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Effects of Expression of Exogenous Cyclin G1 on Proliferation of Human Endometrial Carcinoma Cells

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Abstract

Cyclin G1 is the only cyclin that has either positive or negative effects on cell growth. Our previous study found decreased expression of cyclin G1 in human endometrial carcinoma tissues compared with normal endometrial tissues. The study aimed to evaluate cyclin G1 expression and its effect on proliferation of human endometrial carcinoma cells (ECCs). Cyclin G1-GFP (green fluorescence protein) plasmid was constructed and transfected into various differentiated human ECCs, including Ishikawa, HEC-1-B and KLE cells, and proliferation of the transfected cells was determined by the CCK-8 method. Exogenous cyclin G1 mRNA and protein were measured by RT-PCR and Western blot, respectively, and GFP signal was monitored by fluorescence microscopy. Chinese hamster ovary (CHO) cells were transfected with the same constructs as a cell control. Cyclin G1-GFP-transfected Ishikawa cells were further treated with MG132, an inhibitor of proteasome, to analyze if low expression of cyclin G1 is related to its abnormal degradation in ECCs. Ectopic expression of exogenous cyclin G1 was found to significantly suppress the proliferation of Ishikawa and HEC-1-B cells but not KLE cells. Compared with cyclin G1-transfected CHO cells, exogenous cyclin G1 protein expression was low in Ishikawa and HEC-1-B cells, and was undetectable in KLE cells. However, all ECC lines and CHO cells expressed similar levels of exogenous cyclin G1 and GFP mRNA. MG132 treatment increased cyclin G1 protein expression in cyclin G1-GFP-transfected Ishikawa cells. This is the first study to present evidence to suggest that cyclin G1 exerts negative control on proliferation of ECCs. Exogenous cyclin G1 shows different protein expression levels in ECCs with different malignancies, and cyclin G1 protein is highly unstable and is rapidly degraded in ECCs.

Key Words: cell proliferation, cyclin G1, endometrial carcinoma

Introduction

Cyclin G1 has been identified as a transcriptional target of p53 with strong homology to the cyclin family members (13, 20). Although cyclin G1 has neither a PEST sequence nor a destruction box (14), cyclin G1 protein is highly unstable and undergoes ubiquitination- and proteasome-mediated degradation (16, 24). Unlike other cyclins, cyclin G1 has either positive or negative effects on cell growth in different cells. Some reports indicated that over-expression of cyclin G1 promoted the growth of cancer cells, such as leukemia (9), heaptic tumor (6), leiomyoma (1) and cervical carcinoma (10). Introduction of anti-sense constructs of cyclin G1 suppressed the growth of those cancer cells (5, 7, 19). Conversely, cyclin G1

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Table 1. Primers used for RT-PCR analyses

mRNA	Primer Sequences (5'-3')[a]	Product Size (bp)
Cyclin G1	TCTAAGCTTATGATAGAGGTACTGACAAC TTTGAATTCTGTAATAATCCAGTTAAGG	909
GFP	CTGGTCGAGCTGGACGGCGACG CATGGTCCTGCTGGAGTTCGTG	430
GAPDH	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA	480

[a]Primer sets were obtained from Life Technologies, Inc. (Grand Island, NY, USA)

has been shown to inhibit cell proliferation by inducing cell-cycle arrest in other cells (15, 17, 18). For example, exogenously expressed cyclin G1 induces G1-phase arrest in NIH3T3 and U_2Os osteosarcoma cells (16, 24).

Our previous studies found that cyclin G1 expression was progesterone-dependent in peri-implantation mouse uterus (23) and it was expressed mainly in normal human endometrial epithelial cells (EECs) at the secretory phase (2). Progesterone treatment induced cyclin G1 expression and inhibited proliferation of cultured primary mouse EECs (12). Moreover, immunohistochemistry study showed decreased expression of cyclin G1 in human endometrial carcinoma tissues (21). These findings suggest that up-regulation of cyclin G1 by progesterone is involved in the growth suppression of normal EECs. In this research, we studied the effects of ectopic expression of exogenous cyclin G1 on endometrial carcinoma cells (ECCs) to further evaluate the functional role of cyclin G1 in ECCs. The mechanism of low-level expression of exogenous cyclin G1 was also investigated.

Materials and Methods

Reagents

DMEM medium, fetal bovine serum (FBS), Trizol, Lipofectamine 2000, incision enzyme EcoRI, BamHI and SuperScript II Reverse Transcriptase Kit were purchased from Invitrogen (Carlsbad, CA, USA). The vector plasmid pGFP-N1 was purchased from Clontech Laboratories (Palo Alto, CA, USA). Recombinant $GluT_4$ (glucose transporter 4)-GFP plasmid was a gift from Dr. Luo Dixiang of Kang Hong Pharmaceutical Company (Chengdu, Sichuan, PRC). CCK-8 kit was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). G418, MG132, urea, PMSF and leupeptin were purchased from Sigma (Saint Louis, MO, USA). Antibodies against cyclin G1 (SC-320), β-actin (SC-130656) and goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (SC-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Broadford protein assay kit, agarose gel, Immun-Star HRP chemiluminescent kits and densitometry were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA).

Cell Lines and Culture Conditions

Four cell lines were used for this experiment: Chinese hamster ovary (CHO), well-, moderately- and poorly-differentiated human endometrial carcinoma cell lines, Ishikawa, HEC-1-B and KLE, respectively (ATCC-LGC Standards GmbH, Wesel, Germany). Cells were cultured in DMEM medium supplemented with 10% FBS and 100 units/ml pen/strep. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

Cyclin G1-GFP Expression Plasmid Construction and Transfection

Human cyclin G1 cDNA was obtained by polymerase chain reaction (PCR) using a human cDNA library. The sequences of the primers are listed in Table 1. A 909-bp fragment of cyclin G1 cDNA, including the complete coding sequence, was cloned into pEGFP-N1 at the EcoRI and BamHI sites. For transfection, cells were seeded in 6-well-plates and allowed to grow for 24 h before the cells were transfected with 4 μg of cyclin G1-GFP plasmid or an empty vector or $GluT_4$-GFP plasmid using Lipofectamine 2000. Forty-eight hours after transfection, G418 was added at 800 μg/ml and cultured for two weeks to select stably-transfected cells. G418 was removed and the fluorescence emitted by the transfected cells was observed by a fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan).

Cell Proliferation Assay

Cyclin G1-GFP- or empty vector-transfected

ECCs were plated in 96-well plates at a density of 2 × 10^3 per well. Cell proliferation was evaluated at the first, third, fifth and seventh day by CCK-8 assay following the manufacture's manual. Briefly, 10 μl CCK-8 reagent was added to each well and incubated for 1 h at 37°C. The absorbance at 450 nm was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The experiments were repeated at least thrice.

Western Blot

Cyclin G1-GFP-transfected ECCs and CHO cells were collected with trypsin/EDTA solution and homogenized in lysis buffer containing 8 M urea, 1 mM PMSF and 1 mg/ml leupeptin. The lysates were centrifuged at 12,000 × g for 15 min at 4°C and the supernatants were stored at -70°C. Protein concentration was determined using the Broadford protein assay and extracts equivalent to 50 μg of total protein was separated by SDS-polyacrylamide gels. Proteins were transferred to a PVDF membrane with a plate electrode apparatus. The membrane was firstly blocked with 5% fat-free dried milk in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) overnight at 4°C. The membrane was then incubated for 2 h at room temperature with a rabbit anti-cyclin G1 antibody or anti-β-actin antibody, washed three times in TBS/0.05% Tween-20, and incubated with a HRP-conjugated goat anti-rabbit secondary antibody. Signals were detected by enhanced chemiluminescence and quantitatively analyzed by densitometry.

Analysis of mRNA Expression by Semi-Quantitative RT-PCR

RNA was extracted from cells using TRIzol reagent followed by isopropanol precipitation. RNA concentration was determined by measuring absorbance at 260 nm. Total RNA (2 μg) was converted to cDNA using the SuperScript II Reverse Transcriptase Kit. The primer sequences for cyclin G1, GFP and GADPH are listed in Table 1. To amplify human mRNA of *cyclin G1*, *GFP* and *GAPDH*, cDNA corresponding to 200 ng of total RNA was used for PCR with the following parameters: (a) a first denaturation step at 95°C for 2 min; (b) 25 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 45 s; (c) a final extension step for 10 min at 72°C. The number of cycles was optimized after examining a range of 20-30 cycles. After PCR, 7 μl of each PCR product was subsequently separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining, followed by quantitatively analyzed by densitometry.

Protein Degradation Assay

Cyclin G1-GFP-transfected Ishikawa cells were dispersed on 6-well plate and were allowed to grow for 24 h. The cells were treated with 0.1 μM MG132 (15) for 24 h. Cell fluorescence was observed by fluorescence microscopy and the amount of cyclin G1 protein was measured by Western blot.

Statistical Analysis

Results are reported as means ± SD. Comparison between two groups was performed using two-tailed Student's *t*-test. Statistically significant results are defined as $P < 0.05$.

Results

Cyclin G1 Inhibited the Proliferation of Human ECCs

To check the functional effect of cyclin G1 on ECCs, we transfected cyclin G1-GFP plasmid, or an empty vector, into three differentiated human endometrial carcinoma cell lines, and cell proliferation rates were examined. It was found that transfection of a cyclin G1 expression plasmid significantly inhibited the proliferation rate of Ishikawa and HEC-1-B cells (Fig. 1, A and B) compared to the empty vector-transfected cells. To our surprise, transfection of the cyclin G1-expressing plasmid had no suppression effects on the poorly-differentiated KLE cells (Fig. 1C).

Exogenous Cyclin G1 Protein Expression Is Low and Absent in Human ECCs

It is interesting to see over-expression of cyclin G1 has different effects on various differentiated ECCs. To explore the underlying mechanisms, we firstly checked the expression of the exogenous cyclin G1 gene. In our cyclin G1 expression plasmid construct, GFP was tagged to the carboxyl-terminus of cyclin G1, so the fluorescence intensity of GFP could be used as an indicator of the cyclin G1-GFP fusion protein expression. Surprisingly, only weak fluorescence signal was observed in cyclin G1-GFP plasmid transfected Ishikawa and HEC-1-B cells; no fluorescence signal was visible in the transfected KLE cells (Fig. 2). In order to exclude the influence of transfection efficiency, empty vector was transfected to the three human ECCs as controls, and CHO cells were also similarly transfected as a cell control. To further exclude the possibility of the inserted exogenous gene influencing GFP expression, $GluT_4$-GFP expression plasmid was also transfected to the human ECCs and CHO cells, and fluorescence signal

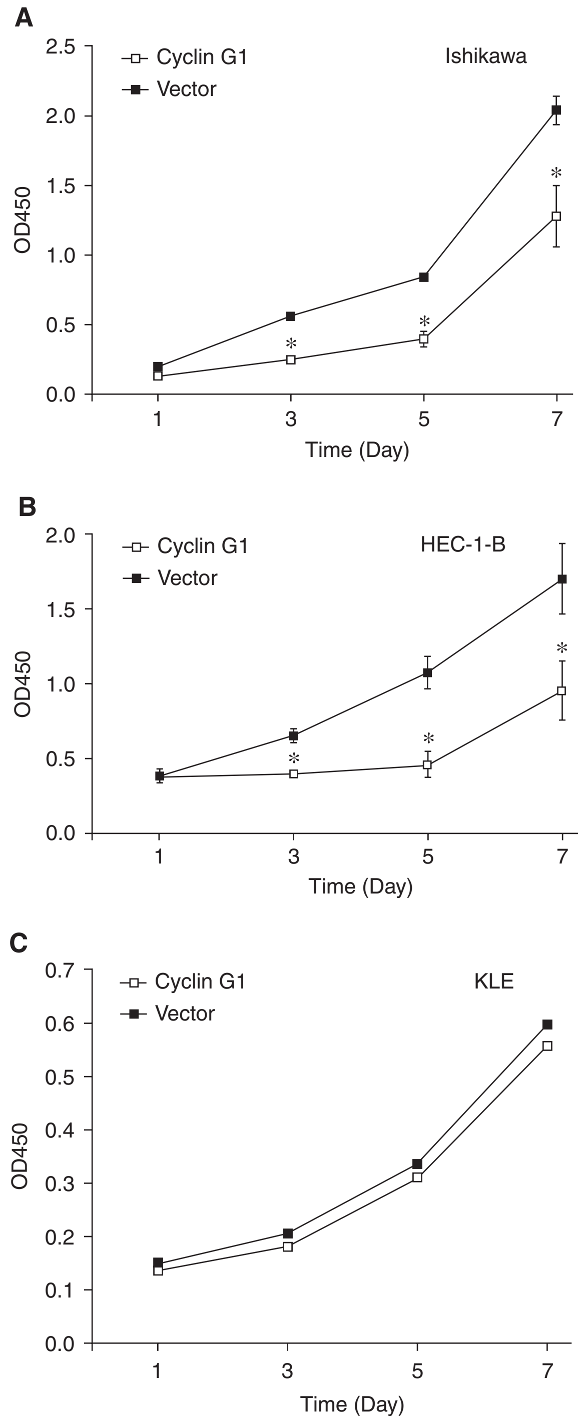

Fig. 1. Ectopic expression of cylcin G1 suppressed proliferation of ECCs. Empty vector (closed squares) or cyclin G1-GFP construct (open squares) was transfected into Ishikawa (A), HEC-1-B cells (B) and KLE cells (C), and cell proliferation was assessed by the CCK-8 method. $^{*}P < 0.05$ as compared with corresponding empty vector.

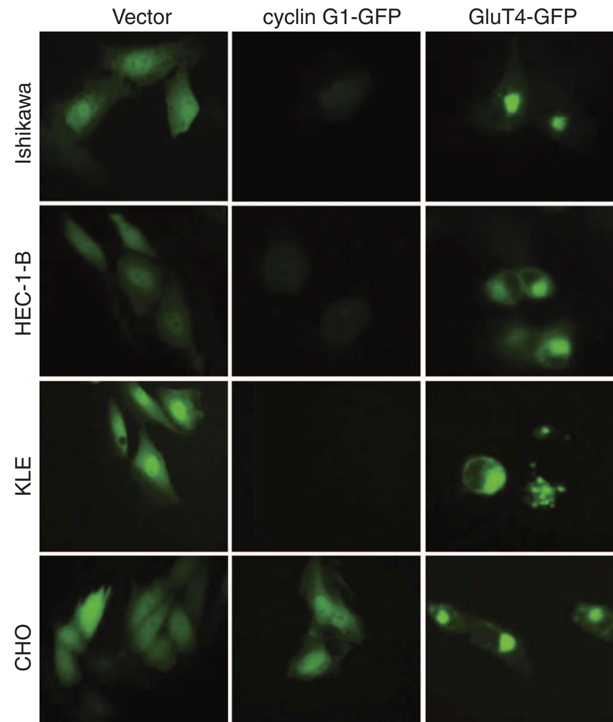

Fig. 2. Cyclin G1-GFP construct showed differential fusion protein expression levels in ECCs and CHO cells as indicated by GFP signals. Ishikawa, HEC-1-B, KLE and CHO cells were transfected with an empty vector or the indicated constructs, and green fluorescence was viewed under a fluorescence microscope (400 ×).

of GFP was monitored. Cyclin G1-GFP-transfected CHO cells gave strong green fluorescence signals. All of ECCs and CHO cells transfected with the empty vector or the $GluT_4$-GFP plasmid also gave strong fluorescence (Fig. 2). These results suggest that the expression of the exogenous cyclin G1-GFP is low specifically in endometrial carcinoma cells.

To confirm the expression of exogenous cyclin G1, Western blot was performed. The exogenous cyclin G1 expression level was high in cyclin G-transfected CHO cells, but was much lower in Ishikawa and HEC-1-B, and undetectable in KLE cells (Fig. 3).

Exogenous Cyclin G1 *and* GFP *mRNA Expression Had no Difference between Human ECCs and CHO Cells*

We next compared the mRNA levels of the cyclin G1-GFP gene in the transfected cells to explore the reason of low expression levels of cyclin G1 protein in ECCs. As shown by semi-quantitative RT-PCR, the mRNA levels of exogenous *cyclin G1* and *GFP* did not show marked differences between the three ECCs and CHO cells (Fig. 4). The relative ratio of cyclin G1 and GAPDH band intensity was 0.75: 0.79: 0.74: 0.76 in the four cell lines (CHO *vs.* ISK *vs.* HEC *vs.* KLE); *GFP* mRNA expression was similar to *cyclin G1* with relative ratio of 1.01: 0.93: 0.95: 0.96, respectively.

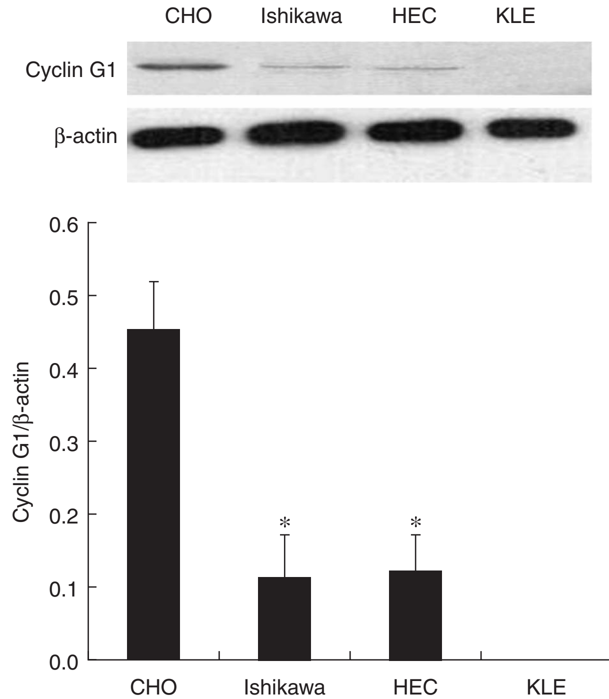

Fig. 3. Cyclin G1 protein was differentially expressed in cyclin G1-GFP construct-transfected CHO cells and human ECCs. Transfected cells were lysed and the cell lysate was subjected to SDS-PAGE and immunoblotting using specific antibodies against either cyclin G1 or β-actin. A: Representative blots are shown. B: Chemiluminescent signals of Western blot were quantified using β-actin as control to normalize total protein loading. Data shown in the bar graph are means ± SD. $^*P < 0.05$.

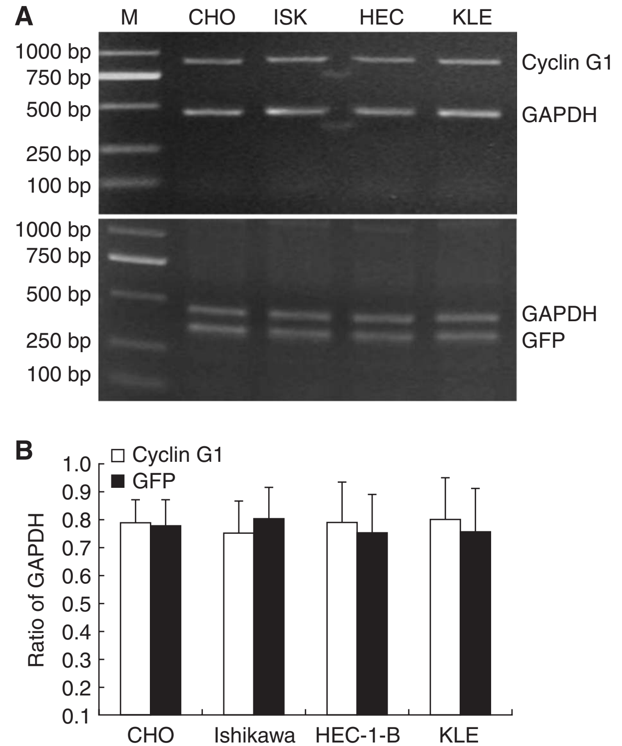

Fig. 4. mRNA expression levels of *cyclin G1* and *GFP* in cyclin G1-GFP construct-transfected CHO cells and human ECCs. Total RNA of the transfected cells was prepared and the mRNA levels of *cyclin G1*, *GFP* and *GAPDH* were quantified by semi-quantitative RT-PCR. A: Representative gel data are shown. B: Density of cyclin G1 or GFP RT-PCR product was normalized to that of GAPDH; the ratios are shown in the bar graph (means ± SD).

Exogenous Cyclin G1 Protein Expression Was Unstable in Human Endometrial Carcinoma Cells

It has been indicated that cyclin G1 protein is unstable and is degraded by proteasome (16, 26). After excluding regulation at the transcription level in endometrial carcinoma cells, we postulate that low expression levels of exogenous cyclin G1-GFP might be due to protein degradation. To confirm this, MG132, an inhibitor of proteasome, was used to treat transfected Ishikawa cells and expression of cyclin G1-GFP was monitored by fluorescence microscopy as well as in Western blots. The results showed that after MG132 treatment for 24 h, both fluorescence signals of GFP and cyclin G1 protein expression increased significantly (Fig. 5). It is proposed that there is an abnormal fast cyclin G1 protein degradation machinery in the endometrial carcinoma cells.

Discussion

Our previous studies have suggested cyclin G1 as a negative cell-cycle regulator, inhibiting proliferation of normal endometrial epithelial cells (12, 23). In order to further study the effect of cyclin G1 on the proliferation of human endometrial carcinoma cells, a cyclin G1 expression plasmid was constructed for transfection into human ECCs to monitor cell proliferation. The results showed that cell proliferation was inhibited significantly in well- and moderately-differentiated ECCs. However, over-expression of cyclin G1 did not show cell proliferation suppression in KLE cells, a poorly-differentiated ECCs. Subsequently, we noticed that, compared with cyclin G1-GFP-transfected CHO cells, exogenous cyclin G1 protein expression was weak in cyclin G1-GFP transfected ECCs, and was undetectable in KLE cells. Previous researches on human endometrial carcinoma mostly have focussed on cyclins that are positively regulating cell cycle, such as cyclin A, cyclin D and cyclin E (3, 11, 22). Here, we report for the first time that cyclin G1 exerts negative control in proliferation of human ECCs. A human endometrial carcinoma-bearing mice model and transfection of cyclin G1

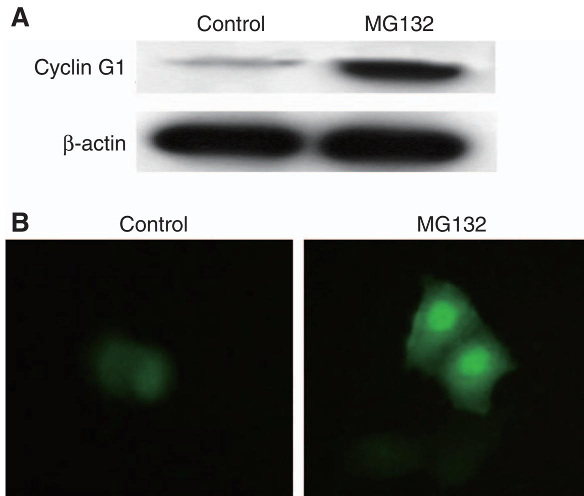

Fig. 5. Effects of MG132 on expression of cyclin G1 protein and fluorescence intensity in cyclin G1-GFP construct-transfected Ishikawa cells. Cells were transfected with cyclin G1-GFP construct and treated with 0.1 μM MG132 for 24 h. Cells were then lysed for Western blot assay using an anti-cyclin G1 or anti-β-actin antibody (A), or were viewed under a fluorescence microscope (B).

expression plasmid will be used in further study to confirm cyclin G1 anti-cancer activity *in vivo*.

In the study, CHO cells were transfected with the same vector used as a control because the normal human endometrial epithelial cell line was unavailable. Compared with the cyclin G1-GFP construct-transfected CHO cells, the mRNA levels of exogenous *cyclin G1* and *GFP* in the three human ECCs did not show significant difference, but the expression of cyclin G1 protein was dramatically lower in ECCs or undetectable in poorly-differentiated ECCs. These findings suggested exogenous expression of *cyclin G1* mRNA was not consistent with its protein expression levels in cyclin G1-transfected human ECCs. The observation suggests that the expression of the cyclin G1 protein is not controlled at the transcriptional level but is controlled at the post-transcriptional level in endometrial carcinoma cells.

It has been reported that cyclin G1 was unstable and it is easily degraded by proteasome (16, 24). We also found the fluorescence intensity decreased more rapidly in cyclin G1-GFP-transfected ECCs compared with the transfected CHO cells. It seems that the cyclin G1 protein has a much shorter half life in ECCs. Treating cyclin G1-GFP-transfected Ishikawa cells with MG132, an inhibitor of proteasome, markedly restored cyclin G1 protein expression suggesting that low expression levels of exogenous cyclin G1 in ECCs was due to rapid protein degradation. Given that cyclin G1-GFP construst-transfected ECCs had different cyclin G1 expression levels according to the degree of differentiation of the ECCs, it will be interesting to compare the cyclin G1 protein stability in the three variously differenentiated human ECCs and investigate the mechanism regulating dagradation of cyclin G1, which may be linked to the maligancy of human endometirial carcinoma. In addition, the possibility that there is abnormal low cyclin G1 protein sythesis in human ECCs is also worthy of further investigation.

Normal endometrium is controlled by estrogen and progesterone. Estrogen stimulates the proliferation of glandular cells, whereas progesterone inhibits their growth and induces secretory changes (4). Once the balance is destroyed, insufficient progesterone will result in unopposed estrogen action and lead to the development of endometrial carcinoma (8). Our previous studies discovered that cylin G1 protein expression is progesterone-dependent in normal EECs (2, 12, 23). We also observed increased expression of the cyclin G1 protein in human ECCs after treatment with progesterone (unpublished data). Our present research showed that even on over-expression of the cyclin G1 gene, cycline G1 was still unable to be expressed normally in ECCs, indicating that the reason of low or lacking expression of cyclin G1 is not only due to progesterone defects.

Taken together, cyclin G1 was shown in this study to exert negative regulation on the proliferation of ECCs. Loss of cyclin G1 might be involved in the development and progression of endometrial carcinomas. Decreased expression of cyclin G1 protein in ECCs is possibly related to accelerated protein degradation. Cyclin G1 might be a useful biomarker in diagnosis and in being used in the development of new treatment strategy for endometrial carcinomas.

Acknowledgments

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