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TITLE: The Role & Action of Prohibitin, an Antiproliferative Gene, in Ovarian Cancer

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The experiments proposed in this application aim at understanding the mechanisms by which prohibitin induces growth arrest in ovarian cancer. We hypothesize that the prohibitin gene product will substantially induce arrest in ovarian cancer cells and enhance differentiation. Accordingly, we will characterize the expression pattern and function of this gene. In specific aim 1, we will define the spatial, temporal and stage specific cellular expression pattern of prohibitin in normal ovary, ovarian tumors of patients with early (FIGO stage I) and advanced (FIGO stage II-IV) stages of ovarian cancer, using immunohistochemistry, in situ hybridization, Western and Northern blot analyses. In specific aim 2, we will determine whether prohibitin over-expression results in the inhibition of ovarian cancer cell growth, using human ovarian cancer cell lines, OVCAR-3, OVCAR-8, TOV-112D, and OV-90. Lastly, in specific aim 3, we will identify and characterize the expression of variant prohibitin mRNA species in ovarian cancer. Clearly, from a clinical perspective, an understanding of prohibitin gene involvement in ovarian somatic cell growth and differentiation during the normal physiological development as well as its role in the etio-pathogenesis of ovarian tumorigenesis is important. This understanding would lead to development of novel strategies for diagnostic or prognostic therapeutics in the treatment of ovarian cancer and in the design of a more rational basis for drug development.
INTRODUCTION

Ovarian cancer is the fourth most common form of cancer in females in the United States. It accounts for 4% of the total number of cancer cases and 25% of cases occurring in the female genital tract. Because of its low rate of cure, however, it is responsible for 5% of all cancer deaths in women and approximately half of deaths due to cancer of the female genital tract. Treatment of ovarian cancer is effective when confined to the ovary with a cure rate approaching 90%. Unfortunately, most patients are diagnosed when cancer cells metastasize. At this stage, ovarian cancer is very difficult to treat and most patients die within 5 years. Therefore, research efforts that are directed at reducing incidence of and mortality from ovarian cancer are of paramount importance.

Cancer arises from the accumulation of multiple mutations and these mutations can arise in oncogenes, tumor suppressor or DNA repair genes. Alterations in tumor suppressor genes are associated with a variety of cancers. In this study, we target a protein that may function as a tumor suppressor in ovarian cancer, prohibitin.

The major focus of this proposal is to understand the tumor suppressive/antiproliferative properties of prohibitin; a gene located on human chromosome 17q21 close to the ovarian and breast carcinoma susceptibility gene BRCA1. Prohibitin was originally cloned based on its ability to induce growth arrest in mammalian fibroblasts and HeLa cells. This gene can induce a G1/S arrest in cells, and prohibitin mutations have been found in a subset of sporadic breast cancers. However, the prohibitin gene is not mutated in epithelial ovarian cancer. Despite the fact that the prohibitin gene is not mutated in ovarian carcinogenesis, there is no information available on the mechanisms of action for prohibitin gene products in ovarian tumor ontology. Recent developments have implicated prohibitin and retinoblastoma tumor suppressor gene product (Rb) to regulate the transcriptional factor E2F function. Moreover, it has been demonstrated that Rb regulates the expression of the murine and human BRACA1 genes through its ability to modulate E2F transcriptional activity. These results provide the rationale to support the goal of this proposal to determine the functional significance of prohibitin in ovarian cancer biology. Our laboratory was the first to identify and characterize the expression of prohibitin gene products during ongoing differentiation of primary rat ovarian granulosa cells isolated from preantral and early antral follicles. Subsequently, we have also demonstrated the cellular distribution of prohibitin during the transitional stages of follicular differentiation in vivo. Within the ovary, increased prohibitin expression has been demonstrated in terminally differentiated cells and cells of atretic follicles. Our recent preliminary data demonstrate that the majority of prohibitin expression is sequestered to the inner mitochondrial membrane of a conditionally immortalized granulosa cells, and its expression increases during cellular differentiation. In addition, preliminary results presented in this proposal revealed for the first time the localization of prohibitin protein pattern in normal and diseased ovarian epithelial cells. We hypothesize, therefore, that over-expression of prohibitin in ovarian cancer cells will substantially induce arrest in cell growth and promote differentiation. The exact functions of prohibitin as well as its mode of regulation in mammalian cells are currently unknown. The ability to negatively regulate cell proliferation is a necessity for all living organisms. The failure in a multicellular organism to provide adequate negative growth control in the developmental period may result in a malformation. This malformation may result in neoplasia. Because negative control is so critical, specific genes have evolved whose role is actively antiproliferative.

In this study, we propose to examine the role of prohibitin in ovarian cancer as a means of investigating the mechanism(s) by which this antiproliferative/tumor suppressor gene product participate in growth, differentiation and cell death. We hypothesize that prohibitin expression plays a role in the regulation of ovarian cancer development. We intend to test this hypothesis by first determining the spatial and stage specific expression pattern of prohibitin in normal ovary, ovarian tumors of patients with early and advanced stages of ovarian cancer and then examine whether prohibitin over-expression results in the inhibition of ovarian cancer growth. Lastly, we will identify and characterize the expression of variant prohibitin mRNA species in ovarian cancer. The data generated by these experiments will begin to address fundamental questions in the field of ovarian cancer biology. “What is the relative expression of prohibitin in various stages of ovarian cancer?”, “How does prohibitin affect the cell cycle subsequently leading to growth arrest and/ or differentiation?” and, “Does prohibitin participate in programmed cell death?”
APPROVED STATEMENT OF WORK

Task 1. Define the spatial, temporal and stage specific cellular expression pattern of prohibitin in normal ovary, ovarian tumors of patients with early (FIGO stage I) and advanced (FIGO stage II-IV) stages of ovarian cancer, using immunohistochemistry, in situ hybridization, Western and Northern blot analyses. [Month 1-12]

   a. Measure the expression of prohibitin protein and RNA content in ovarian cancer specimens. [Month 1-4] 
   b. Localization of prohibitin protein and RNA content in ovarian tumors, using immunohistochemistry and in situ hybridization techniques. Correlate the expression of prohibitin with other well-known tumor suppressor, proliferative, cell survival and apoptotic markers. [Month 1-12] 
   c. Prepare adenovirus vectors for task 2. [Month 1-12]

Task 2. To determine whether prohibitin over-expression results in the inhibition of ovarian cancer cell growth, using human ovarian cancer cell line, OVAR-3 and OVAR-8. [Month 12-24]

   a. Infect human ovarian cell lines with recombinant adenovirus containing prohibitin sense cDNA and anti-sense cDNA. Assay for cell cycle perturbation. FACS analysis and Western blotting to evaluate the levels of the cell cycle regulatory molecules, PCNA, cyclin D2, p16, p16INK4A, p21, Rb and E2F1 and correlate there expression levels with prohibitin. [Month 13-24]
   b. Examine the steady-state levels of PCNA and correlate its expression with prohibitin using Northern/RNAse protection assays. [Month 13-24] 
   c. Construct glutathione S-transferase (GST) fusion protein for prohibitin and REA. [Month 20-24]

Task 3. To determine the role of prohibitin and REA (repressor of estrogen receptor activity) in growth suppression by estrogen antagonists. [Month 24-36]

   a. Determine the precise subcellular distribution of prohibitin and REA in ovarian cancer cells [Month 20-24]
   b. Complete construction of glutathione S-transferase (GST) fusion protein for prohibitin and REA. [Month 24-30]
   c. Western blot analysis of protein extracts. [Month 30-32]
   d. Infect human ovarian cell lines with recombinant adenovirus containing prohibitin sense cDNA and RNAi constructs.
   e. Cell proliferation assays – cell growth curves and tetrazolium assay. [Month 30-36]
   f. Flow cytometry. [Month 33-36]
Immunohistochemical analyses revealed that prohibitin was highly expressed in the cytoplasm and perinuclear region of ovarian cancer cells. This pattern of prohibitin immunostaining appeared to be inversely related to that of Ki67 expression, a well-known marker of cellular proliferation (figure 1). This observation supports the notion that prohibitin may serve a cytoprotective role in ovarian cancer cells.

Figure 1. Immunolocalization and Western blot analyses of prohibitin in normal and ovarian cancer tissues. A. Immunolocalization of prohibitin (red), DAPI (blue) and Ki67(green) in normal ovarian epithelium, endometrioid, and serous ovarian tumors. Ovarian tissues were fixed in 10% formalin, embedded in paraffin, and sectioned for immunohistochemical analyses as described in Materials and Methods. Immuno-colocalization of endogenous prohibitin detected with Alexa fluor 594 (red) labeled antibody and the proliferative marker Ki67 detected with Alexa fluor 488 (green) labeled secondary antibody. Nuclear DNA was counterstained with 4',6'-diamidino 2-phenylindole, DAPI (blue). Images D, E, and F were higher magnification, demonstrating staining specificity. Bar = 100 μm (A, B, and C) and 50 μm (D, E, and F). B, Western blot analyses of protein levels for prohibitin and XIAP in normal ovarian epithelium (lane 1), endometrioid (lane 2), and serous (lane 3) ovarian tumors. Fifty micrograms of protein from normal and cancerous tissue samples were applied to each lane and analyzed for prohibitin and XIAP protein expression levels by Western blot analyses. Cyclophilin A served as an internal control for sample loading.
negative regulator of cell growth in ovarian cancer (Gamble et al., 2004; Wang et al., 2004; Mishra et al., 2005). These findings were further corroborated by the results of our Western blotting analysis showing a direct correlation with prohibitin protein content with that of X-linked inhibitor of apoptosis in ovarian tumors (figure 1). These results provide further experimental evidence suggesting that prohibitin may function as a cell survival or anti-apoptotic factor, and thus play an important role in determining the chemosensitivity of ovarian cancer cells. Alternatively, however, these results may also reflect the increased metabolic activity occurring within the mitochondria of ovarian cancer cells. It is well known that prohibitin is isomorphic with the mitochondria and highly expressed in cells showing a particular reliance on mitochondrial metabolism, including those in prostate (Gamble et al., 2004; Gamble et al., 2007), melanoma (Coates et al., 2001), thyroid (Coates et al., 2001), gastric (He et al., 2004), and bladder carcinomas (Asamoto and Cohen 1994; Wu et al., 2007). Prohibitin have been shown to be overexpressed in all the above tumor cells, suggesting it has an important role in tumorigenesis. Whether prohibitin plays a direct role in modulating proliferation or mitochondrial metabolism in ovarian cancer cells awaits further investigation.

Although the physiological role of prohibitin is not fully understood within the ovarian cell types, we have recently demonstrated that overexpression of prohibitin in ovarian granulosa cells suppress cell death (Chowdhury et al., 2007). To investigate the possible role of prohibitin in mediating cell fate in ovarian cancer cells, we utilized the ovarian cancer cell lines OVCAR3 and OVCAR8 (Ha et al., 2000) as our in vitro models. Immunocytochemistry and Western blot analysis of subcellular fractions of OVCAR3 and OVCAR8 cells revealed that prohibitin is primarily associated with the mitochondria (figure 2). We also observed an immunoreactive prohibitin-like molecule that was recognized by the prohibitin antibody in the nuclear fraction of the ovarian cancer cells. These results are in agreement with our previous studies using rat primary granulosa cells (Chowdhury et al., 2007). The localization of prohibitin in both the mitochondria and nucleus further support the notion that prohibitin has dual function within a cell that may depend on the physiological status. Indeed, mammalian prohibitin may be involved in regulating mitochondrial function and transcription in the nucleus. When OVCAR8 cells were infected with an adenoviral sense PHB1 cDNA, greater than 90% of cells in culture exhibited adenovirus expression of GFP reporter gene. Because the viruses infect virtually all cells in culture, direct analysis of virally mediated overexpression of specific protein on cell function can be assessed. Infection with adenoviral PHB1 expression vector that ensured overexpression of prohibitin in these cells resulted in suppression of cell growth. This growth suppression in the infected OVCAR8 cells was due to an accumulation of cells at the G1 phase of the cell cycle (Table 1). These results are in agreement with previous findings indicating that prohibitin overexpression inhibited cells growth in both breast and prostate cancer cells (Wang et al., 2004; Gamble et al., 2004). A plausible molecular mechanism for the growth repression by prohibitin observed in ovarian cancer is through interactions of corepressors (i.e., nuclear corepressor and BRG1/BRM) with the prohibitin-E2F1 complex (Wang et al., 2004). To test this hypothesis, we are designing studies to investigate the role of prohibitin in the repression of E2F1 activities in ovarian cancer cell growth.

Recently, we reported that when apoptosis was induced with STS to activate the mitochondrial intrinsic apoptotic pathway, a corresponding increase in prohibitin expression was observed in granulosa cells (Chowdhury et al., 2007). This increase in prohibitin expression corresponded with an increased processing of procaspase-3 to the active enzyme that resulted in an increase caspase-3 enzymatic activity. We therefore performed additional studies to evaluate whether

**Figure 2.** Localization of endogenous prohibitin in OVCAR3 and OVCAR8 cells. A. Immunofluorescence analysis of endogenous prohibitin detected with Alexa fluor 594 (red). The DNA was counterstained with 4',6'-diamidino 2-phenylindole, DAPI (blue). Note that the majority of the immunostaining of prohibitin is isomorphic with the mitochondrial reticular network. B and C. Location of prohibitin in subcellular fractions of OVCAR3 and OVCAR8 cells. Fifty micograms of protein from the cytosol (cyto), mitochondria (mito), and nuclei (nuc) of OVCAR3 and OVCAR8 cells were applied to each lane and subjected to Western blot analysis as described in Materials and Methods. All blots were probed with porin, tubulin, and lamin A/C antibodies.
Table 1. Influence of prohibitin overexpression on OVCAR8 cell cycle after 72 h post infection.

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<th>G₀/G₁ (%)</th>
<th>S (%)</th>
<th>G₂/M (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>58.03±2.16</td>
<td>26.72±1.36</td>
<td>12.16±0.92</td>
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<tr>
<td>Ad-eGFP</td>
<td>60.12±2.24</td>
<td>30.95±1.22</td>
<td>10.02±0.18</td>
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<tr>
<td>Ad-PHB1-eGFP</td>
<td>75.70±0.39</td>
<td>15.56±0.59</td>
<td>8.74±1.12</td>
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P<0.05 a, b, and c; mean±SD of 3 independent experiments

Table 1. Influence of prohibitin overexpression on OVCAR8 cell cycle after 72 h post infection. OVCAR8 cells were infected with 40 MOI of adenovirus vectors for 72 h followed by FACS analysis of infected cells. Values are mean ± standard deviation of triplicate samples stained with propidium iodide.

over-expression of prohibitin delayed STS-induced apoptosis in ovarian cancer cells. In this study similar results were obtained when OVCAR8 cells that were treated with STS. We demonstrated that infection of OVCAR8 cells with adenoviral vectors that direct the overexpression of the PHB1 gene, tilted the balance in favor of survival by decreasing the activation of caspase-3 cleavage (figure 3). Infection of OVCAR8 cells with adenoviral vectors lacking the prohibitin gene resulted in apoptosis after STS treatment. These results provided evidence for prohibitin playing a role in suppressing apoptosis in the ovarian cancer cell line OVCAR8. Although the molecular mechanism(s) involved are currently unkown, a possible explanation is that prohibitin is able to inhibit the intrinsic apoptotic pathway through the stabilization of the mitochondrial dynamics via post-translational modifications such as phosphorylation (Nijtmans et al., 2000; Rajalingam et al., 2005; Chowdhury et al., 2007), that contribute to maintaining the integrity of the cell. Future studies assessing the expression of downstream elements involved will provide further information on where prohibitin directly exerts its effects along the apoptotic pathway. The protective nature of prohibitin in OVCAR8 cells in response to the cell stressor, such as STS, is in agreement with previous studies on camptothecin-induced apoptosis (Fusaro et al., 2002) as well as growth factor withdrawal-induced apoptosis (Vander Heiden et al., 2002; Chowdhury et al., 2007).

Previous studies have shown that small interfering RNAs can effectively induce sequence specific post-transcriptional target gene silencing in a number of cancer cell lines (Bourguignon et al., 2007; Whitehurst et al., 2007). Accordingly, we have used this strategy to knock-down prohibitin gene expression in OVCAR8 cells to determine the impact of prohibitin gene silencing on apoptosis. Our result showed that reducing the expression of the prohibitin gene resulted in the fragmentation of the mitochondrial network (figure 4). Previous studies have shown that mitochondrial
fragmentation is associated with apoptosis possibly by inducing changes in mitochondrial morphology (Karbowski and Youle, 2003). This is consistent with our result that revealed a transition from a reticular network to vesicular punctiform mitochondrial morphology following a reduction in prohibitin expression. This decrease in mitochondrial reticular network connectivity has been previously shown to occur early in the apoptotic process (Karbowski and Youle, 2003). These results are in agreement with previous studies in Hela cells (Kasashima et al., 2006), yeast (Tatsuta et al., 2005) and C. elegans (Artal-Sanz et al., 2003), suggesting that prohibitin is an essential mitochondrial protein involved in maintaining mitochondria integrity. Indeed, prohibitin knockdown resulted in an early onset of apoptosis in STS treated cells as evidenced by the cleavage of pro-caspase-3 to its active caspase-3 enzymatic form. Although, this study and others have shown that prohibitin can participate in the mitochondrial fusion/fission machinery, the mechanism(s) involved in the mitochondrial fragmentation leading to apoptosis is not known.

In summary, our findings indicate that overexpression of prohibitin promotes cell survival in ovarian cancer cells. Knockdown of the prohibitin gene expression therefore, is likely to enhance the chemosensitivity of ovarian cancer cells and promote cytotoxic induced programmed cell death. These findings provide experimental evidence that an siRNA-based strategy to reduce prohibitin content in ovarian cancer cells may be useful in combination with conventional chemotherapy in therapeutic treatment of chemoresistant ovarian cancers.

Figure 6. Effects of silencing the prohibitin gene in OVCAR8 cells. A, OVCAR8 cells were infected with Ad-siRNA prohibitin (MOI = 80) or Ad-eGFP (MOI = 80) for 2 h and maintained in culture for 48 h. Expression of prohibitin protein level was assayed using Western-blotting techniques. B, Effect of Ad-siRNA-PHB1 on the morphology of the mitochondria. Two days after infection with vector only (a) or PHB1 siRNA (b), OVCAR8 cells were fix and immunostained with prohibitin antibody. Note arrow depicting long tubular mitochondrial network (a) and arrowhead showing punctiform mitochondria (b). C, Western blot analysis was conducted to detect levels of prohibitin and cleaved caspase-3, 0.5, 1, 2, and 3 h post-treatment with 1uM STS. Tubulin was used as an internal control. Prohibitin knockdown enhances apoptosis in OVCAR8 cells. Data are representative of 3 individual experiments. Bar = 20 μm.
We have completed task 1 and 2 as proposed in the initial Ovarian Cancer Idea Award as demonstrated in the above-mentioned studies. The information provided below completes task 3. Our initial goal was to identify and characterize the expression of variant prohibitin mRNA species and determine the functional role of these variant in ovarian carcinoma cells. However, due to recent findings indicating that the repressor of estrogen (REA) activity is a prohibitin spliced variant, we designed experiments to determine the role of prohibitin and REA (repressor of estrogen receptor activity) in growth suppression by estrogen antagonists.

Estrogen antagonists comprise the current endocrine therapy of choice in ovarian cancer, and lead to decreases in the rates of both disease recurrence and death. Antagonist therapy is limited, however, by the inevitable development of cellular resistance. The molecular mechanism by which estrogen antagonists inhibit cellular proliferation are not fully defined in ovarian cancer, and this lack of information impedes the rational design of improved anti-ovarian cancer drugs. Recently, it has been shown that REA directly interacts with the liganded estrogen receptor (ER), suppresses ER-activated gene transcription by estrogens, and competes with coactivators for binding to ER. It has also been reported that estrogen antagonists-induced growth suppression of breast cancer cells requires prohibitin and co-repressors, Brg1/Brm. However, there are no reported studies that demonstrate a similar functional mechanism (s) for prohibitin and REA actions in the pathogenesis of ovarian cancer. In this study we have utilized the estrogen receptor-positive ovarian cancer cell lines, OVCAR3 and OVCAR8, and the estrogen receptor-negative ovarian cancer cell line A2780 to determine whether prohibitin and REA are required for growth suppression and the induction of apoptosis by estrogen antagonists. A determination of the precise subcellular distribution of prohibitin and REA in ovarian cancer cells is important for complete characterization of prohibitin and REA function in these cells. Immunocytochemical analysis revealed that REA and prohibitin are associated with mitochondria in all three cell lines. To ask whether interactions could be observed between REA and prohibitin, OVCAR8 cell extracts partitioned into subcellular fractions of mitochondria and nuclear were applied to glutathione-Sepharose columns previously loaded with GST or with GST-REA and/or GST-prohibitin. These experiments reveal that endogenous REA was pulled down by GST-prohibitin in both the mitochondria and nucleus but not by GST alone. Similarly, endogenous prohibitin was pulled down by GST-REA but not by GST alone. Further analysis by two dimensional gel and Western blot analyses of mitochondria and nuclear extracts of OVCAR3, OVCAR8, and A2780 cell showed that there are three isoforms of prohibitin in the mitochondrial extract and one isoform in the nucleus. To begin to clarify the function of prohibitin in ovarian cancer cells treated with tamoxifen, we performed adenovirus mediated overexpression and knock-down studies. OVCAR3, OVCAR8 and A2780 cells were infected or un-infected with adenovirus containing prohibitin cDNA (Ad-PHB1) and adenovirus containing small interfering RNA for prohibitin (Ad-siRNA PHB1). All cell cultures were stimulated with tamoxifen (1 μm) for 48 h. Quantitative real-time PCR confirmed that the mRNA for prohibitin significantly increased in cells infected with Ad-PHB1, while cells infected with Ad-siRNA-PHB1 showed markedly decreased prohibitin mRNA expression. Overexpression or knockdown of prohibitin mRNA expression levels had no effects on the repressor of estrogen receptor activity (REA) mRNA levels. Although the adenovirus constructs that mediated overexpression or knockdown of prohibitin were specific at the mRNA level, the protein levels for both prohibitin and REA were either increased or reduced by Ad-PHB1 or Ad-siRNA-PHB1 in a dose dependent manner, respectively. This observation was not surprising because the prohibitin and REA expression are interdependent in HeLa cells, C. elegans and yeast so that amplification or deletion of one decreases the protein level of the other. Knockdown of prohibitin content in the estrogen receptor positive (OVCAR3 and OVCAR8) and estrogen receptor negative (A2780) cell lines treated with 1 μm tamoxifen resulted in cytotoxic induced-apoptosis. Whereas overexpression of prohibitin protect the cells from cytotoxic induced-cell death. Previous studies have shown that low dosage of tamoxifen induced cell-cycle arrest at the G1 phase. Similar results were observed using FACS analysis in these studies.

These results provide compelling evidence that prohibitin and REA are expressed in OVCAR3, OVCAR8, and A2780 cells and physically interact with each other in both the mitochondria and nucleus. As mentioned above, these finding provide experimental evidence that an siRNA-based strategy to reduce prohibitin content in ovarian cancer cells may be useful in combination with conventional chemotherapy in therapeutic treatment of chemoresistant ovarian cancers.

KEY RESEARCH ACCOMPLISHMENTS

- Determine the immunolocalization of prohibitin protein in normal and ovarian cancer tissues
- Development of recombinant adenoviruses containing prohibitin sense and antisense cDNA, and recombinant adenovirus containing only EGFP.
- Construction of Silencing interference RNA (siRNA) Constructs.

REPORTABLE OUTCOMES
CONCLUSIONS

We anticipate that the results from these studies will provide significant novel information concerning the importance of prohibitin in ovarian cancer growth and apoptotic mechanisms. We will begin to gain insight to the possible mechanism(s) of action for prohibitin within the ovarian cancer cells.