Small interference RNA-mediated suppression of overexpressed cyclin E protein restores G1/S regulation in NIH-OVCAR-3 ovarian cancer cells

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Abstract. The development of ovarian cancer, unlike that of most human tumors, is rarely dependent upon the mutually exclusive loss of RB and p16 cell cycle proteins. RB$^+$/p16$^+$ ovarian cancer cell lines are, however, insensitive to the growth-suppressive effects of ectopically expressed p16 protein, which suggests that they harbor as yet unidentified defects that compromise cell cycle regulation in late G1/S. In the current study, we used Western blotting to analyze cyclin E protein expression in a panel of normal and tumor ovarian tissues and ovarian cancer cell lines (including the p16-insensitive RB$^+$/p16$^+$ ovarian cancer cell line, NIH-OVCAR-3). Both the NIH-OVCAR-3 cell line and 70% of RB$^+$/p16$^+$ ovarian tumors showed abnormally elevated levels of the full-length cyclin E protein (EL1) in addition to several low molecular weight (LMW) isoforms of cyclin E. Using small interference RNA (siRNA), we have inhibited the synthesis of cyclin EL1 protein by approximately 80% and eliminated the LMW isoforms in NIH-OVCAR-3 ovarian cancer cells. Associated with the down-regulation of cyclin E expression, we observed both a marked shift in RB protein expression to the active, hypophosphorylated state and barely detectable expression of cyclin A (which is usually expressed upon entry into S-phase). Consistent with the protein expression data, cell cycle distribution analysis indicated that the NIH-OVCAR-3 cells had undergone a marked accumulation in G1 phase of the cell cycle. These data indicate the therapeutic potential of targeted RNA interference in the treatment of ovarian cancer patients whose tumors overexpress cyclin E protein.

Introduction

Owing to the insidious nature of ovarian cancer progression and the lack of reliable diagnostic markers, patients often present at a stage too late to benefit from conventional therapies. The need for the development of novel diagnostic, prognostic and therapeutic interventions for ovarian cancer patients is critical and depends upon the identification of relatively consistent molecular defects that can be readily assayed and, in the case of gene-based therapies, compensated for through the introduction of a functional gene or suppression of an aberrantly expressed gene.

In common with other cancers, ovarian tumors exhibit uncontrolled cellular proliferation, prompting researchers in recent years to assess the contribution of cell cycle deregulation to ovarian tumorigenesis. As a consequence of these efforts, loss of control at the restriction point in late G1 phase has been identified as a likely precondition for the development of ovarian cancer although the precise mechanism(s) of deregulation have yet to be fully elucidated (reviewed in ref. 1).

In normal cells, active, hypophosphorylated RB blocks transit into the DNA synthetic (S)-phase by complex and not yet fully understood mechanisms. These include binding and inactivation of E2F-family transcription factors (2,3) and active repression of E2F-regulated genes (reviewed in ref. 4). Progression through G1 into S-phase of the cell cycle is achieved through the sequential phosphorylation (and inactivation) of the RB protein by cyclin D/CDK4/6 and cyclin E/CDK2 complexes, respectively. This results in the release of active E2F transcription factors. The cyclin-dependent kinase inhibitor (CDI), p16, negatively regulates G1 progression by binding to CDK4 and CDK6. The subsequent disruption of cyclin D/CDK4/6 complexes and the redistribution of the CDIs, p21 and p27, to cyclin E/CDK2 complexes inhibits cyclin E/CDK2 kinase activity, contributing to G1 arrest (reviewed in ref. 5).

The most frequently observed mechanisms of G1/S cell cycle deregulation in human malignancies involve the mutually exclusive loss of RB or p16 expression (6). In contrast, structural alterations of the RB and p16 genes are uncommon in ovarian cancers as is the loss of protein expression (reviewed in ref. 1). Indeed, we have previously shown that 82% of ovarian tumors and cell lines retain the expression of both RB and p16 proteins with the majority overexpressing p16 protein (7). Despite the presence of these two negative regulators of G1-phase, however, RB$^+$/p16$^+$ ovarian cancer cell lines are defective with regard to G1/S regulation as indicated by their insensitivity to the growth suppressive effects of ectopically overexpressed functional p16 protein (7).

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It is likely that deregulation of one of the G1 cyclin/CDK complexes is responsible for overriding RB function in RB+/p16+ ovarian cancers. Indeed, overexpression of cyclin D protein has been reported in multiple types of cancers including non-small cell lung, breast, and head and neck tumors (reviewed in ref. 8) although this overexpression is usually in association with the loss of p16 expression (not observed in ovarian cancers). In contrast, several groups have reported low frequencies of cyclin D overexpression in primary ovarian tumors, ranging from 26 to 32% (9-11).

Recent studies suggest that abnormally elevated cyclin E protein expression may play a more significant role than that of cyclin D in ovarian tumorigenesis. Overexpression of cyclin E has been detected at frequencies from 45 to 88% of primary ovarian cancers and is associated with tumors of a more advanced stage and grade (12-19). Indeed, Sui *et al* (12) reported a correlation between increasing levels of cyclin E protein and ovarian cancer progression with only 9.1% of benign tumors versus 70.2% of malignant tumors showing elevated levels of cyclin E protein. Several groups have established a link between overexpression of cyclin E and a poor prognosis in ovarian cancer patients (12-13,16), with a further increase in the risk of mortality in those patients whose tumors also lack p27 (12).

In addition to the full-length (50 kDa) form of cyclin E (EL1), several tumor-specific low molecular weight (LMW) isoforms of the protein have been identified (20-22). Proteolysis of cyclin EL1 by either elastase (23) or calpain (24) results in 4 of the 5 known LMW isoforms (EL2, EL3, EL5 and EL6) whereas the remaining LMW isoform, EL4, is produced from an alternate translation start site (23-25). Transfection of the LMW isoforms, EL2 and EL3, into normal mammary epithelial cells and into the breast and ovarian cancer cell lines, MCF-7 and MDAH-2774, respectively, was found to result in elevated cyclin E/CDK2 kinase activity (hyperactivity) and accelerated entry into S-phase relative to cells overexpressing the full-length cyclin E protein, EL1 (15,26,27). Furthermore, the LMW-associated kinase activity was completely resistant to inhibition by p21 and p27 (15,26,27). Using Western blot analysis, three studies have reported overexpression of the LMW isoforms of cyclin E in 68, 84 and 88% of primary ovarian cancers, respectively. In each study, the presence of the LMW isoforms were associated both with an advanced stage and grade of tumor and poor patient prognosis (15,16,18).

As mentioned earlier, we previously reported the insensitivity of RB+/p16+ ovarian cancer cell lines to adenoviral-mediated overexpression of p16 protein (7). The inability of p16 to induce a G1 arrest in one of the cell lines, NIH-OVCAR-3, was associated with the persistence of RB phosphorylation, suggesting deregulation of G1 cyclin-associated kinase activity in these cells. Analysis of cyclin D and its kinase partners, CDK4 and CDK6 did not reveal abnormalities in expression nor, in the case of CDK4, did we find mutations that have been reported to inhibit p16 binding in NIH-OVCAR-3 cells (7,28,29).

In the present study, however, we identified abnormally high levels of both the full-length and LMW isoforms of cyclin E protein in the p16-insensitive NIH-OVCAR-3 cells and in 70% of RB+/p16+ primary ovarian tumors, consistent with the high frequency of cyclin E overexpression in ovarian cancers reported by other groups (12-19). The above expression data clearly support a role for the deregulation of cyclin E expression in ovarian tumorigenesis. However, there are no reports, to our knowledge, of functional studies that address the effects of siRNA-targeted suppression of cyclin E upon the cell cycle distribution of ovarian cancer cells. Studies of this sort are essential for assessing the therapeutic potential of aberrantly expressed cyclin E protein depletion in ovarian cancer patients. Thus, we have used siRNA to substantially reduce the level of full-length cyclin E protein and eliminate the LMW isoforms in NIH-OVCAR-3 cells. The consequent loss of RB phosphorylation and accumulation of cells in G1 phase demonstrates that the suppression of endogenous, overexpressed cyclin E protein is capable of restoring RB-mediated control in late G1 phase.

Materials and methods

Cell lines and tissues. The ovarian cancer cell lines Caov-3, ES-2, NIH-OVCAR-3, PA-1 and SK-OV-3, and the breast epithelial cell line, HBL-100 were obtained from the American Type Culture Collection. All of the culture media was purchased from Gibco BRL. Caov-3 was cultured in DME medium supplemented with 0.45% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum (FBS). ES-2 and SK-OV-3 were cultured in McCoy's 5a medium supplemented with 2 mM L-glutamine and 10% FBS. NIH-OVCAR-3 was cultured in OPTI-MEM I medium supplemented with 2 mM L-glutamine and 10% FBS. PA-1 and HBL-100 were cultured in MEM medium supplemented with 2 mM L-glutamine and 10% FBS. All cells were grown in a humidified incubator at 37˚C in the presence of 5% CO_2.

Two matched pairs of frozen normal and tumor ovarian tissues and nine unmatched frozen ovarian tumor tissues were obtained from the University of Colorado Tissue Procurement Core (Denver, CO, USA). Eight of the tumors were carcinomas, two were benign epithelial tumors and one was a teratoma.

Antibodies. The following primary antibodies were used: anti-cyclin E (HE12) (Santa Cruz Biotechnology), anti-cyclin A (AC-40) (Upstate Biotechnology), anti-RB (14001A) (BD Biosciences) and anti-actin (I-19) (Santa Cruz Biotechnology). The horseradish peroxidase-conjugated secondary goat anti-mouse and goat anti-rabbit antibodies were obtained from Bio-Rad.

siRNA transfections. An ON-TARGETplus SMARTpool (L-003213-00-0005) containing four siRNAs that targeted different regions of the cyclin E gene and a non-targeting (control) siRNA were obtained from Thermo Scientific Dharmacon. Duplicate 10-cm dishes of NIH-OVCAR-3 ovarian cancer cells were grown to ~50% confluence and transfected with 100 nM cyclin E-specific siRNA or 100 nM non-targeting (control) siRNA using the DharmaFECT I lipid transfection reagent, according to the manufacturer's instructions. Additional transfection controls included incubation of the cells either in the DharmaFECT I lipid (no siRNA) or OPTI-MEM I medium supplemented with 2 mM

L-glutamine (no siRNA or DharmaFECT I lipid). After 5 h at 37˚C, the medium was replaced with OPTI-MEM I supplemented with 10% FBS and 2 mM L-glutamine. At 48 and 72 h post-transfection, the cells were harvested both for protein extraction and cell cycle distribution analysis by fluorescence activated cell sorting (FACS).

Protein isolation and Western blot analysis. Cells were washed twice with PBS and harvested with 0.25% trypsin/ 1 mM EDTA (or PBS/1 mM EDTA for NIH-OVCAR-3 transfectants). Following centrifugation, the cells were lysed in Laemmli sample buffer (30), boiled for 3 min and sheared through a 26-gauge syringe needle. Protein was extracted from frozen normal and tumor ovarian tissues as previously described (7). Approximately 50 μg of each protein extract was subjected to SDS-PAGE (30) on 7% (for detection of RB) or 10% (for detection of cyclin E and cyclin A) gels and electrophoretically transferred to PVDF membranes for 45 min at 0.45 A. Immunodetection was performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Fluorescent activated cell sorting analysis. NIH-OVCAR-3 transfectants and controls were washed twice with PBS and harvested with PBS/1 mM EDTA. Following centrifugation, the cells were washed in ice-cold PBS, fixed in ice-cold ethanol, subjected to centrifugation and resuspended at $5x10^5$ cells/ml in 0.040 mg/ml propidium iodide. Cell cycle distribution profiles were determined by FACS on a Beckman-Coulter (Fullerton, CA) FC500 utilizing a 20 mW 488 nm blue solid-state laser source. The propidium iodide emission was detected by a standard photomultiplier tube with a 620 nm short-pass filter. Aggregate discrimination was employed using peak vs area signals. Post acquisition analysis was performed with Phoenix Flow Systems (San Diego, CA) Multicycle software. The FACS analysis was conducted at Texas A&M Health Science Center College of Medicine, Temple, TX, USA.

Results

Overexpression of cyclin E protein in NIH-OVCAR-3 ovarian cancer cells. To determine the nature of the G1 cell cycle defect underlying the p16-insensitivity of the RB+/p16+ NIH-OVCAR-3 cells, we assessed cell extracts for cyclin E protein expression by Western blotting. For comparison, we included an additional four ovarian cancer cell lines, of which two, ES-2 and PA-1, coexpress RB and p16 proteins and two, Caov-3 and SK-OV-3, express RB but lack p16 expression (7). In addition, the breast epithelial cell line, HBL-100, which overexpresses p16, lacks functional RB (due to the binding of SV40 T Antigen) and expresses a high level of cyclin E protein, acted as a positive control. NIH-OVCAR-3 showed a marked overexpression of the full-length cyclin EL1 protein (relative to the other cell lines in the panel) in addition to several LMW isoforms (Fig. 1). The remaining four ovarian cancer cell lines expressed readily detectable cyclin EL1 with levels ranging from low (in PA-1 cells) to high (in Caov-3 cells) relative to the HBL-100 normal breast epithelial cell line (Fig. 1). Further, in the Caov-3 and ES-2
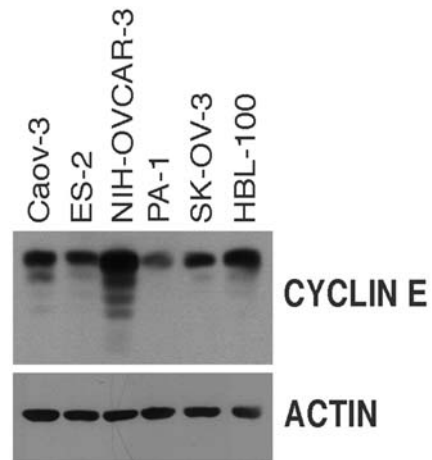

Figure 1. Overexpression of cyclin E protein in the NIH-OVCAR-3 ovarian cancer cell line. Extracts were prepared from logarithmic phase cultures of the indicated ovarian cancer cell lines and equal amounts of protein subjected to Western blotting using anti-cyclin E and anti-actin (as a loading control) antibodies. The HBL-100 cell line acted as a positive control.
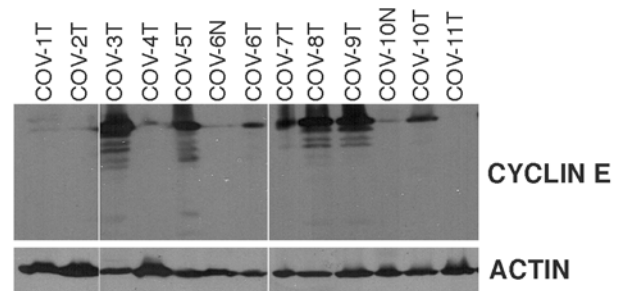

Figure 2. Frequent overexpression of cyclin E protein in primary ovarian tumors. Extracts were prepared from frozen ovarian tissues and subjected to Western blotting using anti-cyclin E and anti-actin (as a loading control) antibodies. Normal and tumor tissues were designated by 'N' and 'T' suffixes, respectively.

cell lines we detected fewer, less intense LMW isoforms of cyclin E relative to NIH-OVCAR-3 (Fig. 1).

Cyclin E overexpression in primary ovarian cancers. To determine if the cell line data were representative of primary ovarian tumors, we assessed the expression of cyclin E protein in a panel of ovarian tissue extracts (including two matched pairs of normal and tumor ovarian tissue extracts, and nine unmatched ovarian tumor tissue extracts). Like NIH-OVCAR-3, ten of the eleven ovarian tumors in our panel were previously shown to express both RB and p16 proteins and, of these, only three showed concomitant cyclin D protein overexpression (7). This low incidence of defective cyclin D expression implied a potential role for cyclin E deregulation in compromising RB function in the RB+/p16+ ovarian tumors in our panel. Indeed, we found abnormally elevated expression of the full-length cyclin EL1 protein relative to the two normal ovarian tissue controls in seven of the ten RB+/p16+ tumors (Fig. 2). Furthermore, all seven of these tumors also expressed the LMW isoforms of cyclin E protein with noticeably higher levels of the LMW isoforms in those tumors that expressed higher levels of the full-length
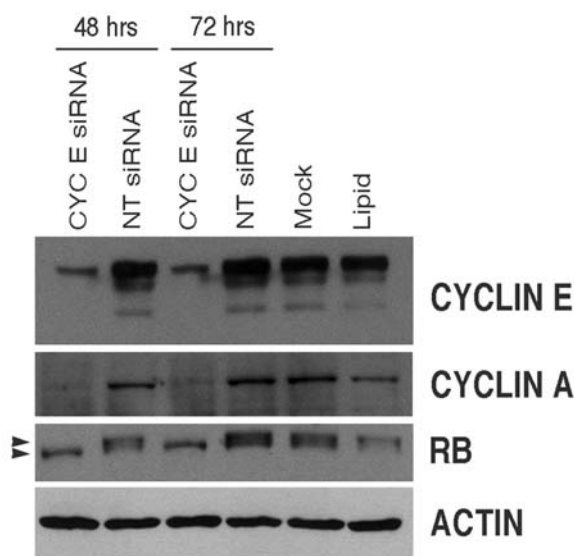
Figure 3. siRNA-mediated suppression of cyclin E protein in NIH-OVCAR-3 cells results in a cell cycle protein expression profile consistent with accumulation in G1. Logarithmic phase cultures of NIH-OVCAR-3 cells were transfected either with cyclin E-specific or non-targeting (NT) siRNA or were mock-transfected (medium only) or treated with the DharmaFECT I lipid only. At 48 and 72 h post-transfection extracts derived from the cells were analyzed for the expression of cyclin E, RB (hyper- and hypophosphorylated forms of RB are indicated by the upper and lower arrows, respectively), cyclin A and actin (loading control) proteins by Western blotting. Identical patterns of expression were obtained at both time-points.
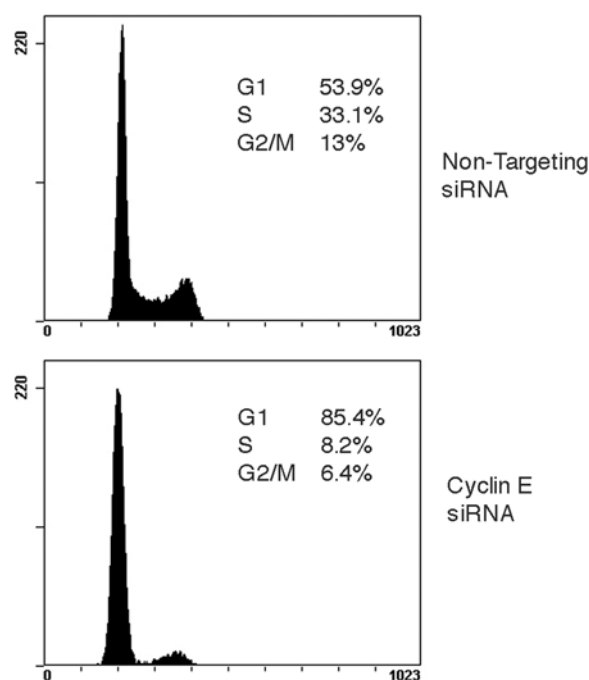

Figure 4. siRNA-mediated suppression of cyclin E protein results in an accumulation of NIH-OVCAR-3 ovarian cancer cells in G1. NIH-OVCAR-3 cells were transfected either with cyclin E-specific or NT siRNA or were mock-transfected (medium only) or treated with the DharmaFECT I lipid only. At 48 and 72 h the cells were harvested for cell cycle distribution analysis by FACS (representative data are shown for the 48-h time-point and control cells transfected with the NT siRNA). Transfection of the cells with the cyclin E-specific siRNA resulted in a marked accumulation of the cells in G1 phase (bottom panel), consistent with the substantial reduction in the expression of the full-length (EL1) form of cyclin E and complete elimination of the LMW isoforms (see Fig. 3). In contrast, the control cells exhibited a normal logarithmic cell cycle profile (top panel).

cyclin EL1 protein (Fig. 2). Notably, the three tumors previously shown to overexpress cyclin D (7) also overexpressed cyclin E, suggesting that deregulation of these two G1-phase cyclins may occur at and contribute to different stages of ovarian cancer progression. Three of the ovarian tumors expressed levels of cyclin E protein that were comparable to the normal tissue controls and one benign serous tumor lacked detectable cyclin E protein expression. The finding of abnormally elevated cyclin EL1 protein accompanied by the presence of LMW isoforms in the majority of RB+/p16+ primary ovarian tumors suggested a clear role for cyclin E deregulation in loss of regulation in late G1/S phase. We therefore hypothesized that a reduction in the level of cyclin E protein would restore cell cycle regulation in ovarian cancer cells. To test this, we used siRNA to suppress the expression of cyclin E in RB+/p16+ NIH-OVCAR-3 ovarian cancer cells and assessed the effect upon cell cycle distribution both by FACS and the analysis of cell cycle regulatory protein expression.

siRNA-mediated suppression of cyclin E restores G1/S regulation to NIH-OVCAR-3 ovarian cancer cells. NIH-OVCAR-3 cells were transiently transfected with either a pool of four cyclin E-specific siRNA duplexes, non-targeting siRNA, DharmaFECT I lipid alone, or were mock-transfected with culture medium. Cells were harvested at 48 and 72 h post-transfection and analyzed both for cell cycle distribution (by FACS), and the expression of the full-length EL1 and LMW isoforms of cyclin E protein by Western blotting. Extracts isolated from the cells transfected with the cyclin E-specific siRNA showed an ~80% decrease in the expression of the full-length cyclin EL1 protein in comparison with

extracts derived from control cells (Fig. 3). Most striking, however, was the lack of detectable cyclin E LMW protein isoforms in cells transfected with the cyclin E-specific siRNA (Fig. 3). The suppression of cyclin E protein was accompanied by an accumulation of cells in G1 phase (Fig. 4) unlike the control cells that maintained a normal logarithmic cell cycle distribution profile (Fig. 4). In keeping with the cell cycle distribution data, NIH-OVCAR-3 cells transfected with the cyclin E-specific siRNA, underwent a shift in RB to the fully hypophosphorylated (active) state and expressed only barely detectable cyclin A - a protein that is ordinarily expressed upon entry into S-phase and maintained throughout G2/M (Fig. 3). In contrast, the control cells expressed both the hyper- and hypophosphorylated forms of RB and readily detectable levels of cyclin A consistent with their unperturbed proliferation (Fig. 3). These data demonstrated that a substantial reduction in the level of full-length cyclin E protein coupled with the elimination of the LMW isoforms was sufficient to reactivate the RB-mediated block to S-phase and thus restore regulation at G1/S in NIH-OVCAR-3.

Discussion

Defective regulation of the G1/S transition appears to be a universal requirement for human malignancy with the majority of cancers showing the loss of RB or p16 expression (5). Ovarian cancers are unusual in that they retain RB and p16

protein expression at a high frequency but are defective in G1/S regulation as indicated by their insensitivity to p16 (7). In the present study, we found that the p16-insensitive RB+/p16+ NIH-OVCAR-3 ovarian cancer cell line markedly overexpressed both the full-length cyclin EL1 protein and its LMW isoforms. Unlike cyclin EL1, the LMW isoforms have been shown to form hyperactive kinase complexes with CDK2 and are resistant to the CDIs, p21 and p27 (15,23,26,27,31). It has also been shown that the LMW isoforms are capable of effectively sequestering p21 and p27, thereby preventing the CDI-mediated inhibition of the full-length cyclin EL1-associated kinase activity (27,31). Thus, our data would suggest that deregulated cyclin E complex activity is responsible for the previously observed persistence of RB phosphorylation and associated uncontrolled cellular proliferation shown by NIH-OVCAR-3 ovarian cancer cells in the presence of ectopically overexpressed functional p16 protein (7).

In addition to our findings in NIH-OVCAR-3, we demonstrated overexpression of cyclin EL1 coupled with the presence of its LMW isoforms in 70% of RB+/p16+ primary ovarian tumors. These data indicated that cyclin E deregulation is an important event in ovarian tumorigenesis *in vivo* and are consistent with the high frequency of cyclin E overexpression reported in primary ovarian tumors (12-19). Notably, whenever Western blot analysis has been used to assess cyclin E protein expression, a clear correlation has been found between the overexpression of cyclin EL1 and its LMW isoforms (15,16,18). To our knowledge, only three published studies in addition to our own have assessed cyclin E expression in ovarian tumors by Western blotting, which has the advantage over the more commonly used immunohistochemical approach in that it is able to distinguish the various isoforms of cyclin E. Thus, it is possible that the contribution of LMW isoform expression to ovarian cancer development (and potentially the development of other types of cancer) has, to-date, been underestimated.

To determine if the targeted down-regulation of overexpressed cyclin E would restore cell cycle regulation in late G1/S in RB+/p16+ ovarian cancer cells, we transfected NIH-OVCAR-3 cells with a pool of four cyclin E-specific siRNAs. The successful suppression of both the EL1 protein (by approximately 80%) and LMW isoforms (by 100%) resulted in the loss of RB phosphorylation and accumulation of cells in G1 phase. These data suggest that the total depletion of the p21/p27-insensitive LMW isoforms of cyclin E, enabled endogenous p21 and p27 to effectively inhibit the residual full-length cyclin E/CKD2 complexes. An earlier report by Li *et al* (32) described a similar decrease in the S-phase population of hepatocarcinoma (HCC) cell lines (that expressed elevated levels of endogenous cyclin E) following the siRNA-mediated suppression of cyclin E protein. These findings indicate the therapeutic potential of using cyclin E-specific siRNA to stem the growth of cyclin E-overexpressing ovarian cancer and hepatocarcinoma, and indeed the multiple types of cancer that have been shown to overexpress cyclin E protein including breast, colorectal and gastric tumors (20-22,33,34).

Rather than directly targeting cyclin E for suppression, Akli and Keyomarsi (25), inhibited elastase, the enzyme that cleaves cyclin EL1 into its LMW isoforms, and found that in the absence of LMW isoform generation, tumor cells undergo a partial G1 arrest. Consistent with our findings, these data indicate that restoration of regulation at G1/S is dependent more upon the elimination of the p21/p27-insensitive LMW isoforms of cyclin E rather than the complete suppression of the p21/p27-sensitive full-length cyclin E protein.

Two studies have addressed the effects of cyclin E deregulation on the responsiveness of ovarian tumors to platinum-based drugs, the standard first line chemotherapy for ovarian cancer, either alone or in combination with other drugs. Farley *et al* (13) focused their analysis upon a group of patients with suboptimally debulked advanced epithelial ovarian cancers. They found that those patients whose tumors overexpressed cyclin E protein showed a decrease in survival following treatment with a combination of cisplatin and taxol. These findings suggest that the suppression of cyclin E might increase the sensitivity of ovarian tumors to cisplatin and taxol, again indicating the potential value of cyclin E-specific siRNA as an adjuvant to existing drug therapies. In contrast, a more recent study by Bedrosian *et al* (18) reported an association between elevated levels of cyclin E/CDK2 activity and increased responsiveness of patients to platinum-based treatments. A similar increase in cisplatin sensitivity was observed in MDAH-2774 ovarian cancer cells transfected with the cyclin E LMW isoforms EL2 and EL3 relative to empty vector control cells and is likely attributable to the increase in the S-phase population, the target of platinum-based drugs, of cells with deregulated cyclin E-associated kinase activity (15). Clearly further studies are needed to clarify the impact of cyclin E protein expression level and cyclin E-associated kinase activity on the effectiveness of platinum-based drugs.

Owing to differences in the responsiveness of ovarian tumors to standard platinum-based chemotherapies, studies are currently underway to find adjuvant or alternative treatment strategies including the use of synthetic steroid agonists or antagonists. Mifepristone (RU486) is a cell type-dependent progesterone agonist or antagonist that has been shown to arrest SK-OV-3 and OV2008 ovarian cancer cell lines in G1 via the induction of p21/p27-mediated inhibition of cyclin E/CDK2 kinase activity (35). As the LMW isoforms of cyclin E are resistant to inhibition by p21 and p27 and capable of sequestering them away from the p21/p27-sensitive full-length cyclin EL1 (15,23,26,27,31), the cyclin E expression status of an ovarian tumor should prove a valuable predictor of its responsiveness to drugs that mediate their effects through p21 and p27. Patients whose ovarian tumors do not express the LMW isoforms of cyclin E might be expected to respond well to treatment with drugs such as Mifepristone. In contrast, the relatively high proportion of patients whose ovarian tumors overexpress both cyclin EL1 and its LMW isoforms (including 70% in the present study) would likely be less responsive to treatment with Mifepristone. Evidence from studies involving other drugs that act through the induction of p21 and p27 support this latter prediction. Akli *et al* (27) reported that ovarian cancer cells transfected with the EL2 and EL3 LMW isoforms of cyclin E were insensitive to both the anti-estrogen ICI 182,780 and lovastatin, which mediate their growth suppressive effects through the induction of p21 and p27. This poses a potential limitation in the

treatment of patients with deregulated cyclin E expression, which might arguably be resolved by the use of RNA interference or elastase inhibitors (25) (to target the p21/p27-insensitive LMW isoforms) in combination with Mifepristone (or other drugs that act through the induction of p21 and p27).

In conclusion, we have found abnormal overexpression of cyclin E protein associated with the presence of LMW isoforms in 70% of RB$^+$/p16$^+$ primary ovarian tumors and in the p16-insensitive RB$^+$/p16$^+$ NIH-OVCAR-3 ovarian cancer cell line, consistent with a major role for cyclin E deregulation in the pathogenesis of ovarian cancer. Using siRNA, we have shown that the suppression of both the full-length and LMW isoforms of cyclin E in NIH-OVCAR-3 cells is sufficient to reactivate RB and restore regulation in late G1/S. These findings indicate the potential therapeutic value of cyclin E-targeted RNA interference strategies for ovarian cancer and tumors of other types that exhibit deregulated cyclin E expression.

Acknowledgements

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