Extracellular Signal-Regulated Protein Kinase, But Not c-Jun N-Terminal Kinase, Is Activated by Type II Gonadotropin-Releasing Hormone Involved in the Inhibition of Ovarian Cancer Cell Proliferation

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Although a novel second form of GnRH (GnRH-II) has been reported to have an antiproliferative effect on gynecologic cancer cells, its biological mechanism remains to be elucidated. We have previously demonstrated that GnRH-II activates p38 MAPK. There is accumulating evidence that activation of MAPKs by GnRH-I and -II is important for cell proliferation, differentiation, and apoptosis. In the present study, we further investigated the involvement of GnRH-II in the inhibition of cell proliferation and activation of ERK1/2 and c-Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) in ovarian cancer cells, OVCAR-3. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays revealed that treatment with GnRH-II suppresses cell proliferation of ovarian cancer cells. Western blot analysis demonstrated that ERK1/2 was activated by GnRH-II (100 nM). Moreover, PD98059 (10 μM), an inhibitor of a MAPK/ERK kinase, reversed the activation of ERK1/2 induced by GnRH-II. The activation of ERK1/2 by GnRH-II subsequently phosphorylated Elk-1 as a downstream pathway, which was blocked by PD98059. On the other hand, it is not likely that GnRH-II activates the JNK/SAPK pathway. Taken together, these results indicate that the ERK1/2 pathway is involved in the effect of GnRH-II on antiproliferation and may be an important target for ovarian cancer therapy. (J Clin Endocrinol Metab 90: 1670–1677, 2005)

GnRH, ALSO KNOWN as LHRRH, is a key molecule of the hypothalamus-pituitary-gonadal axis in the reproduction of vertebrates. In addition to its well known physiological roles in the regulation of gonadotropins in the brain, GnRH has also been used for the therapy of reproductive cancers (1). It has been reported that agonists of a classical form of GnRH (GnRH-I) suppressed various tumors by blocking the secretion of FSH and LH from pituitary gland, called chemical castration. In addition, direct application is considered important for the induction of apoptosis or suppression of proliferation (2, 3). Because a second form of GnRH (GnRH-II) was identified in mammals, the role and possible mechanism of action of GnRH-II has been investigated in normal physiology and cancer cells (4). Although it has been noted that GnRH-II induced the secretion of human chorionic gonadotropin (hCG) in cytotrophoblastic cells, the normal physiological function of GnRH-II is poorly understood (5). In mammals, GnRH-II receptors have been found to be more widely expressed than GnRH-I receptors in the body, suggesting that GnRH-II may have additional various functions (6). The expression and potential antiproliferative effect of GnRH-II indicate that GnRH-II, similar to GnRH-I, may have a growth-regulatory effect in normal and neoplastic ovarian surface epithelial cells (7). Furthermore, a recent study demonstrated that GnRH-II has an antiproliferative effect in gynecologic tumors and may exert a stronger anti-proliferative effect than GnRH-I in ovarian cancer cells, suggesting that GnRH-II could be considered as a novel target for antiproliferative therapeutic approaches (4, 8). However, in contrast to GnRH-I, the biological mechanism of GnRH-II remains obscure.

MAPK cascades are activated via two distinct classes of cell surface receptors, receptor tyrosine kinases and G protein-coupled receptors. Signals transmitted through these cascades induce the activation of diverse molecules that regulate cell growth, survival, and differentiation (9–11). ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are activated by mitogenic stimuli and represent a group of the most extensively studied members. In contrast, c-Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK1) and p38 MAPK are activated in response to stress such as heat shock, osmotic shock, cytokines, protein synthesis inhibitors, antioxidants, UV light, and DNA-damaging agents (12–15). MAPK family members are directly regulated by kinases known as MAPK kinases, which activate MAPKs by phosphorylation of tyrosine and threonine residues (15, 16). It has been reported that ERK1/2 is involved in cell cycle arrest and the inhibition of growth (17–19) as well as cell survival and differentiation (9). The role of the MAPK family in the antiproliferative effect of GnRH in the CaOV-3 ovarian cancer cell line has been demonstrated (20). In our previous reports,
we demonstrated that FSH stimulated activation of the ERK1/2 cascade and phosphorylated Elk-1 in neoplastic ovarian surface epithelial cells (21) and that the p38 MAPK pathway is involved in the antiproliferative effect of GnRH-II in ovarian cancer cells (4).

Although it is difficult to define each of the mechanisms involved in the regulation of MAPKs in response to external stimuli, it is important to clarify the specific signaling pathways used by GnRH-II. Increasing our understanding of GnRH-II signaling pathways may improve the efficacy of chemotherapy using these agonists in ovarian cancer treatment. Thus, in the present study, we examined the effect of GnRH-II on the activation of ERK1/2 and JNK/SAPK1 in ovarian cancer cells. In addition, the possible involvement of the ERK1/2 pathway in mediating the antiproliferative effects of GnRH-II was investigated in ovarian cancer cells.

Materials and Methods

Materials

A GnRH-II analog, d-Arg6-Azagly10-NH2, was purchased from Peninsula Laboratories (Belmont, CA). PD98059, a MAPK/ERK kinase (MEK) inhibitor, was purchased from New England Biolabs Inc. (Beverly, MA) and was dissolved in dimethylsulfoxide (DMSO).

Cell culture and treatment

Human ovarian adenocarcinoma cell lines, OVCAR-3 and SKOV-3, were cultured as previously described (4, 22, 23). The non-tumorigenic SV40 Tag-immortalized ovarian surface epithelium (OSE)-derived cell line, and IOSE-80 post crisis (IOSE-80PC), were cultured in the above mentioned culture conditions and used in the present study. Briefly, the cells were cultured in medium 199:MCDB 105 (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Life Technologies, Inc., Rockville, MD) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Cells were trypsinized with 0.06% trypsin (1:250)/0.01% EDTA (Life Technologies) in Mg2+/Ca2+-free Hank’s balanced salt solution and seeded at a density of 2 × 104 cells in 35-mm dishes (Falcon; Becton Dickinson, Franklin Lakes, NJ) and cultured for 2 d. Cells were washed once with medium and serum starved for 6 h before GnRH-II treatment. To investigate the direct effect on ERK1/2, the cells were pretreated with 10 µM PD98059 for 1 h and then treated with GnRH-II (100 nM).

Immunoblot analysis

Immunoblot analysis was carried out as previously described (4, 24). The extracts were run on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was immunoblotted using a rabbit polyclonal antibody for phosphorylated ERK1/2 and JNK/SAPK (Biosource International Inc., Camarillo, CA) (25, 26) with protein molecular marker (New England Biolabs, Inc., Mississauga, Ontario, Canada). Alternatively, the membrane was probed with pan ERK1/2 and pan JNK/SAPK antibodies (Biosource International), which detect total ERK1/2 and JNK/SAPK1 level, respectively. The intensity of signals was quantified by densitometry (BioDocAnalyze, Biometra, Germany). Alternatively, the membrane was probed with pan ERK1/2 and JNK/SAPK1 antibodies (Biosource International Inc., Camarillo, CA) (25, 26) with protein molecular marker (New England Biolabs, Inc., Mississauga, Ontario, Canada). The activity of ERK1/2 and JNK/SAPK was represented as a ratio of phosphorylated MAPKs (P-MAPKs) to total MAPKs (T-MAPKs). The intensity of signals was quantitated by densitometry (BioDocAnalyze, Biometra, Germany). Alternatively, the membrane was probed with pan ERK1/2 and JNK/SAPK1 antibodies (Biosource International Inc., Camarillo, CA) (25, 26) with protein molecular marker (New England Biolabs, Inc., Mississauga, Ontario, Canada). The activity of ERK1/2 and JNK/SAPK was represented as a ratio of phosphorylated MAPKs (P-MAPKs) to total MAPKs (T-MAPKs).

In vitro MAPK assay

OVCAR-3 cells were seeded at a density of 4 × 105 cells in 60-mm dishes (Corning, Corning Laboratory Sciences Co., Corning, NY) and cultured for 2 d. After treatment with GnRH-II in the presence or absence of PD98059, protein extracts were prepared under the conditions described above. Cellular protein (200 µg) was immunoprecipitated with an immobilized phospho-ERK1/2 MAPK monoclonal antibody. The in vitro MAPK assay was performed using an Elk-1 fusion protein as a substrate for activated MAPK, according to the manufacturer’s suggested procedure (New England Biolabs).

Proliferation assay

The proliferation assay was performed using [3H]thymidine incorporation as previously described (7, 27, 28). Briefly, 2 × 104 OVCAR-3 cells were plated in 24-well dishes in 0.5 ml medium as described above. GnRH-II was appropriately diluted with medium, and the cells were treated with a final concentration of GnRH-II (100 nM) after 2-4 h of incubation. Cells were treated for 4 d with medium changes every 24 h. After treatment with GnRH-II, the cells were then incubated with medium containing 1 µCi [3H]thymidine (0.5 Ci/mmol; Amersham Pharmacia Biotech Inc., Piscataway, NJ) and collected after 6 h incubation. To block the activation of ERK1/2, the cells were pretreated with PD98059 (10 µM) for 1 h, followed by the addition of GnRH-II (100 nm final concentration) or vehicle.

MTT assay

Cell viability was estimated by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazoliumbromide] (MTT) (Sigma-Aldrich Corp.) assay. OVCAR-3 cells were seeded onto 96-well dishes. The MTT colorimetric assay was performed to detect tumor cell viability after 96 h of incubation (29). The cells were incubated at 37 C with 50 µl MTT solution (2 mg/ml in PBS) for 4 h. The supernatants were removed and the cells were solubilized in DMSO (200 µl) for 30 min. The OD at 570 nm was determined using an ELISA reader (Fisher Scientific Ltd., Ottawa, Canada).

Statistical analysis

The results of three separate experiments are presented as the mean ± sp. Each individual experiment was performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison test. P < 0.05 was considered statistically significant.

Results

Effects of GnRH-II on the activation of ERK1/2 in ovarian cancer cells

To investigate the activation of ERK1/2 in ovarian cancer cell lines and immortalized OSE-derived cell lines, the cells were treated with GnRH-II (100 nM) in a time-dependent manner. The phosphorylation of ERK1/2 was examined using the antibodies targeting ERK1/2 and T-ERK1/2 (activated plus inactivated forms). Treatment with GnRH-II (100 nM) activated ERK1/2 in OVCAR-3, SKOV-3, IOSE-80PC, and IOSE-80 cell lines in a time-dependent manner (Fig. 1A). As seen in Fig. 1B, GnRH-II activated ERK1/2 significantly in OVCAR-3 cells, and the maximal level of ERK1/2 activation was observed (2-fold increase) at 10 min. These results indicate that GnRH-II activates ERK1/2 in both ovarian carcinomas and IOSE cell lines.

JNK/SAPK1 activation by GnRH-II in ovarian cancer cells

To determine the activation of JNK/SAPK1 by GnRH-II, we treated OVCAR-3 cells with GnRH-II (100 nM) in a time-dependent manner. In contrast to the activation of ERK1/2, no difference was observed in P-JNK/SAPK1 levels after treatment with GnRH-II (Fig. 2). In addition, GnRH-II had no effect on the activation of JNK/SAPK1 in SKOV-3, IOSE-80, and IOSE-80PC cells (data not shown).

Further elucidate the direct effect of GnRH-II on the activation of ERK1/2 through MEK1/2, OVCAR-3 cells were pretreated with the MEK inhibitor PD98059 (10 µM), fol-
followed by treatment with GnRH-II (100 nM) for 10 min. As seen in Fig. 3, pretreatment with PD98059 attenuated GnRH-II-induced phosphorylation of ERK1/2, whereas no significant difference was observed in DMSO-treated cells or cells treated with only PD98059.

**Activation of Elk-1 by GnRH-II involved in ERK1/2 signal pathway**

To investigate whether GnRH-II-induced activation of ERK1/2 leads to phosphorylation of Elk-1 in vitro as a downstream pathway of MAPK, the cells were treated with GnRH-II (100 nM) for 10 min in the presence or absence of PD98059 (10 μM) for 1 h. As shown in Fig. 4, the treatment with GnRH-II resulted in a significant increase in Elk-1 phosphorylation, whereas pretreatment with PD98059 significantly reduced GnRH-II-induced Elk-1 phosphorylation in OVCAR-3 cells.

**Inhibition of cell proliferation in GnRH-II-treated ovarian cancer cells**

To determine the role of GnRH-II in ovarian cancer, we further examined the effect of GnRH-II on proliferation by
Fig. 2. Effect of GnRH-II on the activation of JNK/SAPK1. The T-JNK/SAPK1 and P-JNK/SAPK1 levels were analyzed by immunoblot assay. JNK/SAPK1 level is expressed as a fold change relative to basal level. Values are represented as the mean ± SD of three individual experiments.

Fig. 3. Effect of GnRH-II on ERK1/2 activation in the presence or absence of PD98059. OVCAR-3 cells were pretreated with PD98059 (10 μM) for 1 h, followed by treatment with GnRH-II (100 nM) for 10 min. A control was treated with vehicle. The T-ERK1/2 and P-ERK1/2 levels were analyzed by immunoblot assay. ERK1/2 level is expressed as a fold change relative to basal level. Values are represented as the mean ± SD of three individual experiments. a, P < 0.01 vs. control.
thymidine incorporation and MTT assays in OVCAR-3 cells. The cells were treated for 4 d with 100 nM GnRH-II followed by 24 h culture. As illustrated in Fig. 5, treatment with GnRH-II (100 nM) induced a decrease in cell proliferation in OVCAR-3 cells as assessed by thymidine incorporation assay. To confirm the antiproliferative effect of GnRH-II, cellular viability was measured by MTT assay. Treatment with GnRH-II (100 nM) resulted in a significant reduction in cell viability after 4 d of treatment (Fig. 6). Furthermore, to elucidate the relevance of ERK1/2 activation in the proliferation of ovarian cancer cells, we challenged OVCAR-3 cells with 10 μM PD98059. Pretreatment with PD98059 (10 μM) completely abolished the antiproliferative effect induced by GnRH-II (Fig. 5), suggesting that the MEK/MAPK pathway mediates the antiproliferative effect of GnRH-II in ovarian cancer cells.

**Discussion**

Ovarian carcinoma is one of the most frequent gynecologic cancers after breast, lung, and colorectal cancer (30). However, the biological causes of this disease remain unknown. Treatment with hormone, including GnRH, is known to have potential benefits for patients with ovarian cancer that do not respond to chemotherapy (31). In addition to its important role in the reproduction system to regulate FSH and LH in the pituitary, GnRH-I, a classical form of GnRH, has an inhibitory effect on cell growth in human mammary, ovarian, endometrial, and prostate tumors and has been implicated as an antiproliferative regulator of gynecologic cancers (30, 32–34). Recently, it has been shown that a GnRH-II analog is stable and binds to high-affinity binding sites in the mammalian ovary, suggesting that GnRH-II may be a potent regulator of ovarian function (35). Although the biological function of GnRH-II is poorly understood, approaches to define the mechanism of action of GnRH-II may yield an important therapeutic clue in the treatment of ovarian cancer.

In our previous study, we demonstrated that the antiproliferative effect of GnRH-II in ovarian cancer cells involve p38 MAPK, which led us to investigate the role of other MAPK family members. Over 12 different types of MAPKs have been identified in mammalian cells. ERK1/2, JNK/SAPK1, and p38/SAPK2 are three of the best-characterized MAPK family members that exert their effects via the activation of transcription factors resulting in cellular responses such as cell proliferation or apoptosis (13–16).

In this study, we demonstrated that treatment with GnRH-II activated ERK1/2 in immortalized OSE and ovarian cancer cells. To confirm the antiproliferative effect of GnRH-II, cellular viability was measured by MTT assay. The cells were seeded into 96-well plates at a density of 10^4 cells per well. The cells were treated with GnRH-II (100 nM) for 4 d, and cell viability was measured by MTT assay. Values are represented as the mean ± SD of three individual experiments. a, P < 0.05 vs. control.
cancer cell lines in a diverse pattern. It is of interest to note that GnRH-II appears to activate ERK1/2 in a different time manner in these cell lines, indicating that different signal pathways may be involved in the GnRH-II-induced ERK1/2 activation in different ovarian cell types. Moreover, PD98059, an inhibitor of MEK, markedly attenuated the activation of ERK1/2 by GnRH-II in OVCAR-3 cells. This is in agreement with previous studies that demonstrated that GnRH-I activates ERK1/2 in normal and ovarian cancer cells (20, 22). The present results indicate that the ERK1/2 pathway might be an important signaling pathway mediating the effects of GnRH-II in ovarian cancer cells. It has been shown that treatment of CaOV-3 cells with GnRH results in an activation of ERK at 5 min with maximal activation occurring at 3 h and sustained until 24 h, whereas GnRH had no effect on the activation of the JNK (20). In addition, ERK1/2 kinase was also activated, and an increase in phosphorylation of son of sevenless (Sos) and Shc was observed after GnRH treatment. Treatment with a MEK inhibitor, PD98059, reduced the antiproliferative effect of GnRH analog and the GnRH-induced dephosphorylation of the retinoblastoma protein, indicating that the activation of ERK may play an important role in the antiproliferative effect of GnRH (20). In parallel with the previous study, ERK may play a critical role in GnRH-II-induced antiproliferation in ovarian cancer cells. Furthermore, GnRH-I agonist activated the JNK pathway in endometrial cancer cells (38) and the αT3-1 gonadotroph cell line (39) but not in ovarian cancer cells (20). In this study, JNK/SAPK1 was not activated by GnRH-II in OVCAR-3 cells. This is in agreement with a previous report using GnRH-I agonist in CaOV-3 cells (20). In addition, JNK/SAPK1 was not activated by GnRH-II in SKOV-3 cells, an ovarian cancer cell line (data not shown), suggesting that the JNK/SAPK1 pathway may not be involved in GnRH-I and -II signaling to induce cellular responses. We have also examined the effect of GnRH-II on TE671, a human breast tumor cell line, using an antibody to phospho-JNK/SAPK1, and demonstrated that GnRH-II phosphorylates JNK/SAPK1 in this cell line until 60 min of treatment (unpublished data). Taken together, these results indicate that the effects of GnRH-I and GnRH-II on the activation of ERK1/2 but not JNK/SAPK1 may be identical in ovarian cancer cells.

Activated MAPKs translocate from the cytoplasm to the nucleus and activate transcription factors. ERK1/2 induces gene expression by the activation of transcription factors including the phosphorylation of ternary complex factors such as Elk-1 and SAP-1 (40–42). Elk-1, an Ets family transcription factor, is a physiological substrate for ERK1/2 and mediates the c-fos and other coregulated gene activity through the serum response element. Therefore, the ability of GnRH-II to activate a downstream pathway of ERK1/2 was examined using the immunoprecipitation method. In this study, the treatment with GnRH-II resulted in substantial phosphorylation of Elk-1 fusion protein in vitro. Furthermore, PD98059, an inhibitor of MEK, abolished the effect of GnRH-II on the phosphorylation of Elk-1, suggesting that GnRH-II-induced ERK1/2 activation resulted in the phosphorylation of Elk-1, possibly mediating cellular response in ovarian cancer cells. In our previous study, we demonstrated the in vitro effect of GnRH-II in regard to inhibited cell growth and induced apoptosis in the ovarian cancer cell line OVCAR-3 (4). To confirm its effect on the inhibition of tumor growth, [3H]thymidine incorporation and MTT assays were performed. After a 4-d treatment, GnRH-II (100 nm) inhibited cell growth and cell viability. The importance of MEK-MAPK in cell proliferation and apoptosis is now widely recognized (43). It has been reported that inhibition of the ERK1/2 signaling pathway with PD98059 may affect the growth of prostate and breast tumors (44, 45), and PD98059 restored cell proliferation inhibition (46). In addition, it has been observed that PD98059 reduced the antiproliferative effect GnRH-I, suggesting that the MEK-MAPK pathway has a critical role in the effect of GnRH-I (20). Therefore, we investigated the involvement of the MEK-MAPK pathway in the antiproliferative effect of GnRH-II on ovarian cancer cells. In the present study, GnRH-II-induced growth inhibition in ovarian cancer cells was completely abolished by PD98059, suggesting that ERK1/2 mediate the antiproliferative effect of GnRH-II and that the MEK-MAPK pathway may be an integral mediator of GnRH-induced functions such as cell growth and/or apoptosis. In the previous study, p38 MAPK is involved in the GnRH-II-induced inhibition of cell growth through activator protein-1 (AP-1) activation, which may be related to induction of apoptosis in ovarian cancer cells (4). It has been known that PD98059 is a specific inhibitor for MEK1 and does not block p38 MAPK (47), and SB203580 is also a specific inhibitor of p38 MAPK (48). Furthermore, we have tested the effect of PD98059 or SB203580 to examine the specificity of these inhibitors and found that PD98059 could not block an activation of p38 in the preliminary experiment. It can be hypothesized that p38 MAPK is involved in the GnRH-II-induced apoptotic pathway via AP-1 transcriptional factor and that ERK1/2 MAPK is involved in GnRH-II-induced cell growth inhibition via Elk-1 transcriptional factor in ovarian cancer cells as proposed in Fig. 7. However, we cannot rule out the possibility that the ERK1/2 pathway activated by GnRH-II may induce subsequent AP-1 transcriptional activation or that the p38 MAPK pathway by GnRH-II may result in Elk-1 phosphorylation in this cell type. It has been reported that the MAPK pathways are regulated by other signaling pathways (49); thus, further study is necessary to investigate a possible involvement of other pathways in this cellular response.

GnRH-I and -II receptors belong to the G protein-coupled receptor family, which is known to regulate the MAPK cascade (50). However, the issue of whether the GnRH-I receptor mediates the effects of GnRH-II or of the GnRH-II receptor is functional in humans remains unsolved. It has been observed that the human gene homolog of GnRH-II receptor has a stop codon and a frame shift (51), suggesting that GnRH-II signaling may be mediated through the GnRH-I receptor (52). However, the expression of GnRH-II receptor mRNA was more widely observed than GnRH-I receptor in the body (6, 37, 51), leading to the notion that GnRH-II receptor may be functional in specific cell types (36, 37). In addition, it has been reported that the antiproliferative effect of GnRH-II might be mediated through GnRH-II receptors in gynecologic tumors (8). In addition, GnRH-II was more stable than GnRH-I in mammalian ovary, and the existence of a GnRH-II receptor was proposed (35). However, it has been
mediated by the activation of these MAPKs. Furthermore, the antiproliferative effect of GnRH-II appears to be activated transcriptional factors such as AP-1 and Elk-1, respectively. Our previous and present studies. The activation of p38 and ERK in cancer. GnRH-II activates p38 MAPK (4) and ERK1/2 but not JNK in neoplastic ovarian cancer cells. GnRH-II activates p38 MAPK but not JNK in ovarian cell lines, OVCAR-3 and IOSE-80PC, were kindly provided by Drs. T. C. Hamilton and A. Godwin (Fox Chase Cancer Center, Philadelphia, PA), respectively.

noted that treatment with antide completely blocked the growth inhibitory effect of GnRH-II in neoplastic ovarian surface epithelial cells (7). Therefore, the possibility that the effect of GnRH-II is mediated by functional GnRH-II receptors remains to be determined.

In conclusion, the present study demonstrates that treatment with GnRH-II induces the phosphorylation of ERK1/2 in OVCAR-3 cells, which was reduced by a MEK inhibitor, PD98059. In an *in vitro* kinase assay, treatment with GnRH-II resulted in the phosphorylation of Elk-1, and this effect was also blocked by PD98059. This suggests that Elk-1 may mediate cellular responses to GnRH-II-induced ERK1/2 activation. Furthermore, the growth inhibitory effect of GnRH-II was attenuated by PD98059. These results, taken together with our previous study, strongly suggest that GnRH-II-induced MAPK activation, including ERK1/2 and p38 but not JNK/SAPK1, mediate cellular responses such as growth inhibition and induction of apoptosis in ovarian cancer cells and may be a potential target in the treatment of ovarian cancer.

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**References**


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