Cyclin G1 inhibits the proliferation of mouse endometrial stromal cell in decidualization

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Abstract: Uterine stromal cell decidualization is a dynamic physiological process in which cell proliferation, differentiation and apoptosis are orchestrated and occur in a temporal and cell-specific manner. This process is important for successful embryo implantation. Many cell-cycle regulators are involved in decidualization. The protein cyclin G1 is a unique regulator of the cell cycle with dual functions in cell proliferation. It was reported that cyclin G1 is expressed in mouse uterine stromal cells during the period of peri-implantation. To prove the function of cyclin G1 in mouse uterine stromal cells during this period, immunohistochemistry was used to stain mouse uterine tissues on days 4-8 of pregnancy. The results showed obvious spatial and temporal expression of cyclin G1 in uterine stromal cells, and that it is expressed in the cells of the primary decidual zone (PDZ) on day 5 and secondary decidual zone (SDZ) on days 6 and 7, when the stromal cells experienced active proliferation and differentiation was initiated. Applying the decidualization model of cultured primary stromal cells *in vitro*, we further revealed that the expression of cyclin G1 is associated with decidualization of stromal cells induced by medroxyprogesterone acetate (MPA) and estradiol-17β (E2). RNA interference was used for the knockdown of cyclin G1 in the induced decidual cells. Flow cytometry analysis indicated that the proportion of cells in the S stage was increased, and decreased in the G2/M phase. Our study indicates that cyclin G1, as a negative regulator of the cell cycle, plays an important role in the process of decidualization in mouse uterine stromal cells by inhibiting cell-cycle progression.

Key words: cyclin G1; decidualization; stromal cell; proliferation; mouse

INTRODUCTION

Implantation is the process whereby blastocysts are successfully attached to the endometrium. In rodents and primates, decidualization is a significant reaction of the uterus for implantation. The extensive proliferation of endometrial stromal cells (ESCs) is followed by the differentiation of these cells into a wide variety of decidual cells [1,2]. Endometrial decidualization is crucial for the development of early embryo and early pregnancy maintenance [3].

Mouse ESC decidualization has obvious spatial and temporal characteristics that are accompanied by regular cell proliferation and differentiation [4,5]. During blastocyst attachment at day 4 of mice pregnancy, ESCs surrounding the implanting blastocyst undergo intense proliferation. From late day 5 to 6, stromal cells near the implanting blastocyst cease to proliferate and initiate their differentiation to form the primary decidual zone. After two or three days, cells within the PDZ subsequently undergo apoptosis and form spaces for further growth of the implanted blastocysts. During days 6 to 7 of pregnancy, stromal cell proliferation and differentiation continue outside the PDZ, to form the secondary decidual zone. Then, the SDZ also undergoes apoptosis, which enlarges the implantation chamber to accommodate the growing embryo.

The process of decidualization is complicated and it is related to the activities of cell cycle which are controlled by many regulators. Cyclins, as cell-cycle regulators linked to cell-cycle events, are likely to participate in this process [6]. Recent studies have provided evidence that cyclins are a potentially important player during implantation and decidualization [7,8]. Most of members in the cyclin family, such as cyclin D3, cyclin B and cyclin E2, usually combine with a certain subtype of cyclin-dependent kinase (CDK) to play a role in the decidualization of ESC [9-11]. Besides positive molecules, negative regulators coordinate cell proliferation, differentiation and apoptosis during the process of decidualization. However, the

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modes of their operation in the cell cycle of ESC remains poorly understood.

Cyclin G1 is a subtype of cyclin G, which is the latest discovered cyclin member. Cyclin G1 is also a unique regulator that has both positive and negative effects on cell proliferation [12-14]. Our previous study showed that as a negative cell-cycle regulator, cyclin G1 plays important role in the inhibitory effect of progesterone on the proliferation of endometrial epithelial cells [15], and the expression of cyclin G1 mRNA is in a temporal and cell-specific manner in the endometrium of pregnant mice [16]; thus, we speculate that cyclin G1 may play an important role during stromal cell decidualization.

In the present research, multiple approaches were employed *in vivo* and *in vitro*, and the aim was to demonstrate the spatiotemporal characteristics of cyclin G1 expression in decidual stromal cells during peri-implantation, and further to explore its negative regulatory function for cell proliferation in the process of decidualization. This work should clarify the molecular mechanism of stromal cell decidualization.

MATERIALS AND METHOD

Ethics statement

Female Kunming mice were used in this study. All animal procedures were conducted under the protocol approved by the Ethics and Scientific Research Committee in Sichuan University. All efforts were made to minimize suffering.

Animal treatments and tissue preparation

The 8- to10-week-old Kunming mice were housed under a controlled environment with a photoperiod of 12 h light (0700-1900 h) and 12 h dark, and a temperature ranging from 20-25°C. Food and tap water were available *ad libitum*. To obtain the uterine tissues during peri-implantation, female mice were caged with a fertile male mouse (2:1) overnight. The day the copulation plug was found was designated as day 1 of pregnancy. Day 4 of pregnancy was confirmed by recovering embryos from the uteri. The implantation sites on day 5 were visualized through intravenous injection of 0.3 ml of 1% Chicago blue dye (Sigma, St. Louis, Missouri) in saline. On days 6, 7 and 8, implantation sites are distinct and their identification does not require any special manipulation.

To determine the proliferation activity of stromal cells during peri-implantation, mice on days 4-8 of pregnancy were intraperitoneally injected with 5-ethynyl-2′-deoxyuridine (EdU, 5 mg/kg body weight) between 8:00 and 9:00, and were killed by cervical dislocation 4 h later. Uteri were removed to isolate implantation sites, which were fixed in 4% paraformaldehyde in PBS for 12-48 h. After dehydration in increased concentrations of ethanol, specimens were cleared in xylene and embedded in paraffin. Then, they were serially cut to 4μm-thick sections, which were mounted onto APES Plus slides for immunohistochemistry.

Isolation of mouse uterine stromal cells and inducing decidualization *in vitro*

Mouse uterine stromal cells were isolated and cultured as previously described with some modifications [10,17]. The uterine horns of 3-4 mice on pregnant day 4 were cut into 1-3-mm pieces. Tissue pieces were firstly digested in 3.2 ml of fresh medium containing 0.05% collagenase (Sigma, St. Louis, Missouri) and 0.5% trypsin (Sigma, St. Louis, Missouri) at 37°C for 20 min, then the supernatant was removed. The remaining tissue sedimentation was digested in the medium as the first step at 37°C for 20 min. The supernatants were collected to obtain the stromal cells. This process was repeated 3 times. Cells were plated on 12- or 6-well plates, containing phenol red-free DMEM/F-12 medium (Gibco, Carlsbad, California) with 10% charcoal-stripped fetal bovine serum (CS-FBS) and antibiotic. After culture for 3 h, the medium was replaced with fresh medium (DMEM/F-12, 1:1) with 10% CS-FBS to remove epithelial cells. Immunostaining of vimentin (sc-sc-373717; Santa Cruz Biotechnology, Santa Cruz, California; dilution 1:100) revealed that the isolated primary stromal cells exhibited nearly 98% purity. MPA combined with E2 were used to induce decidualization of the cells, which could be determined through observing their appearance changes and examining prolactin (PRL), a marker of decidual cells [18] at different time of the induction.

Immunohistochemistry and immunocytochemistry

The procedure was performed as described previously with some modifications [19]. Briefly, sections of uterine horns from all experimental animals were deparaffinized, hydrated and irradiated in the microwave oven for antigen retrieval. Nonspecific reaction was blocked by incubation in 10% non-immune serum. Sections were incubated with rabbit anti-cyclin G1 polyclonal antibody (sc-7865; Santa Cruz, dilution 1:100) primary antibody at 4°C for 16 h. Immunohistochemical staining was performed using biotinylated secondary antibody/horseradish peroxidase-conjugated streptavidin (SP-9001; Zhongshan Golden Bridge Biotechnology Co., LTD. Beijing, China) according to the manufacturer's instructions. Diaminobenzidine (DAB) staining was used to identify positive cells. The negative control was stained with nonspecific IgGs from the same isotypes instead of primary antibodies.

Cultured mouse stromal cells with induced decidualization were fixed at 0, 24, 48, 72 and 96 h after being induced decidualization with MPA and E2. Rabbit anti-cyclin G1 polyclonal antibody and rabbit anti-PRL polyclonal antibody (bs-0508R; Bioss Institute of Biotechnology, Beijing, China; dilution 1:1000) were used.

Western blot

Western blot was performed as described previously [20]. Cultured stromal cells were collected in RIPA buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate). Protein concentration was measured by the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Samples were run on a 12% polyacrylamide gel electrophoresis gel and transferred onto PVDF membranes. After being blocked with 5% non-fat milk in phosphate-buffered saline (0.01M, pH7.4) with 0.1% Tween 20 (PBST) for 1 h, the membranes were incubated with primary antibody overnight at 4°C. Rabbit anti-cyclin G1 polyclonal antibody (sc-7865; dilution 1:1000) and rabbit anti-β-actin polyclonal antibody (sc-130656; dilution 1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). After 3 washes in PBST for 10 min each, the membranes were incubated with matched goat anti-rabbit IgG horseradish peroxidase conjugate (sc-2054; Santa Cruz Biotechnology, Santa Cruz, California; dilution 1:5000) for 1 h followed by 3 washes in PBST for 10 min each. The signals were developed in enhanced chemiluminescence (ECL). The relative expression level of cyclin G1 in the Western blot was quantified using the analysis software Quantity One.

Real-time RT-PCR

Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously [21]. Briefly, total RNA from cultured cells was isolated using Trizol (Invitrogen, Carlsbad, California) according to the manufacturer's recommendations and 1 μg of total RNA was reverse transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). Real-time PCR analysis was performed using CFX 96 Real-time PCR Detection System (Bio-Rad, Hercules, California). Each reaction was performed in triplicate and consisted of 25 ng of cDNA, 1×Ssofast SYBR Green Supermix (Bio-Rad, Hercules, California), and 500 nM of gene-specific primers. The fold change in gene expression was calculated using the comparative cycle threshold method with the housekeeping gene GAPDH as the internal control. Relative gene expression data were calculated by the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used for quantitative PCR were: mouse cyclin G1 (186bp) sense: 5'-TTCTGTGCTGGCGCTATCTA-3', antisense: 5'-CCGTTAGGTTTGGAGCACTT-3'; mouse PRL(141bp) sense: 5'-AGCCAGAAATCACTGCCACT-3', antisense: 5'-TGATCCATGCACCCATAAAA-3', GAPDH (233bp) sense: 5'-GGTGAAGGTCGGTGTGAACG-3', antisense: 5'-CTCGCTCCTGGAAGATGGTG-3'.

siRNA Transfections Experiment

To ascertain the role of cyclin G1 on stromal cell decidualization, we employed RNA interference for knockdown of its gene expression. siRNA was used to specifically target cyclin G1 mRNA. Primary stromal cells were isolated from the uteri of day-4 pregnant mice as described previously and transfected with siRNA targeted specifically to the cyclin G1 mRNA. The annealed siRNA duplexes were transfected into cultured primary uterine stromal cells following the protocol of Lipofectamine RNAiMAX Reagent (In-

vitrogen, Carlsbad, California). Briefly, 4 µl of transfection reagent was mixed with 30 nmol of siRNA duplexes to form complexes and dispersed into 6-well cell-culture plates. The red fluorescence observed by fluorescence microscopy in the cytoplasm indicated that transfection was successful. The cell-cycle progression of the ESCs was assessed using flow cytometry. The specific siRNA targeting cyclin G1 (cyclin G1-siRNA: 5'-GGAGAAACGAUCUGAAUUUdTdT-3', 3'-dTdTCCUCUUUGCUAGACUUAAA-5') and non-specific control siRNA (control-siRNA: 5'-UUCUCCGAACGUGUCACGUdTdT-3', 3'-dTdTCUCCGUUCACGAGCUCUAU-5') were designed and synthesized by RiboBio (Guangzhou, China).

Flow cytometry

The experimental cells were suspended in cold 70% ethanol for 10 h at 4°C and then centrifuged at 1000 rpm for 5 min. The pellets were washed twice with cold PBS (0.01 M, pH7.4) and resuspended PBS, and incubated with 5 µl of RNase (20 µg/ml final concentration) at 37°C for 30 min. The cells were then chilled over ice for 10 min and stained with propidium iodide (PI, 50 µg/ml final concentration) for 5 min for analysis by flow cytometry. Flow cytometry was performed with an Epics Elite ESP instrument (Beckman Coulter, California). The samples were checked and the DNA histograms were further used for analysis of cell distribution at different stages of the cell cycle.

5-Ethynyl-2′-deoxyuridine(EdU) incorporation

To observe cell proliferation during early pregnancy *in vivo*, we employed 5-ethynyl-2′-deoxyuridine (EdU) incorporation. Mice were intraperitoneally injected with EdU (50 mg/kg body weight) 4 h prior to termination of pregnancy. The incorporation of EdU into DNA of actively dividing cells was determined using a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio Co. Ltd., Guangzhou, China). Briefly, after deparaffinization, samples were washed in 0.2% glycine for 10min, treated with 0.5% Triton X-100 in PBS for 20 min, washed in PBS for 10 min, then incubated with 1X Apollo reaction cocktail for 30min. Coupling of EdU to the Alexa Fluor substrate was then observed using fluorescence microscopy (Olympus IX71; Olympus, Tokyo, Japan).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)assay

To evaluate cell proliferation activity of cultured cell *in vitro*, cells were seeded in 96-well culture plates at a density of 1×10^4 cells/well. After 0-4 days (specific to experiment) of *in vitro* decidualization, the media were replaced with 110 µl of media containing 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] labeling reagent. After 4 h of incubation at 37°C, the media with MTT were then removed from the wells, and 100 µl of dimethyl sulfoxide (DMSO) were added. Absorbance at 570 nm was measured and converted to cell survival percentage as compared to cells without induced decidualization. Each measurement was performed in triplicate.

Statistical analysis

Statistical analysis was performed with SPSS17.0 program. Comparison of means was performed using the Independent-Samples t Test. Data are shown as means±SEM.

RESULTS

Spatial-temporal expression of the Cyclin G1 protein is coincident with stromal cell decidualization during early pregnancy

We examined the expression profile of the protein cyclin G1 in mouse uterus during early pregnancy by immunohistochemistry (Fig. 1, A-E). Cyclin G1 expression was undetectable in uterine stromal cells obtained from day-4 pregnant mice. Distinct cyclin G1 immunostaining was visible in the stromal cells of the PDZ surrounding the implanted embryo in the uterine sections of mice on day 5 of pregnancy. Later, on days 6 and 7, cyclin G1 signal decreased in the stromal cells in the immediate vicinity of the implanted embryo but was still observed in the proliferating predecidual cells in the SDZ, especially in the mesenteric cells. As pregnancy progressed to day 8, cyclin G1 expression in the SDZ decreased significantly, with only a low level of expression in the cells at the mesometrial side.

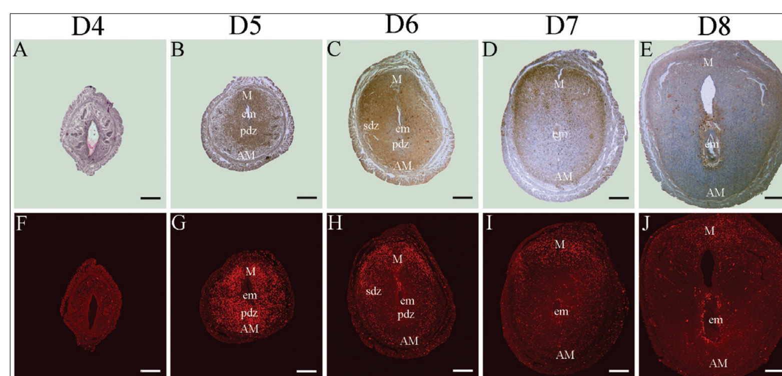

Fig. 1. The expression of cyclin G1 protein and cell proliferation activity in mouse uterus during peri-implantation. The photomicrographs of representative uterine sections are shown for cyclin G1. Brown staining indicates the localization of immunoreactive proteins (**A-E**). The red dot indicates proliferative cells (**F-J**). scale bar=125μm. M – Mesometrial pole; AM – anti-mesometrial pole; e – embryo; PDZ – primary decidual zone; SDZ – secondary decidual zone. These experiments were repeated at least three times with similar results.

The sections that were used for detecting EdU signals and cyclin G1 expression were serially cut from the same paraffin-embedded specimen. The results showed that on day 4 of pregnancy, EdU signals were only scattered in individual stromal cells, thus suggesting that only a few cells were in a proliferative state. On day 5, proliferative cells were mainly distributed in the PDZ surrounding the implanted blastocysts. On days 6 and 7, the EdU signals were mainly detected in the SDZ and also obvious in the cells at the mesometrial side. On day 8, only very weak signals focused on the mesenteric stromal cells in the SDZ, whereas the embryonic cell proliferation signal was obvious (Fig. 1, F-G). These results suggested the expression of cyclin G1 is spatiotemporal-specific in stromal cell decidualization and may be involved in regulating decidualization of the cells.

Cyclin G1 expression is coincident with MPA-induced decidualization *in vitro*

In the decidualization model of mouse endometrial stromal cells *in vitro*, the cells started to display the distinct morphological characteristics of decidual cells within 24 to 96 h of MPA and E2 treatment. (Fig. 2A). The cells became larger and rounder and the nuclei became larger as well, and the boundaries between cells were gradually unclear.

The result of real-time PCR analysis showed that the expression of PRL was significantly upregulated from 48 to 96 h after MPA and E2 treatment ($p<0.05$) (Fig. 2B). Likewise, immunocytochemistry results showed that PRL was expressed 48 h after *in vitro*-induced decidualization, with the staining intensifying from 48 to 96 h (Fig. 2C). Thus, the decidualization process was recapitulated morphologically and biochemically in primary cultured preimplantation uterine stromal cells *in vitro*.

The detection of cyclin G1 expression by immunocytochemistry and real-time PCR showed that before decidualization induction, cyclin G1 expression was low. After decidualization was induced, the cyclin G1 protein expression became more evident. It was significantly elevated from 24 to 96 h after *in vitro* induction of decidualization (Fig. 3, B and C). The expression of cyclin G1 was exactly consistent with the process of decidualization.

In the MTT assay, as shown in Fig. 3A, the level of proliferative activity was strongly increased from 24-48 h after decidualization compared with that of the control, keeping almost the same level as that at 48 h. This revealed that the stromal cell proliferation rate is fast in the early stages of decidualization *in vitro*. With the development of decidualization, the cell proliferation rate gradually decreased.

These results suggest that the expression of cyclin G1 is consistent with MPA-induced decidualization *in vitro*, as it is *in vivo*, and cyclin G1 may be involved in the regulation of the proliferation of decidual cells.

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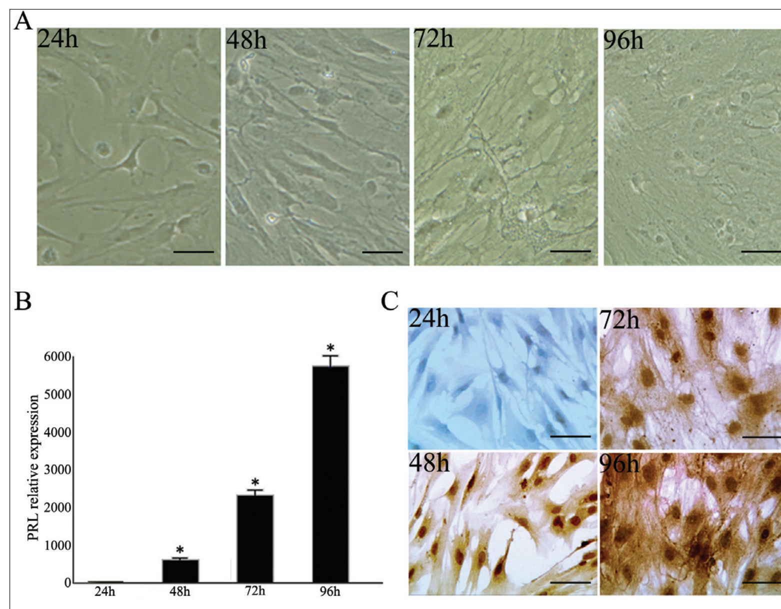

Fig. 2. Primary cultured mouse endometrial stromal cells induced to decidualization *in vitro*. **A** – stromal cells isolated from the mice at day 4 of pregnancy; the cells were plated and allowed to attach for 3 h; after attachment, the cells were collected at 24-h intervals, cultured in the presence of MPA and E2 and their morphology was examined under a microscope at different time. **B** – real time-PCR analysis to monitor PRL mRNA expression in the stromal cells cultured up to 96 h. * – The relative fold induction of PRL mRNA expression at each time point compared with that at 24 h sample is shown. ($p<0.05$). **C** – cells fixed and subjected to immunocytochemical staining using anti-PRL antibody. Brown staining indicates the localization of PRL. scale bar=100 μm.

The effect of cyclin G1 knockdown on the cell cycle of stromal cells *in vitro*

In siRNA transfection *in vitro*, the overall transfection efficiency was greater than 85% for 30, 50 and 100 nmol/L siRNA. A concentration of 30 nmol/L was selected as the experimental concentration (Fig. 4A).

We observed that the cells transfected with cyclin G1 siRNA exhibited more than 60% reduction in cyclin G1 mRNA expression compared with cells transfected with Luc siRNA at 24 h. At 36 h post-transfection, there was 75% reduction in cyclin G1 mRNA expression compared with cells treated with control siRNA. At 48 h, the cells transfected with cyclin G1 siRNA exhibited more than 87% reduction in cyclin G1 mRNA expression compared with cells transfected with control siRNA (Fig. 4B).

Western blot analyses revealed that the cells transfected with cyclin G1 siRNA exhibited a drastic decrease in cyclin G1 expression, whereas transfection with control siRNA did not affect this gene expression (Fig. 4C). At 48 h after transfection, the ratio of cyclin G1 in cyclin G1 siRNA and the control siRNA group was 0.43 and 0.98, respectively, based on the electrophoretic bands.

The result of flow cytometry to determine the cell-cycle profile was that the percentage of the cell in the cyclin G1 siRNA group distributed in each phase of the cell cycle changed significantly compared with the corresponding points in the control siRNA group. The number of cells in S stage in the cyclin G1 siRNA group were increased 24, 36 and 48 h post-transfection, whereas cell proportion in the G2/M phase was decreased in the group 24 h, 36 h, and 48 h after transfection compared to the control (Fig. 5, A and B).

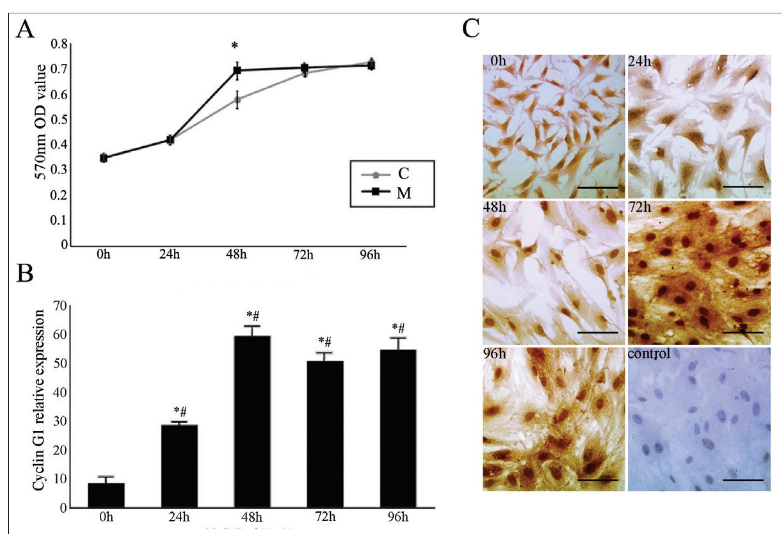

Fig. 3. The proliferation of stromal cells and their cyclin G1 expression in decidualization *in vitro*. **A** – MTT test showed the level of proliferative activity of stromal cells was strongly increased from 24 to 48 h after induced decidualization compared with that of the control, then it was almost kept at the same level as that at 48h. C – control group; M – decidualizing group *, compared with control (P<0.05). **B** – real time-PCR analysis to detected cyclin G1 mRNA expression in the stromal cells cultured up to 96 h. * – The relative fold induction of cyclin G1 mRNA expression at each time point compared with that of 0-h sample is shown. # – The relative fold induction of cyclin G1 mRNA expression at each time point compared with that of 24-h sample is shown (p<0.05). **C** – immunostaining of cyclin G1 in cultured stromal cells that were induced to decidualization *in vitro*. The photomicrographs of stromal cells are shown for cyclin G1. Brown staining indicates the localization of cyclin G1 proteins. Scale bar=100μm.

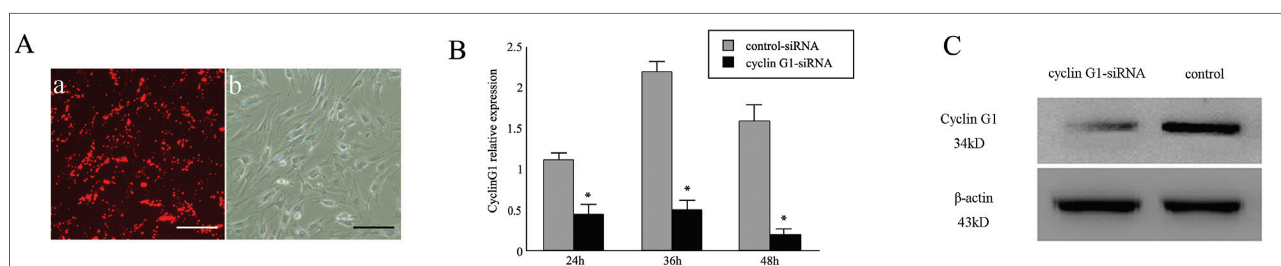

Fig. 4. RNA interference for knockdown of cyclin G1. **A** – fluorescence images showing the transfection efficiency of Cy5-siRNA in ESCs. a – the red fluorescent signal points to successful transfection of siRNA; b – cells under an ordinary light microscope; scale bar=100μm. **B** – RT PCR analysis of cyclin G1 mRNA at 24, 36 and 48 h after transfection. Cyclin G1 mRNA level in the cyclin G1-siRNA group was significantly weaker than in the control at 24, 36 and 48 h after transfection. * – compared with control-siRNA group, p<0.05. **C** – Western blot analysis of cyclin G1 at 48 h after transfection. The level of cyclin G1 in the cyclin G1-siRNA group was significantly weaker than in the control.

DISCUSSION

The process of decidualization in endometrial stromal cells (ESCs) is complicated. During the period of peri-implantation, the cells proliferate in an orderly manner, differentiate into decidual cells with polyploidy, and finally undergo apoptosis to offer adequate space for the growth of embryo [22]. As regards mice, the proliferation, differentiation and apoptosis events for the same regions or cells may overlap in time and space, as well as coordinate with each other, to ensure the development and implantation of embryos [23]. These complicated functional activities of the cells, which are associated with cell cycle, are obviously subject to the

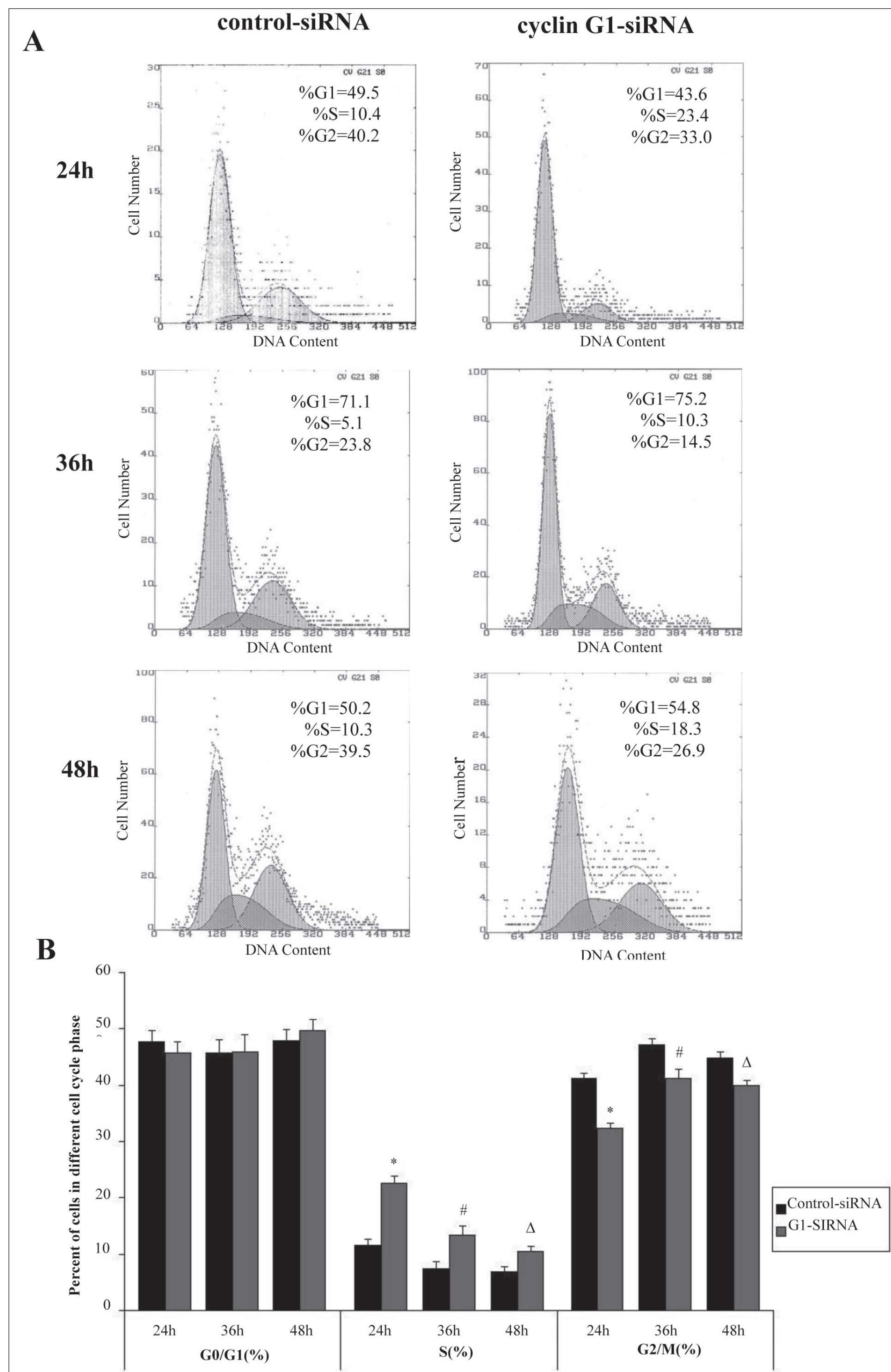

Fig. 5. Flow cytometric analysis of the cell cycle of endometrial stromal cells transfected with siRNA. Mouse stromal cells (from day-4 pregnant uterus) were transfected after plating for 24 h with siRNA (30 nM) which targeted cyclin G1, followed by decidualization induction. **A** and **B** – the cell population in the S phase treated with cyclin G1 siRNA is higher than in the control at 24, 36 and 48 h after transfection. The cell population in the G2/M phase treated with cyclin G1 siRNA is lower than that in control at 24, 36 and 48 h after transfection. * – compared with the control siRNA group after transfection $p<0.05$.

co-regulation of positive and negative factors. Generally, when stromal cells proliferate to a certain extent, some pro-differentiation and pro-apoptotic-related factors will be generated and/or activated to limit their further proliferation and promote their differentiation and apoptosis [20,24]. For example, heparin-binding EGF-like growth factor (HB-EGF) induced high expression of cyclin D3/CDK6 block cell cycle G2/M, which is related to the occurrence of polyploidy of decidual cells [10]. When cyclin A is downregulated, cyclin B/CDK1 may also cause G2/M phase blocking. Given the cyclin A/CDK2 synergy, DNA replication continues; however, the cells are not capable of dividing, with the decidual cells becoming polyploid [25].

As a special group of protein molecules in cell-cycle progression, most members of the cyclin family are positive regulators of the cell cycle in decidualization. Cyclin G1 is the only known cyclin to date that has dual positive and negative regulation on cell proliferation [26]. As a negative regulator of cell proliferation, cyclin G1 slows down the progression of cell cycle in endometrial epithelial cells by inducing G1 stage blocking to mediate the inhibitory effect of progesterone on the proliferation of endometrial epithelial cells [15]. It was reported that the expression of cyclin G1 mRNA has space-time characteristics during mouse implantation [16]. Our results showed that cyclin G1 was expressed in the same manner as its mRNA in spatial and temporal distribution during decidualization, and its expression is significantly consistent with the spatial and temporal development of decidualization. Meanwhile, the result of the EdU incorporation test showed that cyclin G1 was always expressed in distinct regions that had active cell proliferation. This suggests cyclin G1 may act as a regulator of cell proliferation in the decidualization of ESCs.

In our study, primary cultured ESCs *in vitro* were further used to detect cyclin G1 expression and cell proliferation. ESCs first experienced rapid proliferation at 24 h to 48 h of induced decidualization, followed by cell proliferation at a relatively stable state, when the shuttle-type stromal cells began to transform into polygonal decidual cells, which suggested that the cells had entered the differentiation process. Immunocytochemistry and real-time quantitative PCR were conducted to detect changes in cyclin G1 expression during this process. Results showed that cyclin G1 expression was significantly increased after induced decidualization, which was also consistent with the temporal characteristics of active cell proliferation. This trend further confirms the relationship between cyclin G1 expression and cell proliferation in decidualization. The combined results of *in vivo* and *in vitro* experiments imply that cyclin G1 is mainly involved in the regulation of cell proliferation during decidualization.

During mouse endometrial decidualization, some pro-differentiation and pro-apoptotic-related factors would be activated only after stromal cells proliferate to a certain degree, and this activation could further limit the proliferation of stromal cells and cause their apoptosis and degradation to provide adequate space for the growth of the embryo [9,24]. To prove the role of cyclin G1 on cell proliferation in decidualization, RNA interference was applied to inhibit the cyclin G1 expression in ESCs' induced decidualization. At 24, 36 and 48 h after the transfection of specific cyclin G1-siRNA, the stromal cells in phase S significantly increased, whereas the stromal cells in the G2/M phase decreased compared with those transfected with control siRNA at the same time points. This fact indicates that cyclin G1 inhibits cell proliferation of stromal cells in decidualization *in vitro*. The cells were blocked at G2/M, thus demonstrating the role of cyclin G1 as a negative regulator of the cell cycle of stromal cells.

As regards previous studies, cyclin G1 is highly expressed in well-differentiated tissue, such as skeletal muscle, brain and kidney tissue. As a negative cell-cycle regulator, it inhibits cell growth [26,27]. Cyclin G1 also serves as a negative regulator of cell proliferation in endometrial epithelial cells, and in progesterone-mediated inhibition of endometrial epithelial cell proliferation [15]. These results also support our conclusions based on the RNA interference experiments that cyclin G1 serves as a negative regulator in cell proliferation.

CONCLUSION

In our current study, we observed that cyclin G1 served as an important negative regulator, coordinating the biological behavior of endometrial epithelial

and stromal cells during peri-implantation. On the one hand, cyclin G1 inhibited the proliferation of endometrial epithelial cells for their differentiation into cells with a secretory function, while in actively proliferating stromal cells, it antagonized the roles of cell-proliferation promotors to inhibit the proliferation of stromal cells and promote them to differentiate into decidual cells that enable embryo implantation.

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Conflict of interest disclosure: The authors confirm that the content of this article bears no conflict of interest that would prejudice the impartiality of the information.

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