

# Prostasin may contribute to chemoresistance, repress cancer cells in ovarian cancer, and is involved in the signaling pathways of CASP/PAK2-p34/actin

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Ovarian cancer is the deadliest of gynecologic cancers, largely due to the development of drug resistance in chemotherapy. Prostasin may have an essential role in the oncogenesis. In this study, we show that prostasin is decreased in an ovarian cancer drug-resistant cell line and in ovarian cancer patients with high levels of excision repair cross-complementing 1, a marker for chemoresistance. Our cell cultural model investigation demonstrates prostasin has important roles in the development of drug resistance and cancer cell survival. Forced overexpression of prostasin in ovarian cancer cells greatly induces cell death (resulting in 99% cell death in a drug-resistant cell line and 100% cell death in other tested cell lines). In addition, the surviving cells grow at a much lower rate compared with non-overexpressed cells. *In vivo* studies indicate that forced overexpression of prostasin in drug-resistant cells greatly inhibits the growth of tumors and may partially reverse drug resistance. Our investigation of the molecular mechanisms suggests that prostasin may repress cancer cells and/or contribute to chemoresistance by modulating the CASP/P21-activated protein kinase (PAK2)-p34 pathway, and thereafter PAK2-p34/JNK/c-jun and PAK2-p34/mlck/actin signaling pathways. Thus, we introduce prostasin as a potential target for treating/repressing some ovarian tumors and have begun to identify their relevant molecular targets in specific signaling pathways.

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**Subject Category:** Cancer

It is estimated that about 22 000 new cases of ovarian cancer and 15 000 deaths from this malignancy will occur in the United States in 2013.<sup>1</sup> Chemotherapy after surgically is the standard treatment for ovarian cancer, which may result in complete clinical remission in up to 75% of cases.<sup>2</sup> However, a majority of responders will relapse within 18–28 months and only 20–40% of women will survive beyond 5 years.<sup>3–5</sup> Ovarian cancer is the deadliest of gynecologic cancers, largely due to the development of drug resistance in chemotherapy and late-stage diagnosis.

Prostasin, also known as PRSS8 (protease serine 8), is a trypsin-like serine peptidase expressed in epithelial cells, with the highest expression in the normal prostate gland and seminal fluid and lesser amount in various tissues.<sup>6,7</sup> The expression of prostasin has been shown to malexpress in ovarian, prostate, breast, and gastric cancers<sup>8–11</sup> and to be a potential biomarker for ovarian cancer early detection alone or in combination with CA125.<sup>9,12,13</sup> Prostasin has been suggested to inhibit cancer cell proliferation and invasion.<sup>14</sup> Prostasin is also termed channel-activating protease 1, which is implicated in the regulation of sodium and fluid levels via

proteolysis of the epithelial sodium channel, and thus has important functions in blood pressure being a target for regulating hypertension.<sup>15–17</sup> In addition, prostasin has been found to have important roles in the epidermal barrier function, skin phenotypes, and embryonic viability.<sup>18,19</sup> Thus, prostasin is implicated in a wide spectrum of physiological and pathophysiological conditions.

P21-activated protein kinase (PAK) 2 is a serine/threonine kinase, which has critical roles in various cytoskeletal functions, such as cell motility and membrane blebbing during apoptosis.<sup>20,21</sup> Full-length PAK2 can be activated either by the small GTPases CDC42 and Rac or CASPs, which stimulates cell survival or induction of cell death in response to many apoptotic stimuli.<sup>21</sup> PAK2-p34 is a constitutively active 34-kDa PAK2 C-terminal kinase fragment cleaved by several CASPs members.<sup>22</sup> Recombinant expression of PAK2-p34 induced morphological changes characteristic of apoptotic cell death in a variety of cell lines and induced apoptotic cell death.<sup>23,24</sup> In addition, accumulation of PAK2-p34 by ubiquitin inhibits degradation results in a dramatic increase in cell death.<sup>25</sup> Therefore, PAK2-p34 is involved in cellular death

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**Abbreviations:** O432 (Ovca432), human ovarian cancer parental cell line; O432-RP (Ovca432-RP), drug-resistant sub-line generated by repeatedly treated Ovca432 cells with paclitaxel; ERCC1, excision repair cross-complementing 1

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regulation and execution of programmed cell death, and caspase activation stimulated by apoptotic stimuli appears to turn the antiapoptotic activity of PAK2 into a proapoptotic activity of PAK2-p34. Aberrant activity/level of PAK2 or PAK2-p34 has been linked with human cancer, Alzheimer's, and Parkinson's disease.<sup>23</sup>

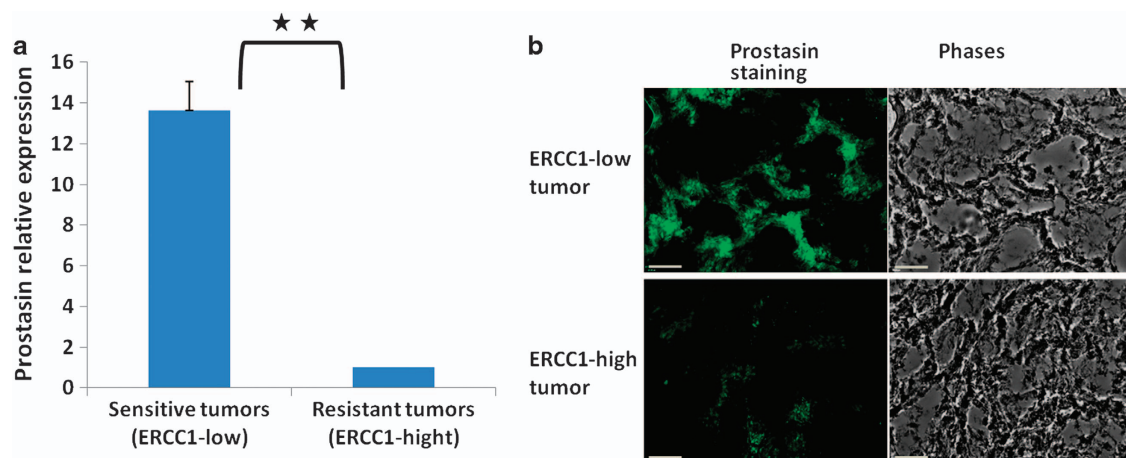
In this work, we focus on the functions of prostasin in chemoresistance and tumor repression. Our investigations revealed that prostasin has critical roles in chemoresistant development and cell survival. Forced overexpression of prostasin greatly reduces cancer cell survival, and may partially reverse chemoresistance. Therefore, prostasin may be a potential target for treating some ovarian tumors, including chemoresistant tumors. Investigations of the signaling pathways suggest that prostasin may repress cancer cells and/or contribute to chemoresistance by modulating the CASP/PAK2-p34 and thereafter PAK2-p34/JNK/c-jun and PAK2-p34/mlck/actin signaling pathways.

## Results

**Prostasin expression is decreased in ovarian cancer patients with high expression of excision repair cross-complementing 1 (ERCC1), a marker for chemoresistance in ovarian cancer.** ERCC1, the essential nucleotide excision repair component, is a biomarker for chemoresistance and a target to overcome chemoresistance in cancer therapy.<sup>26–30</sup> We proposed that prostasin may have role in chemoresistance because it is malexpressed in various types of cancers.<sup>8–11</sup> To investigate the possible association, we examined prostasin expression in ovarian tumor samples with known ERCC1 expression, representing chemoresistant phenotype.<sup>31</sup> Real-time quantitative PCR data showed that prostasin expression was significantly lower in tumor samples from ERCC1-high group ( $n=18$ ), compared with ERCC1-low group ( $n=31$ ), and that prostasin mRNA is greatly reduced in potentially chemoresistant tumors compared with chemosensitive tumors (Figure 1a,  $P<0.01$ ).

We examined prostasin protein level in these tumor samples using immunohistochemistry. The data show that prostasin protein is decreased in chemoresistant/ERCC1-high tumors compared with chemosensitive/ERCC1-low tumors (Figure 1b), which is consistent with prostasin mRNA level. These results demonstrated that prostasin is decreased in ovarian tumors with high ERCC1 expression, the chemoresistant phenotype. Therefore, prostasin may have an important role in chemoresistance in ovarian cancer.

**Prostasin is decreased, has key roles in chemoresistance and cell survivals in ovarian cancer cells.** To study the function of prostasin in chemoresistance, we examined prostasin expression in ovarian cancer paclitaxel-resistant cell line O432-RP (it also cross-resistant to cisplatin), a cell line we generated previously from ovarian cancer cell Ovca432 (O432),<sup>32</sup> and performed the functional investigations. Western blot analysis showed that prostasin is decreased in drug-resistant O432-RP cell line, compared with the parental-sensitive O432 cell line (Figure 2a), suggesting prostasin may be involved in acquired chemoresistance in ovarian cancer cells. We then examined chemoresistant phenotype after knockdown prostasin in sensitive O432 cell and overexpression of prostasin in resistant cell O432-RP. siRNA knockdown prostasin in O432 cells obtained greatly reduced prostasin cells (O432-pro-D: Figure 2b) compared with the control cells. These cells were treated with paclitaxel at several doses and drug sensitivity was assessed by cell survival assays. We observed that O432-pro-D cells have a higher survival rate compared with O432 cells, which transfected with no-targeting siRNA or reagent (Figure 2c). The data demonstrated that O432-pro-D cells are more resistant to paclitaxel treatment compared with the control cells, suggesting that downregulation of prostasin alone may result in increasing chemoresistance. In contrast, we overexpressed prostasin by transfecting prostasin cDNA containing vector into O432-RP cells. To our surprise, we observed that forced



**Figure 1** Reduced prostasin expression in patients with high expression of ERCC1, a marker for chemoresistance in ovarian cancer. (a) Prostasin mRNA levels were compared in tumors with high ERCC1 levels (ERCC1 average = 1.54,  $n=18$ ) representing the chemoresistant phenotype, and in tumors with low ERCC1 levels (ERCC1 average = 0.44,  $n=31$ ) representing the chemosensitive phenotype<sup>26,27</sup> by real-time qPCR analysis. Relative levels of prostasin in chemoresistant *versus* chemosensitive tumors are shown, respectively. \*\* $P<0.01$ . Mean  $\pm$  s.d. are given, and  $P$  values were calculated using the two-sided Student's  $t$ -test. (b) Expression of prostasin by fluorescent immunohistochemistry in (patient-derived) ovarian tumor samples. Prostasin is decreased in ovarian ERCC1-high tumors compared with the ERCC1-low tumors

overexpression of prostasin resulted in inducing cell death dramatically, with more than 99.99% cells dead after successful transfection (Figure 2d). Less than 0.01% of the surviving cells (named O432-RP-pro-O), which expressed higher prostasin (Figure 2e), were found to have a very low growth rate compared with the control cells O432-RP and O432-RP-C (transfected with empty vector; Figure 2f). These three cell sub-lines were then treated with paclitaxel at several doses and were assessed for the drug sensitivity by cell survival assays. The survival rate of O432-RP-pro-O was dramatically reduced compared with the control cells (Figure 2g), suggesting that chemoresistance may be reversed. We also tried to overexpress prostasin in other ovarian cancer cell lines, such as Uci101, OVCAR-3, and Caov-3. As seen in the O432-RP cell line, cell death was greatly induced upon prostasin overexpression. However, we did not detect any surviving cells in these cell lines after prostasin cDNA was successfully transfected, which was assessed by geneticin selection and control transfections (Figure 2e). These results indicate that forced overexpression of prostasin in ovarian cancer cells dramatically induces cell death – 100% in some cell lines and more than 99.99% in the other cell line – and represses the cell growth of surviving cells, and may partially reverse chemoresistance. These functional studies indicate prostasin may have critical roles in cell survival and chemoresistance in the studied cell models.

**Overexpression of prostasin represses chemoresistant tumors.** We extended our *in vitro* finding to a mouse tumor model to explore the potential of prostasin as a therapeutic target for treating/repressing some ovarian tumors. The tumor cells of O432-RP-pro-O and O432-RP-C were implanted into each flank of mice. These tumor-bearing animals were treated with paclitaxel or vehicle PBS when the tumor volume reaches about 100 mm<sup>3</sup> (the tumor volume of O432-RP-C is about 100 mm<sup>3</sup> at the time of treatment; however, the volume of O432-RP-pro-O tumor is only about 50 mm<sup>3</sup> because of low growth rate of cells). At the end of treatment, we compared the tumor volumes of O432-RP-C and O432-RP-pro-O in the presence and absence of paclitaxel, respectively. In the absence of paclitaxel treatment (e.g., just vehicle PBS), we observed that O432-RP-pro-O tumors were significantly smaller compared with that of O432-RP-C control tumors ( $P < 0.01$ , Figures 3a and b). We believe this is probably due to the low growth rate of this cell line, which overexpressed prostasin. In addition, we observed that the O432-RP-pro-O tumors became further smaller when treated with paclitaxel ( $P < 0.001$ , Figures 3a and b), and paclitaxel-treated O432-RP-pro-O tumors were slightly smaller than PBS-treated O432-RP-pro-O tumors ( $P < 0.1$ , Figures 3a and b). The data suggest that restoration of prostasin in chemoresistant cells inhibits growth of these tumors and may partially reverse chemoresistance, which is consistent with our *in vitro* findings.

**Prostasin regulates a network involving CASP/PAK2-p34 and thereafter mlck/actin, and JNK/c-jun pathways in ovarian cancer cells.** To uncover the signaling pathways for prostasin in cell survival and chemoresistance, we

compared the expression profile of O432-RP-pro-O and O432-RP-C cells using PCR arrays analysis.

$\beta$ -Actin was found to be decreased in O432-RP cells compared with O432 cells in our study (we initially used  $\beta$ -actin as a loading control for western blot analysis; however, we found  $\beta$ -actin is not consistent when we used GAPDH as loading control for equal loading of samples (Figure 4a)). With the previous finding that  $\beta$ -actin expression changed in breast cancer drug resistance cells, this prompted us to hypothesize that  $\beta$ -actin and cytoskeletal genes may be involved in the prostasin-directed chemoresistance development as actin gene is believed to be a central player of cell shape and movement and a key component of cytoskeleton.<sup>33</sup> We examined gene expression of cytoskeleton pathway using PCR array in these cells. The PCR array data showed that PAK and mlck increased, and  $\beta$ -actin decreased in O432-RP-pro-O cells, compared with control O432-RP-C cells (Figure 4b). Western blot analysis further demonstrated that protein levels of mlck and  $\beta$ -actin changed, which were consistent with mRNA levels (Figure 4a). However, we did not see significant difference for PAK proteins. Instead, we observed that PAK2-p34, a 34KD C-terminal fragment of PAK2, which is cleaved by CASPs,<sup>34,35</sup> is increased in O432-RP-pro-O cells. PAK2-p34 has been shown to regulate JNK expression during apoptosis,<sup>20</sup> so we examined JNK and thereafter target c-jun expression. The western blot analysis showed that JNK and c-Jun both increased in O432-RP-pro-O cells. The data suggested that prostasin regulates PAK2-p34 and thereafter JNK and c-jun signaling in these cells. In addition, mlck has been shown to be a downstream target of PAK2/PAK2-p34, and upstream target of actin. Thus, PAK2-p34 seems to be an important mediator of prostasin in these cells and appears to regulate JNK/c-jun and mlck/act sub-pathways.

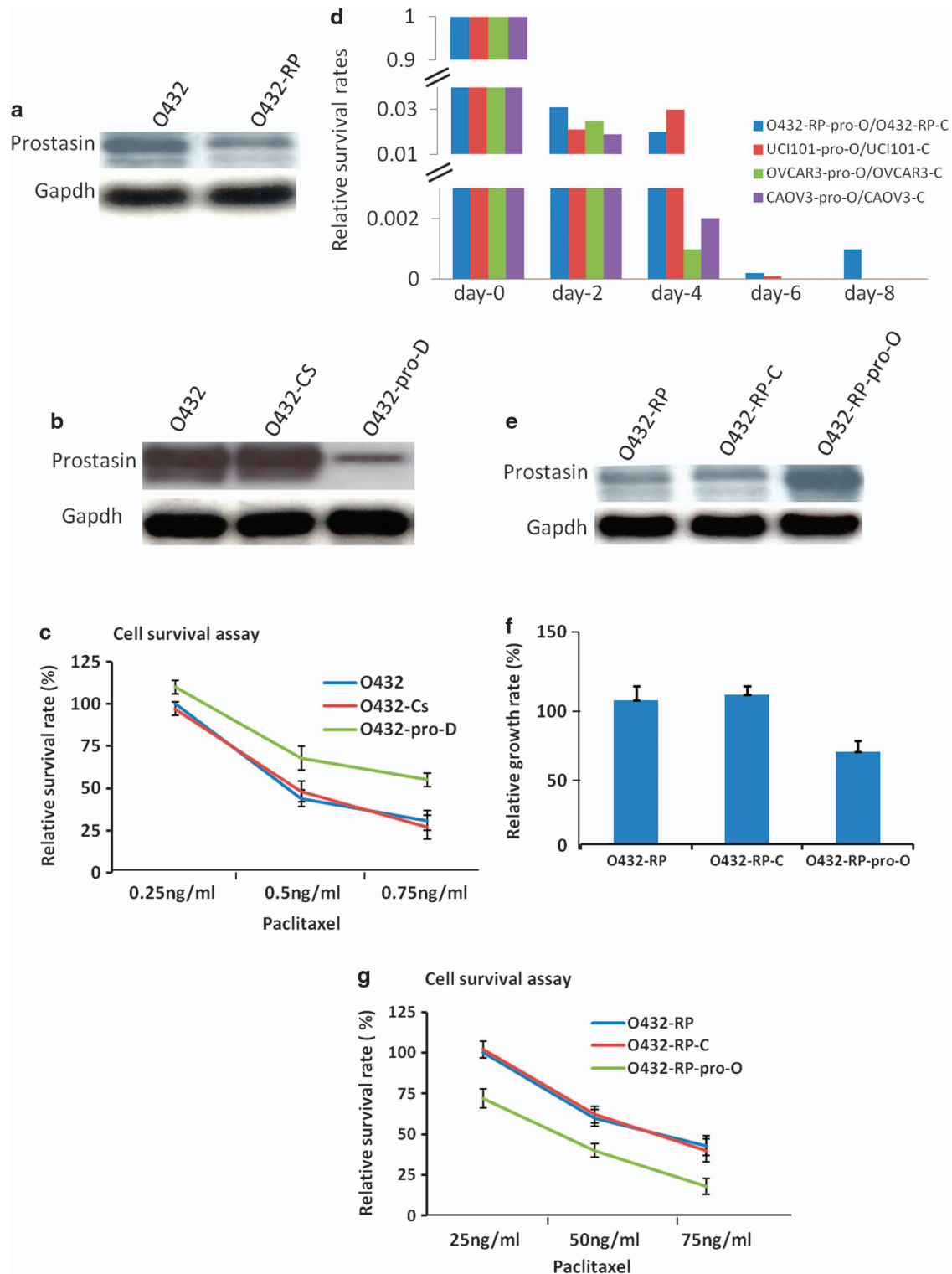
To explore the upstream targets of PAK2-p34, we examined expression of several CASP genes, as PAK2-p34 is specifically cleaved by CASPs.<sup>34,35</sup> Interestingly, several CASPs were found increased in O432-RP-pro-O cells compared with O432-RP-C cells (Figures 4c and d). To confirm that PAK2-p34 is cleaved by CASPs, we blocked CASPs activity using CASPs inhibitors. We observed that the cleavage was blocked (PAK2-p34 decrease or disappear) as early as 12 h after incubating the cell with CASPs inhibitor (Figure 4e). Thus, CASPs are upstream targets of PAK2-p34 and downstream target of prostasin in our experimental system. We tried to identify the upstream signaling of CASPs in these cells, but were unsuccessful. We also checked the expression level of these prostasin downstream targets in prostasin knockdown cells O432-pro-D compared with the control, and a reverse pattern was revealed (Figure 4f). The data suggest that prostasin may affect chemoresistance and/or cell survival through regulating the CASPs/PAK2-p34 axis and thereafter JNK/c-jun and mlck/actin signaling pathways (Figure 5).

## Discussion

Dramatic advances have been made in understanding cancer's basic mechanisms during recent decades. Despite these advances, ovarian cancer is still a deadly disease with a

less than 50% 5-year survival rate.<sup>1</sup> Antitumor drug resistance or non-response to chemotherapy is one of the major challenges to successful treatment of ovarian cancer. In this study, we show that prostasin expression is significantly decreased in both ovarian cancer chemoresistant cells and in

tumor tissues of high ERCC1-expressing ovarian cancer patients. ERCC1 has been well-described as a potential biomarker for drug resistance.<sup>26-29</sup> Prostasin appears to have an important role in chemoresistance. The functional investigations demonstrate that forced overexpression of prostasin





in ovarian cancer cells induces more than 99.99% cell death and represses the cell growth of surviving cells, and may partially reverse chemoresistance. This indicates that prostasin may have very strong power in repressing cancer cells and have roles in chemoresistance. Thus, may be a target for treating/repressing some ovarian tumors in gene therapy.

Prostasin may have an important role in the development of various types of cancers, including ovarian, prostate, breast, and gastric cancers, and expression of prostasin has been shown to be changed in these cancer patients.<sup>8–11</sup> In addition, restoration of prostasin in prostate cancer cells results in the inhibition of cell proliferation and invasion.<sup>14</sup> Therefore, prostasin is believed to be a tumor suppressor in prostate and breast cancers. For ovarian cancer, the role of prostasin is complex. It is overexpressed in ovarian cancer patients suggesting it is an oncogene.<sup>9</sup> Our findings show that prostasin is decreased in potentially chemoresistant ovarian cancer patients and chemoresistant cell line, and forced overexpression of prostasin in ovarian cancer cells greatly induces cells death, which behaves like a tumor suppressor. Prostasin is overexpressed in ovarian cancer patients with all stages. However, the levels change significantly from early to late stages. Prostasin increases and reaches a peak level at stage II and III and then decreases sharply at stage IV (the level is still slightly higher than in normal tissue).<sup>9</sup> Therefore, decreased prostasin (advanced stage compared with early stage patients) may be required for advanced ovarian cancer cells, or prostasin has different roles in different stage cancer cells. Prostasin is a glycosylphosphatidylinositol-anchored membrane protein as well as a secreted protein from epithelial cells, where it is bound to the cell surface, secreted, or both.<sup>10</sup> Studies showed that the membrane-anchored form of prostasin has important roles in tumor or invasion suppression of prostate or breast cancers, but not the secreted or recombinant prostasin.<sup>10</sup> Our finding is consistent with these observations, which demonstrate that forced overexpression of prostasin inhibits cancer cells growth, but the recombinant prostasin has no effect on cell growth or death in ovarian cancer cells (data not shown). Therefore, membrane-anchored prostasin has critical roles in cell survival and/or chemoresistance, and overexpression of prostasin triggers cell death in some ovarian cancer cells. We propose that prostasin is a potential target for treating some ovarian tumors in gene therapy, which overexpress prostasin in cells, but not by infusion of secreted/recombinant prostasin. Overexpression of prostasin can be done either by traditional transgene methods or newly identified gene-regulation mechanisms to

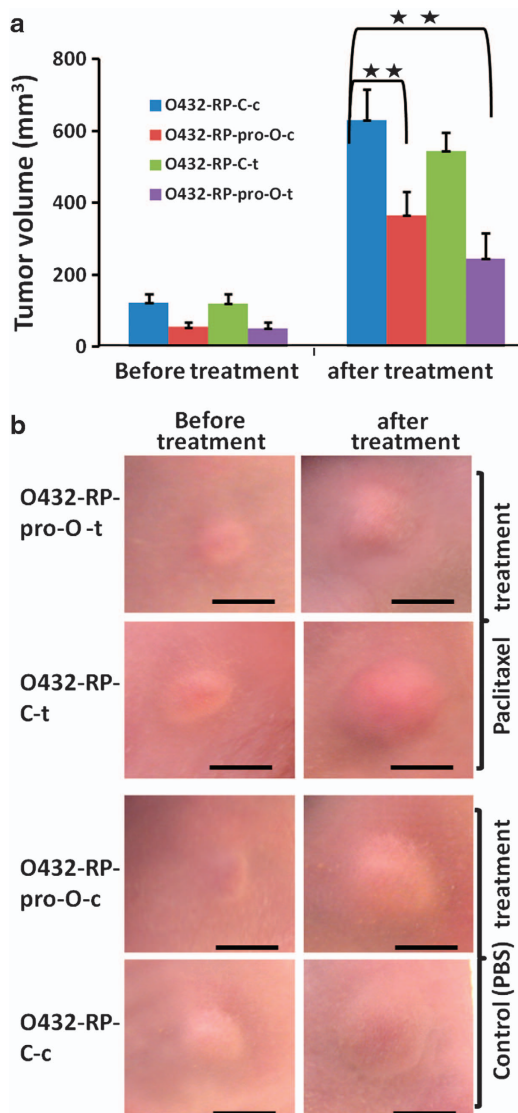
enhance gene expression through transfection of promoter-associated siRNAs.<sup>36</sup>

The signaling pathways that we investigated show that prostasin controls the downstream axis of CASPs/PAK2-p34, and thereafter mlck/actin and JNK/c-jun sub-signalings, in ovarian cancer cells. The finding suggests a new mechanism by which prostasin induces cell death and/or contribute to chemoresistance through regulating these pathways (Figure 5). The CASPs are believed to be the core effectors of apoptosis.<sup>37</sup> PAK2-p34 has been shown to induce cell death may be by increasing signal of JNK and regulating cytoskeletal dynamics of mlck pathways.<sup>20</sup> All these genes and signaling pathways<sup>33,38,39</sup> have essential roles in apoptosis and transcription. Therefore, prostasin may contribute to chemoresistance and repress cancer cells through these mechanisms. Study showed that overexpression of PAK2-p34 in cancer cells induced apoptosis and cell death.<sup>20</sup> We also observed that forced overexpression of PAK2-p34 in chemoresistant cell O432-RP greatly inhibited cell survival (Supplementary Figure 1). We propose that PAK2-p34 is a key executor of prostasin, and prostasin induces cancer cell death and/or contributes to chemoresistance by regulating these signaling pathways in our experimental system. Further functional studies should build the direct link between prostasin and these pathways. Our pathway analysis is focused on the apoptosis. Surely, the functions of prostasin in cell death and chemoresistance may involve in other pathways, such as cell cycle or stem cell development, and future study may focus on these topics.

PAK2 has been shown to contribute to chemoresistance in breast cancer cells by decreasing levels of active caspase-3 and thereafter PAK2-p34 (PAK2 and PAK2-p34 have opposing role in cell apoptosis).  $\beta$ -Actin was also found to be altered in these cells.<sup>36</sup> We proposed that alteration of  $\beta$ -actin should result from CASP/PAK2 (PAK2-p34)/mlck regulation, as mlck is one main target of PAK2-p34.<sup>15</sup> Together with our findings, the data suggest CASP/PAK2-p34 and sub-pathways thereafter may have an important role in chemoresistance in different cancer types (Supplementary Figure 2). In addition, changes in  $\beta$ -actin have been shown both in breast cancer drug-resistant (cisplatin and paclitaxel) cells<sup>36</sup> and our ovarian cancer drug-resistant cells. Involvement of  $\beta$ -actin in drug resistance in different types of cancer precludes it as an internal control in the future study.

In summary, our study points to prostasin as a potential target for treatment/repress of some ovarian cancers, including chemoresistant tumors. The pathway and mechanism

**Figure 2** Prostasin has important roles in chemoresistance and cell death in cell culture model. (a) Prostasin decreased in paclitaxel-resistance cancer cell line. O432: ovarian cancer cell line Ovca432 (sensitive to paclitaxel); O432-RP: paclitaxel resistance cell line generated from Ovca432. The prostasin protein levels in O432 and O432-RP cells are shown in immunoblots with specific antibodies. (b) Prostasin siRNAs transfection reduced prostasin. O432-pro-D cells (transfected with prostasin siRNA) express lower prostasin, compared with control cells of O432 (transfected with reagent only) and O432-Cs (transfected with no-targeting siRNA). The prostasin protein levels are shown in immunoblots with specific antibodies. (c) Downregulation of prostasin in O432 cells resulted in increase of chemoresistant activity. Cells were treated with paclitaxel at different concentrations for 24 h (starting 48 h after siRNAs transfection) and cultured with normal medium for an additional 7 to 10 days before cell survival was assayed. Relative cell survival rates of each cell line are shown. (d) Overexpression of prostasin greatly induces cell death in ovarian cancer cells. The cell survival rates are shown after forced overexpression of prostasin in several cell lines from day-0 to day-8, respectively. (e) Prostasin cDNA transfection resulted in overexpression of prostasin in chemoresistant O432-RP cells. O432-RP-pro-O cells (transfected with prostasin cDNA) express higher prostasin compared with control cells O432-RP and O432-RP-C (transfected with control vector). The prostasin protein levels are shown in immunoblots with specific antibodies. (f) Forced overexpression of prostasin represses growth of chemoresistant cells. Relative cell growth rates are shown for O432-RP-pro-O and control cells O432-RP and O432-RP-C. (g) Forced overexpression of prostasin in O432-RP cells re-sensitizes chemoresistant cells. Cells were plated at about 10–20% confluence and treated with paclitaxel at different concentrations for 24 h, cultured with normal medium for additional 7 to 10 days, then assayed for cell survival. Relative survival rates of cell lines are shown



**Figure 3** Forced restoration of prostasin represses and re-sensitizes chemoresistant tumors. (a) Overexpression of prostasin represses and re-sensitizes chemoresistant tumors. O432-RP-pro-O cells (stably transfected with prostasin cDNA) or control cells O432-RP-C (stably transfected with control pCI-neo vector) were injected into the left and right flanks of mice, respectively. The animals were treated with paclitaxel (15 mg/kg/week) or control vehicle PBS for 2 weeks after the tumors reached about 100 or 50 mm<sup>3</sup>. Tumor volumes before and after treatment are shown. O432-RP-*pro-O-c* and O432-RP-*pro-O-t*: PBS- and paclitaxel-treated O432-RP-*pro-O* tumors; O432-RP-*C-c* and O432-RP-*C-t*: PBS- and paclitaxel-treated O432-RP-*C* tumors.  $n = 6$  per group,  $**P < 0.01$ . Mean  $\pm$  s.d. are given, and the  $P$ -values were calculated using the two-sided Student's  $t$ -test. (b) Tumors in mice before and after treatments. Scale bar = 5 mm

findings suggest prostasin may regulate cancer cell survival and/or chemoresistance by controlling CASPs/PAK2-p34 and thereafter signaling through apoptosis mechanisms.

## Materials and Methods

**Human samples and ethics statement.** Frozen GOG (Gynecologic Oncology Group) samples from ovarian cancer patients were obtained from the Cooperative Human Tissue Network, Pediatric Division (Children's Hospital, Columbus, Ohio). All patient identifiers of these samples had been stripped of before shipping to ensure that the presented molecular laboratory data cannot be

linked with any subject in this study. The tumor specimens were collected at primary surgery, fresh frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until RNA/DNA extraction. All samples were evaluated by pathologists.

## Cell lines, cell culture and generation of drug-resistance sub-line

Cell lines, cell culture and generation of drug-resistance sub-line are described as previously.<sup>32</sup> All cell lines were propagated as an adherent monolayer in MEM (Invitrogen, Life Technologies, Inc., Carlsbad, CA, USA) at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  supplemented with 10% heat inactivated FBS, 100  $\mu\text{g}/\text{ml}$  penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Human ovarian cancer cell lines of Ovca432 and Uci101 were provided by Dr. Liu T from the Chinese Academy of Sciences. OVCAR-3 and Caov-3 were purchased from ATCC (Manassas, VA, USA). The Ovca432-RP drug-resistance sub-cell line was generated from Ovca432 cells with paclitaxel (Sigma-Aldrich, Inc., St. Louis, MO, USA) treatment at increasing concentrations for several cycles (each cycle including 24-h exposure to the drug, and subsequent recovery culture with normal medium for about 10 days) until cells were found resistant to treatment.

## RNA extraction and cDNA generation

Total RNA from tumors of ovarian cancer patients was partially purified by hot phenol/chloroform extraction as previously reported.<sup>40</sup> Through reverse transcription, using the Super Script Preamplification System (Life Technologies, Inc.), cDNA was generated with oligo-dT primers from 5  $\mu\text{g}$  of total RNA per sample (Life Technologies, Inc.). Total RNA was extracted from cells using Trizol. The cDNA was synthesized using Taqman Reverse Transcription (PE Applied Biosystems, Foster City, CA, USA) from 1  $\mu\text{g}$  of total RNA. cDNAs were used for the gene expression profiling.

## Real-time reverse transcription quantitative-PCR

Real-time quantitative PCR was performed with an ABI PRISM 7900 or 7500 instrument (PE Applied Biosystems) according to the manufacturer's instructions as previously described.<sup>32</sup> PCR cycling conditions were set as follows:  $50^{\circ}\text{C}$ , 2 min;  $95^{\circ}\text{C}$ , 10 min; and 40 cycles for the melting ( $95^{\circ}\text{C}$ , 15 s) and annealing/extension ( $60^{\circ}\text{C}$  for 1 min) steps. qPCR reactions for each template were carried out in duplicate or triplicate in 96-well plates. Comparative Ct (PE Applied Biosystems) determined relative expression in each sample using 18S or GAPDH as endogenous controls.

## Western blot

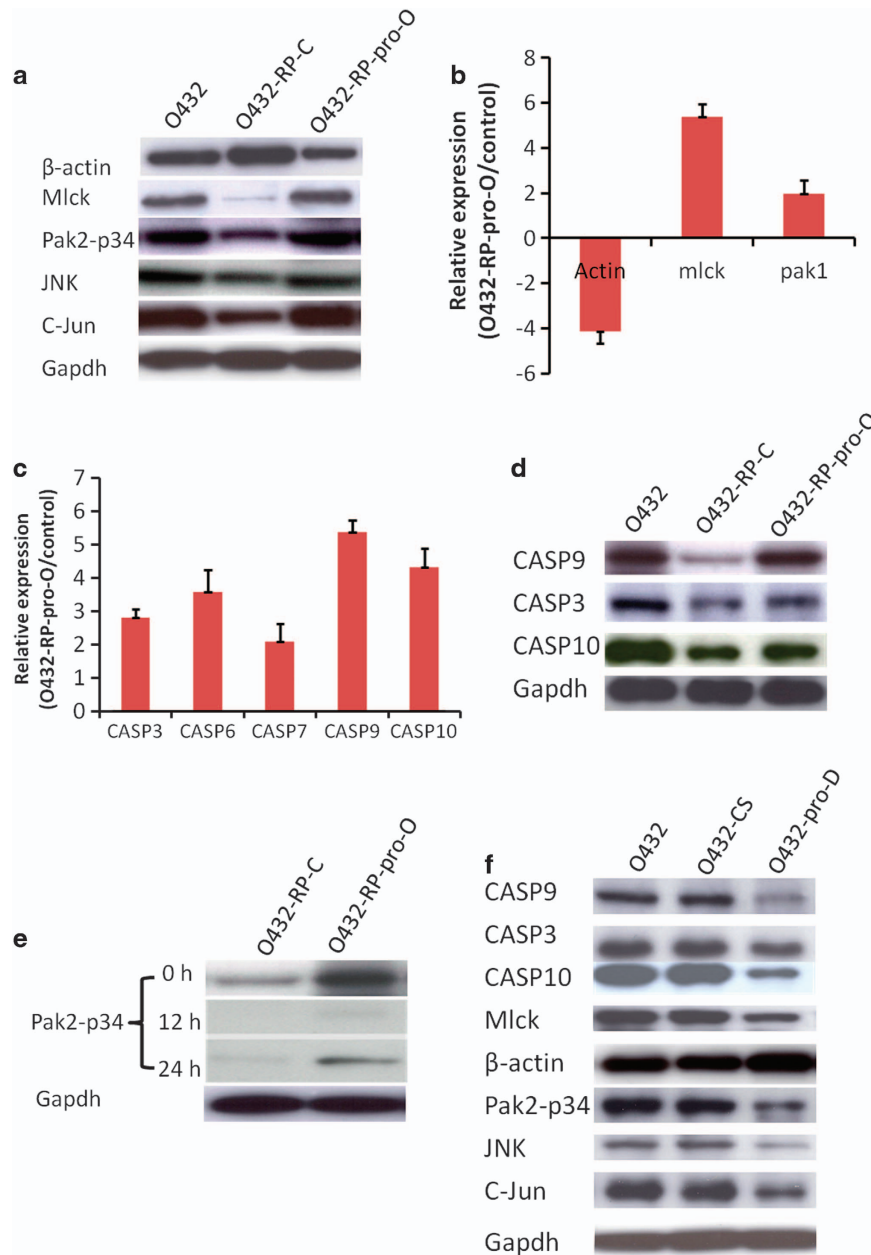
Western blot analysis was performed as previously described.<sup>32</sup> Briefly, cells were rinsed twice with PBS and total proteins were solubilized in lysis buffer (150 mM sodium chloride; 50 mM Tris hydrochloride, pH 7.5; 1% glycerol; 1% Non-idetp-40 substitute solution). Equal amounts of proteins were loaded and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane, blocked by 5% skim milk, and probed with the specific (primary) antibodies, followed by treatment with secondary antibody conjugated to horseradish peroxidase (1 : 5000). Proteins were visualized by (enhanced) chemiluminescence detection (Pierce Biotechnology, Rockford, IL, USA) and exposure to X-ray film.  $\beta$ -Actin or Gapdh proteins were detected by normal chemiluminescence detection (Pierce Biotechnology) and exposed to less-sensitive X-ray film, which may result in a relatively weaker signal. All antibodies used in this study were obtained from Abnova (Taipei, Taiwan) or Abcam (Cambridge, MA, USA).

## siRNA and cDNA transfection

O432 cells were transfected with prostasin siRNA duplexes (Ambion, Austin, TX, USA) and O432-RP cells with prostasin or PAK2-p34 cDNA-containing vectors (cDNAs were amplified by reverse transcription PCR and then cloned into a pCI-neo vector) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For siRNA experiments, mock transfections and no-targeting siRNA were used as negative controls. Cells were treated for 72 h to allow for maximum knockdown, after which they were lysed for western blot analysis, RNA preparation or other assays. For cDNA transfection, a pCI-neo vector was used as a negative control. Geneticin was used to select stably expressing cells.

## Cell survival assay

Cell survival assay was performed as previously described.<sup>32</sup> Cells were counted and plated in culture dishes at about 10–20% confluence on the day before treatment. Paclitaxel or control PBS was added for about 20 h and removed, then the cells were cultured with normal medium for recovery and continuous propagation for 10–14 days. To quantify final cell numbers, cells were stained with 0.25% crystal violet/20% ethanol and counted, or

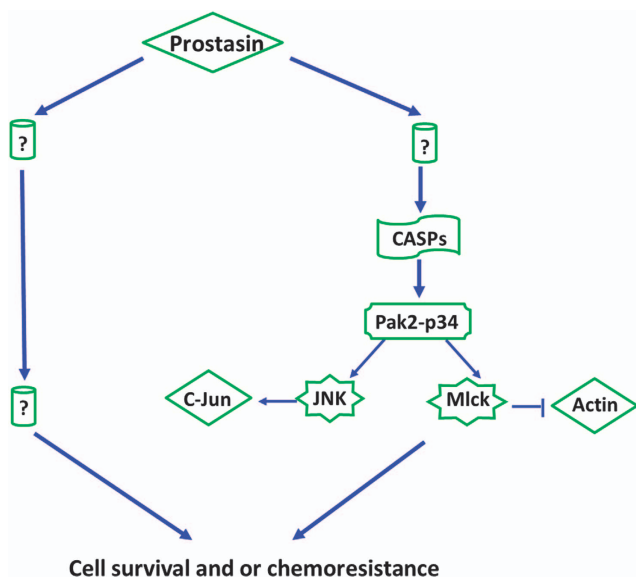


**Figure 4** Prostasin regulates CASPs-PAK2-p34 axis and thereafter downstream signaling. (a) Immunoblot of mlck,  $\beta$ -actin, PAK2-p34, JNK, C-Jun in O432-RP-pro-O (O432-RP cells transfected with prostasin cDNA which express higher levels of prostasin) control O432-RP-C cells (O432-RP cells transfected with pCI-neo vector). Mlck, PAK2-p34, JNK, C-Jun expressions increase and  $\beta$ -actin decreases in O432-RP-pro-O cells when compared with control O432-RP-C. (b) Comparison of actin, mlck, and pak1 expression between O432-RP-pro-O and control O432-RP-C cells by real-time qPCR. mRNA levels of actin decreases and mlck and pak1 increase in O432-RP-pro-O cells compared with control O432-RP-C cells. (c) mRNA levels of CASPs detection by real-time qPCR in O432-RP-pro-O cells and control O432-RP-C cells. Several CASP expression increase in O432-RP-pro-O cells compared with the controls. (d) Immunoblot of CASP3, 9, and 10 in O432-RP-pro-O and O432-RP-C cells. CASP3, 9, and 10 protein levels increase in O432-RP-pro-O cells compared with control O432-RP-C cells. (e) CASP inhibitors block CASP activity and thus PAK2 cleavage. PAK2-p34 protein was examined by immunoblot after CASP inhibitors were added to the medium. PAK2-p34 was seen decreased or lost when CASPs were inhibited at several time points. (f) Immunoblot of mlck,  $\beta$ -actin, PAK2-p34, JNK, C-Jun, CASP3, 9, and 10 in prostasin knockdown O432-pro-D cells and controls O432 (mock transfected with reagent only) and O432-Cs (transfected with no-targeting siRNA). Reverse expression patterns for these genes are revealed in O432-pro-D cells and controls compared with O432-RP-pro-O cells and controls

the proliferation rate was measured using a Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions. Briefly, MTS/PMS solution (at final concentrations of 333  $\mu$ g/ml MTS and 25  $\mu$ M PMS) was added to each well and cells were incubated for 2–3 h at 37  $^{\circ}$ C. The absorbance was determined at 490 nm using a 96-well plate ELISA reader. Culture medium was used as background control. The experiments were repeated at least three times.

**Immunofluorescence.** The tumor sections were deparaffinized in Histo Clear II (Electron Microscopy Sciences, Hatfield, PA, USA), hydrated in gradient alcohol (passed through gradient alcohol washes), and antigen retrieval treated in a microwave oven for 10 min at 'Power 20%' in citrate buffer (0.01 M, pH = 6.0, 0.05% Tween-20), then cooled for 20 min. The sections were incubated with blocking buffer (2% goat serum, 5% BSA, 0.1% cold fish skin gelatin, 0.1% Triton





**Figure 5** Proposed model of prostasin-regulated signaling network affecting cell survival and/or chemoresistance. Prostasin appears to regulate cell survival and/or chemoresistance may be through CASPs/Pak2-p34 axis and thereafter PAK2-p34/JNK/c-jun and PAK2-p34/mlck/actin signaling pathways

X-100, 0.05% Tween-20, 0.05% sodium azide, pH 7.2) for 2 h. Thereafter, the slides were incubated with mouse monoclonal anti-prostasin antibody (1:20 dilution) overnight at 4 °C. The primary antibody was detected using anti-mouse IgG Alexa Fluor 488 (Life Technologies, Inc.). Slides were mounted with the SlowFade Gold AntiFade reagent with DAPI (Life Technologies, Inc.). In the negative control tissue sections, the primary antibody was replaced by isotype-specific non-immune mouse IgG. Immunoreactivity was visualized and photographed using a Nikon confocal microscope (Nikon Elipse90i, Deutschland, Germany) at the appropriate wavelength.

**Tumor mouse model study.** We performed mouse experiments as previously described<sup>32</sup> and in compliance with the published guidelines of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985; <http://grants1.nih.gov/grants/olaw/references/phspol.htm>) and the Care and Use of Laboratory Animals (National Research Council, 1996). Four- to six-week-old female BALB/cnu mice (Charles Rivers Laboratories, Wilmington, MA, USA and Laboratory Animal Research Center, Shanghai, China) were used to generate xenograft tumors.  $1.0 \times 10^7$  cells were suspended in 200  $\mu$ l saline solution and injected into both flanks of mice. The mice were treated with paclitaxel (15 mg/kg) or vehicle PBS by intratumoral injection for 2 weeks when tumor volume reached about 100 or 50 mm<sup>3</sup>. Tumor volume and mouse weight were monitored accordingly.

**CASPs inhibition experiment.** CASP family inhibitor (MBL International, Woburn, MA, USA) was added to the culture medium at 1000-fold dilution to inhibit caspase activity. Cells were lysed after 12 and 24 h post inhibitor treatment and subjected to western blot analysis.

**Statistical analysis.** Statistical analysis was conducted using Student's *t*-test. Statistical significance was defined as  $P < 0.05$ . All statistical tests and corresponding *P*-values were two-sided.

### Conflict of Interest

The authors declare no conflict of interest.

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