Expression of p16\(^{\text{INK4a}}\) in mouse endometrium and its effect during blastocyst implantation

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Abstract: The expression of tumor suppressor gene p16\(^{\text{INK4a}}\) in mouse endometrium during early pregnancy and its possible role in blastocyst implantation were investigated in the present study. Real-time fluorescent quantitative PCR (FQ-PCR) and immunohistochemistry were applied to detect p16\(^{\text{INK4a}}\) mRNA and protein expressions in endometrium of un-pregnant and pregnant mice on day 2, 3, 4, 5, 7, respectively. In addition, p16\(^{\text{INK4a}}\) antibody was injected into the horns of uteri in pregnant mice on day 3 and its effect during blastocyst implantation was detected \textit{in vivo}. The higher expressions of p16\(^{\text{INK4a}}\) mRNA and protein were observed in pregnant mice compared with that in un-pregnant mice, with a steady increase from day 2 to day 5 and reaching the maximal level on day 5 of pregnancy and then decreasing. p16\(^{\text{INK4a}}\) antibody decreased the number of implanted blastocysts compared with that of saline-injected group. The results suggest that p16\(^{\text{INK4a}}\) may be associated with apoptosis of luminal epithelial cells and decidual cells, coordinating decidualization of endometrium and invasion of trophoblastic cells. Thus, we presume that p16\(^{\text{INK4a}}\) participates in the process of blastocyst implantation in mice.

Key words: blastocyst implantation; endometrium; p16\(^{\text{INK4a}}\); real-time fluorescent quantitative PCR; immunohistochemistry; \textit{in vivo}

p16\(^{\text{INK4a}}\) in早孕小鼠子宫内膜的表达及其对胚泡着床的影响

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摘要：本研究旨在检测肿瘤抑制基因p16\(^{\text{INK4a}}\)（抑制细胞周期依赖性激酶4a）在早孕小鼠子宫内膜中的表达规律，探讨p16\(^{\text{INK4a}}\)在小鼠胚胎着床过程中的作用。采用荧光定量PCR（FQ-PCR）和免疫组织化学方法分别检测未孕小鼠及孕小鼠第2、3、4、5、7天子宫内膜p16\(^{\text{INK4a}}\) mRNA和蛋白的表达；子宫角注射p16\(^{\text{INK4a}}\)抗体观察胚泡着床数。FQ-PCR结果显示孕小鼠子宫内膜组织p16\(^{\text{INK4a}}\)mRNA的表达高于未孕小鼠，且随着妊娠天数的增加呈现表达逐渐增强的趋势，到妊娠第5天达到最高，后渐降。免疫组织化学分析显示p16\(^{\text{INK4a}}\)蛋白在子宫内膜的表达规律与mRNA结果一致。子宫角注射p16\(^{\text{INK4a}}\)抗体后胚泡着床数明显减少。以上结果提示，p16\(^{\text{INK4a}}\)在妊娠早期子宫内膜持续表达，可能参与胚胎着床。

关键词：胚胎着床；子宫内膜；p16\(^{\text{INK4a}}\)；实时荧光定量PCR；免疫组织化学；体内实验

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p16\(^{\text{INK4a}}\)，位于人染色体9p21，是一种肿瘤抑制基因包括2个内含子和3个外显子和编码一个156-氨基酸酸蛋白，对视网膜母细胞(Rb)细胞周期调节途径具有重要作用[3]。该基因缺失的细胞可以检测到在大多数早期肿瘤和肿瘤细胞[5]。p16\(^{\text{INK4a}}\)的表达显著增加与衰老在各种组织和人中，但p16\(^{\text{INK4a}}\)反义寡核苷酸可以延长细胞周期表明p16\(^{\text{INK4a}}\)是细胞衰老参与的[4-6]。此外，p16\(^{\text{INK4a}}\)可以触发细胞凋亡，细胞凋亡在肿瘤细胞中被转导由p16\(^{\text{INK4a}}\)腺病毒载体可以被去-
tected\cite{7,8}. Recently, p16INK4a was also detected in mouse endometrium during blastocyst implantation\cite{9}.

Early pregnancy of rodents is characterized by a progressive interaction between the blastocyst and the maternal compartment. And this interaction includes apposition of the blastocyst, adhesion of the trophoblast and invasion of the trophoblast\cite{10,11}. But the effect of p16INK4a on blastocyst implantation is still unknown. The present study was aimed to investigate the contributions of p16INK4a to blastocyst implantation by real-time fluorescent quantitative PCR (FQ-PCR), immunohistochemistry and in vivo experiment.

1 MATERIAL AND METHODS

1.1 Animal and preparation
Female depuratory NIH mice (6-8 weeks old, 23-25 g) were obtained from Experimental Animal Center of Chongqing Medical University. Mice were kept under 12 h-light/12 h-dark cycle in the animal care facility. Female mice were mated overnight with males of proven fertility. Pregnancy was identified by the vaginal plug. Un-pregnant mice and pregnant mice on day 2, 3, 4, 5, 7 were killed by cervical dislocation. A minimum of 20 un-pregnant or pregnant animals on day 2, 3, 4, 5, 7 were investigated, and recorded as d0, d2, d3, d4, d5, d7. The endometria were collected under sterile conditions. Tissues were rapidly flash frozen and kept at -80 ºC for subsequent FQ-PCR or fixed in 4% paraformaldehyde for paraffin blocks.

1.2 Isolation of total RNA and reverse transcription-PCR
Total RNA was extracted in Trizol reagent (Invitrogen) and its concentration was determined by UV spectrophotometer. Total RNA were reversely transcribed into cDNA in a total volume of 20 μL. The master Mix reagents were used as follows: 5 μL total RNA, 1 μL M-MLV (200 U/μL, Promega, USA), 0.5 μL RNAsin (40 U/μL, Promega, USA), 500 ng Oligo-dT (TaKaRa Biotech., Dalian, China), 1 μL dNTPs (10 mmol/L, TaKaRa), added DEPC-treated water to 20 μL. The reaction was carried out as follows: 70 ºC for 3 min, 42 ºC for 60 min, and 70 ºC for 15 min. cDNA was kept at -20 ºC.

1.3 Standard templates for FQ-PCR
A conventional PCR was carried out using cDNA with p16INK4a-out-primers (Table 1) to get a PCR product. p16INK4a plasmids were constructed with an 242-bp genomic sequence including p16INK4a PCR product, by ligating the relevant in a sense orientation into a vector, PMD18-T. The p16INK4a plasmids were quantified at the ultraviolet (UV) wave length of 260nm (\(A_{260}\)), divided into aliquots, and frozen before use. The standard templates were diluted by 5 orders of magnitude, including 1×10⁷, 1×10⁶, 1×10⁵, 1×10⁴, 1×10³ copies/μL. The FQ-PCR assay showed excellent linearity between the log of target input and threshold cycle (Ct) value, suggesting that the assay has a dynamic range of 5 logs and is capable of detecting as few as 1 000 copies of p16INK4a in the reaction, and the correlation coefficient was 0.998 (Fig. 1).

1.4 FQ-PCR
FQ-PCR was performed using a Taqman PCR protocol on an Applied Biosystems 7000 sequence detection system (Applied Biosystems). The 25 μL of PCR mixture included 2.0 μL cDNA, 2.0 μL Mg²⁺ (25 mmol/L, TaKaRa),

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**Table 1. The sequence of primers and probes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>p16INK4a-out-FP</td>
<td>5’-CACTGAATCTCCGCGAGGAA-3’</td>
</tr>
<tr>
<td>p16INK4a-out-RP</td>
<td>5’-CAGTTCAGATGAGCCTCCAGGAGGA-3’</td>
</tr>
<tr>
<td>p16INK4a-TAMRA-FAM</td>
<td>5’-TGCTCCAGATGAGCCCTCCAGGAGGA-3’</td>
</tr>
<tr>
<td>p16INK4a-TAMRA-FP</td>
<td>5’-TGCTCCAGATGAGCCCTCCAGGAGGA-3’</td>
</tr>
<tr>
<td>mGADPH-TAMRA-FAM</td>
<td>5’-CCTCACCACCATCCTGATGATGTT-3’</td>
</tr>
<tr>
<td>mGADPH-TAMRA-FP</td>
<td>5’-CATCACCATCTCCAGGAGGA-3’</td>
</tr>
<tr>
<td>mGADPH-TAMRA-RP</td>
<td>5’-CATCACCATCTCCAGGAGGA-3’</td>
</tr>
</tbody>
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Fig. 1. The stand curve of gene p16INK4a.
2.0 μL dNTPs (2.5 mmol/L, TaKaRa), 2.5 μL 10×PCR buffer, 0.3 μL rTag DNA polymerase (5 U/μL, TaKaRa), 1.125 μL amplification forward primer (20 μmol/L), 1.125 μL amplification reverse primer (20 μmol/L), 0.625 μL TaqMan probe (20 μmol/L), added deionized water to 25 μL. All the primers and probes were designed and synthesized according to the sequence of NM_001040654 (GenBank) by Shanghai GeneCore Biotechnologies Co. Ltd. (Table 1). The reaction mixtures were incubated at 95 ºC for 5 min, followed by 40 amplification cycles of 95 ºC for 30 s and 60 ºC for 1 min. The signal detection figure of p16INK4a was shown in Fig 2. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. TaqMan Ct values were converted into absolute copy numbers using a standard curve from standard templates. We also quantified transcripts of glyceraldehyde-3-phosphate dehydrogenase (GADPH) as the endogenous RNA control.

1.6 In vivo experiment
To find out whether p16 INK4a plays a role in blastocyst implantation, we blocked the function of p16INK4a by intrauterine injection of p16INK4a antibody. Twenty pregnant mice on day 3 were randomized to control group and experiment group. Each horn of the uterius was fixed with forceps, and the needle was carefully introduced as close as possible into cervix of the uterine horn. The mice in control group received the injection of normal saline into both horns of each uterus (10 mice) and the mice in experiment group received the injection of p16INK4a antibody (stock solution, 1.5 μg in 8 μL) into left horn of each uterus and normal saline (8 μL) into right horn. The mouse uteri were removed by laparotomy on day 8 of pregnancy and the number of blastocysts was counted.

1.7 Statistical analysis
All the data are presented as mean±SD. The results were analyzed by one-way ANOVA and paired t-test, which were performed with SPSS13.0 for windows software. P<0.05 was considered statistically significant.

2 RESULTS
2.1 p16INK4a mRNA expression in mouse endometrium
p16INK4a mRNA was expressed in endometrial of un-pregnant and pregnant mice and the relative value of the p16INK4a mRNA/GADPH mRNA ratio in each group was shown in Table 2. The p16INK4a mRNA/GADPH mRNA ratio in pregnant group was higher than that in un-pregnant group. It showed an increasing trend as day passed, and the p16INK4a mRNA reached the maximal level on day 5 of pregnancy and then decreased. Analysis of variance showed there were significant differences in the expression of p16INK4a mRNA between every two groups (P<0.05) except day 0 vs day 7, day 2 vs day 3 (Table 2).
Fig. 3. Expression of p16INK4a protein in mouse endometrium of un-pregnant (A) and pregnant mice on day 2 (B), 3 (C), 4 (D), 5 (E), 7 (F), respectively. le: luminal epithelium. s: stromal cell. ge: gland epithelium. Scale bar, 50 μm.

Fig. 4. *In vivo* experiment using uterine horn injection of p16INK4a antibody. A: Control group. B: Experiment group. Scale bar, 1 cm.
2.2 p16^{INK4a} protein expression in mouse endometrium
The p16^{INK4a} protein was expressed in mouse endometria of un-pregnant and pregnant mice. Positive staining particles were observed in the cytoplasm of luminal epithelial cells and glandular epithelial cells, and more intense on the tip of the cells. The immunohistochemistry result showed higher expression of p16^{INK4a} protein in the pregnant group than that in the un-pregnant group ($P < 0.05$) and it had an increasing trend of p16^{INK4a} positive staining in stromal cells on day 3, 4, 5 of pregnant group. p16^{INK4a} protein expression reached the maximum on day 5 of pregnancy (Fig. 3).

2.3 The results of in vivo experiment
The number of implanted blastocysts in the left horn (p16^{INK4a} antibody) of the mouse uterus was less than the right horn (normal saline) in the experiment group ($P < 0.05$), while the difference in two horns (normal saline) of the mouse uterus in the control group was not significant (Fig. 4, Table 3).

3 DISCUSSION
The mammalian uterus changes dramatically in response to the blastocyst implantation during the early pregnancy. Dynamic changes in the uterine endometrium are a type of homeostasis and proceed with proliferation and exclusion of cells in which apoptosis is significant. Animal studies show that apoptosis of luminal epithelial cells facing the trophoblasts can exclude the barrier of blastocyst permeation and the regression of the decidual cells allows a restricted and coordinated invasion of trophoblast cells into the maternal compartment. Apoptosis is in connection with retaining mater-fetal immune tolerance, too. Endometrial apoptosis during the implantation window and early pregnancy in some species has been reported. Endometrial apoptosis, triggered by blastocyst through paracrine secretion or justacrine, starts with apposition of the blastocyst and adhesion of the trophoblast in the receptive uterine epithelium and is more dramatic in the uterine endometrial epithelia surrounding the blastocyst.

p16^{INK4a}, as a tumor suppressor gene, has been investigated in many tumor lines, and considered that it can induce apoptosis in many kinds of cells. But there is no report about whether it also participates in the process of blastocyst implantation. The apposition of blastocyst to endometrium happens at 11-12 pm on day 4 of pregnancy, and the uterine epithelial cells situated around the attachment site undergo apoptosis in the initial phase of implantation in order to go across the epithelial barrier, facilitate the trophoblastic cell exposed to basal layer, and access to maternal blood supply. Our results showed the expression of p16^{INK4a} reached the maximal level on pregnant day 5, which indicated that apoptosis induced by p16^{INK4a} might contribute to trophoblast invasion during “window period” of endometrium. As initiation of blastocyst apposition, the stromal cells under epithelial cells develop into decimal cells, and extend to the whole uterus. Then uterine stromal cells closest to the implantation chamber first commit suicide to make room for the rapidly developed blastocyst. The result that positive staining could be detected in basal cells on day 3, 4, 5 suggested that p16^{INK4a} related to apoptosis was one of the factors which participated in the dynamic changes of basal layer to accommodate the limited invasion of blastocyst. To investigate whether p16^{INK4a} has influence on embryo implantation, we carried out in vivo experiment. Our experimental evidence suggested that p16^{INK4a} antibody could partly inhibit blastocyst implantation. p16^{INK4a}, an important cell cycle regulator, is one of the cell cycle-dependent kinase inhibitors cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CDKIs) and cyclins control the process of cell cycle by regulating the checkpoints in cell cycles. In most tissues, p16^{INK4a} decrease CDK4/6 kinase activity and Rb hypophosphorylation which leads to the transcription factor E2F repression and growth arrest of G_{1}-S period. The deletion of p16^{INK4a} can be detected in most of tumors, while it is a key step leading to cell apoptosis and aging by arresting growth through Rb pathway. It was reported recently that the region-specific expression pattern of CDK4 in wild-type...
mice at the mesometrial pole and of CDK6 at the antimesometrial pole is completely lost in Hoxa-10 null decidua in early pregnancy[23]. That p16INK4a protein expressed in early pregnancy mice and reached the maximal level on day 5 indicated that p16INK4a might induce apoptosis through binding to CDK4/CDK6 to inhibit Rb phosphorylation and repress an important transcript regulator E2F, then the cell growth arrested in G1-S period which followed by apoptosis. Thus, we propose that the regular expression of p16INK4a in mouse endometrium in early pregnancy is closely related to endometrial dynamic changes in response to apposition and invasion of blastocyst. In conclusion, the apoptosis of luminal epithelial cells and decidual cells induced by p16INK4a may participate in the process which endometrium changes to the receptive one to accommodate blastocyst implantation.

The investigation of expression rule of p16INK4a is helpful to understand the regulation of implantation and infertile etiopathogenesis, but detailed mechanisms of p16INK4a in inducing apoptosis of the endometrial cells and interaction with other factors that influence the embryo blastocyst implantation need further researchs.

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REFERENCES


