Inhibiting the Expression of DNA Replication-Initiation Proteins Induces Apoptosis in Human Cancer Cells

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ABSTRACT

DNA replication-initiation proteins are expressed in cancer cells, whereas some of these proteins are not expressed in nonproliferating normal cells. Therefore, replication-initiation proteins may present attractive targets for anticancer therapy. Using selected antisense oligodeoxynucleotides and small interfering RNA molecules targeted to the mRNA encoding the DNA replication-initiation proteins hCdc6p, hMcm2p, and hCdc45p, we show that the target genes could be effectively and specifically silenced and that, consequently, DNA replication and cell proliferation were inhibited in cultured human cells. In addition, silencing of these genes resulted in apoptosis in both p53-positive and -negative cancer cells but not in normal cells: cancer cells entered an abortive S-phase, whereas normal cells arrested mainly in G0 phase. Our studies are the first to suggest that inhibiting the expression of selective replication-initiation proteins is a novel and effective anticancer strategy.

INTRODUCTION

DNA replication is one of the most fundamental cellular processes. To ensure proper genome duplication and inheritance, eukaryotic cells exert strict control over DNA replication by regulating a series of replication-initiation proteins. Although obligatorily expressed in cancer cells, a subset of initiation proteins for DNA replication, such as Cdc6p, a subset of initiation proteins for DNA replication, such as Cdc6p, MCM proteins (2), and Cdc45p, are not expressed in nonproliferating normal cells. Inhibiting the expression of these proteins should therefore effectively abate DNA replication, thus stopping cancer growth while leaving most normal cells in the body largely unaffected. Thus, these proteins may present attractive targets for development of effective anticancer drugs with few side effects.

To date, at least six groups of initiation proteins, including ORC (Orc1p through -6p; Refs. 3–7), Noc3p (8), Cdc6p (9), MCM proteins (2), and Cdc45p, are known to be required for eukaryotic DNA replication. These proteins are conserved in eukaryotes, and homologues of most of these gene products, thus stopping DNA replication and cell proliferation, have been identified in model organisms from yeast to humans. To test the idea that inhibiting the expression of replication-initiation proteins can stop cancer cell growth, we have designed, screened, and tested antisense ODNs and siRNA molecules targeted to three human DNA replication-initiation genes, hCdc6, hMcm2, and hCdc45. We found that selective antisense ODNs and siRNAs not only significantly reduced the mRNA and protein levels of the target genes, thus stopping DNA replication and cell proliferation, but also resulted in apoptosis of both p53-positive and -negative cancer cells. Furthermore, silencing of these genes does not cause death of cells derived from normal tissues.

MATERIALS AND METHODS

ODNs and siRNAs. Two strategies were used to run the mfold program (24) to predict the secondary structures of the target mRNA. The first was to input the entire sequence of a target gene but to limit the range of allowable base pairing to 250 or 500 nucleotides (in two separate runs). The second was to input 500–700 nucleotide segments of a target gene for each run with 200 nucleotides of overlapping sequences between two adjacent segments, without limiting the range of base pairing. The first 10–15 lowest free-energy structures from the output of each run were compared and used to design antisense ODNs to target mRNA areas of 14–22 nucleotides that were predicted to be at least 60% unpaired. Some of the 170 antisense ODNs were designed based on two other considerations: (a) 10 ODNs were designed to span areas containing the tetranucleotide GGGG sequence on the target mRNA, which has been suggested to be a target motif for antisense ODNs (25); and (b), at least one ODN was designed to encompass the start codon of each gene. For these two classes of ODNs, the numbers of unpaired bases on the target sites were also maximized based on the predicted mRNA structures, but the unpaired nucleotides could be <60%. It is of note that two ODNs, M2-47-as and C31-30-as, that were targeted to GAAA-containing sites were among the nine highly active antisense ODNs, and none of the ODNs encompassing the start codon had high activity.

All ODNs were custom-synthesized and purified to <99% (MWG Biotech, Ebersberg, Germany), and the 16 phosphorothioate-end-modified antisense ODNs and control ODNs were further ethanol-precipitated twice to remove small-molecule impurities. Synthetic siRNAs (Dharmacon, Lafayette, CO) were further ethanol-precipitated twice. Following are the regions of the genes targeted by the 16 end-modified antisense ODNs and 8 siRNAs, as specified by the nt numbers of cDNA sequence entries (hCdc6, U77949; hMcm2, NM_004526; hCdc45, AF053704) in databases: The antisense ODNs (C6-, targeted to hCdc6; M2-, targeted to hMcm2; C45-, targeted to hCdc45) were as follows: C6-30 (nt 1006–1025), C6-33 (nt 1163–1181), C6-35 (nt 1123–1146), C6-39 (nt 1550–1576), M2-33 (nt 1686–1705), M2-34 (nt 1716–1731), M2-47 (nt 2330–2346), M2-61 (nt 2568–2583), C45-8 (nt 455–469), C45-18 (nt 874–893), C45-22 (nt 1040–1059), and C45-30 (nt 1200–1215). The siRNAs (siC6., targeted to hCdc6; siM2., targeted to hMcm2; siC45., targeted to hCdc45; siC6.1 (nt 1234–1254), siC6.2 (nt 1085–1105), siC6.3 (nt 2509–2529), siM2.1 (nt 643–663), siM2.2 (nt 2727–2747), siM2.3 (nt 2804–2824), siC45.1 (nt 1189–1192), and siC45.2 (nt 456–460), the sequences of the mismatched control ODNs and RNAs that appear in the figures are as follows: C6-35-mm, 5′-AAGATGATTTAGTGCAAAA-3′; M2-47-mm, 5′-TCCTAGGTTGAAGCG-3′; C45-30-mm, 5′-AAGGAGTTGTCCTCCTCCC-3′; siC6.1 (the sense strand); siC6.1 (the sense strand); same for other control RNAs), 5′-ATT- TTCCGGCTTTATACCCAGA-3′; siC6.2 mm, 5′-AAGACCTTAGTTTG- AGCACA-3′; siM2.1 mm, 5′-AAGGAGCGCCTCCGGAACGGAC-3′; and siC45.1, 5′-AAGGAGTTGTCCTCCTCCAGT-3′.

Cell Culture, Transfection, and Cell Viability Assay. All culture media and transfection agents were from Invitrogen (Carlsbad, CA). Cells were grown in various media supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2, HeLa (cervical adenocarcinoma), Hone1 (nasopharyngeal cancer), T-Tn (esophagel cancer), and HepG2 (hepatocellular carcinoma) cells were grown in DMEM. NCI-H446 (small cell lung carcinoma), NCI-H460 (large cell lung carcinoma), Chang (from liver; a gift from M. Lung, Hong Kong University of Science and Technology, Hong Kong, China), who obtained the cells from R. S. Chang, University of California at Davis, Davis, CA, who generated the line; the cells we used are...
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tumorigenic, as determined by non-contact-inhibited growth and xenograft growth in nude mice; data not shown), BEL-7402 (hepatocellular carcinoma), and L-02 (normal human liver cells; Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Shanghai, China) were cultured in RPMI (1640). Hep3B (hepatocellular carcinoma; p53−/−) were maintained in MEM. One day before transfection, cells were trypsinized, diluted in growth medium, and seeded in 96-well plates. At 70–80% confluence, cells were transfected with a complex of ODNs (final concentrations during transfection, 0.7 μM for HeLa, Henl, T-Tn, and Chang cells and 1.0 μM for other cells) and 0.7 μl (for HeLa, T-Tn, and Chang cells) or 1.0 μl (for other cells) of Lipofectamine 2000 (Invitrogen) in 100 μl of OptiMEM medium for 4 h. Cells were then incubated in FBS-containing growth medium for 44 h, before WST-1 assays were performed as described previously (26). Counted numbers of cells were used to construct the standard curves. siRNAs were transfected into cells at 40–50% confluence with a complex of siRNA (final concentration during transfection, 100 nM) and 1.0 μl of Oligofectamine (Invitrogen) in 150 μl of OptiMEM medium for 18 h. The cells were then incubated in FBS-containing growth medium for 54 h before the WST-1 assay.

Rescue by the Silently Mutated hCdc6 Gene. The sense sequence targeted by C6-35-as was changed from 5′-TTGAACTTCCCACCTT-3′ to 5′-CTCAATTITTCGCCGT-3′. The silently mutated and wild-type hCdc6 genes were cloned into pcDNA3.1/Zeo (Invitrogen) to obtain pC6WT, pC6SM, or pC6SM together with 0.2 μg of pcDNA3.1/Zeo (Invitrogen) in 100 μl of OptiMEM medium for 4 h. Cells were then incubated in FBS-containing growth medium for 44 h, before WST-1 assays were performed as described previously (26). Counted numbers of cells were used to construct the standard curves. siRNAs were transfected into cells at 40–50% confluence with a complex of siRNA (final concentration during transfection, 100 nM) and 1.0 μl of Oligofectamine (Invitrogen) in 150 μl of OptiMEM medium for 18 h. The cells were then incubated in FBS-containing growth medium for 54 h before the WST-1 assay.

RESULTS

Design and Initial Screening of Antisense ODNs. The primary action of antisense ODNs to inhibit gene expression is to bind to the complementary target mRNA and activate the endogenous RNase H to cleave the mRNA. We first applied the computer program mFold (24) to predict the secondary structures of the mRNAs of the target genes. The areas on the mRNA predicted to have 60–100% unpaired nucleotides were chosen as potential target sites for antisense ODNs. A total of 170 antisense ODNs of 14–22 nucleotides each were designed, of which 66 (named C6-1-as through C6-66-as) were targeted to hCdc6, 64 (M2-1-as through M2-64-as) to hMcm2, and 40 (C45-1-as through C45-40-as) to hCdc45. Some of the 170 antisense ODNs were designed based on other considerations, including targeting areas containing the tetranucleotide GGGA sequence (25) and the AUG start codon on the target mRNA (see “Materials and Methods” for more details).

Because silencing of replication-initiation genes was expected to result in inhibition of DNA replication and thus of cell proliferation, the 170 antisense ODNs were first individually subjected to initial screening for their abilities to inhibit cell proliferation in human cell lines derived from liver, esophageal, and cervical cancers. Sixteen of the antisense ODNs were initially considered active and chosen for further analysis (see below) because each inhibited cell growth by 50–70% (i.e., viable cell numbers were 30–50% compared with untreated cells) in all of the cell lines tested, whereas the transfection agent alone inhibited cell growth by ~20% (data not shown), as measured by the WST-1 cell viability assay (26).

Selected Antisense ODNs and siRNAs Can Inhibit Target Gene Expression. To investigate the effects on inhibition of target gene expression, DNA replication and cell proliferation, the 16 ODNs that showed antiproliferation activities in the initial screening were modified with phosphorothioate linkages at both the 5′ and 3′ ends to increase resistance to exonucleases. Similarly modified sense and mismatched ODNs were used as the controls. The mismatched ODNs had the same base compositions of the corresponding antisense ODNs, but one in every five nucleotides on average in each ODN was chosen as potential target sites for antisense ODNs, but one in every five nucleotides on average in each ODN was chosen as potential target sites for antisense ODNs.
initiation proteins in addition to the target gene and the internal control \(\beta\)-actin gene was coamplified with each of the three replication-initiation genes.

The results show that the mRNA and protein product levels of the target genes were significantly and specifically reduced by several antisense ODNs in all cell lines tested. In Chang cells (Fig. 1A) and L-02 cells (Fig. 1B), for example, C6-35-as targeted to hCdc6 lowered the level of hCdc6 mRNA (Fig. 1, A and B, Lane 5, top panels), but not the mRNA levels of two other replication-initiation proteins, hMcm2p and hCdc45p, in the same cells transfected with C6-35-as (Fig. 1, A and B, Lane 5, middle and bottom panels). Similarly, M2-47-as targeted to hMcm2 diminished the level of hMcm2 mRNA (Fig. 1, A and B, Lane 8, middle panels) without affecting the levels of the mRNAs encoding hCdc6p and hCdc45p (Fig. 1, A and B, Lane 8, top and bottom panels), and C45-30-as targeted to hCdc45 reduced the level of hCdc45 mRNA (Fig. 1, A and B, Lane 11, bottom panels) but did not affect the levels of hCdc6 and hMcm2 mRNAs (Fig. 1, A and B, Lane 11, top and middle panels). The transfection agent (Lane LF) alone and the sense (Lane ss) and mismatched (Lane mm) control ODNs did not noticeably alter the expression levels of the genes analyzed (Fig. 1, A and B). Similar results were obtained for all other cell lines tested (data not shown). In addition, six other antisense ODNs, C6-60-as, M2-61-as, M2-33-as, C6-33-as, M2-34-as, and C45-18-as, also significantly reduced (by \(\geq 60\%\)) the mRNA levels of the corresponding target genes (Fig. 1C).

As the mRNA levels were specifically reduced by the antisense ODNs, the protein levels of the target genes were also correspondingly lowered (Fig. 1, D–F). For example, as shown in Fig. 1D, the level of hCdc6p (top panels), but not of hMcm2p (middle panels) were reduced by C6-35-as (Lanes 3, 7, and 11) in three cell lines, whereas the transfection agent (Lanes 2, 6, and 10) and C6-35-mm (Lanes 4, 8, and 12) had no effect. Similarly, M2-47-as, but not the transfection agent or M2-47-mm, reduced the hMcm2p level (Fig. 1E), and C45-30-as, but not the transfection agent or C45-30-mm,
lowered the hCdc45p level (Fig. 1F). Together, these data suggest that the antisense ODNs effectively and specifically inhibited the expression of the corresponding target genes.

We also used a different gene-silencing strategy, RNA interference. Three siRNAs (siC6.1, siC6.2, and siC6.3) targeted to hCdc6, three (siM2.1, siM2.2, and siM2.3) to hMcm2, and two (siC45.1 and siC45.2) to hCdc45 were designed based on general strategies (29), except that siC6.1 and siC45.1 were targeted to areas overlapping with the target sites of two active antisense ODNs, C6-35-as and C45-30-as, respectively. These two siRNAs still obeyed the general siRNA selection guidelines (29). All eight siRNAs were able to reduce target gene expression by 60–90% as measured by RT-PCR (Fig. 1G) and immunoblotting (Fig. 1H), whereas the transfection agent (Lane OF) and the control RNAs (Lane mm), with two to four mismatched bases, did not significantly affect the mRNA or protein levels of the target genes (Fig. 1, G and H).

DNA Replication Is Inhibited by the Antisense ODNs and siRNAs That Can Silence the Target Genes. Using BrdUrd incorporation assays, we showed that silencing of replication-initiation genes by antisense ODNs (Fig. 2, A and B) and siRNA (Fig. 2C) led to specific inhibition of DNA replication. For example, C6-35-as significantly reduced the number of HepG2 cells that incorporated BrdUrd, whereas the transfection agent alone and C6-35-mm had no effect (Fig. 2A). Similar results were also obtained for several other cell lines tested, as shown quantitatively in Fig. 2B. Approximately 25–45% of untreated cells and of those treated with the transfection agent alone or C6-35-mm incorporated BrdUrd, indicating that these cells were in S phase during the labeling period, as expected for exponentially growing human cells in culture. In contrast, only ∼5% of cells were able to incorporate BrdUrd after transfection with C6-35-as in all cell lines tested (Fig. 2B). Therefore, C6-35-as inhibited DNA replication by 70–90% when the results were normalized to those for the untreated cells. Similar results were also obtained for other antisense ODNs tested, including M2-47-as, C45-18-as, and C45-30-as (data not shown). siRNAs inhibited DNA replication to degrees similar to those for the antisense ODNs (Fig. 2C). The results from both antisense ODNs and siRNAs are consistent with hCdc6p, hMcm2p, and hCdc45p being required for DNA replication in human cells.

Cell Proliferation Is Inhibited by the Antisense ODNs and siRNAs That Can Silence the Target Genes. The effects of the antisense and control ODNs on the proliferation of cancer and normal cells were measured by use of the WST-1 assay. The antisense ODNs C6-35-as, M2-47-as, and C45-30-as inhibited the proliferation of cancer cells (HepG2, Hep3B, and Chang cells) by 60–90% (i.e., the...
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We first confirmed that hCdc6p was expressed from both HA-tagged and untagged versions of pC6SM and pC6WT (the wild-type hCdc6 gene) after transient transfection by immunoblotting with anti-HA and anti-hCdc6 antibodies (data not shown). After transfection with C6-35-as, the level of hCdc6p expressed from C6-35-as was much reduced as expected, whereas that from pC6SM was only moderately reduced (data not shown). However, possible rescue of cell viability could not be easily observed by measuring the cell number of the entire cell population because the transfection efficiency for plasmid DNA (~20%, as measured in a plasmid expressing GFP; data not shown) was much lower than that for ODNs. To overcome this problem, we cotransfected pC6SM with a plasmid expressing GFP (at a 4:1 molar ratio in favor of pC6SM or pC6WT) and monitored the percentage of GFP-positive cells in the population as the indication of cell viability after further transfection with C6-35-as. If the expected rescue occurred, the percentage of GFP-positive cells transfected with C6-35-as (normalized to cells not further treated) should have been higher for cells pretransfected with pC6SM than for those pretransfected with the vector control (pcDNA3.1) and those pretransfected with pC6WT.

As expected, pretransfection of the vector pcDNA3.1 did not rescue the cells from the effects of C6-35-as (Fig. 3C; ~18% GFP-positive cells, or ~82% growth inhibition). On the other hand, pretransfection with pC6SM increased the number of GFP-positive cells to ~45%, whereas pC6WT rescue was much lower, with ~25% GFP-positive cells (Fig. 3C). The reason that pC6SM did not fully rescue the cells (in which case the GFP-positive cells would be ~80%, as for the cells treated with the transfection agent instead of C6-35-as) is probably because some GFP-positive cells were under- or untransfected by pC6SM. As controls, the percentages of GFP-positive cells treated with the transfection agent alone or the mismatched control ODN (C6-35-mm) were not significantly affected by pretransfection with pC6SM, pC6WT, or pcDNA3.1 (Fig. 3C). These results strongly suggest that the inhibition of cell proliferation was the result of gene silencing by the antisense ODN.

Apoptosis Occurs in Both p53-Positive and -Negative Cancer Cells But Not in Normal Cells When the Target Genes Are Silenced. Not only was cell proliferation inhibited, but most cancer cells transfected with the antisense ODNs and siRNAs died, as ob-
served under the microscope (data not shown). We then determined that the mode of cell death was apoptosis by several assays (Figs. 4 and 5). We first detected apoptosis by the TUNEL assay (32) in HepG2 cells after transfection with C6-35-as but not with the transfection agent or C6-35-mm (Fig. 4A). Similar degrees of apoptosis were induced in all of the cancer cell lines tested (HepG2, Hep3B, Chang, and L-02) cells transfected with C6-35-as. C. average percentages (±SD; bars) of TUNEL-positive cells in different cell lines transfected with C6-35-as. LF and C6-35-mm were used as controls. D. TUNEL assays in HeLa cells transfected with the transfection agent (OF), siC6.1, or siC6.1 mm. E. Annexin V (Ann-Cy3) staining plus esterase (6-CFDA) assays in HeLa cells. Green fluorescence represents live or early apoptotic cells and red fluorescence represents apoptotic or necrotic cells. Cells positive for both represent early apoptotic cells. F. average percentages (±SD; bars) of Annexin V–Cy3-positive cells in different cell lines. G. apoptosis induced by the antisense ODNs was attenuated by the caspase inhibitor, Z-VAD-fmk. Chang cells were transfected with different antisense ODNs as indicated or also treated with Z-VAD after transfection. Cell viability was measured by the WST-1 assay. Bars, SD.
assays. In addition, our data (Fig. 4E) also suggest that cell death induced by the antisense ODNs was not necrosis because the cells were still positive for 6-CFDA, which measures the activity of cellular esterases (which convert 6-CFDA to 6-carboxyfluorescein; Ref. 34) and should therefore be positive for early apoptotic cells, as well as for viable cells, but not for necrotic cells.

To determine whether apoptosis induced by the antisense ODNs proceeded via caspase activation, we added the caspase inhibitor Z-VAD to cell cultures immediately after transfection with the antisense ODNs and observed significantly reduced cell death as measured by the WST-1 assay (Fig. 4G) and microscopic observations (data not shown). Without Z-VAD, the numbers of viable cells after transfection with the four antisense ODNs tested were 18–30% of the numbers of untreated cells, whereas Z-VAD increased the percentages to 40–65% (Fig. 4G). These results suggest that apoptosis induced by the antisense ODNs was caspase dependent.

Apoptosis of cancer cells was also observed by flow cytometric analysis of DNA content. HepG2 cells had a sub-G1 (<2C DNA) population (34.2%) after transfection with C6-35-as (Fig. 5A). On the other hand, most normal cells (L-02) showed G1 arrest (68.7%) with a reduction of the S and G2-M populations, and apoptosis was probably prevented as a consequence (Fig. 5B). As controls, the transfection agent and C6-35-mm did not significantly affect cell cycle distributions compared with the untreated cells in either the HepG2 or L-02 cell line (Fig. 5, A and B).

To examine the effects of inhibiting the expression of replication-initiation proteins in different phases of the cell cycle, we performed flow cytometry experiments with synchronized HeLa cells, which showed good synchrony with double thymidine block in G1 and subsequent release (Fig. 5C). Most cells had not entered the S phase 1 h after release, but most had completed the S phase by 6 h after release and then finished mitosis after 12 h. We selected the time points of 1 and 6 h after release from G1 block to transfect the cells with C6-35-as, using the transfection agent alone and C6-35-mm as the controls, and then analyzed the cells 8 h after transfection (with a transfection time of 4 h, the cells at harvest were at 12 h after release from G1). When the cells were treated with the transfection agent or C6-35-mm at 1 h after release (when the cells had not entered S phase, according to the data shown in Fig. 5C), they were able to complete DNA replication and mitosis by the harvest time, as did the untreated cells (Fig. 5D), consistent with unimpeded cell cycle progression shown in Fig. 5C. In contrast, the cells transfected with C6-35-as at 1 h after release were blocked in the S and G2-M phases (Fig. 5D; 35.3% in S and 40.2% in G2-M phase), and apoptosis was observed at a later time point (20 h post-transfection; data not shown). On the other hand, when the cells were transfected with C6-35-as at 6 h after release from G1 (when they had completed DNA replication, according to the results shown in Fig. 5C), they were able to undergo mitosis to become G1 cells by the harvest time, as did the untreated cells and those treated with the transfection agent or C6-35-mm (Fig. 5E). Therefore, our data (Fig. 5, C–E) are consistent with the replication-initiation protein being required for DNA replication but not for mitosis, indicating that the antisense ODN did not have significant nonspecific effects on the cells, at least during mitosis.

**DISCUSSION**

We have shown that selected antisense ODNs and siRNAs targeted to three human DNA replication-initiation genes effectively and specifically inhibited target gene expression, DNA replication, and cell proliferation. Moreover, the antisense ODNs and siRNAs induced apoptosis in both p53-positive and -negative cancer cells but not in normal L-02 cells. Given that various antisense ODNs and siRNAs, targeted to different replication-initiation genes, brought about similar effects on a variety of cancer cell lines, we conclude that a novel and effective anticancer strategy would be to inhibit the expression of selective replication-initiation proteins with antisense ODNs and siRNAs.
Our data show that inhibition of cell proliferation and induction of apoptosis is consequently linked to silencing of the target genes by the antisense ODNs and siRNAs, as summarized below. (a) Several antisense ODNs and siRNAs largely silenced the corresponding target genes while leaving the expression of other functionally related replication-initiation proteins unaffected. (b) The 16 antisense ODNs targeted to three replication-initiation genes showed good agreement between their abilities to silence the target genes and to inhibit cell proliferation. (c) A silently mutated gene significantly lessened the effects of the antisense ODN, providing very strong evidence that the inhibition of cell proliferation and induction of apoptosis did not result from unintended inhibition of an unknown cellular target(s). (d) The antisense ODN did not block mitosis when synchronized cells were transfected after they had completed S phase, indicating that the antisense ODN did not cause any detectable harmful effect on cells in the G2–M phase. (e) Inhibition of the expression of the target genes by two different gene-silencing strategies, antisense and RNA interference, had similar effects on DNA replication, cell proliferation, and cell viability.

Because the vast majority of human body cells are nonproliferating and thus do not express or need most replication-initiation proteins (1, 2), antisense ODNs, siRNAs, and other agents that can silence these genes should not harm, or affect the cellular functions of, most normal cells in the body. This should provide the first level of selectivity of these agents as potential anticancer therapeutics. As for the small fraction of normal, proliferative body cells, they should still be able to perform their duties while arresting their cell cycles instead of entering into a fatal S phase when the expression of a replication-initiation protein is inhibited. This should provide another level of selectivity for cancer therapy: stopping proliferation of normal cells without interfering with their functions or killing them. We therefore suggest that inhibition of replication-initiation genes is an effective anticancer strategy that should not cause serious side effects.

It has been reported that gene silencing of Orc6p (a subunit of the initiator protein ORC, which is expressed in both stationary and cycling cells) by siRNAs results in deregulation of the cell cycle, including a block in mitosis and appearance of multinucleated cells (35). It has also recently been shown that a fragment of the geminin protein, which inhibits the activity of the replication-initiation protein Cdt1p, can inhibit DNA replication and cell proliferation and lead to apoptosis of cancer cells in culture (36). As proposed (36), although geminin or a fragment thereof cannot be targeted to body cells, small molecules that may inhibit the activities of replication-initiation proteins are potential anticancer drugs. On the other hand, antisense ODNs (reviewed in Refs. 37–40) and siRNAs (41–43) can inhibit gene expression and have been shown to exert expected biological actions in animals as well as in cultured cells. Therefore, antisense ODNs and siRNAs that can inhibit the expression of selective replication-initiation proteins may become effective anticancer agents. In addition, appropriate carriers (reviewed in Refs. 44 and 45) and/or chemical modifications may further increase the in vivo stability, delivery, cellular uptake, and thus efﬁcacy of these potential anticancer drugs.

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REFERENCES


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