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Induction of matrix metalloproteinase-9 and -2 activity in mouse blastocyst by fibronectin-integrin interaction

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Abstract   Fibronectin, a major extracellular matrix, plays an important role in embryo implantation by mediating embryo adhesion and outgrowth. In this work, mouse blastocysts produced pro-matrix metalloproteinase-9, pro-matrix metalloproteinase-2 and 64 ku matrix metalloproteinase-2 when they were co-cultured with fibronectin. In contrast, mouse blastocysts did not produce these proteinases without fibronectin. Focal adhesion kinase is a fundamental molecule of integrin signaling pathway and its antisense oligodeoxynucleiotide inhibited blastocyst matrix metalloproteinases expression induced by fibronectin. The results indicated that fibronectin triggered matrix metalloproteinase-9 and -2 expression in mouse blastocyst through its integrin receptors and subsequent signaling pathway, which enhanced the synchronization of blastocyst invasiveness and uterine receptivity and ensured the accuracy of events relative to implantation in timing and spatiality.

Keywords: **fibronectin, focal adhesion kinase (pp125[FAK]), blastocyst, matrix metalloproteinase-9, matrix metalloproteinase-2.**

Fibronectin is a heterodimer composed of two peptides through a pair of disulfide bonds on their C-terminal and a major extracelluler matrix (ECM)[1]. By interacting with integrin, fibronectin regulates cell behavior, such as adhesion, migration, proliferation and differentiation[2], which plays a vital role in morphorgenesis, wound healing, tissue repairing and remodeling as well as malignant metastasis[3].

Embryo implantation is a complicate process consisting of blastocyst adhesion, local ECM hydrolysis and trophoblast invasion[4]. During early implantation, fibronectin is highly expressed in uterus, especially at maternal-fetal interface. Meanwhile, integrin $\alpha5\beta1$, $\alpha4\beta1$ and $\alpha3\beta1$, which are the receptors of fibronectin, present on blastocyst[5]. *In vitro* experiments indicate that fibronectin mediates embryo adhesion through integrins $\alpha5\beta1$, $\alpha4\beta1$, $\alpha3\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha4\beta7$, $\alpha IIb\beta3$, $\alpha v\beta6$ and $\alpha v\beta8$[6,7].

Recently, Berthold[8] demonstrated that immerse ECM distributed around invasive trophoblast cells and at least three different matrix metalloproteinases (MMPs), namely MMP-2, MMP-3 and MMP-9, existed in the areas composed of fibronectin-positive fibrils embedded in heparin sulfate and vitronectin, which implies that fibronectin not only mediates embryo adhesion, but also has a tight relationship with MMPs. MMPs catalyze ECM hydrolysis and contribute the major invasiveness of embryo, so it is necessary for elucidating embryo implantation mechanism to identify the relationship of fibronectin and MMPs. In this work, MMPs zymography and focal adhesion kinase (pp125[FAK]) antisense oligodeoxynucleotide (ODN) were used to investigate the influence of fibronectin on blastocyst MMPs expression and its mechanism.

1   Materials and methods

Adult female mice of Kunming white strain were supplied by Institute of Zoology, the Chinese Academy of Sciences and raised at 25°C in a constant photoperiod (light : dark cycle, 14: 10 h). They were allowed free access to water and food.

After routine superovulation with PMSG and hCG, female mice were caged with the same strain male overnight. In the following morning, those having vaginal plugs were designated as the first day of pregnancy. On the fourth day of pregnancy, the mice were killed, their uteri were flushed with Hank's solution. Blastocysts were sorted out and cultured in Ham's F-12 (Gibco BRL Co.) complemented with 1.6 mg/mL NaHCO$_3$, 0.3 mg/mL L-glutamine, 0.24 mg/mL calcium lactate, 400 U/mL gentamycin and 0.5% bovine serum albumin (Gibco BRL Co.).

Culture dishes were precoated with fibronectin (Sigma Co., 1 mg/mL, 10 μL/droplet) in the experimental groups and not precoated in control groups. Blastocysts were cultured in droplets of

Ham's F-12 under mineral oil (37℃, 5% CO_2). The ratio of blastocyst to media was 1 blastocyst to 1 μL media in droplets. According to designation, culture media samples were collected and blastocyst adhesion and outgrowth were investigated at 6, 12, 24 and 36 h respectively. While culture dishes were rotated gently, blastocysts that kept attached to dish bottom were recorded as adhesion and blastocysts with macrotrophoblast cells were recorded as outgrowth. The numbers of hatched blastocysts were recorded in order to calculate adhesion rates and outgrowth rates. All the media samples were stored at -20℃.

Based on mouse pp125^{FAK} cDNA sequence[9], the antisense ODN of mouse pp125^{FAK} was synthesized as follows (Institute of Microbiology, the Chinese Academy of Sciences): 5′-AAG ATA AGC AGC TGC CAT TCT TTT-3′. Culture media added with 50 μg/mL pp125^{FAK} antisense ODN were used in experimental groups while culture media of control groups were not added with pp125^{FAK} antisense ODN.

MMPs zymography was carried out as Martelli[10] described. Briefly, gelatin (Sigma) at a final concentration of 0.5 mg/mL was mixed into the running gel containing 10% acrylamide, 25% 1.5 mol/L Tris(hydroxymethyl)-aminomethane pH8.8, 0.4% SDS and 0.3% ammonium peroxodisulfate (APS). A stacking gel was layered, which contains 2% acrylamide, 0.05% N,N′-methylene-bis- acrylamide, 25% Tris buffer, 0.4% APS and 0.1% N,N,N,N-tetramethylethylendiamine (TEMED). Samples were standardized by protein concentration and 50 μg protein per sample was infused on gel. Electrophoresis ran at 100 V for 0.5 h and at 150 V for 2.5 h in 25 mmol/L Tris-HCl containing 0.19 mol/L glycine and 0.1% SDS pH 8.6. After electrophoresis, the gel was washed six times for 60 min in 2.5% Triton X-100 at 36°C on a moving platform and then incubated in PBS pH7.4 containing 0.9 mmol/L $CaCl_2$ overnight. After incubation, gels were stained with Coomassie brilliant blue G 250 for 1 h and destained in 5% methanol with 7.5% acetic acid. The MMPs activity was quantified with personal densitometer.

The data were presented as means ± SD and analyzed with analysis of variance.

2　Results

(i) Influence of fibronectin on blastocyst adhesion and outgrowth.　By 12 h of culture, blastocysts on fibronectin layers started to adhere and the adhesion rates increased gradually along with culture time. Blastocysts cultured without fibronectin adhered occasionally and their adhesion rates did not trend to increase obviously. The adhesion rates of groups cultured on fibronectin layers were significantly higher than those of the groups without fibronectin all the times ($P < 0.01$, $n = 3$) (fig. 1).

Outgrowth occurred in the groups on fibronectin by 12 h of culture and the numbers increased as culture proceeded, but outgrowth was observed till 36 h of culture in groups without fibronectin. Although the outgrowth rates of the groups cultured on fibronectin layers were obviously higher than those of groups without fibronectin, the difference was statistically significant only after 36 h ($P < 0.01$, $n = 3$) (fig. 2).
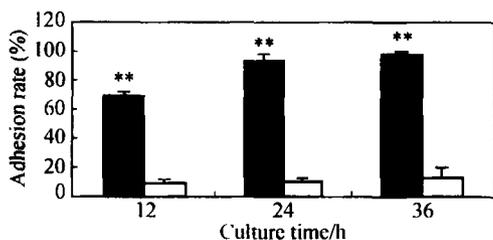
Fig. 1.　The influence of FN on blastocyst adhesion. **, Compared with control groups $P < 0.01$; □, treatment; ■, control.
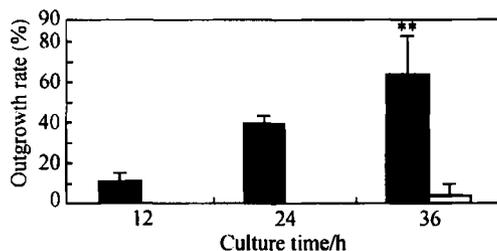
Fig. 2.　The influence of FN on blastocyst outgrowth. **, Compared with control groups $P < 0.01$; □, treatment; ■, control.

(ii) Induction of MMPs expression by fibronectin.　MMPs activity was detected only in the groups cultured on fibronectin layers and it was detectable by 12 h of culture. At 12 h, zymography

showed two proteolytic bands corresponding to 66 ku and 64 ku molecular weight; at 24 h, a new band appeared and from then on, no other bands emerged. According to their molecular weight, the three bands were pro-MMP-2, 64 ku MMP-2 and pro-MMP-9 (figs. 3 and 4). Incubation with the inhibitors of MMPs verified this determination. Zymography did not demonstrate any MMPs activity in control groups, and MMPs relative activity is shown in fig. 5.

(iii) Inhibition of pp125^{FAK} antisense ODN on MMPs expression. In this experiment, media samples from the groups cultured with or without pp125^{FAK} antisense ODN were examined to verify the influence of the antisense ODN on blastocyst MMPs expression. The antisense ODN of pp125^{FAK}

Fig. 3. Inhibition of MMPs activity by inhibitors. Lanes 1, 2 and 3 are groups hatched with 1, 10-phenathroline, PMSF or EDTA, respectively. Lanes 4 and 5 are normal group (control, and standard of protein molecule weight.

significantly attenuated the MMPs activity in blastocyst culture media all the times examined, but inhibition did not show specificity to any MMP ($P < 0.05$, $n = 3$). In antisense ODN treated groups, pattern and sequence did not change except that proteolytic bands were narrowed because of the decrease in MMPs activity (figs. 4 and 5).
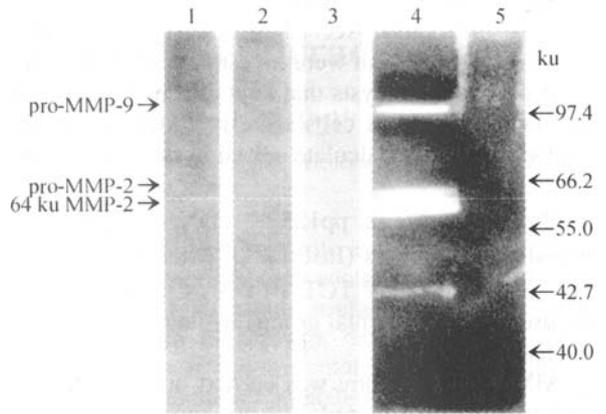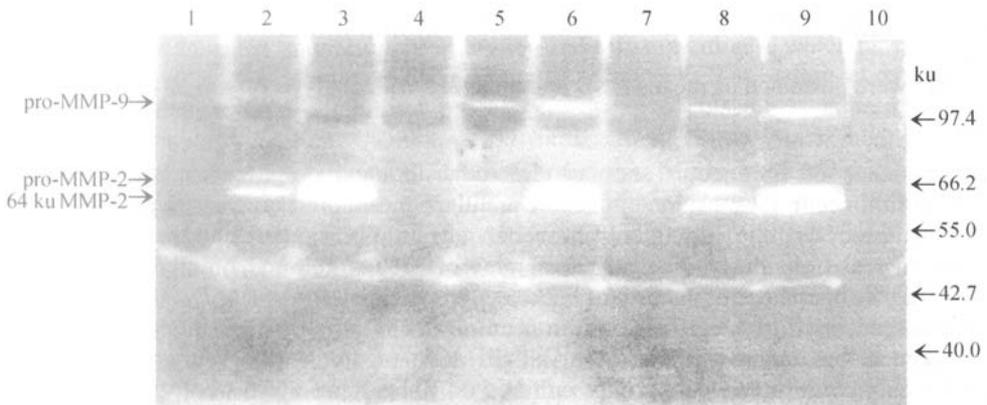

Fig. 4. The influence of fibronection and pp125^{FAK} antisense ODN on blastocyst MMPs. Lanes 1, 2 and 3, samples of 12 h of culture; lanes 4, 5 and 6, samples of 24 h; lanes 7, 8 and 9, samples of 36 h; lane 10, protein molecular weight markers. Lanes 1, 4 and 7, control groups; lanes 2, 5 and 8, pp125^{FAK}-treated groups; lanes 3, 6 and 9, fibronectin-treated groups.

3 Discussion

Analyzing the influence of fibronectin on blastocyst MMPs expression, we found that fibronectin triggered the MMP-9 and -2 expression in mouse blastocyst while blastocyst cultured without fibronectin did not produce any active MMP-9 and -2. The result first indicated that fibronectin triggered MMP-9 and -2 expression in mouse blastocyst through integrin receptors. At the same time, blastocyst adhesion and outgrowth rates of the groups on fibronectin layers were significantly different from those of
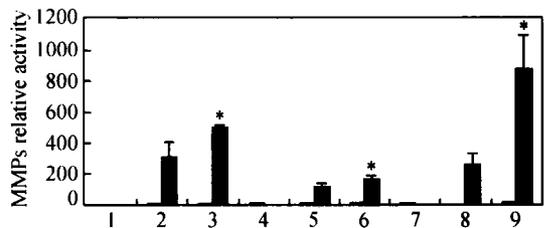

Fig. 5. The influence of FN and pp125^{FAK} antisense ODN on blastocyst MMPs. 1, 2, 3, pro-MMP-9 activitiy; 4, 5, 6, pro-MMP-2 activity; 7, 8, 9, 64 ku MMP-2 activity; 1, 4, 7, control groups; 2, 5, 8, pp125^{FAK} antisense ODN-treated groups; *, compared in groups $P < 0.05$.

the groups without fibronectin. It indicated that fibronectin, as an important ECM, regulates mouse blastocyst adhesion and outgrowth and supports the concept that ECM regulates blastocyst behavior, such as adhesion, migration, proliferation and differentiation. And it also indicated that fibronectin takes a vital role in implantation, which has been evidenced with human trophoblast[11].

Integrins $\alpha5\beta1$, $\alpha4\beta1$, $\alpha3\beta1$, $\alpha v\beta1$, $\alpha v\beta3$, $\alpha4\beta7$, $\alpha IIb\beta3$, $\alpha v\beta6$ and $\alpha v\beta8$ mediate blastocyst attachment to fibronectin[6]. After integrin is bound to fibronectin, the cytoplasmic domain of β subunits couple with α-actin, taxin, paxilin, vinculin and tensin to form focal adhesion. pp125^{FAK} is a 125 ku protein tyrosine kinase and localizes to focal adhesion through focal adhesion targeting domain, which results in autophosphorylation of Tyr397 and activation of pp125^{FAK}[12]. Src family kinase catalyzes phosphorylation of Tyr407, Tyr576, Tyr577 and Tyr925, and fully activates pp125^{FAK} by binding to phosphorylated Tyr397[13]. Phosphorylation of pp125^{FAK} at Tyr925 creates an SH$_2$-binding site for Grb2 which may link pp125^{FAK} to the activation of Ras/MAP kinase pathway[14]. It has been speculated that this signal transduction pathway regulates "early gene expression" after cell attachment. In this work, pp125^{FAK} antisense ODN significantly inhibited blastocyst MMP-9 and -2 expression, which indicated that this signal transduction pathway may also be responsible for induction of blastocyst MMP-9 and -2 expression by fibronectin.

Embryo implantation is a well-organized process and relative events consisting of the process are strictly regulated spatially and temporally. According to previous studies, gonadal steroids and growth factors are regarded as the main modulators of implantation[15], but in this study we found that fibronectin-integrin interaction triggered expression of blastocyst MMP-9 and -2 , which implied that besides gonadal steroids and growth factors, ECM and integrin also formed a local delicate regulatory mechanism. Implantation consists of blastocyst adhesion, local ECM hydrolysis and trophoblast invasion[4]. Integrin on blastocyst recognizes and adheres to ECM on uterial epithelium, blastocysts are anchored on epithelium and then adhesion is performed, whilst MMP-9 and -2 expression in blastocyst is also initiated. It ensures accuracy of subsequent local ECM hydrolysis and invasion in regard of timing and spatiality, and avoids wasting the relative proteinases from blastocyst.

Implantation depends on embryo invasiveness, uterus acceptivity and their synchronization. Former reports indicated that under the maternal gonadal steroid direction, thickness of the glycocalyx of uterine epithelial cells reduced. Luminal epithelium obtained polarity in distribution pattern and ECM were remodeling[16—18]. As a result, uterus gained receptivity. At the same time, zygote accepted regulation of maternal steroids, expressed specific adhesive molecules corresponding to ECM and acquired adhesive ability[7], which made blastocyst invasiveness and uterine acceptivity synchronous to some extent. After embryo entered uterus, they "crosstalked" through local modulators, like growth factors and cytokines, and enhanced the synchronization[2]. However, our results in this study showed that they did not reach full synchronization until they contacted with each other. In other words, blastocyst acquired its full invasive ability through directly attaching to receptive uterine epithelium, which may be the final step of the synchronization process.

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