Expression, distribution and function of the focal adhesion kinase ($pp125^{FAK}$) during murine ectoplacental cone outgrowth *in vitro*

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Abstract Mouse embryo implantation is a complex process that includes trophoblast cells derived from ectoplacental cone (EPC) adhesion to and migration through the extracellular matrix (ECM) of uterine endometrium and invasion into the decidua. At the time of implantation, fibronectin (FN) is abundant in the decidua and is distributed pericellularly around each individual stromal cell, and its receptor (integrin $\alpha_5\beta_1$) expression on trophoblast populations is up-regulated. The focal adhesion kinase, a 125 ku protein tyrosine kinase ($pp125^{FAK}$), is tyrosine phosphorylated upon integrin engagement with its ECM ligand, and its tyrosine phosphorylation sites then serve as the binding sites which couple it with cellular proteins that contain Src SH2 or SH3 domains. Through these linkages, $pp125^{FAK}$ may integrate multiple signals triggered by integrins. The model of EPC culture *in vitro* was used to study the expression, distribution and function of $pp125^{FAK}$ during EPC outgrowth on FN. Results indicated that, $pp125^{FAK}$ primarily expressed and distributed in cellular focal adhesions of the front edge of trophoblast outgrowth from EPC, and was localized in the peripheral region of the individual migrating trophblast cell; antibody or antisense oligodeoxynucleotide to $pp125^{FAK}$ inhibited EPC attachment and outgrowth, as well as trophoblast cells spreading and migration. This experiment demonstrated that $pp125^{FAK}$ as an integrin-mediated signaling molecule was involved in EPC outgrowth *in vitro*, and played an important role during trophoblast cells interaction with FN.

Keywords: fibronectin, integrin, focal adhesion kinase ($pp125^{FAK}$), ectoplacental cone, attachment, outgrowth.

MOUSE embryo implantation is a complex process that includes trophoblast cells derived from ectoplacental cone (EPC) adhesion to and migration through the extracellular matrix (ECM) of uterine endometrium and invasion into the decidua. Studies have indicated that fibronectin (FN) is a main ECM molecule in the decidua and its receptor (integrin $\alpha_5\beta_1$) expression on trophoblast cells is up-regulated during trophoblast invasion. FN-integrin interaction has been demonstrated to be very important for embryo implantation. Monoclonal antibodies to α_5 and β_1 integrin subunits or synthetic peptides containing an arginine-glycine-aspartic acid sequence (RGD) which was FN active site recognized by integrin blocked first trimester human trophoblast binding to FN and purified decidual stromal cell monolayers *in vitro* system[1]. Besides acting as adhesion substrates, ECM proteins can also transmit signals from extracellular to nuclear which control a variable of cellular

behaviors, including cell adhesion, motility, proliferation and apoptosis. This signal pathway is mainly mediated by their integrin receptors[2]. ECM induced integrin activation leads many kinase pathways activation which include Ras/MAP kinase, Rho/PI-3 kinase and PKC. Interestingly, after ECM-integrin interaction, a 125 ku nonreceptor protein tyrosine kinase called focal adhesion kinase ($pp125^{FAK}$) is phosphorylated, and its tyrosine phosphorylation sites then serve as binding sites that couple it with cellular proteins that contain Src SH2 and SH3 domains, such as Src kinase[3]. Through these linkages, $pp125^{FAK}$ may integrate multiple signals triggered by integrins, which may control cell adhesion, spreading, migration, survival and proliferation[4].

Although ECM-integrin interaction induced $pp125^{FAK}$ activation has been deeply studied, its role is not clear during trophoblast invasion which is a key process in embryo implantation and placentation. In this work we first used an *in vitro* model of murine EPC culture to study the expression, distribution and function of $pp125^{FAK}$ during EPC outgrowth on FN.

1 Materials and methods

Fibronectin (FN) was supplied by Department of Cell Biology, Beijing Medical University. Rabbit Anti-$pp125^{FAK}$ IgG was purchased from Santa Cruz Biotechnology, Inc., and fluorescein isothiocyanate (FITC) labelled goat anti-rabbit IgG antibody was from Sigma. (Ham) F-12 nutrient mixture (Ham's F-12) and fetal calf serum (FCS) were the products of Gibco BRL.

The young adult female mice (25—30 g) of Kunming white strain were obtained from the Feed Lot of Zoo, Institute of Zoology, Chinese Academy of Sciences. They were superovulated by the routine method of PMSG-hCG injection, then impregnated with the same strain male (1:1) overnight. The appearance of a vaginal plug on the following morning was designated Day 1 of pregnancy.

EPCs were isolated as previously described[5]. Briefly, Day 8.5 embryos were removed from the decidual capsules. EPCs were dissected at the junction of the extraembryonic ectoderm and transferred randomly to culture dishes by means of siliconized pipettes. EPCs were cultured in Ham's F-12 supplemented with 1.6 mg/mL $NaHCO_3$, 0.3 mg/mL L-glutamine, 0.24 mg/mL Ca-lactate, and 400 U/mL gentamycin. 24-well dishes were precoated with FN (0.8 mg/mL) (10 μL/well) at room temperature for 3 h in the air clean bench.

EPCs were cultured for 48 h in Ham's F-12 medium with 3%—4% FCS[6]. EPC outgrowths were fixed in 4% paraformaldehyde, 60 mmol/L sucrose in PBS for 10 min, permeabilized for 1 min at 4℃ in 0.05 mol/L Tris buffer (sodium (TBS, pH 7.4) containing 0.5% Triton X-100, and washed twice with PBS. Cells were incubated with rabbit anti-$pp125^{FAK}$ IgG (1:100) in a humid box for 60 min at 37℃, washed, and then incubated similarly by goat anti-rabbit IgG antibody (1:200) conjugated with FITC for 30 min. The wells were mounted in PBS: glycerol (1:1), and viewed on an OLYMPUS BH2-RFCA fluoresence microscope. Micrographs were taken on a LUCKYPAN 400 black and white film. PBS containing 1 mg/mL BSA was incubated as a control for nonspecific staining.

EPC culture media added with anti-$pp125^{FAK}$ IgG (1 μg/mL) were used as experimental

groups, while the control groups were added by rabbit IgG of the same concentration.

On the basis of mouse pp125^{FAK} cDNA sequence[7], we synthesized the sense oligodeoxynucleotide (ODN) that includes ATG initiation codon and its corresponding antisense ODN:

(i) pp125^{FAK} sense ODN 5′-AAA AGA ATG GCA GCT GCT TAT CTT-3′(104—127);

(ii) pp125^{FAK} antisense ODN 5′-AAG ATA AGC AGC TGC CAT TCT TTT-3′.

These ODNs were synthesized by BECKMAN OLIGO 1000M DNA Synthesizer and purified by desalting (The Lab of American BECKMAN Biotechnology Demonstration, The Center of Gene Engineering, Institute of Microbiology, Chinese Academy of Sciences).

EPC culture media added with 50 μg/mL pp125^{FAK} antisense ODN were used as the experimental groups, while the control groups were added by sense ODN at the same concentration. In some experiments, 50 μg/mL pp125^{FAK} antisense ODN was mixed with its sense ODN in an equal amount and incubated at 42℃ for 8 h and then was added into the EPC culture media.

EPC attachment and outgrowth were observed and recorded under OLYMPUS inverted phase-contrast microscope at 24—72 h, and the areas of EPC outgrowth were measured by eyepiece grid. The differences between experimental and control groups were examined by t-test.

2 Results

2.1 Expression and distribution of pp125^{FAK} during EPC outgrowth *in vitro*

Anti-pp125^{FAK} IgG is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to ammino acids 1 033—1 052 mapping at the carboxy teminus of pp125^{FAK} of hu-

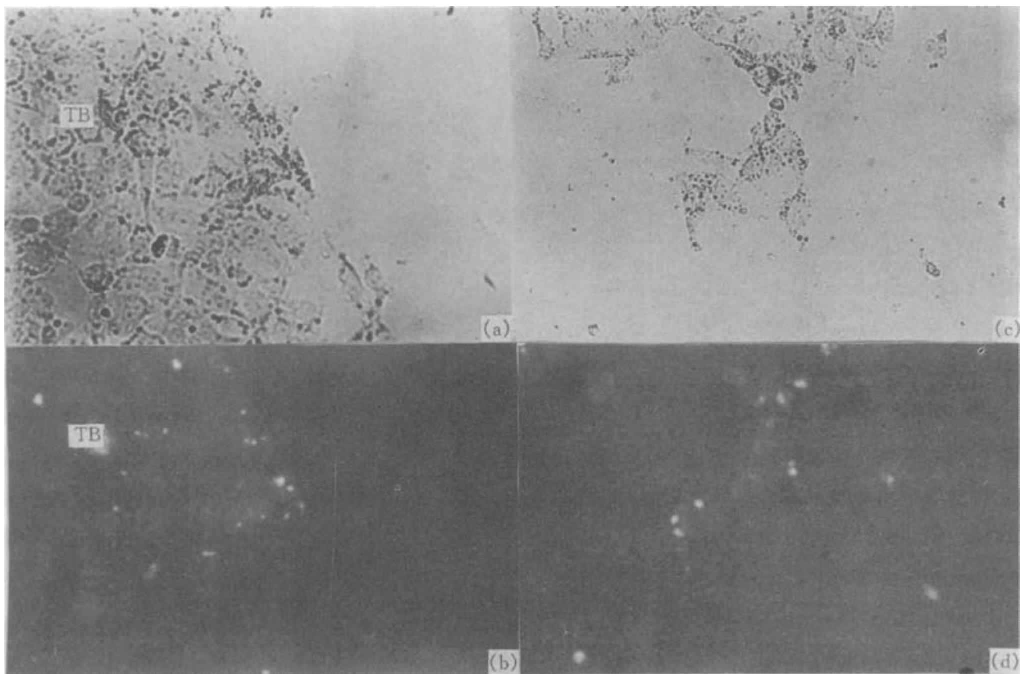

Fig. 1. Expression and distribution of pp125^{FAK} during EPC outgrowth *in vitro*. TB, Trophoblast. ×200

man origin, reacts with pp125^{FAK} of mouse, rat, human and chicken origin by Western blotting, and is non cross-reactive with other protein tyrosine kinases (Santa Cruz Biotechnology, Inc.). As shown in fig. 1, pp125^{FAK} was primarily expressed and distributed in cellular focal adhesions of the front edge of trophoblast outgrowth from EPC (fig. 1(a), (b)), and was localized in the peripheral region of the individual migrating trophoblast cell (fig. 1(c), (d)).

2.2 Effects of anti-pp125^{FAK} IgG on EPC attachment and outgrowth *in vitro*

Antibody (100 μg/mL) to pp125^{FAK} binds to its C-terminal region (AA 1 033—1 052) that contains a part sequence of focal adhesion targeting (FAT) domain (AA 919—1 042), which is required for efficient recruitment of pp125^{FAK} to the focal adhesions. Its work concentration is about 1:100 (Santa Cruz Biotechnology, Inc.). Percentages of EPC attachment and outgrowth were significantly inhibited by the addition of 1 μg/mL pp125^{FAK} IgG (fig. 2). In control groups, trophoblast cells derived from EPC migrated and spreaded normally (fig. 3(a)); while in the experimental groups, migration and spreading of trophoblast cells

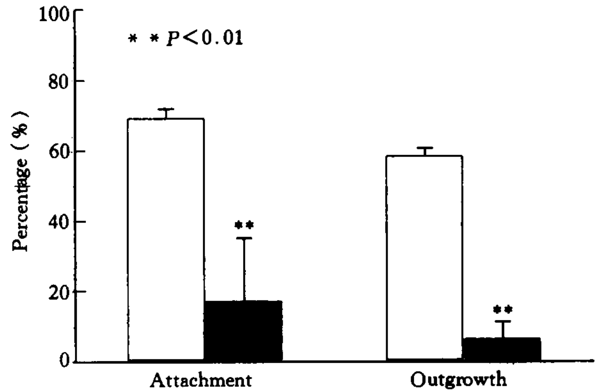

Fig. 2. Effect of anti-pp125^{FAK} IgG on EPC attachment and outgrowth on FN. □, Control; ■, anti-pp125^{FAK} IgG.

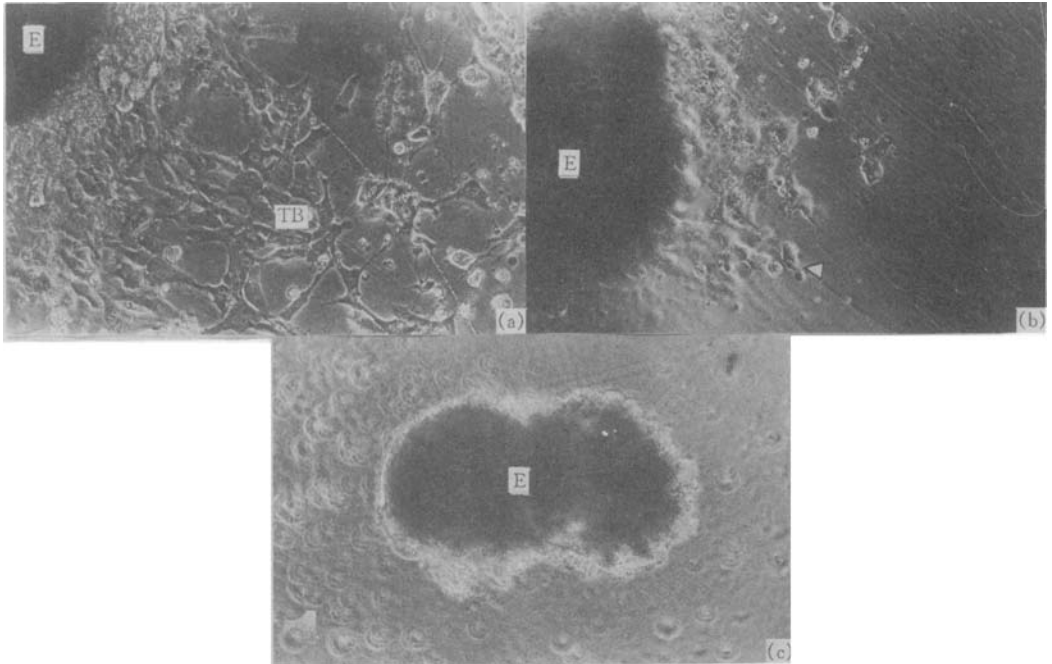

Fig. 3. Growth of EPCs in the presence or absence of anti-pp125^{FAK} IgG *in vitro*. E, ectoplacental cone (EPC); TB, Trophoblast. ×200

were inhibited and cells did not normally spread but became round (shown in fig. 3(b) by arrowhead), even the EPCs floated on the culture medium (fig. 3(c)). The experiment was repeated 3 times.

2.3 Effect of pp125^{FAK} antisense ODN on EPC attachment, outgrowth and area of outgrowth *in vitro*

To further confirm the importance of pp125^{FAK} during EPC attachment and outgrowth *in vitro*, the effect of pp125^{FAK} antisense ODN on EPC attachment, outgrowth and area of outgrowth were examined at 48 h. 50 μg/mL antisense ODN to pp125^{FAK} significantly inhibited the percentage of EPC attachment and outgrowth, as well as the area of outgrowth. However, the inhibitory effect of antisense ODN was neutralized by its corresponding sense ODN when they were preincubated at 42℃ for 8 h (fig. 4). The concentration of antisense or sense ODN in the experiments referred to Sakae Tanaka's work[8]. Each experiment repeated at least 3 times.

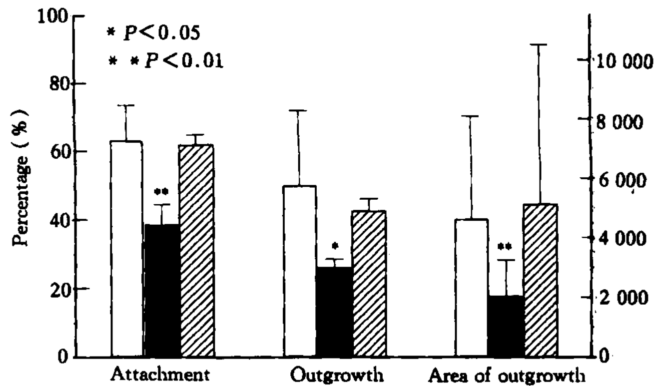

Fig. 4. Effect of pp125^{FAK} antisense ODN on EPC attachment, outgrowth and area of outgrowth on FN (48 h). □, Sense; ■, antisense; ▨, sense + antisense.

3 Discussion

As the main receptors for ECM, integrins both provide a physical link to the cytoskeleton and transfer extracellular signals. Formation of focal contacts, rearrangement of cytoskeleton, tyrosine and serine phosphorylation of a number of proteins, elevation of intracellular Ca^{2+} levels and pH value and changes in phospholipid metabolism are induced by the binding of integrins to ECM. By this way, ECM proteins may affect and regulate cell adhesion, spreading, motility, signaling and gene expression[9].

The recently discovered focal adhesion kinase (pp125^{FAK}) localizes to focal contacts, which are the sites of integrin clustering, and can physically associate with integrins *in vitro*. As integrins lack intrinsic catalytic activity, pp125^{FAK} is a candidate for a signaling molecule that is recruited by integrins in order to trigger the generation of intracellular second messengers. pp125^{FAK}, a 125 ku protein tyrosine kinase (PTK), is recruited to focal contacts by the focal adhesion targeting (FAT) domain within its C terminus, and binds to the cytoplasmic domain of the integrin β_1, β_2 or β_3 subunit *in vitro* by the sequences within its N-terminal non-catalytic domain[4]. pp125^{FAK} is a unique PTK: Its catalytic domain is located centrally between the non-catalytic amino and carboxyl termini; it is capable of autophosphorylation at one major site tyrosine-397(Tyr397), and does not contain SH2 or SH3 domains unlike many PTKs[9]. The au-

tophosphorylation of pp125^{FAK} at Tyr397 coincides with activation of the pp125^{FAK} that is induced by the binding of integrins to ECM. Binding of activated pp125^{FAK} to the SH2 domain of Src may activate Src family kinase (Src/Fyn)[3]. Through adaptor proteins Grb2 and Crk that contain SH2 domain, activated pp125^{FAK} links to the Ras/MAP kinase pathway[3]. MAP kinase regulates gene expression by phosphorylating and activating transcription factors, or phosphorylates and activates cytoplasmic phopholipase A_2 ($cPLA_2$) that hydrolyzes glycerophospholipids to yield arachidonic acid, which induces leukotriene production and further promotes actin polymerization, ultimately, affects cell adhesion and spreading by regulating cytoskeletal changes. PI-3 kinase may associate with tyrosine-phosphorylated pp125^{FAK} by the SH2 domain of the p85 subunit and possibly involve in cytoskelecton rearrangements[2]. Rho-stimulated actin filament constraction resulting in integrin clustering associated with focal adhesion/stress fiber formation may be a common cellular response leading to pp125^{FAK} tyrosine phosphorylation[4].

Since the process of trophoblast outgrowth on ECM involves three steps: attachment, local proteolysis and migration[10], only the cells at the front edge of trophoblast outgrowth and the peripheral region have more chances to contact with FN active sites to form focal adhesion contacts. On the basis of analysis above, when cells interaction with ECM by their surface integrin receptors, pp125^{FAK} is recruited to focal contacts by FAT domain within its C terminus, and binds to the cytoplasmic domain of the integrin β subunit by the sequences within its N-terminal noncatalytic domain. Therfore it is not difficult to explain why pp125^{FAK} was primarily expressed and distributed in cellular focal adhesions of the front edge of trophoblast outgrowth from EPC, and was localized in the peripheral region of the individual migrating trophoblast cell in our observation. In addition, a research reported that the major protein phosphorylated in malignant trophoblast (JEG-3) cells plated onto FN was the 125 ku protein. In contrast, the predominant tyrosine-phosphorylated protein detected in normal trophoblast after adhesion to FN was a 115 ku protein[1]. The 115 ku protein appears to be an isoform of pp125^{FAK} although its nature is not clear yet.

The consequences of pp125^{FAK} activation on cell behavior are likely to include such well-documented integrin/ECM-mediated effects as spreading and migration, anchorage-dependent survival and proliferation, and changes in gene expression. Compelling studies have emphasized a role for pp125^{FAK} in control cell spreading and migration. Mesodermal cells isolated from pp125^{FAK}(− / −) "knockout" embryos appear more rounded and migrate at a decreased rate, as compared to pp125^{FAK}(+ / +) cells. Since pp125^{FAK}(− / −) cells exhibit an unusually large number of focal adhesions distributed abnormally throughout the ventral surface, the spreading and migration defect was interpreted as a lack of normal focal adhesion turnover. The migration defect may account for the embryonic lethality of the pp125^{FAK} knockout, as null embryos exhibit abnormalities indicative of aberrant cell movement during gastrulation[11]. Antisense attenuation of pp125^{FAK} expression in tumor cells results in apoptosis associated with loss of adherence. While overexpression of pp125^{FAK} in CHO cells was found to increase migration on FN. Moreover, pp125^{FAK} expression appears to be elevated in migrating keratinocytes in repairing burn wounds, in invasive and

metastatic colon and breast tumors, and in rapidly migrating melanoma cell lines. In addition, expression of an activated form of pp125^{FAK} in MDCK epithelial cells confers resistance to "anoikis" (apoptosis resulting from loss of ECM contacts) while permitting growth in soft agar[4]. The antibody to pp125^{FAK} we used binds to the C-terminal region AA 1 033—1 052 of it, which contains a part sequence of the FAT domain that is required for efficient recruitment of pp125^{FAK} to the focal adhesions. Thus, the possible mechanism of anti-pp125^{FAK} IgG inhibited EPC attachment and outgrowth is that it interfered with the efficient recruitment of pp125^{FAK} into nascent focal adhesions, while the inhibition of pp125^{FAK} antisense ODN on EPC attachment, outgrowth and trophblast cells migration (area of outgrowth) may be due to the attenuation of antisense ODN on pp125^{FAK} expression.

Our present results clearly showed that pp125^{FAK} was primarily expressed and distributed in cellular focal adhesions of the front edge of trophoblast outgrowth from EPC, and was localized in the peripheral region of the individual migrating trophblast cell; antibody or antisense oligodeoxynucleotide to pp125^{FAK} inhibited EPC attachment and outgrowth, as well as trophoblast cells migration and spreading. This experiment demonstrated that pp125^{FAK} as an integrin-mediated signaling molecule is involved in EPC outgrowth *in vitro*, and played an important rôle during trophoblast cells interaction with FN. Trophoblast invasion into the decidua is a dynamic process that requires a number of molecular and cellular interactions. Transmission of correct signal to invading trophoblast is likely to be important for implantation and placentation. Future work should be focused on the downstream effects of ECM ligands on trophoblast gene expression and regulation. A detailed analysis of the signals transduced from the matrix and decidual microenvironment will provide a better understanding of the factors that determine the behavior of trophoblast *in vivo*, and may lead to the clinical methods to treat disorders of pregnancy associated with inadequate or over invasion by trophoblast.

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