus™ estern Blotting Detection System (GE Healthcare) on the ImageQuant scanner (GE Healthcare).

Growth assays

To measure the effects of siRNA-mediated RRM1 and RRM2 knockdown on cell growth, cellular DNA content was measured using a SYBR® Green I-based fluorometric assay. At the indicated time points following reverse transfection, replicate plates were frozen at -80ºC for subsequent analysis. For assays in which cells were treated with a combination of siRNA and gemcitabine or hydroxyurea, cells 24 hr post-transfection were treated continuously for 72 hr with the indicated concentrations, after which plates were frozen at -80ºC for later analysis. At the completion of the assay, frozen cells were thawed and cell lysis buffer (10 mM Tris HCl pH 8.0 containing 2.5 mM EDTA, 1%, v/v, TritonTM X-100) and SYBR® Green I (1:4000, v/v) were added to the wells and incubated overnight in the dark at 4ºC. The following day, cell lysates were mixed thoroughly, and DNA fluorescence was measured with a Wallac Victor^2 plate reader (Turku, Finland) set at an excitation frequency of 485 nm and measuring emission at 535 nm.

Flow cytometry

To determine the efficiency with which siRNAs were introduced following transfection, cells were analyzed for uptake of the fluorescent FITC-labeled Block-iT™ oligonucleotides by flow cytometry. The day following transfection, cells were resuspended in FACS buffer (PBS containing 1%, w/v, BSA and 0.1%, w/v, sodium azide) and cell-associated fluorescence was measured using a Becton Dickinson LSR II. For cell cycle analysis post transfection, cells were washed with FACS buffer and fixed on ice with 4% (v/v) methanol solution in PBS, after which samples were stored at -20°C for at least 24 hr prior to staining with propidium iodide (5 µg/ml) for DNA content analysis. Cells were stained for 2 hr at 37ºC prior to flow cytometric analysis using a Becton Dickinson LSR II with a 96-well autosampler attached. Using pulse width/area analysis of the Hoechst fluorescent signal, cycling and apoptotic single cells were identified.

A549 xenograft model

A549 cells *in vitro* were reverse-transfected with Lipofectamine™ RNAiMAX and an RRM1- or RRM2-specific siRNA or a non-targeting control siRNA, at a final siRNA concentration of 10 nM. Untreated cells served as an additional control. The following day, cells were detached from flasks with trypsin-EDTA, and the enzymatic reaction was stopped by the addition of FBS. After two washes with PBS, the cells were resuspended in PBS at a final density of $8x10^7$ cells/ml. Using a 26-gauge needle, groups of CD-1 nude mice were injected subcutaneously on the flank with 100 µl of the cell suspension (equivalent to $8x10^6$ A549 cells). The effect of RRM1 or RRM2 knockdown on tumor growth was assessed at the indicated intervals by measuring tumor size with digital calipers. Measurements were used to calculate the tumor volume using the formula: $0.52 \times a^2b$, where a = minor diameter and b = major diameter.

RESULTS

Effective siRNA delivery into cells by reverse transfection

In many transfection protocols, a substantial number of cells do not receive nucleic acid, and this untransfected sub-population can mask the effects of target knockdown. In order to better measure the effects on cell growth of RRM1 and RRM2 knockdown, we first optimized our transfection conditions to maximize the number of cells receiving siRNA by tracking the uptake of the FITC-labeled Block-

iT™ siRNA by flow cytometry following reverse transfection (cells added to siRNA/L2K mix) or forward transfection (cells plated the day before addition of siRNA/L2K). As seen in Figure 1A, compared with control untransfected cells (top panel) the reverse transfection approach used here achieved close to 100% transfection in A549 cells (bottom panel), whereas with standard forward transfection, a substantial untransfected population remained (middle panel). Transfection rate was also dependent on cell density, as the proportion of transfected cells in the population clearly increased as the number of cells in the transfection reaction decreased (Figure 1B). Compared with untransfected cells (top panel), transfecting cells at a cell density of $20x10^3$ cells per cm^2 yielded 72% transfected cells (second panel), increasing to 93% when 10^4 cells were transfected (third panel), and greater than 99% when a cell density of $5x10^3$ cells/cm^2 was used (bottom panel). Similar results were obtained with HCT-116 cells (data not shown). These data directed the subsequent transfection experiments, in which cells were reverse-transfected at a density of $5x10^3$ cells/cm^2.

Effective knockdown of RRM1 and RRM2 by three independent siRNAs

Using the optimized transfection conditions described above, cells were transfected with three independent siRNAs specific for RRM1 or RRM2 and the reduction in mRNA and protein levels of these genes was analyzed. As seen by real-time PCR in Figure 1C, expression of RRM1 mRNA was reduced by greater than 90% 24 hr following transfection with three independent siRNAs (RRM1-1, -2 and -3). RRM2-targeting siRNAs (RRM2-1, -2 and -3) were also potent inhibitors of mRNA expression, with greater than 85% reduction in RRM2 levels with 5 nM siRNA (Figure 1C). Western blot analysis showed that RRM1 and RRM2 protein levels were also strongly decreased 48 hr after transfection with subunit-specific siRNAs at a concentration of 5 nM (Figure 1D). In each case, knockdown mediated by the RRM1- or RRM2-specific siRNAs was limited to the targeted subunit with no effect on the other subunit (Figure 1D).

RR knockdown compromises cancer cell growth

To investigate the effects of RR silencing on cell growth, A549 cells were transfected with RRM1- or RRM2-targeting siRNAs and their proliferation was followed over time. A strong inhibitory effect on the growth of both cell lines was seen when compared with controls, with cell numbers static for at least 5 days after transfection with 1 nM siRNA (Figure 2A). Growth inhibition was observed for at least 8 days when cells were transfected with 10 nM RRM2-specific siRNAs and for at least 10 days when RRM1 was silenced with siRNAs the same concentration (Figure 2B). The potency of growth inhibition caused by silencing RRM1 or RRM2 was tested by measuring growth of A549 cells after transfection with varying concentrations of RRM1- or RRM2-specific siRNAs. Effects on growth were dose-dependent and silencing was potent. At 96 hR post transfection, 50% growth inhibition was achieved with as little as 25-30 pM RRM1-2 or RRM1-3 siRNA; the third siRNA RRM1-1 required 150 pM for the same effect (Figure 2C). RRM2 silencing was moderately less potent, with transfection of 100 pM of either RRM2-1 or RRM2-3, or 300 pM of RRM2-2, necessary to inhibit growth by 50% (Figure 2D).

As the inhibition of RRM1 or RRM2 clearly had an inhibitory effect on cell growth, we further investigated the mechanism of action of RRM1 and RRM2 silencing using flow cytometry-based cell cycle analysis of A549 cells treated with RRM1 or RRM2-specific, or control siRNA. Arrest of the cell cycle occurred within 48 hr in cells transfected with 1 nM RRM1-2 siRNA, and persisted in those surviving for 96 hr, whereas transfection with 1 nM control siRNA had no effect on cell cycle distribution (Figure 3). When RRM1-2 siRNA was used at a concentration of 0.1 nM,,the initial cell cycle arrest was less pronounced and by 96 hr the cell cycle had begun to return to a normal distribution. In comparison, treating cells with 1 nM RRM2-1 siRNA also led to arrest, but this began to reverse by 96 hr. At a concentration of 0.1 nM, RRM2-1 siRNA did not induce an observable change in cell cycle.

Sub-growth inhibitory knockdown of RRM1 and RRM2 sensitizes cells to drugs

In previous studies, knockdown of RRM1 or RRM2 was shown to reverse the resistance of gemcitabine-selected cells. We also observed a substantial sensitization to gemcitabine in cells transfected with RRM1-targeting siRNA, but only when the siRNA concentration was below that required for growth inhibition (Table 1, Figure 4). A549 and HCT-116 cells were sensitized to gemcitabine in a dose-dependent manner following RRM1 knockdown with a final concentration of 10, 20 or 30 pM RRM1-1 siRNA (Figure 4A). RRM1 silencing increased the sensitivity of A549 and HCT-116 cells 2 to 5-fold compared with control siRNA-transfected cells (Table 1), whereas RRM2 knockdown using a final concentration of 20, 40 or 50 pM RRM2-1 siRNA had no effect on gemcitabine sensitivity (Figure 4B). In both cases, the three different picomolar concentrations of siRNA used were insufficient to inhibit growth, as seen in Figure 3. In contrast, hydroxyurea sensitization was observed after partial knockdown of RRM2, but not RRM1 (Figure 4C and D). Again, the 2 to 6-fold sensitization (Table 1) varied in a dose-dependent manner with the final concentration of siRNA used, with knockdown of either RRM1 or RRM2 at these siRNA concentrations insufficient to inhibit growth in the absence of drug.

Effect of RR knockdown on xenograft tumor growth in nude mice

To investigate the effects of RRM1 knockdown on tumor growth, A549 cells, either untransfected or transfected with 10 nM RRM1-2, RRM2-1 or control siRNA were implanted in CD-1 mice 24 hr after transfection. In mice implanted with the untransfected and control siRNA-transfected cells, tumors formed in 8 of 8 mice injected; in the RRM1 transfected group, tumors formed in only 2 of 8 mice. The two tumors that did form in the RRM1 group were very slow-growing with little increase in volume over the course of the experiment (see Figure 5A). In contrast, when cells transfected with 10 nM RRM2 siRNA were implanted, tumors formed in all 8 mice. These tumors grew, but at a reduced rate compared with the tumors derived from control siRNA-transfected and untransfected cells (Figure 5B).

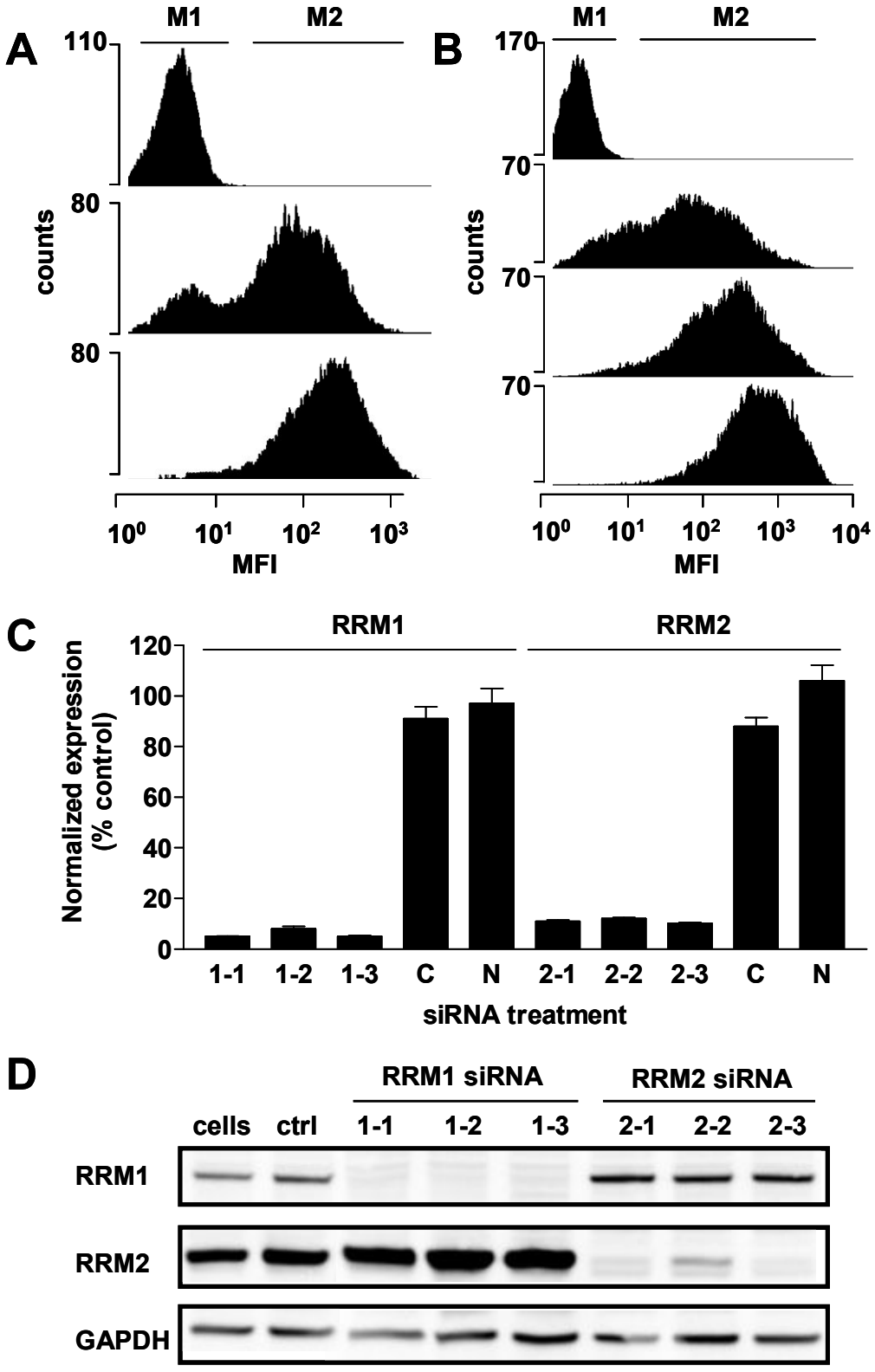

Figure 1. Reverse transfection is highly efficient in delivering siRNAs and enables effective RR knockdown. **(A)** FITC-labeled Block-iT was introduced into cells by either forward (middle) or reverse transfection (bottom) and compared to mock transfection control (top) and cell-associated fluorescence was analyzed by flow cytometry 24 hr later. **(B)** Cells at different plating densities were reverse transfected with Block-iT and cell-associated fluorescence was analyzed by flow cytometry 24 hr later. Cell density was 5, 10 or 20 x 10^3 cells per cm^2 (panels 2-4), with untransfected control in panel 1. M1 and M2 are gates corresponding to untransfected and transfected cells, respectively. **(C)** Cells were transfected with siRNAs (5 nM) targeting RRM1 (1-1, 1-2 and 1-3) or RRM2 (2-1, 2-2 and 2-3), or 81-ctrl control siRNA **(C)**, or were untransfected (N), and RRM1 and RRM2 mRNA levels were quantified 24 hr later by real-time PCR and normalized to the expression of LMNA. **(D)** Protein expression was measured by western blot at 48 hr post-transfection with RRM1- or RRM2-specific or 81-ctrl control siRNAs at a final concentration of 5 nM. Following detection of RRM1 or RRM2, membranes were stripped and probed with GAPDH antibodies.

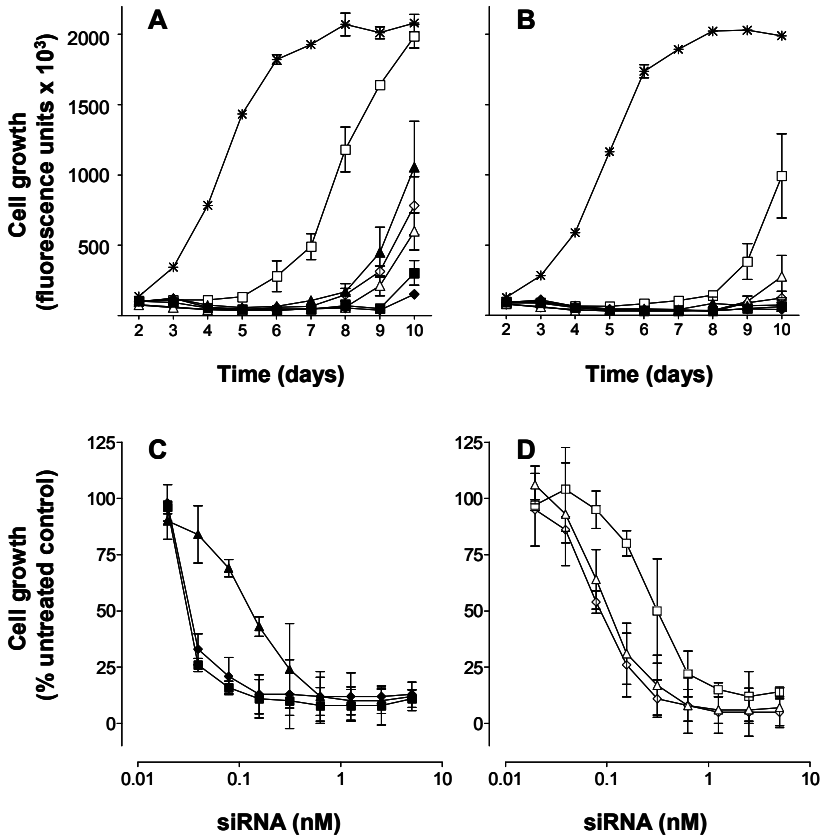

Figure 2. RRM1 and RRM2 siRNAs mediate long lasting and potent growth inhibition. To measure growth inhibition, A549 cells were transfected with 1 nM **(A)** or 10 nM **(B)** of three different RRM1-specific siRNAs [1-1 (▲), 1-2 (■) 1-3 (♦)] or RRM2-specific siRNAs [2-1 (Δ), 2-2 (□), 2-3(◊)] or control siRNA (*) and growth over 10 days was determined by measuring total DNA per well at the indicated time points. To assess the potency of growth inhibition, cells were transfected with siRNA targeting RRM1 **(C)** or RRM2 **(D)** at a range of concentrations (starting at 5 nM with sequential 2-fold dilutions to 19 pM) and growth at 96 hr post-transfection was determined by quantifying total DNA per well and relating this to cells transfected with equivalent concentrations of control siRNA **(A)**. Data are mean ± SEM of values from three wells, and are representative of three independent experiments.

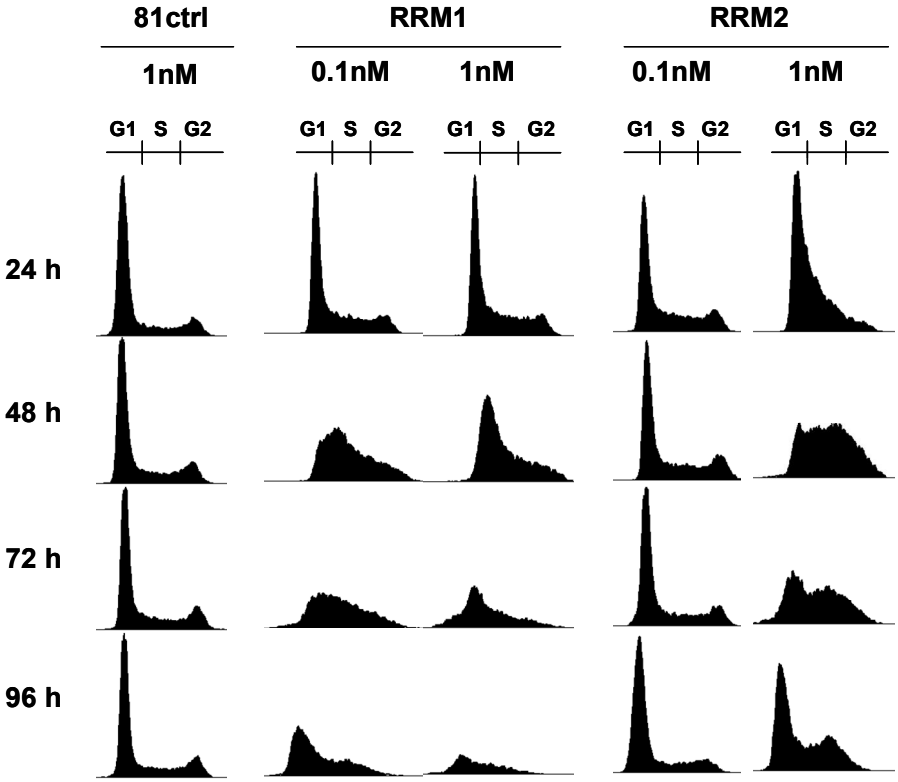

Figure 3. RR knockdown induces cell-cycle arrest and apoptosis. The cell cycle distribution in A549 cells was analyzed over 96 hr following transfection with control siRNA, or siRNAs specific for RRM1 (1-2) or RRM2 (2-1) at a final concentration of 0.1 and 1 nM. After harvesting every 24 hr, cells were fixed in methanol (70%, v/v) and then stained with propidium iodide and analyzed as described in Materials and Methods. The results from one experiment are shown; a second experiment generated similar results.

Table 1. Drug toxicity following RRM1 and RRM2 knockdown

		A549		HCT-116	
	siRNA (concentration)	**IC_{50} #**	**fold change+**	**IC_{50}**	**fold change**
Gemcitabine	RRM1-2 (0.01 nM)	2.03 ± 0.20	2.2*	1.18 ± 0.53	1.8
	RRM1-2 (0.02 nM)	1.13 ± 0.15	3.9*	0.69 ± 0.08	3.0*
	RRM1-2 (0.03 nM)	0.80 ± 0.25	5.5*	0.46 ± 0.10	4.5*
	809-ctrl (1 nM)	4.39 ± 1.08		2.08 ± 0.34	
	RRM2-1 (0.02 nM)	4.09 ± 0.91	1.1	2.26 ± 0.22	0.9
	RRM2-1 (0.04 nM)	3.80 ± 1.99	1.2	2.40 ± 0.30	0.9
	RRM2-1 (0.05 nM)	4.18 ± 1.60	1.1	2.48 ± 0.65	0.8
	809-ctrl (1 nM)	4.39 ± 1.08		2.08 ± 0.34	
Hydroxyurea	RRM1-2 (0.01 nM)	0.22 ± 0.01	1.0	0.35 ± 0.18	1.1
	RRM1-2 (0.02 nM)	0.25 ± 0.03	0.9	0.24 ± 0.14	1.6
	RRM1-2 (0.03 nM)	0.18 ± 0.01	1.2	0.19 ± 0.09	2.1
	809-ctrl (1 nM)	0.22 ± 0.01		0.39 ± 0.12	
	RRM2-1 (0.02 nM)	0.13 ± 0.01	1.7*	0.21 ± 0.04	1.9*
	RRM2-1 (0.04 nM)	0.05 ± 0.02	4.8*	0.06 ± 0.01	6.5*
	RRM2-1 (0.05 nM)	0.04 ± 0.02	6.1*	0.08 ± 0.03	4.8*
	809-ctrl (1 nM)	0.22 ± 0.01		0.39 ± 0.12	

Values are nM for gemcitabine and mM for hydroxyurea. Data are the mean ±SEM from two independent experiments; + The change in IC_{50} value in cells treated with RRM1 or RRM2-specific siRNA compared with those treated with control siRNA; * $p < 0.05$, Student's t-test

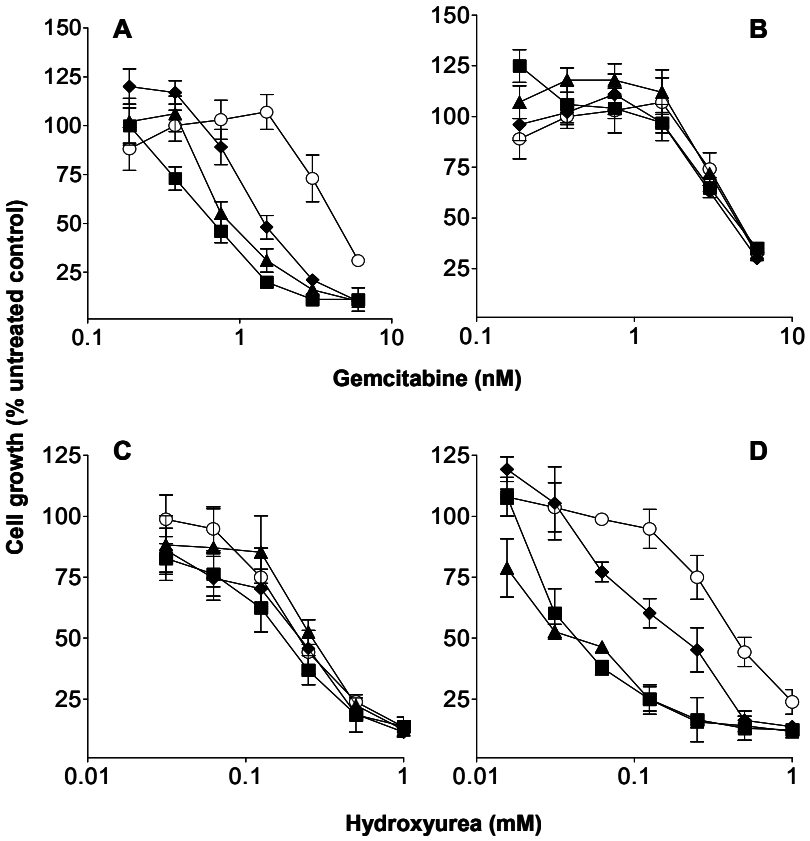

Figure 4. Sensitization to drugs following RR knockdown. A549 cells were transfected with: 10 (♦), 20 (▲) or 30 nM (■) RRM1-specific siRNA (1-2) (A, C); 20 (♦), 40 (▲) or 50 nM RRM2-specific siRNA (2-1) (■) **(B, D)**; or 1 nM 81-ctrl siRNA (○). Following transfection, cells were treated with 0.19-6 nM gemcitabine **(A, B)** or 0.18-1 mM hydroxyurea **(C,D)**, and after 4 days, proliferation was measured by quantifying the DNA content in each well, and was the mean fluorescence ±SEM from three replicate wells. Data are represented as growth of transfected drug-treated cells as a percentage of transfected cells that were not exposed to drug, and are from one of three experiments giving similar results.

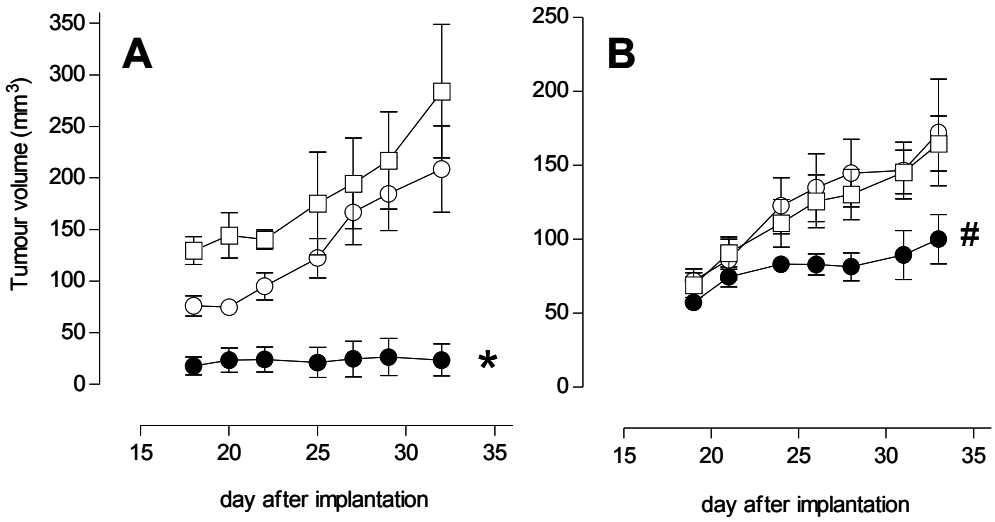

Figure 5. Effect of RRM1 knockdown on xenograft tumor formation. A549 cells were transfected with 10 nM of control siRNA (○), RRM1- or RRM2-specific siRNA (●), or untransfected (□) 24 hr prior to injection into CD-1 nude mice. In **(A)**, cells transfected with RRM1-2 or control siRNA, along with untransfected controls, were implanted into mice, and in **(B)** RRM2-1-transfected cells were used along with controls. Tumor volume was measured on the indicated days. Values represent mean ±SEM of 5 mice. * $p < 0.01$ and # $p = 0.05$ compared with 81-control siRNA-transfected cells (Student's t-test).

DISCUSSION

The enzyme ribonucleotide reductase is essential to maintain the levels of dNTPs necessary to support DNA replication and growth, and has long been an attractive target for anticancer therapy. Although many small molecule drugs have been shown to inhibit the activity of RR directly, only hydroxyurea has reached the clinic. The use of small-interfering RNA or antisense oligonucleotides to inhibit one or more subunits of this enzyme in a specific manner represents an alternative or supplementary approach to small molecule drug treatment.

As both RRM1 and RRM2 are needed to form the active ribonucleotide reductase enzyme, it is not surprising that the efficient knockdown of either subunit presented here led to extensive growth inhibition. In light of the essential role of RR in DNA replication and repair, it was somewhat surprising that early studies using antisense (Chen et al, 2000; Lee et al, 2006) or RNAi (Oguri, et al, 2006; Nakahira et al, 2007) to silence the expression of either RR subunit did not report the level of inhibition of cell growth that we observed. Our results, and those from recent studies targeting RRM2 with siRNAs (Avolio et al, 2007; Heidel et al, 2007), suggest that this is likely due to a combination of incomplete transfection and or inefficient silencing. To accurately assess the effects of knockdown when the target gene is essential for cell growth, it is important to deliver an effective siRNA to as many cells as possible. With our optimized transfection protocol, we were able to transfect >99% of cells as determined with a fluorescently-labeled siRNA, and the effects on cell growth suggest a similar transfection rate in subsequent studies with RRM1- or RRM2-specific siRNAs. Our results comparing forward and reverse transfection contrast somewhat with the pioneering study using FITC-labelled siRNAs as a marker of transfection in which forward transfection was reported to deliver siRNAs to all cells (Holen et al, 2002). Nevertheless, we have seen potent and enduring growth inhibition only with reverse transfection at low cell density. With either forward transfection or higher cell densities, growth inhibition was observed for at most 96 hr (data not shown), suggesting that under these conditions an untransfected (or poorly transfected) population of cells received insufficient siRNA to cause a growth inhibitory reduction of RRM1 or RRM2. Parallels can be seen in the study by Lin and colleagues (Lin et al, 2007) that aimed to generate cells stably transduced with a construct expressing RRM1-specific shRNA; none of the stable transfectants were found to have decreased RRM1 expression, suggesting that reducing RRM1 below a certain threshold is incompatible with cell growth and thus also with the expansion of clones with substantial knockdown.

A number of previous studies have investigated the effects of silencing the genes for one or other RR subunit but none has compared knockdown of each subunit in the same cells. Our results show that following transfection with equimolar concentrations of RRM1- and RRM2-specific siRNAs, RRM1 knockdown *in vitro* results in more effective and longer-lasting growth inhibition than that achieved following the knockdown of RRM2. Not all siRNAs inhibited growth equally, however, with RRM1-1 and RRM2-2 markedly less active than the others. This is consistent with the positional effects of siRNA target site on the extent of knockdown first reported in the case of human tissue factor (Holen et al, 2002). Also, cells recover from RRM2 knockdown faster and xenografts derived from cells transfected with RRM2 siRNA form more readily and grow faster than those resulting from implantation of RRM1-transfected cells. Although we saw

similar levels of knockdown of RRM1 and RRM2 mRNA and protein following transfection of either A549 or HCT-116 cells with subunit specific siRNAs, several possible explanations could account for the increased cytotoxic effects of RRM1 siRNA. First, RRM2 mRNA amplifies more readily in our real-time PCR experiments suggesting that the basal levels of RRM2 mRNA are approximately 4-fold greater than those of RRM1, similar to the results found in patients following treatment with RRM2 targeting AS-ODN (Juhasz et al, 2006). Alternatively, there may be some subtle differences in the knockdown achieved by the siRNAs used in our assays that we have not been able to accurately determine. Regardless of the underlying explanation, it is clear that, at equimolar concentrations, RRM1-targeting siRNA is more growth inhibitory than siRNA targeting RRM2.

In cell lines resistant to gemcitabine or hydroxyurea, upregulation of either RRM1 (Davidson et al, 2004; Jordheim et al, 2005) or RRM2 (Zhou et al, 1995; Goan et al, 1999) has been demonstrated and RRM1 was found upregulated following selection with gemcitabine *in vivo* (Bergman et al, 2005). Our initial attempts to assess whether knockdown of RRM1 or RRM2 sensitize cells to the effects of drugs known to target or interact with these polypeptides were hampered by the efficiency with which the siRNAs we used inhibited cell growth. Only when we reduced the siRNA concentrations to picomolar levels were we able to observe sensitization to gemcitabine and hydroxyurea, drugs known to interact with RRM1 and RRM2, respectively. The 2 to 5-fold increase in gemcitabine sensitivity following sub-growth inhibitory RRM1 knockdown correlates well with a previous study (Bepler et al, 2006), as do the similar increases in hydroxyurea sensitization seen after low-level RRM2 knockdown (Lin et al, 2004). Both of these studies are interesting in that they used stably expressed shRNAs to knockdown RRM1 or RRM2 but did not report (Bepler et al, 2006) or observe (Lin et al, 2004) changes in growth of their stable transfectants despite knockdown of greater than 80%. In contrast, Lin and colleagues failed to generate transfectants stably expressing RRM1 shRNA (Lin et al, 2007). This suggests that there exists a threshold for RRM1 or RRM2 expression, below which cells are unable to survive, and further suggests that the knockdown we observe for RRM1 and RRM2 exceeds this limit.

The inability of small molecules to effectively inhibit RR function has led to silencing of RRM2 being proposed as a possible anticancer treatment, either alone or in combination with gemcitabine (Duxbury et al, 2004; Bepler et al, 2006). More recently siRNAs have been used to silence RRM2 and this has been shown to inhibit tumor cell growth both *in vitro* and *in vivo* (Avolio et al, 2007; Heidel et al, 2007). Our results directly comparing the effects of RRM1 and RRM2 siRNAs suggest that RRM1 silencing is a more effective means of inhibiting cell growth than RRM2 knockdown. It is also apparent from our results that *in vitro* studies of the effects of gene silencing should be carried out with optimized transfection protocols to ensure that the gene in question has been effectively knocked down in the entire population of cells studied. As the majority of cells must be transfected for knockdown of either RRM1 or RRM2 to be most effective *in vitro*, this will likely impact their success in inhibiting tumor growth *in vivo*. In the case of targeting RRM2, even transfecting greater than 99% of cells prior to xenograft formation, only partial tumor growth inhibition was observed. Interestingly, from the view of a therapeutic application, transfection with as little as 1 nM siRNA against either subunit led to growth arrest and apoptosis *in vitro*, whilst concentrations in the picomolar range resulted in a marked sensitization to chemotherapeutic drugs. Taken together our results suggest that, with the appropriate tumor-selective delivery, siRNA targeting RRM1 could form the basis of a therapeutic strategy for cancer, either alone or in combination with gemcitabine.

CONCLUSIONS

- Stealth siRNA-mediated silencing of either RRM1 or RRM2 in cancer cells was growth inhibitory at picomolar concentrations when an optimized transfection method was used, and RRM1 knockdown was more growth inhibitory when cells were treated with equimolar concentrations of siRNA.

- Knockdown of RRM1 or RRM2 using siRNA concentrations lower than those required to inhibit growth sensitized cells to gemcitabine and hydroxyurea, respectively.

- Pre-implantation silencing of RRM1 was more effective than RRM2 knockdown at inhibiting xenograft formation and growth in CD-1 nude mice.

- RNAi-mediated silencing of RRM1 represents a potential strategy for cancer therapy that is both growth inhibitory and drug-sensitizing.

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COMPETING INTERESTS

GR, NCW, RP, FSA, HC and JDW work for and/or have financial interests in Genesis R&D, an RNAi-based therapeutics company.

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