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ORIGINAL PAPER

Prostasin inhibits cell invasion in human choriocarcinomal JEG-3 cells

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Abstract Controlled invasion of the uterine wall by the trophoblast cells is pivotal for the successful pregnancy, and various kinds of protease are involved in this process. Serine protease prostasin has been shown to participate in the proteolytic activation of epithelial sodium channel as well as cleavage of epidermal growth factor receptor extracellular domain in human epithelial cells. Its physiological significance in human placentation has been suggested but not validated. In the present study, we found that prostasin was expressed at a relatively high level in human placenta trophoblasts in early pregnant weeks. In the in vitro cultured human choriocarcinomal JEG-3 cells, treatment with functional antibody against prostasin led to promotion in cell invasion capability, as well as increase in the production of MMP-2, MMP-26, TIMP-1, and TIMP-4. Our data indicated that this serine protease may function as an invasion suppressor in human trophoblast, participating in the invasion-restrictive regulation of trophoblasts to avoid their over-penetration into the uterine wall.

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Introduction

During the process of embryonic implantation, invasion of uterine endometrium by trophoblast cells is one of the critical events. The trophoblast cells mimic the invasion property of tumor cells and migrate to deeper part of the uterus by degrading and reconstructing extracellular matrix (ECM) surrounding uterine stromal cells and spiral arteries (Goldman-Wohl and Yagel 2002). However, different from that of tumor cells, invasion of trophoblast cells are strictly controlled, and thus they are often called pseudo-malignant cells. It has been well accepted that there exists complex regulation on trophoblast behaviors involving the precise interaction of various hormones, growth factors, cytokines, proteases, ECM etc., and both invasion-promoting and invasion-repressing molecules participate in an appropriate stoichiometric amount (Cohen and Bischof 2007; Guzeloglu-Kayisli et al. 2007; Pilka et al. 2003; Salamonsen 1999). Any failure in the regulation will lead to severe pregnancy-associated diseases, such as spontaneous abortion, preeclampsia, hydatidiform mole, and even choriocarcinoma (Murray and Lessey 1999; Soundararajan and Rao 2004).

Prostasin is defined as glycosylphosphatidylinositol(GPI)-anchored serine proteinase (Chen et al. 2001b). In addition, it was generated by cleavage of pro-prostasin between Arg12 and Ile13 into a disulphide linked 12 amino acid light chain and 299 amino acid protease domain (Yu et al. 1995). Prostasin was first purified from seminal fluid in 1994 (Yu et al. 1994). It is widely distributed in tissues, abundant in prostate gland and placenta (Fan et al. 2005;

Yu et al. 1995). As a serine proteinase, accumulating evidence indicates prostasin is involved in the proteolytic processing of the epithelial sodium channel (ENaC) gamma subunit and recent data show persistently activated ENaC, cleavaged by furin and prostasin, contributes to the renal hypertention(Adachi et al. 2001; Bruns et al. 2007; Diakov et al. 2008; Maekawa et al. 2009; Narikiyo et al. 2002; Olivieri et al. 2005; Rotin and Schild 2008; Wang et al. 2003; Zhu et al. 2008). Recently, data in prostate cancer and breast cancer demonstrated the potential of prostasin as an invasion suppressor based on the evidence that over-expression of human prostasin gene in human invasive prostate cancer cells and highly invasive breast carcinoma cells led to reduced cell invasiveness in vitro (Chen et al. 2001a, 2004; Takahashi et al. 2003).

There has been little evidence regarding expression and roles of prostasin in female reproductive processes. Lin et al. (2006) demonstrated the expression of prostasin on rhesus monkey feto-maternal interface during the early gestational stage. They found the production of prostasin in rhesus monkey trophoblast cells and suggested the possible role of this enzyme in regulating trophoblast behaviors.

Based on these evidences, we proposed that prostasin may participate in repressing human trophoblast cell invasion. Therefore, in the present study, we first demonstrate the spatial and temporal expression pattern of prostasin in human placenta at different gestational stages, and then identify the effect of prostasin on human trophoblast cell invasiveness by using a well-accepted trophoblastic cell model—JEG-3 cell line. The influence of prostasin on some invasion-associated molecules is further detected.

Materials and methods

Tissue preparation

Tissues of human chorionic villi or placentae were obtained in Beijing Haidian Hospital (Beijing, China) from patients who underwent surgical termination of pregnancy at 6–9 and 26 weeks, or normal delivery at term, with informed consent of patients and permission of the Local Ethical Committee in the Institute of Zoology, Chinese Academy of Sciences. All the patients received no special medical treatment before termination of pregnancy, and the placental tissues were normal according to pathological diagnosis. The gestational week of specimens during early pregnancy was defined according to morphological observation of the villi and pathological examination, with the record of menstrual cycles as a reference.

Tissues were fixed in 4% paraformaldehyde (PFA) at 4°C for 10 h. Fixed tissues were then gradually dehydrated in ethanol and embedded in paraffin wax. Six μm-thick sections were collected on Super Frost + glass slides (Menzel-Gläser, Braunschweig, Germany). At least three tissue samples at each gestational stage were collected.

Cell culture and treatment

JEG-3 choriocarcinoma cells were purchased from ATCC, and cultured in DMEM media supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine and 1 mmol/L pyruvic sodium. The cells were maintained in humidified incubator at 37°C with 5% CO_2 and 95% air.

For treatment experiment, rabbit polyclonal antibody against human prostasin (Chen et al. 2001b) was added to the culture medium at a dilution of 1:500 when JEG-3 cells grew to 50% confluence. The control cells were treated with non-immune rabbit IgG at the same dilution. Forty-eight hours after treatment, the cells were harvested for further detection as described below.

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate acid buffer (pH 6.0). After immersion in 1% H_2O_2 followed by 10% bovine serum albumin (BSA) in PBS (pH 7.2), the sections were incubated with rabbit anti-human prostasin polyclonal antibody (1:500) (Chen et al. 2001b) at 4°C overnight. Negative controls were performed by replacing the prostasin antibody with preabsorbed rabbit IgG at the same concentration. The sections were further incubated with biotin-conjugated goat anti-rabbit IgG (Vector, 1:300) for 30 min and with horseradish peroxidase streptavidin (Vector, 1:500) for 15 min at 37°C. Final visualization was performed by incubating the sections with DAB (Dako Cytomation, DAB Chromogen) solution with 0.03% H_2O_2 for 15 min. Counterstaining with hematoxylin was performed before the slides were mounted. The assessment of staining intensity was evaluated under an Olympus microscope by three observers who did not know the stage of gestation from which samples had been obtained, and semi-quantitative determined as absent (−), weak (+), moderate (++) and strong (+++).

Transwell insert invasion assay

Transwell insert invasion assay was conducted in 24-well fitted inserts with membranes (8 m pore size, Millipore Corp., Bedford, MA, USA). Briefly, JEG-3 cells were plated in the transwell inserts pre-coated with collagen I (80 μg/ml; Cellmatrix Type I-A, Japan) at 1×10^5 cells in 100 μl culture medium. Lower chambers were loaded with the same medium. After incubating for 24 h, cells on the upper surface of membranes were completely removed, and

the migrated cells were fixed with 2.5% glutaraldehyde and stained with hematoxylin. The number of invaded cells was counted in 10 randomly selected non-overlapping fields of the membranes under light microscope. Meanwhile, in the parallel inserts, total cell numbers were measured by MTT method as reported (Carmichael et al. 1987). Briefly, the cells were incubated with 0.5 mg/ml thiazolyl blue for 4 h followed by addition of 0.1 N HCl-isopropylalcohol. The value of absorbency at 540 nm was recorded with microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA), which corresponds to the equivalence of cell number. Cell invasion index was presented as the number of invaded cells that was adjusted by the total cell number in the parallel insert.

Gelatin zymography

The gelatinolytic activities of MMP-2 and MMP-9 excreted from JEG-3 cells were assayed by gelatin zymography as reported (Xu et al. 2000). Briefly, culture media was mixed with 4× loading buffer (10% SDS, 25% glycerol, 0.05% bromophenol blue, 0.05 mol/L Tris–HCl, pH 6.8) and incubated for 30 min at 37°C, then subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin. The gel was rinsed for 1 h in 2.5% triton X-100, 50 mmol/L Tris–HCl (pH 7.6) to remove SDS, and incubated overnight at 37°C in 50 mmol/L Tris–HCl (pH 7.6) buffer containing 200 mmol/L NaCl, 10 mmol/L $CaCl_2$. The gels were stained with staining buffer (0.1% Coomassie brilliant blue R-250, 30% isopropyl alcohol, 10% acetic acid) for 30 min and destained in 10% methanol, 5% acetic acid. ProMMP-2 was visualized as clear bands at 72kD.

Western blot analysis

The soluble cytoplasmic proteins were extracted by incubating the cells or the homogenized tissues with lysis buffer (20 mM Tris with Mg^{2+} and K^{+}, 1 mM DTT, 0.2% NP-40, 100 M PMSF, 5 μg/ml aprotinin, chymostatin, leupeptin, pristane and trypsin inhibitor; Sigma), and the supernatants were harvested after centrifuging. Protein concentration was measured according to the method of Bradford. Twenty micrograms of protein extract was subjected to 10% SDS-PAGE and subsequent electrotransferring to a nitrocellulose membrane (Amersham Pharmacia biotech, Buckinghamshire, England). The membranes were blocked with 5% defatted milk dissolved in PBS (pH 7.4) with 0.5% Tween-20, and incubated with specific antibodies diluted in 0.1% BSA. The antibodies used included prostasin (1:5000) (Chen et al., 2001a), MMP-26 (1:500, kind gift of Dr. Qing-xiang Amy Sang at Florida State University), TIMP-1 (1:500, Santa Cruz), TIMP-4 (1:500, kind gift of Dr. Qing-xiang Amy Sang at Florida State University), integrin α_1 (1:1000, Chemicon, AB1934), and integrin β_1 (1:500, Santa Cruz, M106). Final visualization was achieved with horseradish peroxidase (HRP)-linked goat anti-mouse IgG (1:2000; PROMEGA, W4021) or goat anti-rabbit IgG (1:2500; PROMEGA, W4011) and subsequent enhanced-chemiluminescence (ECL) reagents (Pierce) according to the manufacture's instruction. The membranes were stripped and re-probed with antibody against β-Actin (1:1000, Neomarker).

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was extracted from the cultured cells with Trizol reagent (Invitrogene) according to the manufacturer's instructions. RNAs were subjected to DNase I digestion to avoid possible genomic DNA contamination, and then reverse transcribed with oligo-dT primers and Superscript II reverse transcriptase (GIBCO). The primer sequences were 5′-AGGTGGCAGCAGTGCAGTC-3′, 5′-ACAGGCAGTTACACGTCTCA-3′ for human prostasin, and 5′-CTCAGACACCATGGGGAAGGTGA-3′, 5′-ATGATCTTGAGGCTGTTGTCATA-3′ for GAPDH. The PCR amplification was performed for 25 cycles at the condition of 30 s at 95°C, 45 s at 60°C (for prostasin) or 55°C (for GAPDH) and 30 s at 72°C.

Statistics

All the experiments were repeated for at least three times with different batches of cells or tissue specimens. Results of gelatin zymography and Western Blotting were scanned and semi-quantitatively analyzed using Gel-Pro Analyzer software (united-bio). Data of Western Blotting were standardized with the value of β-Actin and the relative density was presented as mean ± SD according to three independent experiments. Statistical difference was evaluated by one way ANOVA and value of $p < 0.05$ was accepted as significant.

Results

Expression pattern of prostasin in human placenta at different stages of pregnancy

Distribution and expression of prostasin in human placentae at gestational weeks 6–9, 26, and full-term was detected by immunohistochemistry (Fig. 1) and Western Blotting (Fig. 2). The immunoreactivity of prostasin was observed in various trophoblasts, including villous cytotrophoblast cells, syncytiotrophoblast and column cytotrophoblast

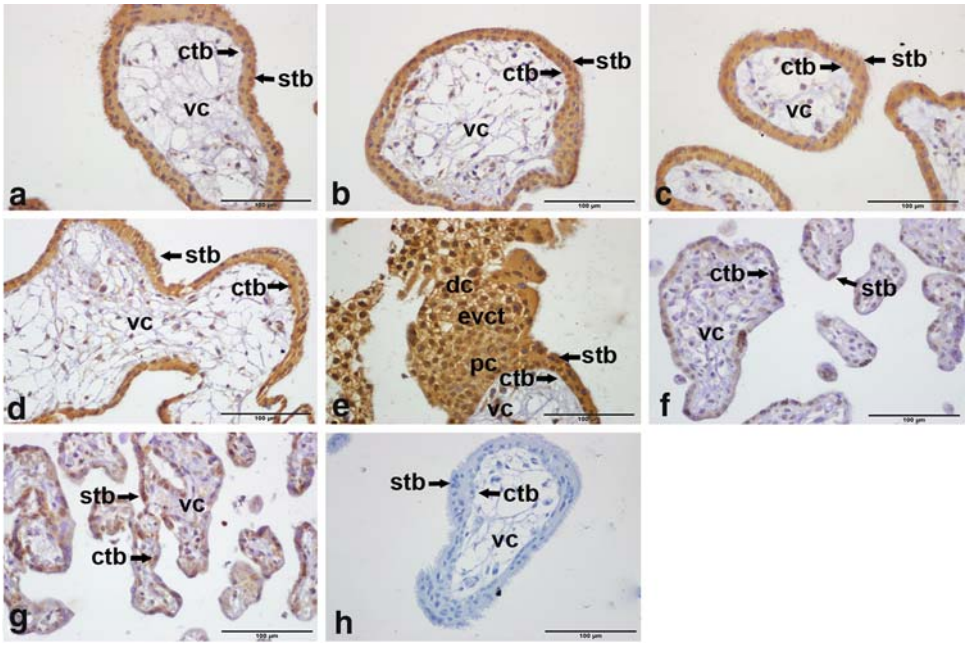

Fig. 1 Immunohistochemistry for prostasin in human placenta at different gestational ages. Prostasin-positive staining was shown as brown. **a–d** Human placenta villi at gestational weeks 6, 7, 8, and 9, respectively; **e** placenta villi at gestational weeks 7 showing the column cytotrophoblast cells; **f** human placenta at gestational weeks 26; **g** human full-term placenta; **h** negative control by replacing prostasin-specific antibody with pre-immune rabbit IgG in the immuno staining procedures. Scale bar equals to 100 μm. *ctb* cytotrophoblast cells, *stb* syncytiotrophoblast, *vc* villous core, *dc* distal column cytotrophoblast, *pc* proximal column cytotrophoblast

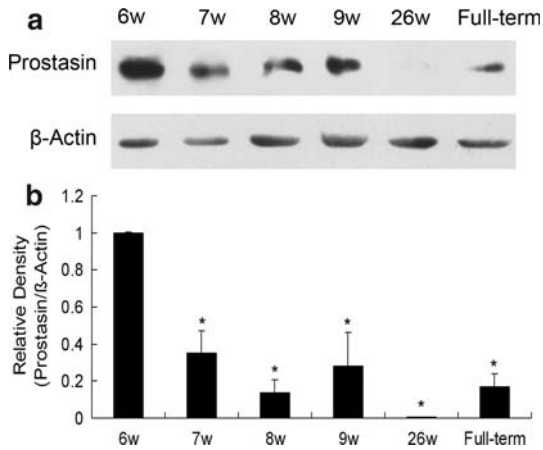

Fig. 2 Western blotting for prostain in human placenta at gestational weeks 6 (6w), weeks 7 (7w), weeks 8 (8w), weeks 9 (9w), week 26 (26w) and term (Full-term). **a** A typical result of Western blotting, **b** bar chart representing the statistical analysis by ANOVA according to three independent experiments. The density of prostasin is adjusted by that of β-Actin, and the values are presented as mean ± SD. *, compared with the relative density at 6w, $p < 0.05$

cells. The staining intensity in these cells was strong at gestational weeks 6–9, being weak at weeks 26, and moderate at full term. Positive staining was also observed in some villous mesenchymal cells, but was relatively weak than that in trophoblasts. Result of Western blotting is in consistence with that of immunohistochemistry, demonstrating that prostasin expression peaked at weeks 6, maintained moderate levels (30–50% of that at weeks 6) at weeks 7–9, and appeared almost undetectable at weeks 26. At full term placenta, prostasin level restored to approximately 10% of that at weeks 6.

Influence of functional prostasin antibody on cell invasiveness in JEG-3 cells

Cultured JEG-3 cells were treated with functional prostasin antibody (JEG/Ab) for 48 h, and treatment with pre-immune rabbit IgG (JEG/preIgG) was included as control. After treatment, we detected prostasin expression in the cells by RT-PCR (Fig. 3c, d) and Western Blotting (Fig. 3a, b). Out of our expectation, prostasin expression in JEG/Ab cells decreased dramatically at mRNA and protein level to 36% and 38%, respectively, of that in JEG/preIgG cells. In JEG/preIgG cells prostasin level was not changed comparing with the untreated cells. The treated cells were seeded into collagen-I coated transwell inserts to conduct cell invasion assay. It was shown that the invasion index of JEG-3/Ab cells was increased by nearly threefolds compared with that of JEG-3/preIgG cells (Fig. 3e, f).

Change of invasion-associated MMPs, TIMPs and integrins production in JEG-3 cells treated with functional prostasin antibody

The changes in the productions of MMP-2, MMP-9, MMP-26, TIMP-1, TIMP-4 as well as integrin α_1, β_1 were analyzed in the JEG-3 cells treated with functional prostasin antibody for 48 h. Gelatin zymography illustrated that production of pro-MMP-2 in JEG/Ab cells increased to 3.7-fold of that in JEG/preIgG (Fig. 4a). Data of Western Blotting revealed that protein levels of MMP-26,TIMP-1 and TIMP-4 in JEG/Ab cells increased evidently, reaching 5.8-, 7.6-, and 3.0-fold of that in JEG/preIgG cells, respectively (Fig. 4b–d). Expressions of integrin α_1 (Fig. 5a) and β_1 (Fig. 5b) did not show obvious changes in JEG/Ab cells.

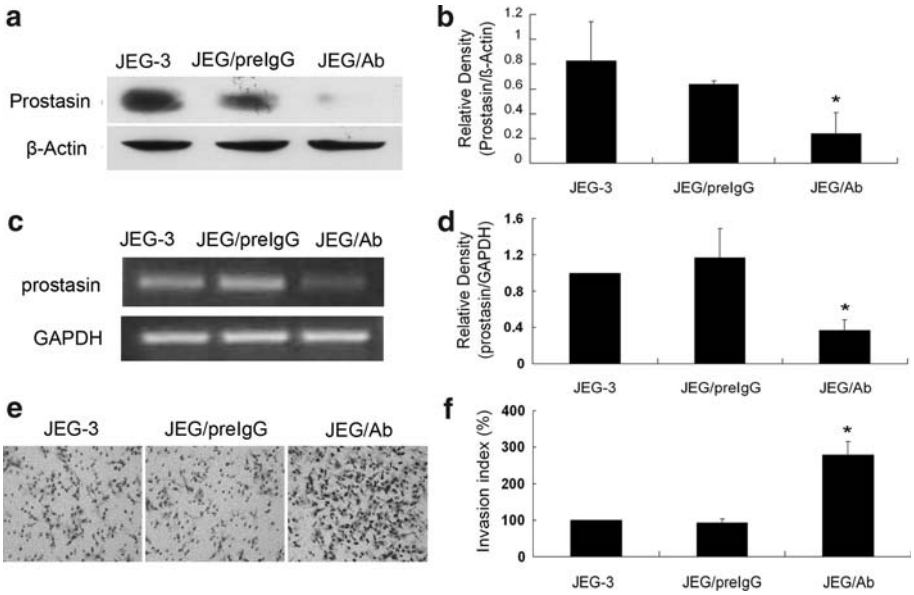

Fig. 3 Effect of prostasin-specific antibody on JEG-3 cell invasion. **a**, **b** Western blotting to show prostasin expression in JEG-3 cells treated with prostasin-specific antibody (JEG/Ab) or pre-immune rabbit IgG (JEG/preIgG). **c**, **d** RT-PCR to show prostasin mRNA expression in JEG/Ab cells. **e**, **f** Transwell insert invasion assay in JEG/Ab and JEG/preIgG cells. Statistical analysis by ANOVA was performed according to three independent experiments, and the *bar charts* were presented in **b**, **d** and **f**. *, compared with the corresponding value of JEG/preIgG, $p < 0.05$

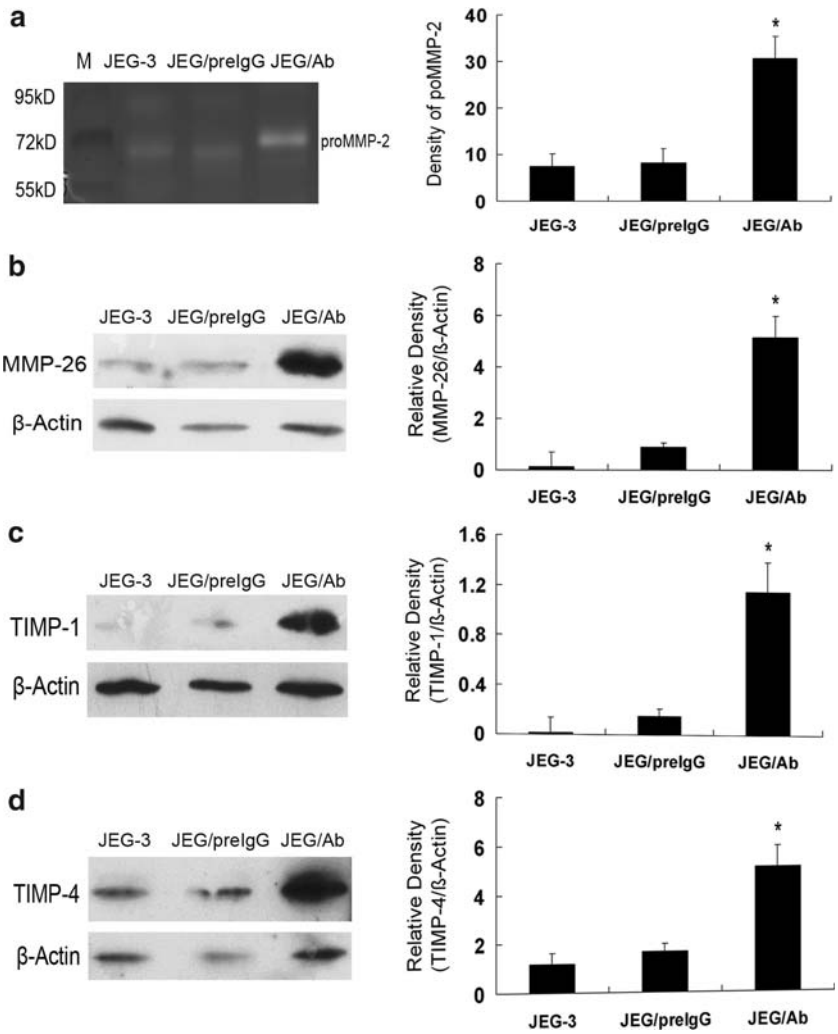

Fig. 4 Influence of prostasin-specific antibody on the production of MMP-2, -9, -26 and TIMP-1, -4 in JEG-3 cells. **a** Gelatin zymorgraphy to measure secretion of MMP-2 and MMP-9 in JEG-3 cells treated with prostasin-specific antibody (JEG/Ab) or pre-immune rabbit IgG (JEG/preIgG). *Left panel* a typical result of gelatin zymorgraphy; *Right panel* densitometric analysis of gelatin zymography. Statistical analysis was performed by ANOVA according to three independent experiments, and the values of proMMP-2 were presented as means ± SD. *, compared with the value in JEG/preIgG, $p < 0.05$. **b–d** Western blotting for MMP-26 (**b**), TIMP-1 (**c**) and TIMP-4 (**d**). *Left panels* typical results of Western blotting; *Right panels* statistical analysis by ANOVA according to three independent experiments. The densities of MMP-26, TIMP-1 and TIMP-4 were adjusted by that of β-Actin, and the values were presented as mean ± SD. *, compared with the corresponding values in JEG-3/preIgG, $p < 0.05$

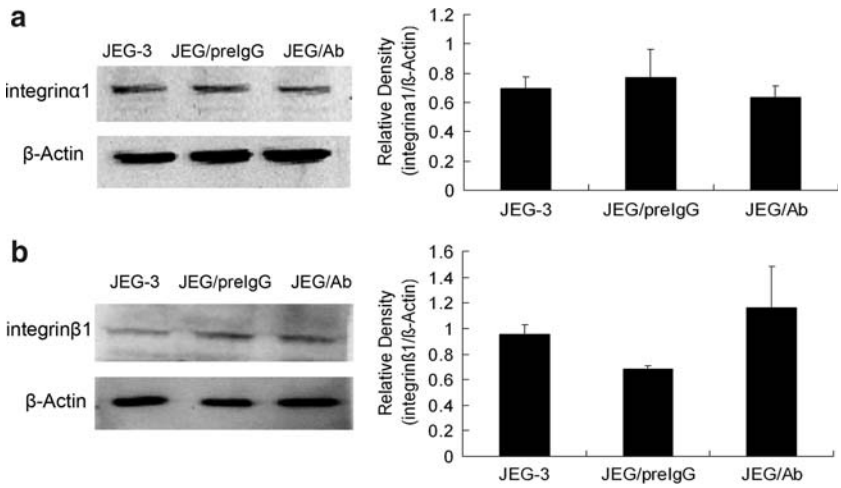

Fig. 5 Influence of prostasin-specific antibody on the production of integrin α_1 (**a**) and β_1 (**b**) in JEG-3 cells. *Left panels* typical results of Western blotting, *Right panels* statistical analysis by ANOVA according to three independent experiments. The densities of integrin α_1 and β_1 were adjusted by that of β-Actin, and the values were presented as mean ± SD. *, compared with the corresponding values in JEG/preIgG, $p < 0.05$

Discussion

The GPI-anchored serine protease, prostasin, was mainly expressed in the prostate with a lesser amount in various tissues, and Lin et al. demonstrated its existence in rhesus monkey placenta. However, little is known regarding its physiological significance in the process of human placentation. In the present study, the expression of prostasin in human normal placenta at various gestational stages was first demonstrated. The evident expression of prostasin in human placenta was observed at the early pregnancy. We showed with immunohistochemistry that prostasin was mainly distributed in various trophoblast cells, including villous trophoblasts and invasive extravillous trophoblast. Trophoblast cell proliferation and differentiation occurs actively in the first trimester of pregnancy, and the differentiation of trophoblasts along the invasive pathway leads to the establishment of placental-uterine circulations that guarantee transportation of nutrients and oxygen to the developing fetus. The temporal and spatial expression pattern of prostasin in placenta seems indicating the active roles of this extracellular serine protease in the behavior modulation of trophoblast cells during the early placentation process. Prostasin was previously shown to be expressed in terminally differentiated epithelial cells, and was reduced in invasive epithelial cancer cells. In vitro investigation in cancer cells revealed prostasin as an invasion suppressor (Chen et al. 2007). We studied the effect of prostasin on human trophoblast cell invasion because these cells share some common properties with tumor cells in the aspect of invasiveness.

We treated choriocarcinomal JEG-3 cells with a functional blocking antibody against prostasin to attenuate prostasin's activation. Our previous work has shown that the antibody could block the complex formation of prostasin and prostasin-binding protein, protease nexin-1 (PN-1) (Chen et al. 2004). Evidences indicated that the PN-1 binding was a reliable method for evaluating prostasin's serine protease activation state in vitro and in vivo(Chen et al. 2001b, 2004; Netzel-Arnett et al. 2006). Here, we observed that the expression of prostasin itself was strongly down regulated by its functional antibody at mRNA and protein levels, accompanied by an evident increase in the invasive capability. The result is in consistence with the observations in prostate and breast cancer cells revealing that re-expression of prostasin exerted anti-invasive effect (Chen et al. 2007). Considering the up-regulation of prostasin in the invasive trophoblast cells, we speculate that this serine protease may participate in the invasion-restrictive regulation in trophoblasts to avoid their over-penetration into the uterine wall.

During normal pregnancy, invasion into uterine wall by trophoblast cells is a strictly controlled process. Coordination of many invasion-associated molecules including various proteases and their inhibitors as well as extracellular matrix proteins and their surface receptors efficiently contributes to the balanced modulation of trophoblast cells invasion. Among them, matrix metalloproteinase (MMP)-2 and MMP-9 are the most well known invasion-promoting substances on the primate feto-maternal interface. The activities of these enzymes can be balanced by their tissue inhibitor, TIMP-1 that preferentially binds and inhibits the latent and active forms of MMP-2 and MMP-9. Recently, we identified the expression of MMP-26 in human placenta, and revealed that this molecule was a strong invasion-enhancer in trophoblast cells (Qiu et al. 2005). TIMP-4 was proposed to be a physiological inhibitor of MMP-26(Zhang et al. 2002). The upregulation of MMP-2 and MMP-26 as well as their tissue inhibitors, TIMP-1 and TIMP-4 in JEG-3 cells treated with prostasin-specific antibody is likely indicating the interaction between prostasin and the up-stream regulators of MMPs and TIMPs. Recent data from Chen et al. revealed that re-expression of prostasin in cultured epithelial cells induced matriptase-dependent

proteolytic cleavages on the extracellular domain of epidermal growth factor receptor (EGFR), leading to the loss of the ligand-binding domains of EGFR and further inactivation of EGF-induced phosphorylation of the extracellular signal-regulated kinases (Erk1/2) (Chen et al. 2008). EGF has been well known to be one of the crucial growth factors regulating trophoblast cells proliferation, differentiation, migration, and invasion. Convincing data demonstrated that EGF could induce MMP-2 and TIMP-1 expression in human trophoblast cells through activation of both the PI3K and MAPK signaling pathways (Knofler et al. 2005; Qiu et al. 2004; Staun-Ram et al. 2004). Therefore, we propose that functional blocking of prostasin in JEG-3 cells might prevent the proteolytic cleavage of EGFR, leading to sufficient activation of EGFR signaling and subsequent up-regulation in MMP-2 and TIMP-1 expressions. Our preliminary data in human trophoblast cells supported the possibility of interaction between prostasin and EGFR signaling (Fu et al. unpublished). On the other hand, though we attribute the EGFR ECD cleavages to matriptase and prostasin in some epithelial cells, but we do not have evidence for a direct interaction between matriptase and EGFR, or between prostasin and EGFR. No defined substrate of prostasin's serine activity has been identified yet. Further investigation is therefore needed to reveal the direct substrate of prostasin to fully understand the molecular mechanisms underlying the modulation of cell invasion by prostasin.

Extracellular matrix (ECM) ligands have been proven to modulate trophoblast cell adhesion, migration, and invasion through the signaling of their corresponding integrin receptors (Damsky et al. 1994; Damsky and Werb 1992; Qin et al. 2003). Integrin expression pattern switches along the invasive pathway of trophoblast differentiation, and integrin $\alpha_1\beta_1$ is mainly produced by the interstitial trophoblasts invading into the uterine wall (Damsky et al. 1992, 1994). In vitro study revealed that interaction of integrin $\alpha_1\beta_1$ with their ECM ligands, laminin and vitronectin, could clearly promote trophoblast cells invasion/migration. However, the data in this work demonstrated that the invasion-suppressing effect of prostasin in trophoblasts did not involve the modulation of integrin $\alpha_1\beta_1$. We still cannot exclude the regulation or proteolytic cleavage of other transmembrane receptors by prostasin, and further investigation is needed to unravel the molecular mechanisms of prostasin effect in trophoblast cells.

In summary, our present study demonstrated the temporal and spatial expression of prostasin in human placenta, and showed its invasion-suppressive function in an in vitro human trophoblast cell model, JEG-3 cells. The data suggested the involvement of this serine protease in the restrict control of trophoblast invasion within the uterine wall.

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