Int J Clin Exp Med 2015;8(4):5144-5149
www.ijcem.com /ISSN:1940-5901/IJCEM0004882

Original Article

P57 and cyclin G1 express differentially in proliferative phase endometrium and early pregnancy decidua

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Received December 15, 2014; Accepted March 20, 2015; Epub April 15, 2015; Published April 30, 2015

Abstract: Objective: To compare the expression of P57 and Cyclin G1 in proliferation endometrium and early pregnancy decidua. Methods: Human endometrial samples were acquired from normal menstrual cycle women undergoing laparoscopy or hysterectomy for fallopian tubes problems. Decidua were acquired from women in early pregnancy who underwent artificial abortion without any endometrial problems. Twelve were in proliferative phase and 13 were deciduas. P57 and Cyclin G1 mRNA and protein were measured by real-time PCR and Western blot. Results: The expression of P57 mRNA and protein were lower in proliferation phase compared with the early pregnancy decidua. Cyclin G1 mRNA and protein expression were slightly higher in decidua than proliferation endometrium but it had no significant difference. Conclusion: P57 and Cyclin G1 may play an important role in the endometrial change during the embryo implantation.

Keywords: Decidual, P57, Cyclin G1, implantation

Introduction

Decidualization is a key prerequisite for successful implantation, which contains secretory transformation of the uterine glands, influx of specialized uterine natural killer cells, and vascular remodeling [1]. In human, decidualization occurs during mid-luteal phase prior to implantation. In this process, fibroblast like stromal cells transform to polygonal cells,induced by estrogen and progesterone, which promotes stromal cells proliferation and differentiation respectively [2]. However, In pregnancy, the decidual reaction extends to the basal endometrial layer and is critical for trophoblast invasion and placenta formation [3]. Although it is recognized that this process is regulated by progesterone and estrogen involving a variety of growth regulatory factors, cytokines and transcription factors [4], the mechanism of decidualization remains poorly understood.

P57 is cyclin-dependent kinase (CDK) inhibitor. It belongs to the CIP/KIP family, which is involved in cell cycle progression, embryogenesis and cancer development [5]. It binds to cyclin-CDK complexes and abrogates their activities which can lead to cell cycle arrest, differentiation, senescence, quiescence or apoptosis. Mice lacking P57 (P57-/-) show increased postnatal mortality accompanied by severe developmental abnormalities such as growth retardation, immaturity of testes and vaginal atresia [5, 6]. Several researches suggested that P57 had an important role in successful implantation. Brar et al. characterized gene expression pattern kinetics during decidual fibroblast differentiation by microarray analysis, revealing that P57 is upregulated during decidualization in vitro [7]. In implantation dysfunction mice with Hoxa-10 mutation, the expression of P57 is increased in a spatiotemporal manner [8]. By using microarray analysis, our previous study revealed that down-regulation of hsa-miR-222 in vitro could lead to obviously increasing of P57 [9].

Cyclin G1 is a member of cyclin family which can bind to and activate CDKs to regulate cell cycle progression. While most of cyclins promote cell cycle progression, Cyclin G1 is the exception. Its function is controversial that in some tissues, it acts as a negative regulatory factor while in some cancer cells it act as a growth promotor

Table 1. Primers used in real-time PCR

Target Size	Forward and Reverse Primer's Sequence (5'-3')	Product
P57	(F)5'CGGCGATCAAGAAGCTGTCC3'	193 kb
	(R)5'CGGGGCTCTTTGGGCTCT3'	
Cyclin G1	(F)5'TTCCAAGATA AATGGCAGAG3'	132 kb
	(R)5'GCAGTACGCCCAGAAACA3'	
β-actin	(F)5'GTCCACCGCAAATGCTTCTA3'	200 kb
	(R)5'TGCTGTCACCTTCACCGTTC3'	

because of its overexpression [10-13]. Yue et al. detected that Cyclin G1 is expressed in a spatiotemporal manner under the regulating of progesterone via its nuclear receptor. Cyclin G1 is primarily associated with epithelial cell differentiation before implantation and stromal cell proliferation and differentiation during decidualization [14]. In human species, Cyclin G1 expresses periodically. The expression of Cyclin G1 is low in proliferation phase then increases at early secretary phase, reaching a peak at mid-late secretary phase [15].

To determine whether P57 and Cyclin G1 are still up-regulated in decidua, we examined the expression of P57 and Cyclin G1 in proliferation phase endometrium and decidua. Our results show for the first time that P57 and Cyclin G1 are up-regulated in decidua regulated by progesterone.

Materials and methods

Tissue collection

Endometrial samples were acquired from normal menstrual cycle women undergoing laparoscopy or hysterectomy for fallopian tubes problems. Deciduas were acquired from women in early pregnancy who underwent artificial abortion for none endometrial problems. Twelve were in proliferation phase and 13 were deciduas. This study was approved by Tongji Hospital Research and Ethics Committee, and we obtained patient's consents before biopsy. Every sample had a pathological report to confirm its phase. Tissues were washed in PBS and kept in -80°C.

Total RNA extraction and real time PCR

Total RNA was extracted from endometrium according to the manufacturer's instructions. Then the RNA was reverse transcribed in a 10 μL reaction system with the parameters: 37°C for 15 minutes and 85°C for 5 seconds, then quench ed at 4°C. Real-time PCR was performed in a 20 μL system containing 10 μL SYBR^{II} (TAKARA, Japan), 1 μL cDNA, 2 μL primers (1 μL sense and 1 μL antisense) and 7 μL ddH_2O. We designed the primers using the OligoPrimer Analysis 5.0 software and analyzed the sequence in BLAST. The primer sequences were shown in **Table 1**. The parameters of Real-time PCR were as follows: 95°C for 2 minutes 30 seconds, followed by 45 cycles of denaturation for 10 seconds at 95°C, annealing for 10 seconds at 55°C, extension for 15 seconds at 72°C. β-actin was used to control the expression levels. All experiments were repeated in three times. Expression relative to control samples was calculated utilizing the ΔΔCT method, and $2^{-\Delta\Delta CT}$ represents mRNA fold change.

Western blot

Proteins were extracted from the samples according to manufacturer's instructions. The concentration was determined by BCA Protein Assay. Protein samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDSHPAGE) (12%). Then they were transferred to polyvinylidene fluoride membrane using a Bio-Rad electroblot apparatus. The membrane was blocked in 5% fat-free milk in TBST, followed by incubation at 4°C overnight with primary antibodies as below: anti-human P57 monoclonal antibody (Abcam, USA; 1:400) and anti-human Cyclin G1 polyclonal antibody (Santa Cruz, CA, USA; 1:200). After washed with TBST, the membranes were incubated at room temperature for 1 hour with secondary antibodies as below: goat peroxidase-conjugated anti-rabbit IgG, (Santa Cruz, CA, USA, 1:2000). Actin was used as loading control. The protein bands were visualize by chemiluminescence using ECL kit (SupreSignalWest Pico; Thermo Scientific), and the results were analyzed with Quantity one software to calculate the gray value.

Statistical analysis

Data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data were presented as mean ± SD. The difference of the mRNA fold change and gray value between pro-

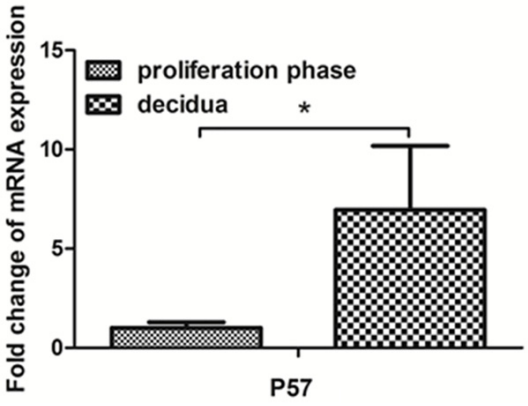

Figure 1. The expression of P57 mRNA by Real-time PCR. *P<0.05.

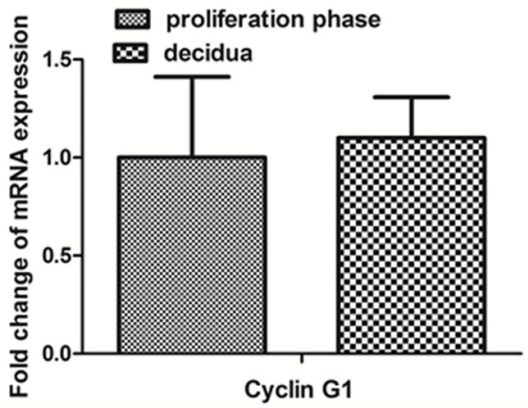

Figure 2. The expression of Cyclin G1 mRNA by Real-time PCR.

liferation phase endometrium and decidua were analyzed by unpaired Student's *t*-test. We considered *P*-value of <0.05 as statistically significant.

Results

P57 and Cyclin G1 mRNA expressions in Human endometrium and decidua

We verified the primers using agarose gel electrophoresis.The product of P57 and Cyclin G1 were 193 kb and 132 kb respectively. The expression of P57 and Cyclin G1 mRNA detected by real-time PCR are shown in **Figures 1** and **2**. In proliferation phase endometrium, P57 mRNA expression was low. However, it was much higher in decidua from women in early pregnancy. Unlike P57, the expression of Cyclin G1 was low in proliferation endometrium. But it was only slightly higher in decidua. By using

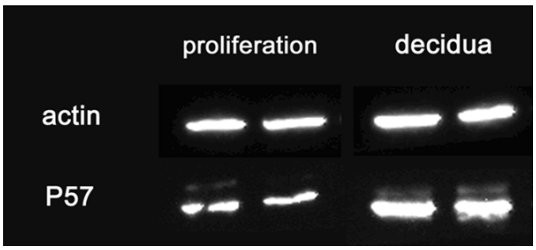

Figure 3. The expression of P57 protein by Western blot.

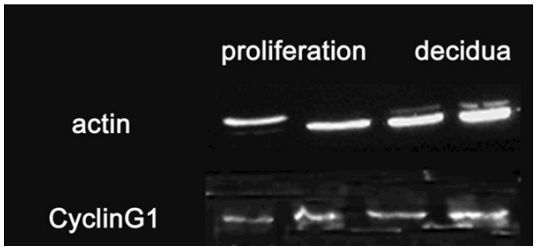

Figure 4. The expression of Cyclin G1 protein by Western blot.

unpaired *t*-test, we found that the difference of P57 between proliferation endometrium and decidua was statistical significant. For Cyclin G1, there was no statistical difference between proliferation endometrium and decidua.

Expressions of P57 and Cyclin G1 proteins in Human endometrium and decidua

The protein expression of P57 and Cyclin G1 were examined by western blot. We used actin as a control.P57 protein was about 52kDa, and Cyclin G1 was 57kDa approximately. The results were shown in **Figures 3** and **4**. In proliferative phase, the level of P57 protein was low. However, in decidua, it was much higher. There was a statistical difference by using unpaired *t*-test. Cyclin G1 protein was at low level in proliferation phase endometrium while in decidua it was up-regulated with a statistical difference by using t-test. These quantification and normalization results were shown in **Figures 5** and **6**. These results are consistent with real-time PCR.

Discussion

CDKs and its regulators have a close relationship with human endometrium

The balance between cell proliferation and differentiation is important for the development of

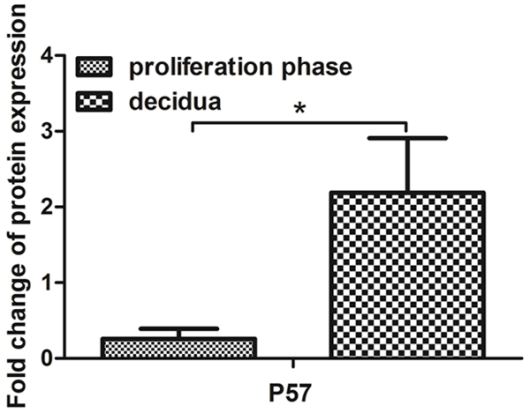

Figure 5. The quantification and normalization of werstern blot bands of P57.

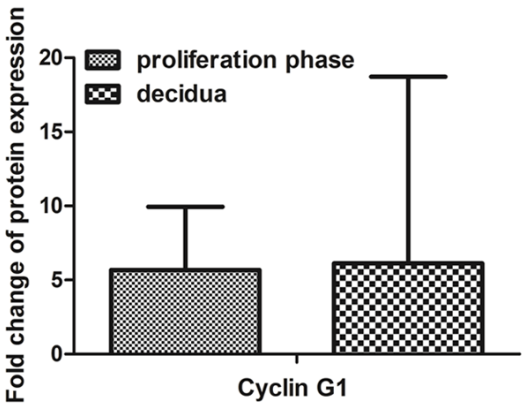

Figure 6. The quantification and normalization of werstern blot bands of Cyclin G1.

tissue. CDKs are a family of protein kinases involved in this progression. The activity of CDK is regulated by cyclin proteins which can bind to CDK and promote cell cycle [16]. For example Cyclin D, which is called G phase cyclin, can bind to CDK4 and CDK6 and promote the entering of cells to S phase [4]. Although most of cyclins can promote cell cycle, there is still exception. Cyclin G1 primarily acts as a negative cell cycle regulator [14]. There are also a kind of negative regulators of CDKs called CKIs. CKIs include two families, the INK4 and CIP/KIP. The CIP/KIP family contains three members, P21, P27, P57 [5]. When they bind to CDKs, they can inhibit cell cycle progression.

During pregnancy, uterus is a dynamic physiological system involving cellular proliferation, differentiation, including the terminal differentiation, and apoptosis. Decidualization is a critical process for successful pregnancy, characterized by the differentiation of endometrial stromal cells into decidual cells. It has been found that cell cycle regulators play important roles in the uterus under the hormonal stimulation [17, 18]. Das et al. found that Cyclin D3 was up-regulated at the sites of blastocyst apposition during the attachment reaction. With the progress of pregnancy, Cyclin D3 level fluctuated [19]. CDK4 and CDK6 were up-regulated during decidualization. But in Hoxa-10 deficiency mouse, their distribution were aberrant in anti-mesometrial [20]. P21 was much higher in mice on D5 of pregnancy when CDK and Cyclin D3 were down-regulated [21]. In the endometrium of women with endometriosis, P27 was down-regulated compared with the normal women [22]. In this study, we observed different levels of two factors of this kind, P57 and Cyclin G1 between proliferative phase endometrium and decidua.

P57 was up-regulated in decidua from early pregnancy women

As we explained above, P57 is an important factor involved in decidualization. We found its expression was at low level in proliferative phase endometrium, which is consistent with other researches, suggesting that P57 was primarily involved in differentiation, not proliferation. In human, P57 was found in the glandular epithelial cells from early to mid-secretory phase and in late secretory phase of decidualized stomal cells, which suggested temporal difference in the expression of P57 between epithelial cells and stromal cells. P57 undergoes cyclic changes during menstrual cycle, which is induced by sex hormones. In vitro, it can be induced by estrogen and progestin, but estrogen alone cannot induce P57 change, which is consistent with the results in vivo [23]. Over-expression of P57 can lead to cell cycle arrest in G1 phase [24]. In human uterus, expression of P57 protein was negligible in endometrial hyperplasia and endometrial cancer. However, it was strongly expressed in cytotrophoblasts, intermediate trophoblasts, villous stromal cells, and decidual stromal cells of complete mole, a malignant proliferation disease, involving in the process of differentiation [23, 25].

We have already known that P57 is up-regulated in decidualized stromal cells in late secreto-

ry phase in which process the stromal cells primarily undergo differentiation, not proliferation [26]. Consistently with other experiments, it suggests that P57 is an important promoter of stromal cells differentiation [23]. In decidua from early pregnancy women, P57 expression was up-regulated in both mRNA and proteins, suggesting that differentiation was still a prior event in early pregnancy decidua, and P57 promoted differentiation in decidua via progesterone mediated pathway.

Expression of Cyclin G1 was up-regulated in decidua from early pregnancy women but had no statistical difference

Cyclin G1 is a member of cyclin proteins, of which most members bind to CDKs and promote cell cycle. But function of Cyclin G1 is not like most of its family members. A research indicated that whether Cyclin G1 promoted or inhibited cell cycle depended on the magnitude of its expression [10]. During the decidualization in mouse uterus, Cyclin G1 was up-regulated in epithelial cells on D3, and it was undetectable at the site of implantation on D5. However, the expression reappeared at the site of implantation primarily in cells of the secondary decidual zone where stromal cells were still proliferating on D6, suggesting that the high expression of Cyclin G1 in these cells restricted their unlimited growth [14]. In human uterus, Cyclin G1 expressed periodically to regulate cell cycle progression of epithelial cells and stromal cells [15]. We found that Cyclin G1 expression was low in proliferation phase endometrium in both mRNA and protein, suggesting that Cyclin G1 was not involved in the process of proliferation of endometrium or had little effect on this process.

We found for the first time that in decidua from early pregnancy women, Cyclin G1 was up-regulated both in mRNA and protein, but the difference had no statistical difference that suggested Cyclin G1 was not a key factor in endometrial differentiation during pregnancy due to it had no signifcant change when the endometrium went through different stages. On the contrary, it was still involved in decidual proliferation since there was similar expression compared with proliferative phase. In addition, along with the high level expression of P57, the proliferation of decidua was inhibited, which may affect the expression of Cyclin G1. According to the negative regulatory role of Cyclin G1 in uterus, we speculates that Cyclin G1 can limit the decidual proliferation or it can prevents hyperplasia of decidua, which can cause some diseases. However, Cyclin G1 may acts as a proliferation promotor in uterus under some circumstances, for example, in vitro-grown uterine leiomyoma cells, reduced Cyclin G1 expression induced apoptosis in a caspase-independent mechanism suggesting Cyclin G1 expression associated with growth promotion [27]. Whether Cyclin G1 can promote or inhibit cell cycle progression remains controversial, further researches are needed to investigate this problem.

In conclusion, P57 and Cyclin G1 are involved in the process of decidualization in human endometrium, and they still play roles in the early pregnancy decidua via a progesterone mediated pathway. Our experiment provides a novel evidence for its function in endometrium, but the mechanism of this process needs more researches to determine.

Acknowledgements

This study was supported by grants from the National Nature Science Foundation of China (No. 81170619).

Disclosure of conflict of interest

None.

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