

Prohibitin as a novel target protein of luteinizing hormone in ovarian epithelial carcinogenesis

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Received August 23, 2010

The exact role of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in ovarian epithelial carcinoma (OEC) development has not been yet characterized. This prompted us to identify particular proteins to better understand the underlying mechanism. Total proteins from ovarian epithelial tumor (OET) cells treated with gonadotropins were analyzed by proteomics. Western blot and immunohistochemistry were used to validate the target protein (prohibitin) and to detect its expression in human ovarian tissue of serous tumors. As the results, prohibitin was found to be significantly up-regulated by LH, with a maximum of 2.5-fold increase at the concentration of 200 mIU/mL. The expression of prohibitin was steadily decreased from benign serous cystadenomas to borderline tumors and serous carcinomas ($P < 0.0001$). The difference between any two groups was significant ($P < 0.001$). Collectively, data from this study indicate that prohibitin is one LH-associated protein and it may be protective of ovarian cancer development and progression, supporting that LH may play an inhibitory role in ovarian tumorigenesis.

Key words: Gonadotropin; Luteinizing hormone; Prohibitin; Ovarian cancer

1 Introduction

Ovarian epithelial cancer (OEC) is the most lethal malignancy of all gynecologic cancers. Although the cell of origin of OEC remains debatable, some investigators still believe that the majority of OECs are derived from ovarian surface epithelium (OSE) and ovarian epithelial inclusions (OEI) [1, 2]. As is clear that OSE and OEI consist largely of a single layer of flat-to-cuboidal epithelial cells with few distinguished features, the mechanisms of malignant transformation of OSE and OEI remain elusive.

There are currently several theories to explain the etiology of sporadic OEC. These include theories of gonadotropins [3, 4], incessant ovulation, sex-steroid hormone, and pelvic chronic inflammation. All these theories may be complimentary

rather than exclusive of each other. The gonadotropin theory proposes that the development of OEC is related to elevated gonadotropin production associated with the onset of menopause. The peak incidence of OEC is 55-65 years, when these women have elevated serum gonadotropins, namely follicle-stimulating hormone (FSH), and luteinizing hormone (LH). These elevated gonadotropins are proposed to facilitate the development of OEC. This hypothesis is strengthened by epidemiological studies showing that pregnancy, oral contraceptive usage and duration of lactation, all of which reduce exposure to FSH and LH, substantially reduce the risk of OEC [5, 6]. The hypothesis has been further supported by studies showing receptors of FSH and LH present in various ovarian epithelial tumors including benign cystadenomas, borderline tumors and cancers, indicating that OECs and their putative precursors are responsive to gonadotropin stimulation. However, the exact role of individual gonadotropins and their corresponding molecular mechanisms in the process of ovarian epithelial tumor development has not been fully characterized.

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; OEC, ovarian epithelial cancer; OEI, ovarian epithelial inclusions; OET, ovarian epithelial tumor; OSE, ovarian surface epithelium;

2 Materials and Methods

2.1 Ovarian epithelial tumor cells and gonadotropin stimulation

MCV152 cell line was derived from SV-40-transformed benign ovarian serous papillary cystadenoma and transfected with telomerase hTERT. The cells were known non-tumorigenic and to contain gonadotropin receptors and responding to gonadotropin stimulation. We selected this benign serous cystadenoma derived cell line as the venue instead of ovarian surface epithelial cells derived cell line mainly because there are many controversial findings in terms of the ovarian surface epithelial cells as sole origin of all OECs [7, 8]. Cell culture condition, growth method, and gonadotropin treatment were described previously [9]. FSH and LH at a final concentration of 50 mIU/mL were used for cell stimulation. The cells were collected for proteomic analysis after 24 h of gonadotropin stimulation.

After proteomic analysis, we identified prohibitin (see below) as one of the target proteins regulated by LH. Further dose-dependent prohibitin expression of MCV152 cells in response to LH stimulation was determined at the concentration of 0, 25, 50, 100, 200 and 400 mIU/mL, for 24 h.

2.2 Proteomic analysis

2.2.1 Protein preparation and two dimensional gel electrophoresis (2DGE)

Protein preparation and 2DGE were performed as described previously [10, 11]. Briefly, cell pellets after gonadotropin stimulations for 2DGE were dissolved into Extraction Buffer II containing 8 M urea, 4% (w/v) Bio-Lyte 4/7, and 2 mM tributyl phosphine (Bio-Rad, Hercules, CA), vigorously vortexed and centrifuged. The supernatant was combined with a rehydration buffer mixture containing Rehydration Buffer [8 M urea, 2% CHAPS, 50 mM DTT, and 0.2% (w/v) Bio-Lyte 4/7 ampholytes, IPG buffer (Amersham Biosciences, Piscataway, NJ)], and bromophenol blue, and subsequently rehydrated overnight with Immobiline Drystrips (pH 4-7, 11 cm; Amersham Biosciences) on a Reswelling Tray (Amersham Biosciences). The isometric focusing for the first dimensional electrophoresis was performed with a Multiphore II Electrophoresis System (Amersham Biosciences). The strips were subjected to voltages at 300-3500 V. Immobilized pH gradient (IPG) strips were equilibrated with Equilibration Buffer I containing 6 M urea, 2% SDS, 375 mM Tris-HCL (pH 8.8), 20% glycerol, and 2% (w/v) DTT; and Buffer II containing 6 M urea, 2% SDS, 375 mM Tris-HCL (pH 8.8), 20% glycerol, and 2.5% (w/v) iodoacetamide (Bio-Rad, Hercules, CA). Precast Excel SDS gels (12-14% Gradient gel; pH4-7, 245 x 180 x 0.5 mm, Amersham Biosciences) were used for the second dimension of protein separation by a Multiphor II Flated System (Amersham Biosciences) under a constant voltage of 700 V. A silver staining kit (Amersham Biosciences) was used to detect protein spots according to the manufacturer's instructions. All samples were run in triplicate.

2.2.2 Image analysis and in-gel digestion

Digital images were acquired for each sample gel and the optical density (OD%) of the protein spots was taken to be proportional to the protein concentration. The protein spots were detected, quantified, and matched using Proteomweaver according to the manufacturer's protocol (Definiens, Munich, Germany). Protein spots of interests were excised and subjected to in-gel digestion with trypsin as previously described [11].

2.2.3 Mass spectrometry and protein identification

Peptides from in-gel digests were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a Proteome X LC/MS system (ThermoElectron, San Jose, CA) operated in the high throughput mode. Reversed phase HPLC was carried out using a BioBasic-18 column (0.18 x 150 mm, ThermoElectron) eluted at 1-2 μ l/min with a gradient of 2-50% B over 30 min. Mobile phase A was H₂O (0.1% FA) and mobile phase B was CH₃CN (0.1% FA). Column effluent was analyzed on the LCQ Deca XP Plus (ThermoElectron) operating in the "Top Five" mode. Un-interpreted MS/MS spectra were searched against a human database utilizing BioWorks and SEQUEST programs (ThermoElectron). A protein identification was accepted when MS/MS spectra of at least two peptides from the same protein exhibited at a minimum the default Xcorr versus charge values set by the program (for Z = 1, 1.50; for Z = 2, 2.00; for Z = 3, 2.50).

2.3 Western blot

The Western blot method was described elsewhere [11]. Membranes with transferred proteins were incubated with primary antibodies against prohibitin (1:400, mouse monoclonal antibody, Labvision Corporation-NeoMarkers, Fremont, CA), at 4°C overnight, followed by exposure to secondary antibody. Anti- β -actin was used as a control. The blots were further developed using the chemiluminescence reagent. Densitometry analysis of the scanned images was done by using Software Image Quant (Molecular Dynamics, Inc).

2.4 Human ovarian tissue and immunohistochemistry

2.4.1 Tissue sections and pathologic features of studied cases

All evaluated ovarian tissue samples were derived from Department of Pathology at the University of Arizona after obtaining an Institutional Review Board approval. A total of 46 ovarian tumor cases including 14 serous cystadenomas, 12 serous borderline tumors, and 20 serous carcinomas were studied as presented in table 1. All tumor cases were diagnosed using criteria of the International Federation of Obstetrics and Gynecology (FIGO) [12].

2.4.2 Immunohistochemistry (IHC) and evaluation

IHC stains were described elsewhere [9]. Representative sections of benign ovary containing developing follicles served as positive controls (highest prohibitin expression level) for the prohibitin antibody [13]. Negative controls were performed by

Table 1. Immunohistochemical expression of prohibitin in ovarian serous tumors

Characteristics	Prohibitin Positivity	Prohibitin Index	P value
	Positive#/total cases # (%)	Mean of Scores	
Benign tumors	13/14 (93)	129	
Borderline tumors	10/12 (83)	66	< 0.001
Carcinoma	9/20 (45)	31	

replacing the primary antibody with non-immune IgG. All IHC slides were reviewed independently by two investigators (W.Z. and X.Y.). The percentage of neoplastic cells that showed dark brown cytoplasmic staining was recorded. The staining intensity was graded for both on the following scale: 0 = no; 1 = weak; 2 = moderate; and 3 = intense. Prohibitin IHC index was generated by multiplying the intensity by the percentage of positive cells in a defined specimen, yielding scores ranging from 0 to 300. Individual IHC score for each case was used for statistical analysis. Cases with at least 10% of the positively stained cells were considered as positive. Duplicate stainings were performed and average scores were used for statistical analysis.

2.5 Statistical analysis

The respective data obtained from 2DGE was verified by the F-test, and the differences among groups were compared by using Student's t-test. The comparison of prohibitin immunohistochemical staining index scores among different types of ovarian tissue was performed using the Wilcoxon signed rank test. Data are presented as means with 95% confidence intervals (CIs). Statistical analysis was performed by using SAS 9.0 (SAS Institute Inc, Cary, NC). A two-sided test with *P* values of less than 0.05 was considered statistically significant.

3 Results

3.1 Comparative proteomic analysis, protein identification and prohibitin validation

Among the protein spots visualized, we isolated and sequenced 10 proteins with at least 2.0-fold amount changes that were uniquely expressed in either gonadotropin-treated OET cells. Representative protein profiles in 2DGE are shown in Figure 1. Prohibitin was one of the proteins up-regulated by LH, not FSH (Fig. 1). Prohibitin was selected as the first target in this study mainly because it is a known cell cycle regulator and the role of prohibitin in the relationship between OEC growth and gonadotropin has never been explored.

To validate the proteomic results, we re-grew the MCV152 cells stimulated by LH and FSH, and analyzed the prohibitin expression by Western blots. As expected, LH stimulation for 24 h successfully induced prohibitin expression when compared with FSH and vehicle control (Fig. 2A). A remarkable more than 2.0-fold increase was observed in cells stimulated

by LH. The prohibitin expression was also shown in a dose-dependent fashion with a maximal 2.5-fold increase at 200 mIU/mL. At the concentration of 50 mIU/mL or above, the induction of prohibitin was not pronounced (Fig. 2B). Prohibitin was also expressed in the epithelial compartment of human ovarian epithelial tumor samples (see below Prohibitin Expression in Human Ovarian Tissue).

3.2 Prohibitin expression in human ovarian tissue

Knowing that prohibitin is regulated by LH, it would be interesting to know if prohibitin expression is present in human ovarian epithelia. If so, this prohibitin expression may help us further understand the role of gonadotropin in the development of OEC. IHC was employed to determine the expression levels of prohibitin in ovarian epithelia tumors. The mean immunostaining scores in tissues from serous cystadenoma, to borderline and to malignant carcinoma were 129, 66 and 31, respectively (Table 1). A significantly higher prohibitin expression was observed in benign serous cystadenoma group than that in borderline and malignant groups ($P < 0.001$). Compared with carcinoma group, prohibitin expression was significantly higher in borderline tumors ($P < 0.001$). This trend of reduction of prohibitin expression from benign cystadenoma to borderline tumor and to carcinoma was statistically significant ($P < 0.0001$). Representative pictures of prohibitin IHC in ovarian tissues are presented in Figure 3.

4 Discussion

The exact individual roles of gonadotropins on OET growth and on ovarian epithelial carcinogenesis are still far from clear, although their roles in steroidogenesis and gametogenesis have been well defined. In this study, one most interesting finding was prohibitin, which was significantly up-regulated by LH, not by FSH. The main reasons we were interested in studying prohibitin expression are due to its differential response to the gonadotropins and it is a cell cycle regulator. Prohibitin, also known as B-cell receptor associated protein-32, is highly conserved and mapped to chromosome 17q12-21 with a ~30 kD protein product [14]. Earlier observations showed that microinjection of prohibitin mRNA could block Hela cell growth [15]. Since then, the cell-cycle function of prohibitin is becoming more attractive with its secondary localization observed in nucleus [16, 17]. In a recent study, overexpression of prohibitin was detected in esophageal squamous cell carcinomas and it was associated with the progression and shorter survival [18]. Other studies in mechanisms found that prohibitin regulates cell cycle via interaction with other tumor suppressor genes such as p53 and Rb [19, 20]. Additionally, it has been shown that it functions in cell cycle arrest in human cervical, bladder, and breast cancers, a role was assigned to its 3' UTR [21, 22]. All of the evidence suggests that prohibitin is a tumor suppressor and has an anti-growth function. Besides, a different role of prohibitin in cancer cells as a crucial prerequisite for cell proliferation and survival was reported, which seemed to be associated with adhesion or

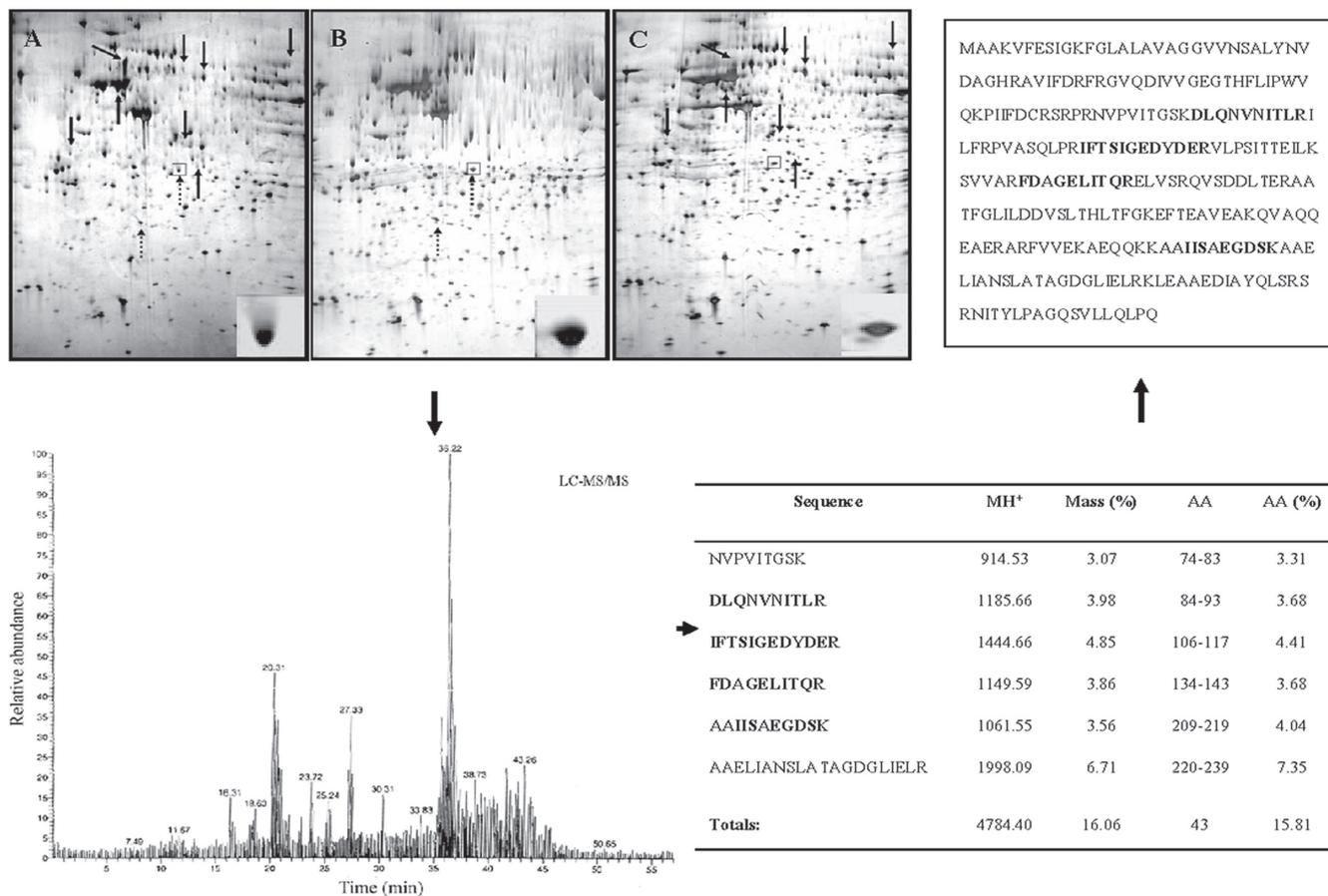


Figure 1. Silver-stained 2DGE images of MCV152 cells treated with gonadotropins and identification of prohibitin from LC-MS/MS. The protein spots were matched, quantified and compared based on the total proteins on the gel background using Proteomweaver. Three-fold increase of prohibitin level was detected in LH treated tumor cells (B) as compared with control (A). FSH treatment did not show a difference of prohibitin induction (C). Solid and broken arrows indicate the protein spots altered in MCV152 cells with FSH and LH treatment, respectively. Boxed areas contain prohibitin, and these are enlarged in the lower right corners. The lower left shows the spectra of LC-MS/MS and the lower right displays the identified peptide sequences. The upper right: prohibitin sequence, bold characters indicate the matching amino acids.

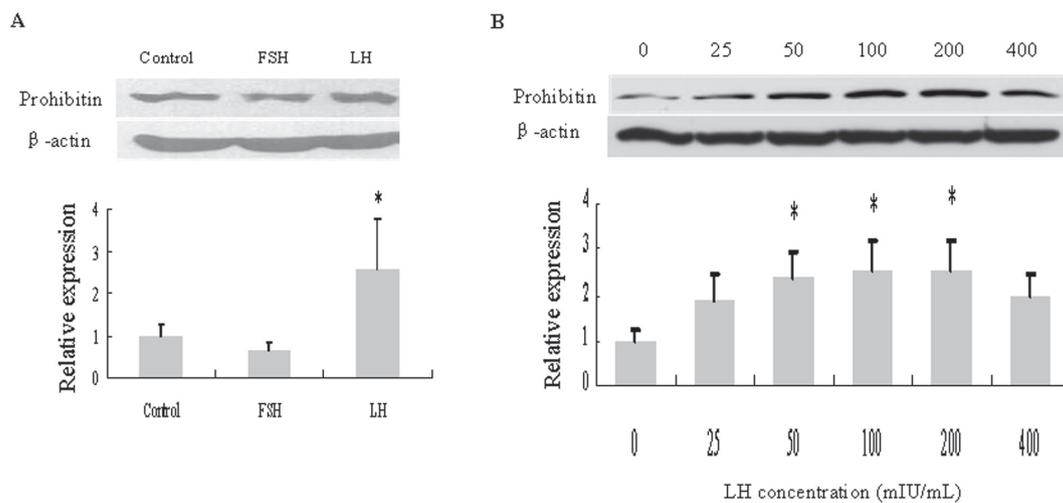


Figure 2. Prohibitin expression in MCV152 cells regulated by gonadotropins. (A) Prohibitin expression was verified in MCV152 cells treated with FSH (50 mIU/ml) and LH (50 mIU/ml) for 24 h. (B) Further prohibitin dose-response was observed in the MCV152 cells after LH treatment for 24 h. * means $p < 0.05$ when compared with control.

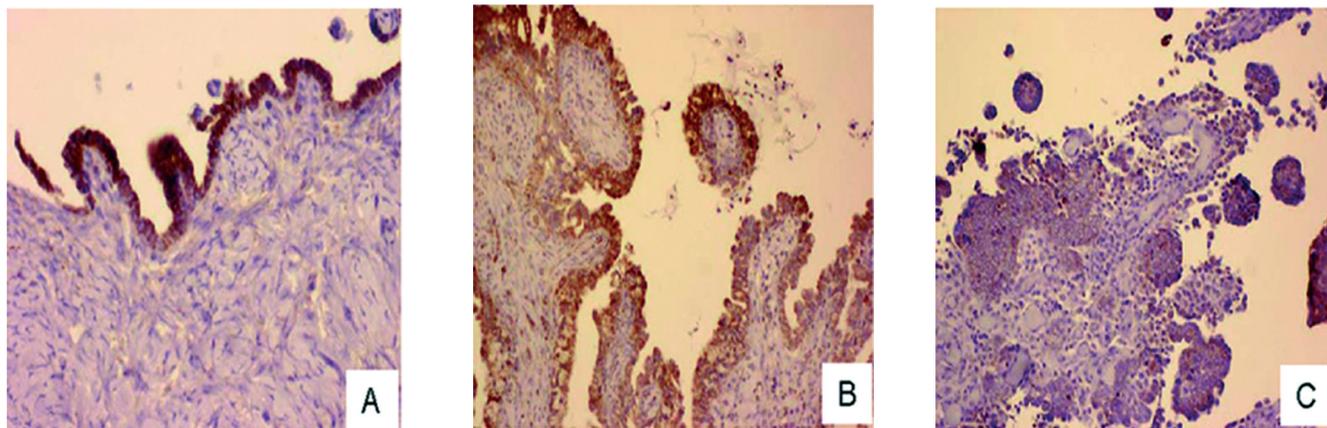


Figure 3. Immunohistochemical staining of prohibitin in benign, borderline and malignant serous ovarian tumors. Serous cystadenomas (A) showed strong positive signals (dark brown), while lesser signal was detected in serous borderline tumors (B) and even negative in serous carcinomas (C). Original magnifications: 200 x.

migration signaling, and most likely as chaperones and scaffolding proteins as in mitochondria [23].

However, it is unknown if prohibitin plays any role in ovarian epithelial cells, particularly in the process of OET development, although prohibitin does have biological function in folliculogenesis [24]. In contrast to those in normal ovarian epithelium, a higher prohibitin expression level was observed in ovarian papillary serous and endometrioid carcinomas, which was inversely associated with cellular Ki-67 expression, a marker of cell proliferation and positively correlated with the cell survival marker X-linked inhibitor of apoptosis protein (XIAP). Prohibitin may be a negative regulator of cell growth and function as an antiapoptotic factor in ovarian cancer cells, as well as play an important role in determining the chemosensitivity of ovarian cancer cells [25]. In this study, we showed that prohibitin was specifically up-regulated by LH in ovarian epithelial cells by a proteomic method. This up-regulation of prohibitin expression was validated by Western blot. Although the inhibitory role of prohibitin in hormone-related diseases such as prostate and breast cancers has been previously reported [17, 26], prohibitin regulation, particularly in association with gonadotropins, in OECs has never been explored. Our finding showed that in OET cells, the prohibitin was up-regulated by LH in a dose-response manner, indicating a LH-specific response in this particular setting. Prohibitin detection in ovarian epithelial cells is a meaningful finding. Our finding suggests that expression of prohibitin may be part of the mechanism maintaining them in non- or minimal dividing status and disturbance of prohibitin expression may provide a suitable opportunity for ovarian epithelial tumor cells to grow, therefore to enhance the susceptibility of malignant transformation. It is of interest to note that the level of prohibitin expression was significantly decreased from benign serous cystadenoma to malignant serous carcinoma. Strikingly, this prohibitin expression pattern in the line of serous OEC development dramatically parallels the LH receptor mRNA expression pattern we reported previously [9]. This further supports that prohibitin expression

in human ovarian epithelial cells is directly regulated by LH and is likely influenced by LH receptor status on those tumor cells. Overall, the steady reduction of prohibitin expression from benign to borderline and to malignant serous ovarian tumors is supportive of the protective role of LH in OEC development. It would be interesting to know if this phenomenon is present in other histologic types of ovarian epithelial tumors since mucinous and endometrioid and other ovarian epithelial tumorigenesis may be different from ovarian serous carcinogenesis [9, 27]. It is also worth exploring the prognostic and diagnostic roles of prohibitin in the future.

Controversies exist in the literature regarding the roles of gonadotropins in the development of OEC. Most studies showed that FSH plays a positive role in growth force. However, cellular effects by LH were dramatically different with some growth promoting results [28], yet in other studies, no association [29] or even reduced proliferation have been observed [30]. Combined with the inhibitory role of prohibitin reported previously, LH is assumed to be inhibitory for OET cell growth.

In summary, this is the first report that prohibitin is particularly up-regulated by LH. The previous observed inhibitory effect of LH in OET cells is likely mediated through up-regulation of prohibitin. Yet in postmenopausal women, it seems logical for both FSH and LH to act simultaneously since both gonadotropins are commonly elevated. However more detailed studies are necessary to further define the combinational effect of gonadotropin in OEC development since elucidation of the mechanism may provide us a new modality of ovarian cancer prevention.

Acknowledgements. Dr. Lin Jia was a co-trained PhD candidate of Shandong University, China. The work was supported partly by the National Natural Science Foundation of China (No. 30901590) to LJ, Arizona Cancer Center Core Grant (P30 CA23074), Arizona Cancer Center Better Than Ever Grant, and University of Arizona Department of Pathology start up fund to WZ. We thank Dr. Jun Wang at Tufts University (Boston, Massachusetts) for her excellent statistical data analysis for this project.

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