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석사학위 청구논문

Cdk2ap1 regulates Proliferation and Decidual Differentiation of Mouse Stromal Cells during Implantation

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성신여자대학교 대학원

생 물 학 과

변 지 현

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이 논문을 석사학위논문으로 제출함.

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생물학과

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논문개요

임신과정 동안 자궁은 착상 위치, 배발생 지원 등을 위한 세포의 증식, 분화, 사멸과 같은 극적인 변화를 겪는다. 이러한 변화에는 '탈락막화' 과정을 포함하는데, 이 과정은 성공적인 배아의 착상과 모체의 보호를 위해 자궁내막이 형태적, 기능적으로 변화하는 것이다. 생쥐의 경우, 착상이 일어나기 전인 임신 3일차까지는 자궁내막의 상피세포가 증식하고, 착상이 일어나기 시작하는 임신 4일차부터는 상피세포의 증식은 멈추고, 기질세포의 증식이 시작된다. 임신 5일차에 탈락막 반응이 시작되며 착상중인 배아 주변의 기질세포들이 활발히 증식한다. 임신 6일차에 배아 부착 부분과 가까운 기질세포는 세포 증식을 멈추고 1차 탈락막 부위를 형성하게 된다. 이후 이 부분은 증식을 멈추고 세포사멸이 일어나는 반면, 그 바깥쪽에 있던 세포는 계속 증식하여 2차 탈락막 부위를 형성하게 된다. 탈락막 세포는 배아 발달에 필요한 성장인자나 사이토카인을 공급하는 역할을 한다. 탈락막의 퇴화는 성공적인 태반 형성과 배아가 정상적으로 발달할 수 있는 공간 형성을 위해 중요하며, 매우 정교하게 조절되는 과정이다. 이러한 기질세포의 탈락막으로의 분화는 여러 요인의 조절 하에 있는데, 그 중 발생과정에서 적절한 수준의 세포 증식이 분화의 기준으로써, 세포 증식의 조절이 탈락막 분화의 중요한 요인으로 여겨질 수 있으나, 아직 그 조절 기작이 잘 밝혀져 있지 않다. Cdk2ap1은 구강상피세포에서 처음 밝혀졌고, 구강암세포 연구를 통해 성장저해인자 기능이 있음이 밝혀졌다. Cdk2ap1은 cdk2의 활성을 억제하여 세포 증식을 저해하는데, 대단위 유전자 발현 연구를 통해 자궁에서도 동정되었다. 따라서, 본 연구에서는 생쥐 배아의 착상 기간 동안 자궁에서의 Cdk2ap1 유전자의 발현 양상을 mRNA 수준과

단백질 수준에서 확인하였고, 탈락막 과정과 관련하여 어떠한 기능이 있는지 살펴 보았다. 실시간 중합효소 연쇄반응 (Real-time PCR)을 통해 분석한 결과, 임신 1일차의 자궁내막조직에서는 *Cdk2ap1* mRNA의 발현 수준이 낮았고, 2일차에서 증가하기 시작하여 5, 6일차까지 높게 유지되었다. 반면 탈락막화 과정의 후반기인 임신 7일차부터 다시 *Cdk2ap1* mRNA의 발현 수준이 감소되었고, 태반이 형성되는 12일차에 다시 약간 증가하였다. Cdk2ap1 발현도 임신 시기 동안 관찰된 전사 변화와 유사하게 착상의 시작, 탈락막의 형성과 쇠퇴 양상과 일치하였다. 면역형광법을 통해 Cdk2ap1 단백질의 발현 부위를 시기적으로 관찰한 결과, 착상전인 3일차까지는 기질세포에서 약하게 표지되었고, 착상시기인 4, 5일차에는 기질세포에서 표지되는 양과 범위가 증가하였다. 6일차에는 1차 탈락막 세포에 주로 표지되었고, 7일차에는 2차 탈락막 세포에 주로 표지 되었다. 탈락막이 소실되고 태반이 나타나는 시기에는 태반이 형성되는 부위에 약하게 표지되었다. 체외에서 자궁내막 기질세포의 1차세포배양시 E_2와 P_4를 처리하여 탈락막 반응을 유도하면서, *Cdk2ap1* shRNA를 형질주입 했을 경우, 대조군에 비하여 자궁내막세포의 수는 유의하게 증가하였고, 탈락막 세포로 분화하는 비율은 상대적으로 적었다. 반면에 Cdk2ap1 발현 DNA를 형질주입 했을 경우, 대조군에 비하여 자궁내막세포의 수는 유의하게 감소하였고, 탈락막 세포로 분화하는 비율은 크게 증가하였다. 이러한 결과를 바탕으로 탈락막 반응이 진행되는 시기에 Cdk2ap1은 기질세포의 증식을 억제함으로써 기질세포가 탈락막으로 분화하게 하는 요인임을 제안한다.

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INTRODUCTION

Implantation is a complex developmental process in mammals and is vital to continued embryonic development within uterus by achieving successful pregnancy. Synchronized development between the embryo to the active stage of the blastocyst and uterine differentiation to the receptive state, and a 'cross talk' between the blastocyst and uterine luminal epithelium are essential to these processes (Das et al., 2009).

During the pregnancy, the cells of the endometrium undergo remarkable remodeling. Luminal and glandular epithelial cells proliferate on days 1 and 2 of pregnancy and cease on day 3. Subsequently, stromal cells and endothelial cells proliferate (Herken et al., 1983). Proliferation of stromal cells is further enhanced on day 4 in response to ovarian estrogen secretion before the initiation of implantation (Huet-Hudson et al., 1989; McCormack et al., 1974). On day 4 of pregnancy, the initiation of blastocyst implantation, the attachment of the blastocyst to the uterine epithelium, induces proliferation of stromal cells surrounding the implanting blastocyst (Dey et al., 1996) and triggers the process of decidualization.

Decidualization is functional and histological changes including cell proliferation, differentiation and apoptosis. By contrast, luminal epithelial cells only at the site of blastocyst apposition progressively undergo apoptosis with the succession of implantation. In the case of mouse, decidualization is normally initiated at the antimesometrial area, and then expands to the mesometrial region (Correia-da-Silva et al., 2005). Between the day 5 afternoon and the day 6 morning, stromal cells immediately surrounding the implanting blastocyst cease proliferation and differentiate into decidual cells, forming the primary decidual zone (PDZ). The PDZ is avascular and epithelioid in nature. The cells at the PDZ subsequently undergo apoptosis and by day 8 most of these cells disappear. However, stromal cells next to the PDZ continue to proliferate and differentiate into polyploid decidual cells forming the secondary decidual zone (SDZ) (Kleinfeld et al., 1983). This pattern persists through days 7 and 8. Eventually the SDZ cells also undergo apoptosis. At later stages, mitosis and development of polyploidy continue in the decidual tissue, but little DNA synthesis or mitosis occurrs in the basal zone of the stroma adjacent to the myometrium (O'Shea et al., 1983). Therefore, the regulation of epithelial and stromal cells proliferation must be properly controlled to retain their roles in the endometrium.

These series of events involving proliferation, differentiation and apoptosis in uterus during implantation have been suggested that cell cycle regulatory molecules play potential roles. But information regarding participation of uterine cell cycle molecules during implantation and decidualization is largely unknown.

Cdk2ap1 [synonyms: $p12^{DOC-1}$, $p12^{CDK2AP1}$, Deleted in oral cancer 1 (Doc-1)] is a growth suppressor identified and isolated from normal keratinocytes (Todd et al., 1995). It is a highly conserved cellular gene. Cdk2Aap1 cDNA has cloned from human, mouse, and hamster. The full-length human and mouse Cdk2ap1 cDNAs are 1.6 kb and 1.2 kb, respectively. Human Cdk2ap1 has one additional amino acid at residue 19, which corresponds to an alanine, and differs from the mouse and hamster Cdk2ap1 at only two other amino acid residues (Ala3Thr at residue 8 and Gly3Ser at residue 100). Human and rodent Cdk2ap1 polypeptides have 97% identity, and the mouse and hamster Cdk2ap1 protein sequences are identical. Human Cdk2ap1 is a 115-amino-acid peptide with a molecular mass of 12.4 kDa (pI, 9.62) (Gordon et al., 1992; Todd et al., 1995; Tsuji et al., 1998).

Ectopic expression of Cdk2ap1 in squamous carcinoma cells led to the reversion of transformation phenotypes including anchorage independence, doubling time, and morphology. Also it alters cell cycle, adhesion, invasion, angiogenesis, and apoptotic gene expression (Daigo et al., 1997; Matsuo et al., 2000; Zolochevska et al., 2009). Cdk2ap1 affected primarily the initiation of DNA replication, not elongation. Cdk2ap1 inhibits DNA replication by binding to DNA polymerase α/primase or negatively regulates cyclin-dependent kinase 2 (CDK2) activities by sequestering the monomeric (inactive) pool of CDK2 and by targeting CDK2 for proteolysis. (Matsuo et al., 2000; Shintani et al., 2000).

In previous studies, Cdk2ap1 mRNA is detected using PCR-select cDNA subtraction in an embryo-implanting uterus (Cheon., 2002) and differential-display RT-PCR (DD RT-PCR) in a delayed-implantation uterus (Lee et al., 2003). These data suggest that Cdk2ap1 may be involved in decidualization. However, Cdk2ap1 is not well characterized with the expression and functional role in the pregnant uterus. In this study, we were profiled spatio-temporal mRNA and protein expression of Cdk2ap1 using real-time PCR (RT-PCR), western blot and immunofluorescence. Using *in vitro* primary endometrial stromal cell culture model for decidualization and technique of transfection with either Cdk2ap1 shRNA plasmid or Cdk2ap1 expression construct, the functional roles of Cdk2ap1 during implantation were examined.

MATERIALS AND METHODS

Experimental animals

All experiment involving animals were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. Animals were maintained under standard condition at Sungshin Women's University diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water ad libitum from weaning at 21 days after birth.

Uterus sampling

Six to eight-weeks old CD-1 females were mated with fertile males of the same strain to induce pregnancy and were checked for vaginal plugs on the following morning. The day on which the vaginal plug was noted was considered as day 1 of pregnancy. The mice were sacrificed to collect uteri on day 1, 2, 3, 4, 5, 6, 7, 9 and 12 of gestation. Pregnancy on day 1–12 was confirmed by recovering embryos from the reproductive tracts at from 10:00 to 11:00 AM. The embryos were removed from oviducts or uteri by flushing with DEPC-treated PBS (PBS).

Total RNA extraction

Total RNAs of uteri were extracted using TRIzol reagent (Invitrogen, San Diego, CA, USA). Briefly, the uterine tissues in TRIzol reagent (100mg/1ml) were homogenized and stored for 10 min at room temperature (RT). The chloroform of 0.2 ml/1 ml TRIzol reagent were added to the homogenates and vigorously shaken for 15 sec. After then, the mixture kept for 15 min at RT and centrifuged 12,000g for 15 min at 4℃. The clear supernatant was transferred to new tube, added 0.5 ml isoprophanol per 1ml TRIzol reagent, mixed softly, kept for 10 min at RT, and centrifuged 12,000g for 8min at 4℃. The supernatant was removed, added 1 ml DEPC-treated 75% ethanol to wash, mixed by inverting, and centrifuged 7,500g for 5 min at 4℃. The supernatant was removed, dried to remove ethanol about 4 min and added 50 ul DEPC-treated water. Quantity of total RNAs was measured up using NanoDrop 2000/2000c Spectrophotometer (Thermo, Wilmington, DE, USA).

First strand cDNA synthesis

In order to reverse-transcription, 5 µg total RANs were used. First strand cDNAs were synthesized using Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA) according to the manual of manufacture. Briefly, reaction reagents were 5 µg total RNA, 5.0 µl Accuscript buffer (10x), 1.0 µl oligo dT primer (0.5 µg/µl), 1.0 µl random primers (0.1 µg/µl), 2 µl dNTP mix (100 mM), RNase-free water. Reaction mixture was incubated at 65℃ for 5min, placed the tube at RT to allow the primers to anneal to the RNA for 10min, after then added 4.0 µl DTT (100 mM), 2.0 µl RNase block ribonuclease inhibitor (40 U/ml), 1.0 µl Accuscript multiple temperature RT. The mixture was incubated at 42℃ for 1 hr and 70℃ for 15 min.

Real-time PCR analysis

Real-time PCR was performed using SYBR Premix Ex Taq^{TM} II and Thermal

Cycler Dice Real Time System TP800 (TaKaRa, Tokyo, Japan). Each reaction was run in triplicate and consisted of 1.0 μl cDNA, 10 μl SYBR Premix Ex Taq^{TM} and 10 pM of the primers listed in Table 2. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping gene, a ribosomal protein, 36B4, as the internal control: $\Delta\Delta Ct = \Delta Ct\ (Target_{treat}\text{-}36B4_{treat}) - \Delta Ct\ (Target_{control}\text{-}36B4_{control})$.

Protein extraction and Western blotting analysis

Before protein extraction, tissue was washed using cold Y-PBS (0.7 mM PMSF, 1 mM Benzamidine-HCl, 4 μg/ml Leupeptin, 2 μg/ml Aprotinin, 2 mM EDTA). Uterine tissues were homogenized in cold homogenization buffer (50 mM Tris-Cl, 150 mM NaCl, 10mM β-mercaptoethaol, 2 mM $CaCl_2$, 0.1 mM PMSF, 1 μM Leupeptin, 1 μM Pepstatin, 0.5 mM EDTA, 15% Glycerol, 0.1% NP-40). The homogenates were centrifuged to remove insoluble materials. The protein concentration was determined using protein dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA) by Bradford assay. 1 mg/ml of protein were boiled in SDS/β-mercaptoethanol sample buffer, and loaded onto each lane of 10% SDS-PAGE. The proteins were separated by electrophoresis and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Richmond, CA) in transfer buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS, 20% Methanol, pH 8.3). The membranes were blocked in 5% skimmed dry milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 hr at RT, and washed three times with TBST. The membranes were incubated for 30 min with rabbit polyclonal Cdk2ap1 antibody (My biosource, USA, diluted 1:500); mouse polyclonal 36B4 antibody (Abcam, Cambridge, UK, diluted 1:500). After incubation, membranes were washed three times and incubated for 50 min with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA, diluted 1:2,000); goat anti-mouse IgG (Santa Cruz Biotechnology, CA, USA, diluted 1:2,000), and washed three times. The bands were detected using ECL solution (GE Healthcare, Little Chalfont, UK) by Kodac Image Station 4000MM PRO.

Immunofluorescence

Dissected mouse uterine horns were cut into 0.5 cm segments, fixed in 4% paraformaldehyde containing 0.16% picric acid for 24 hr and embedded in paraffin. 4 μm sections were mounted on glass slides. After deparaffinization, blocking was performed using PBS containing 1% normal rabbit serum in PBS with 0.1% BSA for 1 hr and then incubated with the Goat polyclonal Cdk2ap1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:200) 1 hr at room temperature. After washing in 0.1%, tissues were incubated with Cy3 conjugated affinipure Rabbit anti-Goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, diluted 1:250) in PBS with 0.1% BSA for 1hr at room temperature. After washing in PBST, tissues were counterstaining with Hoechst 33258(dilution 1:500) for 10 min. Slides were washed and mounted. Specific signal of Cdk2ap1 was observed under the fluorescence microscope (OlympusBX60).

Primary endometrial cells culture

For *in vitro* decidualization of mouse endometrial stromal cells (mESCs), female mice were sacrificed 48 hr post 5 IU/0.1 ml PMSG injection. Uteri cut into

1-mm^3 pieces and incubated in DMEM:F12 supplemented with 2 mg/ml Collagenase Type I (Gibco, Rockville, MD, USA) and 100 μg/ml penicillin/streptomycin (Sigma-Aldrich Corp., St. Louis, MO) for 2 hr at 37℃ in shaking incubator with 150 RPM. After incubation, the solution was passed through 100 μm and 40 μm sieve (BD Falcon, BD Biosciences, San Jose, CA) and the centrifuged at 1000 rpm for 5 min. The mESCs were then resuspended in DMEM:F12 supplemented with 10% charcoal dextran-stripped FBS (cFBS; Sigma-Aldrich Corp., St. Louis, MO). mESCs were plated in 4-well cell culture plates onto glass cover slip at 1×10^5 cells per well and 6-well cell culture plates at $1\text{x}10^6$ per well for immunocytofluorescence and extraction of RNA and protein, respectively. After 30min, medium was aspirated, and fresh media containing 15 nM 17β-estradiol (E_2) and 5% cFBS. Control samples received no hormone supplementation. After 24hr, medium was changed to DMEM:F12 with 1% cFBS without steroid hormone. The next day, transfection was performed using DMEM:F12 with 1% cFBS without antibiotics. After 24 hr, the cells were harvested at 0 hr, 24 hr, 48 hr, 72 hr, 96 hr and 120 hr, respectively.

Construction of plasmid and Transfection

To get *Cdk2ap1* PCR fragment, R1 mouse embryonic stem cell cDNA was used. The following custom primers containing *BamHI* and *XhoI* sites at their 5' and 3’ termini respectively, were used for PCR amplification: 5’ – CCG CTC GAG ATG TCG TAC AAG CCG AAC– 3’ and 5’ – CGC GGA TCC GGA CCT GGC ATT CCG TTC– 3’. The resulting PCR fragment was digested with *BamHI* and *XhoI* and cloned into *BamHI*- and *XhoI*- digested pcDNA 3.1 vector (Invitrogen, Calsbad, CA, USA). The construct was verified by sequencing. Cdk2ap1 shRNA plasmid was purchased (Santa Cruz Biotechnology, CA, USA).

Each of the DNA were transfected into mESCs grown in 6-well plates(plated at 1 × 10^6 cells per well 24 hr before transfection). Transfection was performed using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA).

Proliferation and differentiation analysis of endometrial stromal cells

To evaluate the proliferation of stromal cells, immunofluorescence was performed using ki-67 specific antibody. Briefly, fixed cells using 4% paraformaldehyde containing 0.16% picric acid were incubated with 5% normal rabbit serum in PBS cantaining 0.3% Triton-X100 with 0.1% BSA for 20min and then incubated with the mouse monoclonal ki-67 antibody (NovoCastra, Newcastle, UK, diluted 1:200) overnight at 4℃. After washing in PBS with 0.1% BSA, cells were incubated with Cy3 conjugated affinipure Rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, diluted 1:500) in PBS with 0.1% BSA for 45min at room temperature. After washing in PBS with 0.1% BSA, cells were counterstaining with Hoechst 33258 (diluted 1:200) for 20 min. Slides were washed and mounted. Specific signal of Ki67 was observed under a confocal laser microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany).

Differentiation of endometrial stromal cell was evaluated with real-time PCR using decidual PRL-related protein (dPRP), decidua cells marker.

Statistics

The t-test was used to evaluate the difference between control and experiment group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered significant.

Table 1. Sequence of primers

Gene	**NCBI gene reference**		**Primer sequence (5’-3’)**	**Amplified length (bp)**
Cdk2ap1 *(for Construct)*	NM_013812.2	S	CCGCTCGAGATGTCGTACAAGCCGAAC	342
		AS	CGCGGATCCGGACCTGGCATTCCGTTC	
Cdk2ap1 *(for* real-time PCR*)*	NM_013812.2	S	TGGCTGAAACGGAACGGAAT	277
		AS	GGGGACAAACAATAGCTTGCTG	
dPRP	AF011385.1	S	CTGCTGGTGGTTTCAAACTTGC	281
		AS	GGTGGGTTTGTGACATTAGAGTGG	
36b4	NM_007475	S	CGACCTGGAAGTCCAACTACTTCCT	303
		AS	ATGCTGTTGGCCAATAAGGTGC	

Table 2. Thermal cycler schedule

Step	Temperature (℃)	Time	Cycles
Initial cycle	94	5min	1
	59	30sec	
	72	1min	
Denaturation	94	1min	37
Annealing	59	30sec	
Extension	72	1min	
Final cycle	94	1min	1
	59	30sec	
	72	7min	
Hold	4	Indefinitely	

RESULTS

Expression of *Cdk2ap1* mRNA and protein in mouse uteri during pregnancy

To investigate the quantitative expression level of *Cdk2ap1* mRNA during pregnancy, real-time PCR analysis was performed. The relative quantity of *Cdk2ap1* mRNA was very low on day 1 of pregnancy. Its quantity was significantly increased after day 2 of pregnancy. It peaked on day 3 of pregnancy and decreased continuously until on day 9 of pregnancy. It increased slightly to on day 12 (Fig. 1).

The profiles of Cdk2ap1 protein was analyzed by Western blot. The normalization was performed with β-actin protein. Cdk2ap1 on day 1, 2, 3 of pregnancy was detected in low level and increased after day 4 of pregnancy. It decreased after day 7 (Fig. 2A,B).

From these results, it suggested possible roles of Cdk2ap1 protein that part in the change in the uterine remodeling during pregnancy.

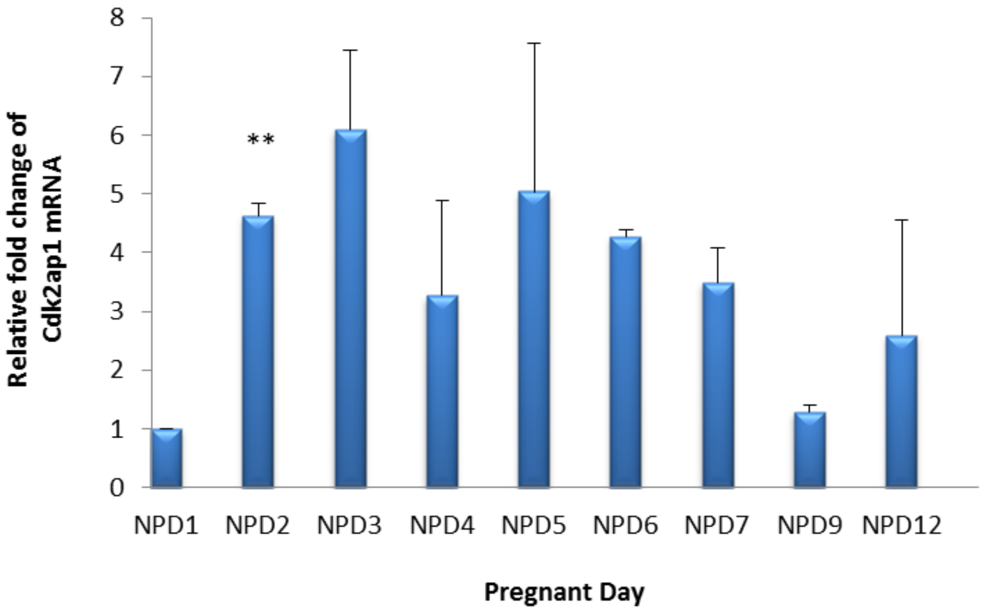


Figure 1. Profiles of *Cdk2ap1* mRNA expression level in mouse uteri during pregnancy.

The relative quantity of *Cdk2ap1* mRNA. Total RNA was extracted from the uteri on day 1, 2, 3, 4, 5, 6, 7, 9 and 12 of gestation. Values represent the mean ± SEM.

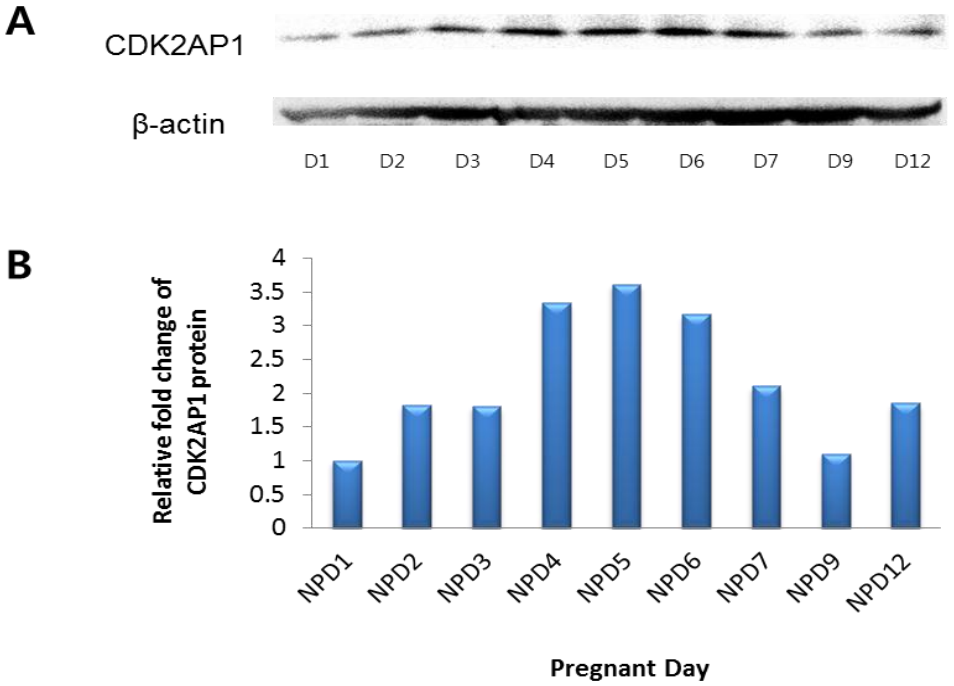


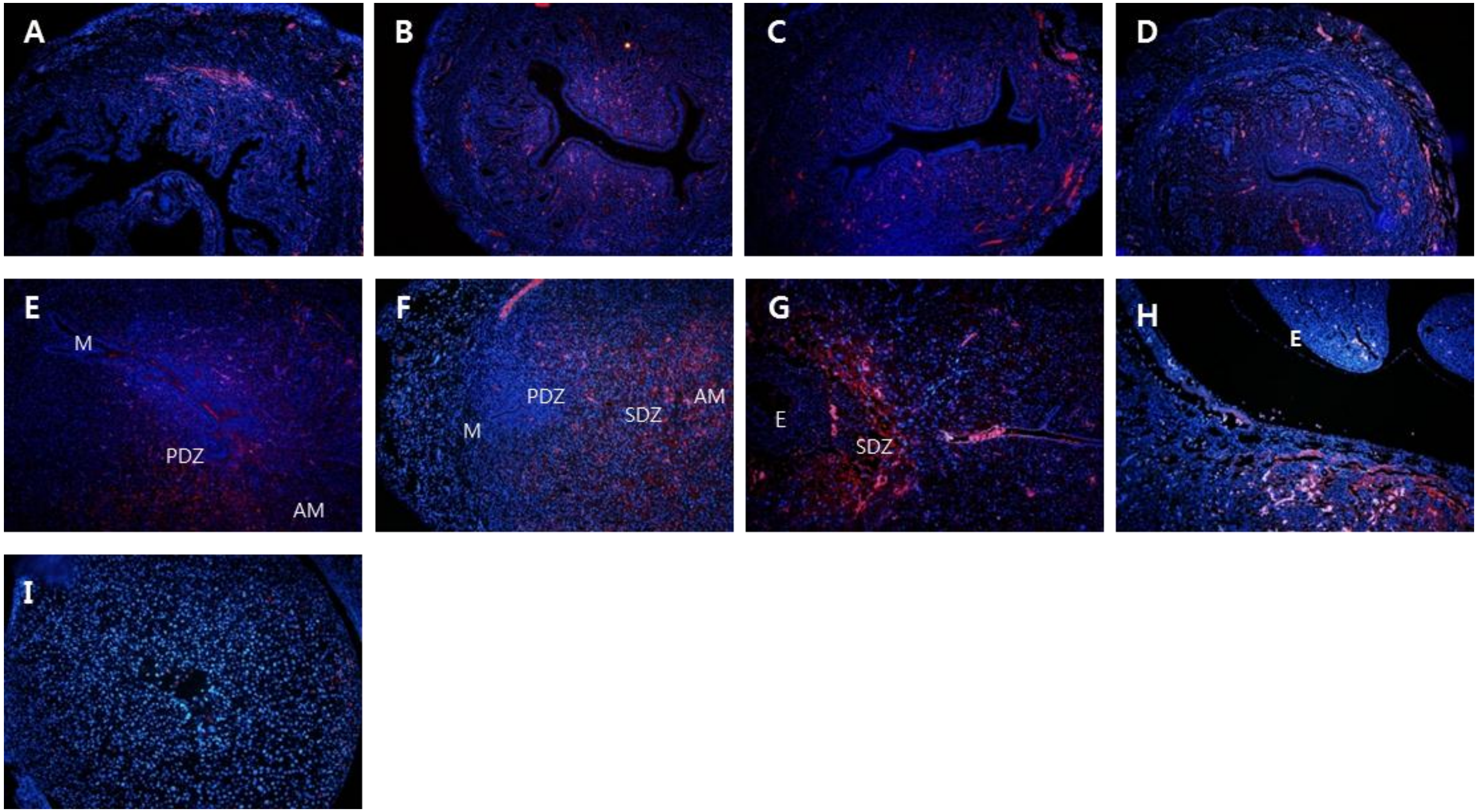
Figure 2. Profiles of Cdk2ap1 protein expression level in mouse uteri during pregnancy.

A. The Cdk2ap1 protein produced in the mouse uterus during pregnancy was detected by Western blot.

B. Cdk2AP1 protein level normalized using β-actin protein level as the housekeeping control. Values represent the mean ± SEM.

Localization of Cdk2ap1 protein in decidua

To evaluate the cell specific expression and stage-dependent expression, immunohystochemistry was performed with Cdk2ap1 specific antibody. Cdk2ap1 was weakly detected from day 1 of gestation (Fig. 3A). Until day 5, Cdk2ap1 was mainly localized in stromal cells next to the epithelial cells (Fig. 3B,C,D). On day 6, its expression was localized in primary decidua zone (PDZ) (Fig. 3E). On day 7, its expression was localized restricted in secondary decidua zone (SDZ) (Fig. 3F). Placental differentiating area was also showed weak Cdk2ap1 intensity (Fig. 3 G,H)



A
B
C
D
E
M
PDZ
AM
F
PDZ
SDZ
AM
M
G
E
SDZ
H
E
I

Figure 3. Localization of Cdk2ap1 protein in decidua

A, day 1; B, day 3; C, day 4; D, day 5; E, day 6; F, day 7; G, day 9; H, day 12; I, negative control on day 9. AM, antimesometrium; M, mesometrium; PDZ, primary decidual zone; SDZ, secondary decidual zone; E, embryo (Magnification A-J, x 100)

Effect of Cdk2ap1 overexpression on proliferation and differentiation of endometrial stromal cells

To gain insight into the potential regulator function of Cdk2ap1 in proliferation and differentiation, the open reading frame (ORF) of Cdk2ap1 was recloned in-frame into the BamHⅠ-XhoⅠsites of pcDNA3 (Fig. 4A). The transfection with Cdk2ap1 expression DNA was verified by western blot (Fig. 4B,C).

The first step of decidualization is proliferation of stromal cells. So we performed immunocytofluorescence in order to examine the expression of the ki-67, which was used as an indicator of proliferation(Fig. 5A). The ratios of ki-67 positive cells were significantly decreased in Cdk2ap1 overexpressed groups in all check-point-times (Fig. 5B). These results mean that Cdk2ap1 can suppress the proliferation of stromal cells

The expression levels of decidual-related prolactin (dPRP) specific mRNA were significantly high in the group of Cdk2ap1 overexpression transfected with Cdk2ap1 expression DNA and inducted decidual after 24 hr compared with that of the control or decidual inducting group (Fig. 6).

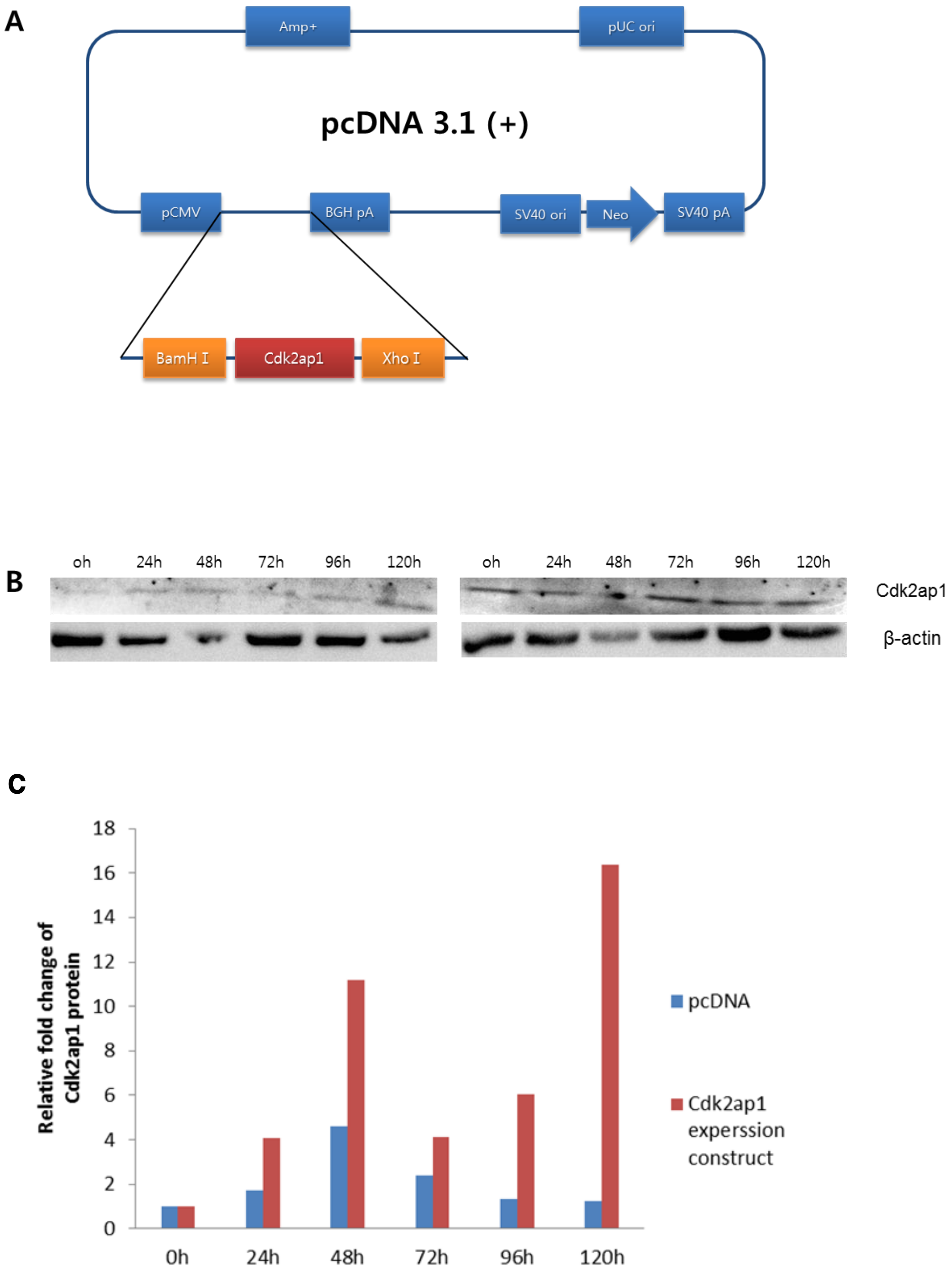
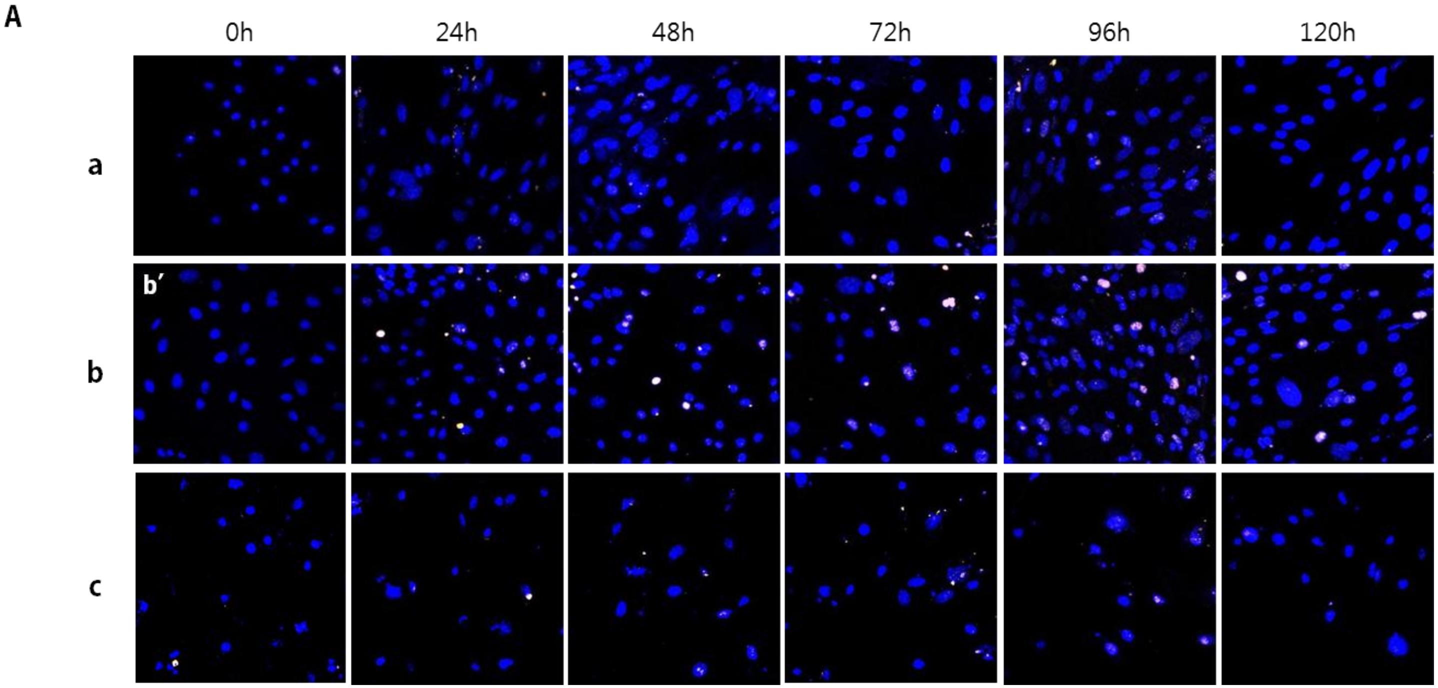

A
Amp+
pUC ori
pcDNA 3.1 (+)
pCMV
BGH pA
SV40 ori
Neo
SV40 pA
BamH I
Cdk2ap1
Xho I
B
oh
24h
48h
72h
96h
120h
oh
24h
48h
72h
96h
120h
Cdk2ap1
β-actin
C
Relative fold change of Cdk2ap1 protein
18
16
14
12
10
8
6
4
2
0
0h
24h
48h
72h
96h
120h
pcDNA
Cdk2ap1 experssion construct

Figure 4. Overexpressions of Cdk2ap1 in endometrial stromal cells transfected with Cdk2ap1 expression construct.

A. Diagram of the Cdk2ap1 expression construct.
B. Western blot of stromal cells transfected with the Cdk2ap1 expression construct (right) and empty vector (left).
C. Cdk2AP1 protein level normalized using β-actin protein level as the housekeeping control.


A
0h
24h
48h
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a
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b′
c

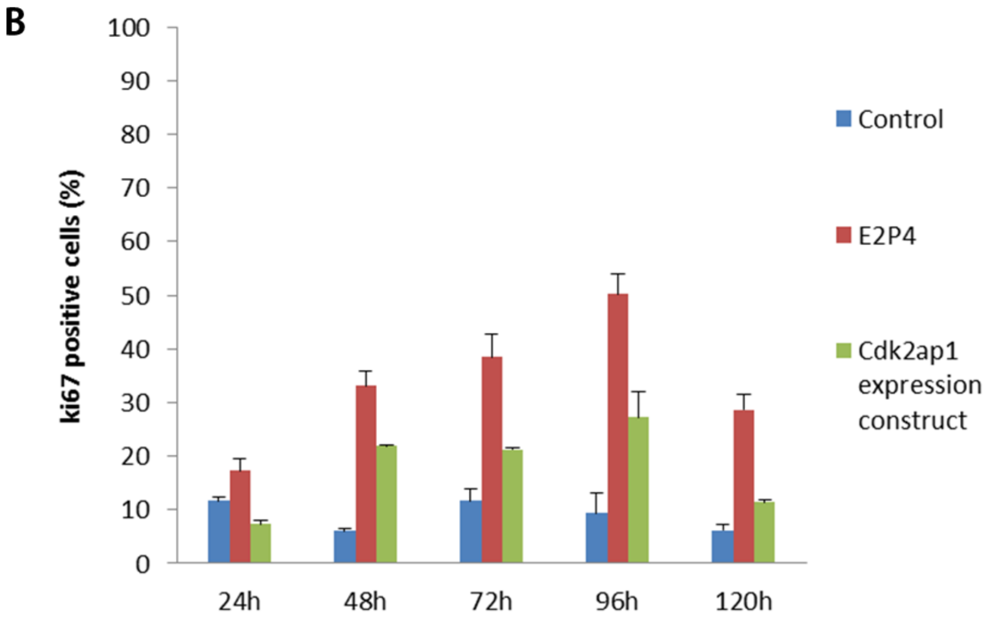

B
ki67 positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
24h
48h
72h
96h
120h
Control
E2P4
Cdk2ap1 expression construct

Figure 5. The Cdk2ap1 overexpression suppressed the proliferation of endometrial stromal cells.

A. Photoimmunofluorescence micrograph of in vitro decidualizing cells. ki-67 specific antibody was used to identify the proliferating cells (orange) and Hoechst33258 was used as control (blue). Immunofluorescence analysis was performed of 0, 24, 48, 72, 96, 120 hr stromal cells transfected with Cdk2ap1 expression construct. Control (a), decidualization induction with E2P4 (b) transfection with Cdk2ap1 expression DNA (c), negative control (b'). Data in the graph represent percentage and as the mean ± SEM of time independent experiments. Magnification; x 200.

B. The relative ratio of ki-67 positive cells number explained the ratio between ki-67 positive and total cells. Data in the graph represent percentage and as the mean ± SEM of time independent experiments.

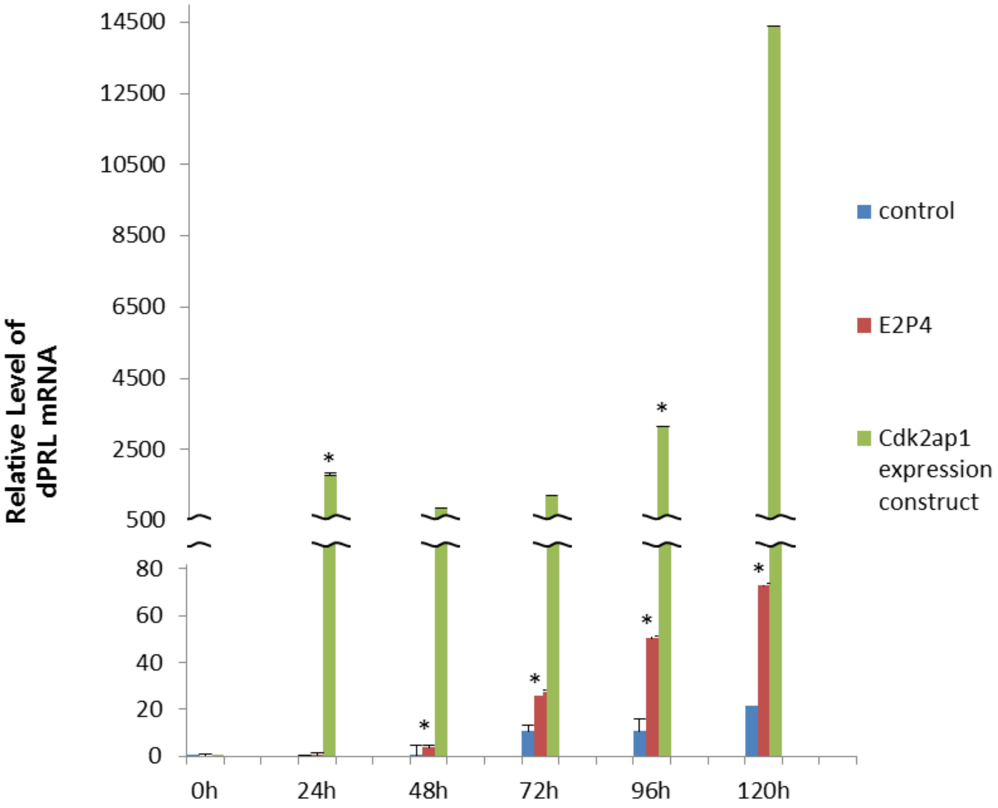


Figure 6. The Cdk2ap1 overexpression enhanced the differentiation of endometrial stromal cells.

Real-time PCR results of Cdk2ap1 overexpression on the differentiation of stromal cells to decidual cells. The expression level of decidual-related prolactin (dPRP) was dramatically increased in Cdk2ap1 overexpressed cell. Values represent the mean ± SEM.

Effects of shRNA-induced Cdk2ap1 knockdown on proliferation and differentiation of endometrial stromal cells

For Knockdown of Cdk2ap1 gene expression, we purchased a Cdk2ap1 shRNA plasmid (Santa Cruz Biotechnology, CA, USA). It is a pool of 3 target-specific lentiviral vector plasmids each encoding 19-25 nt (plus hairpin) shRNAs designed to knockdown gene expression. The transfection with Cdk2ap1 shRNA plasmid was verified by western blot (Fig. 7).

The ratios of ki-67 positive cells were significantly increased in knockdown of group of Cdk2ap1 in all check-point-times (Fig. 8).

The expression levels of decidual-related prolactin (dPRP) specific mRNA were significantly high in group of shRNA-induced Cdk2ap1 knockdown and induced decidual after 24 hr compared with that of the control or decidual inducting group (Fig. 9).

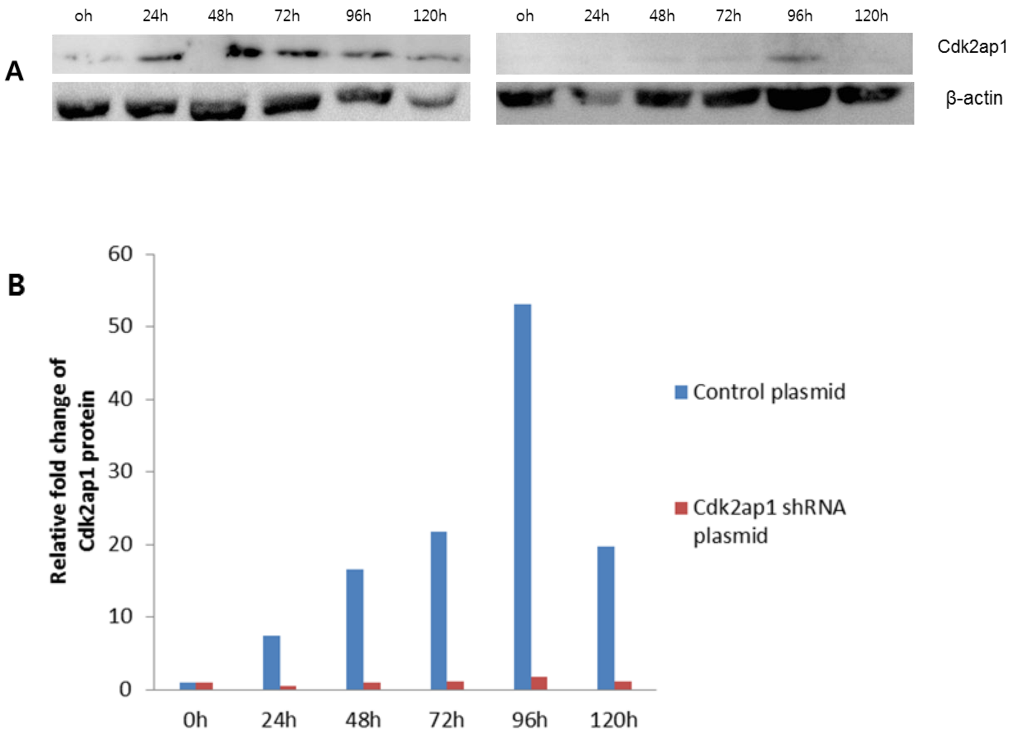
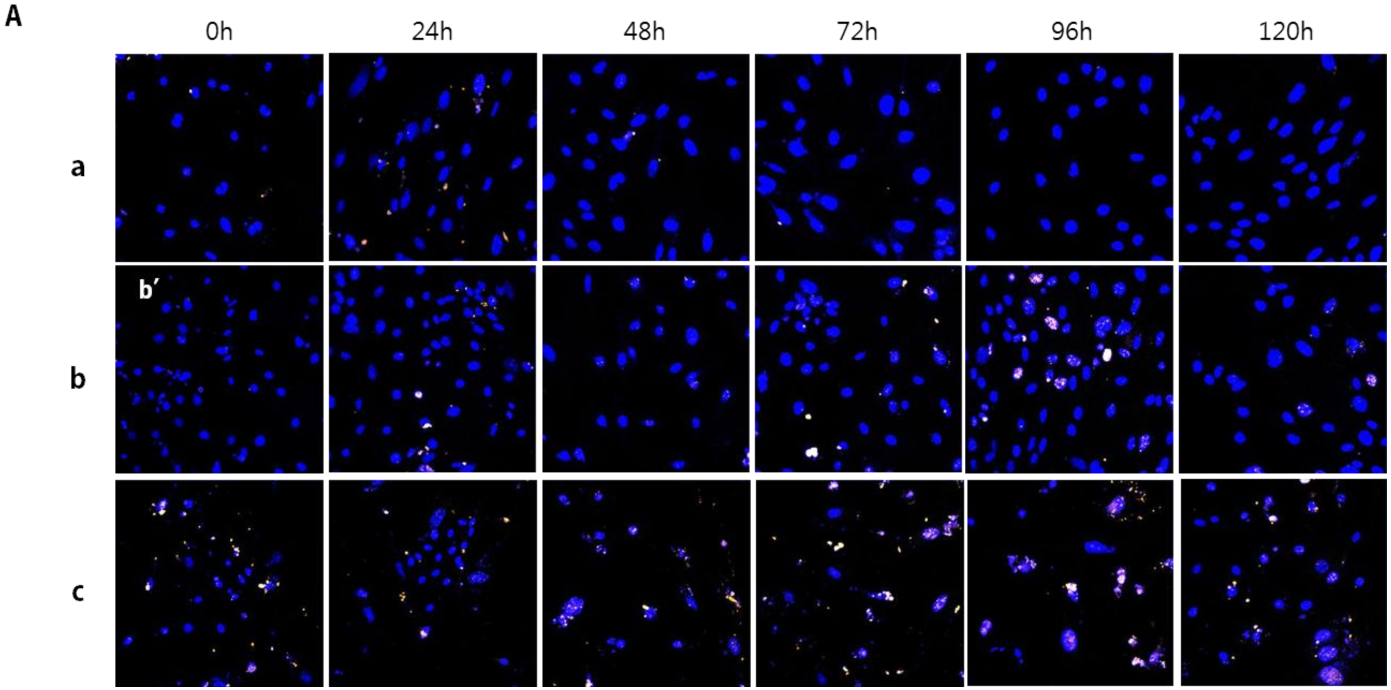


Figure 7. Knockdown of Cdk2ap1 in endometrial stromal cells transfected with *Cdk2ap1* shRNA plasmid.

A. Western blot of stromal cells transfected with the *Cdk2ap1* shRNA plasmid (right) and control shRNA plasmid (left)

B. Cdk2AP1 protein level normalized using β-actin protein level as the housekeeping control. Values represent the mean ± SEM.


A
0h
24h
48h
72h
96h
120h
a
b′
b
c

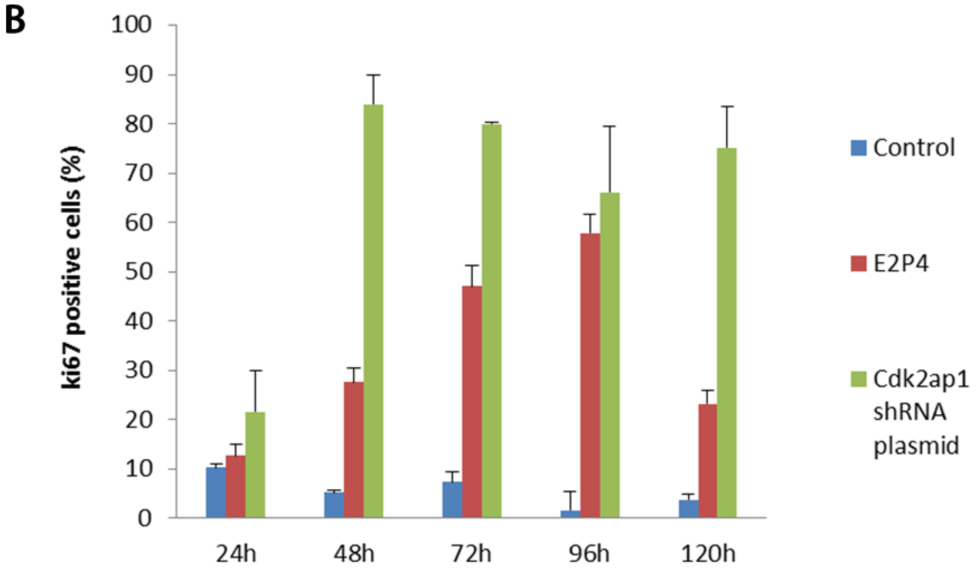

B
ki67 positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
24h
48h
72h
96h
120h
Control
E2P4
Cdk2ap1 shRNA plasmid

Figure 8. Proliferation of the endometrial stromal cells transfected with *Cdk2ap1* shRNA plasmid is enhanced.

A. Photoimmunofluorescence micrograph of in vitro decidualizing cells. Ki-67specific antibody was used to identify the proliferating cells (orange) and Hoechst33258 was used as control (blue). Immunofluorescence analysis was performed of 0, 24, 48, 72, 96, 120 hr stromal cells transfected with *Cdk2ap1* shRNA plasmid. Control (a), decidualization induction with E2P4 (b) transfection with Cdk2ap1 expression DNA (c), negative control (b'). Magnification; x 200.

B. The relative ratio of ki-67 positive cells number explained the ratio between ki-67 positive and total cells. Values represent the mean ± SEM.

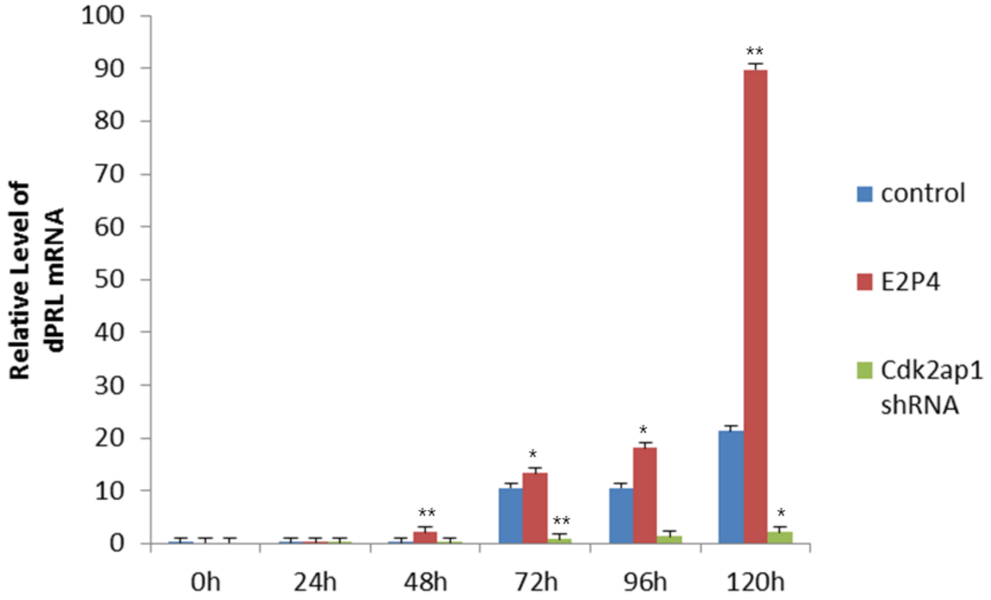


Figure 9. Differeniation of the endometrial stromal cells transfected with *Cdk2ap1* shRNA plasmid is suppressed.

Real-time PCR results of shRNA-induced Cdk2ap1 knockdown on the differentiation of stroma cells to decidual cells. The expression level of decidual-related prolactin (dPRP) was dramatically decreased in shRNA-induced Cdk2ap1 knockdown group. Values represent the mean ± SEM.

DISCUSSION

One of the biggest changes associated with implantation is decidualization. In the case of mouse, implantation of embryo on day 4 of pregnancy triggers the process of decidualization. Then, differentiation and apoptosis continue to followed complex. These are regulated by various factors such as steroid hormones, cytokines, growth factors and cell cycle regulators. Efforts to understand the series of processes that enable the growth of the embryo and the uterine change has led for a long time. Although there is evidence to suggest that cell cycle regulatory molecules play potential roles in the uterus during steroid hormonal stimulation (Geum et al., 1997; Prall et al., 1997) and reproductive cycle (Shiozawa et al., 1998), and in trophoblast differentiation during placentation (Bamberger et al., 1999), very lacked information is available on the molecules involved in cell cycle of uterus during implantation and decidualization.

The cell cycle is a complex process and is primarily controlled by the interplay of cyclins, cyclin-dependent kinases (cdks) and cyclin-dependent kinase inhibitors (CKIs) (Roberts, 1999; Sherr et al., 1999). The cellular growth is critically controlled at two particular checkpoints, G1-S and G2-M and various cell cyclins mediate these transitions via association with specific cdks. In general, the action of cdks is constrained by at least two families of CKI, p16 and p21. The p16 family includes p15, p16, p18 and p19, and they specifically inhibit the catalytic partners of D-type cyclins (cdk4 and cdk6). The p21 family consists of p21, p27 and p57, and they inhibit cdks with a broader specificity. CKIs accumulate in quiescent cells, and are downregulated with the onset of proliferation. Thus, a critical balance between the positive and negative cell cycle regulators is the key decision-maker for cell division (Tan et al., 2002).

In recent study, among the various cell cycle molecules examined, coordinate expression and functional association of cyclin D3 with cdk4 suggest a role for proliferation and, that of cyclin D3 with p21 and cdk6 is consistent with the development of polyploidy during stromal cell decidualization (Tan et al., 2002). In another study, it has been suggested that proper regulation of the cell cycle is a key factor for proper differentiation of mouse embryonic stem cells and that Cdk2ap1 is a mediator of this process (Matsuo et al., 2000). Interestingly, from previous our study, Cdk2ap1 mRNA is a downstream target gene of progesterone and the progesterone receptor in proliferating endometrial cells. In addition to it was expressed in the uterus (Cheon et al., 2010). In here, it is explored that Cdk2ap1 express in pregnant uterus in a spatio-temporal manner. Cdk2ap1 mRNA was expressed highly during decidualization and the profile of Cdk2ap1 protein level was similar to expression pattern of Cdk2ap1 mRNA. Cdk2ap1 was weakly expressed in the stromal cells on day 1 and then increased in the stromal cells next to the epithelial cells until day 5 of pregnancy. Especially, its expression was localized in primary decidua zone (PDZ) and secondary decidua zone (SDZ) on day 6 and 7, respectively.

Cdk2ap1 is a growth suppressor identified and cloned from the oral keratinocyte. And it is a highly conserved cellular gene. Several recent functional studies (Todd R et al., 1995; Tsuji T et al., 1998; Shintani S et al., 2000) support the growth suppressor role of Cdk2ap1. Ectopic expression of Cdk2ap1 in culture cells can lead to growth suppression, arresting cells in G1 of the cell cycle, and suppression of DNA replication (Shintani et al., 2000). The mechanism of action for Cdk2ap1 appears to be through its association with CDK2 and TGF-β1-mediated growth suppression by modulating CDK2 activities and pRB phosphorylation (Hu et al., 2004; Shintani et al., 2000). Cdk2ap1 also associates with DNA polymerase-a/primase, resulting in a direct interface with DNA replication and, hence, cell proliferation (Matsuo et al., 2000). These results suggest for the inhibitory effect of Cdk2ap1 on cell proliferation

Because stromal cell proliferation is a prerequisite for differentiation during implantation, proliferation has to be controlled strictly. We examined the expression of the ki-67 and decidual-related prolactin (dPRP) mRNA as indicator of proliferation and marker of decidualization, respectively. When the stromal cells were cultured in vitro and transfected with Cdk2ap1 expression DNA, decidual differentiation of stromal cells was enhanced while proliferation of stromal cells was suppressed. On the other and, when the stromal cells were cultured in vitro and transfected with Cdk2ap1 shRNA plasmid, decidual differentiation of stromal cells was suppressed while proliferation of stromal cells was enhanced.

In conclusion, we have provided evidence that a Cdk2ap1 regulates proliferation and decidual differentiation of stromal cells during implantation. Cdk2ap1 controls the appropriate proliferation by inhibiting the proliferation during decidualization. This supports the fact that proper proliferation of stromal cells is a prerequisite for decidual differentiation. So we suggest that Cdk2ap1 may be a potential regulator of proliferation and decidual differentiation of stromal cells during implantation.

REFERENCE

Bamberger A, Sudahl S, Bamberger CM, Schulte HM, Löning T. 1999. Expression patterns of the cell-cycle inhibitor p27 and the cell-cycle promoter cyclin E in the human placenta throughout gestation: implications for the control of proliferation. Placenta. 20(5-6):401-406.

Cheon YP. 2002. Expression of doc-1 in pregnant uterus of the mouse. Kor J Fertil Steril 29: 295-302.

Cheon YP, Kim CH. 2010. Progesterone is primary regulator of Cdk2ap1 gene expression and tissue-specific expression in the uterus. J Endocrinol Invest. 33: 650-656.

Correia-da-Silva G, Bell SC, Pringle JH, Teixeira NA. 2005. Patterns of Expression of Bax, Bcl-2 and Bcl-xL in the Implantation Site in Rat During Pregnancy. Placenta 26:796-806.

Daigo Y, Suzuki K, Maruyama O, Miyoshi Y, Yasuda T, Kabuto T, Imaoka S, Fujiwara T, Takahashi E, Fujino MA, Nakamura Y. 1997. Isolation, mapping and mutation analysis of a human cDNA homologous to the doc-1 gene of the Chinese hamster, a candidate tumor suppressor for oral cancer. Genes Chrom. Can. 20:204–207.

Das SK. 2009. Cell cycle regulatory control for uterine stromal cell decidualization in implantation. Reproduction 137:889-99.

Dey SK. 1996. Implantation. In Adashi EY, Rock JA, Rosenwaks Z (eds), Reproductive Endocrinology, Surgery and Technology. New York: Lippincott-Raven Publishers. 421–434.

Geum Geum D, Sun W, Paik SK, Lee CC, Kim K. 1997. Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. Mol Reprod Dev. 46(4):450-458.

Gordon HM, Kucera G, Salvo R, Boss JM. 1992. Tumor necrosis factor induces genes involved in inflammation, cellular and tissue repair, and metabolism in murine fibroblasts. J Immunol 148:4021–4027.

Herken R. 1983. Cell kinetics of early gestation mouse uterus. Cell Tissue Kinet 16: 419-428.

Hu MG, Hu GF, Kim Y, Tsuji T, McBride J, Hinds P, Wong DT. 2004. Role of p12(CDK2-AP1) in transforming growth factor-beta1-mediated growth suppression. Cancer Research 64:490-499

Huet-Hudson YM, Andrews GK, Dey SK. 1989. Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. Endocrinology 125:1683-1690.

Kleinfeld RG, O’Shea JD. 1983. Spatial and temporal patterns of deoxyribonucleic acid synthesis and mitosis in the endometrial stroma during decidualization in the pseudopregnant rat. Biol Reprod 28:691-702.

Lee S, Lee SA, Shim C, Khang I, Lee KA, Park YM, Kang BM, Kim K. 2003. Identification of estrogen-regulated genes in the mouse uterus using a delayed-implantation model. Mol Reprod Dev 64:405-413.

Matsuo K, Shintani S, Tsuji T, Matsuo K, Nagata E, Lerman M, McBride J, Nakahara Y, Ohyama H, Todd R, Wong DT. 2000. p12(DOC-1), a growth uppressor, associates with DNA polymerase alpha/primase. FASEB J 14: 318–324.

McCormack JT, Greenwald GS. 1974. Evidence for a preimplantation rise in estradiol-17β levels on day 4 of pregnancy in the mouse. J Reprod Fertil 41: 297-301.

O’Shea JD, Kleinfeld RG, Morrow HA. 1983. Ultrastructure of decidualization in the pesudopregnant rat. Am J Anat 166: 271-298.

Prall OW, Sarcevic B, Musgrove EA, Watts CK, Sutherland RL. 1997. Estrogen-induced activation of Cdk4 and Cdk2 during G1-S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. J Biol Chem. 18;272(16):10882-10894.

Roberts JM. 1999. Evolving ideas about cyclins. Cell 98, 129–132.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: positive and negative regulations of G1-phase progression. Genes Dev. 13:1501–1512.

Shintani S, Ohyama H, Zhang X, McBride J, Matsuo K, Tsuji T, Hu MG, Hu G, Kohno Y, Lerman M, Todd R, Wong DT. 2000. p12DOC-1 is a novel cyclindependent kinase 2-associated protein. Mol Cell Biol 20: 6300-6307.

Shiozawa T, Li SF, Nakayama K, Nikaido T & Fujii S. 1998. Relationship between the expression of cyclins/cyclin-dependent kinases and sexsteroid receptors/Ki67 in normal human endometrial glands and stroma during the menstrual cycle. Molecular Human Reproduction 2 745–752.

Tan J, Raja S, Davis MK, Tawfik O, Dey SK, Das SK. 2002. Evidence for coordinated interaction of cyclin D3 with p21 and cdk6 in directing the development of uterine stromal cell decidualization and polyploidy during implantation. Mechanisms of Development 111:99-113.

Todd R, McBride J, Tsuji T, Donoff RB, Nagai M, Chou MY, ChiangT, Wong DT. 1995. Deleted in oral cancer-1 (doc-1), a novel oral tumor suppressor gene. FASEB J 9:1362–1370.

Tsuji T, Duh FM, Latif F, Popescu NC, Zimonjic DB, McBride J, Matsuo K, Ohyama H, Todd R, Nagata E, Terakado N, Sasaki A, Matsumura T, Lerman MI, Wong DT. 1998. Cloning, mapping, expression, function, and mutation analyses of the human ortholog of the hamster putative tumor suppressor gene doc-1. J Biol Chem 273:6704– 6709.

Zolochevska O, Figueiredo ML. 2009. Expression of cell cycle regulator cdk2ap1 suppresses tumor cell phenotype by non-cell-autonomous mechanisms. Oral Oncol 45:106-112.

ABSTRACT

Cdk2ap1 regulates Proliferation and Decidual Differentiation of Mouse Stromal Cells during Implantation

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Sungshin Women’s University

The uteri undergo dynamic changes such as cellular proliferation, differentiation and apoptosis during pregnancy. These reactions are termed decidualization that is chacterized by morphological and functional changes in uterine stromal cells and is critical to support the implantation. Until 3 days before implantation begins, the endometrial epithelial cells proliferate. At 4 days starting the implantation is to stop the proliferation of epithelial cells, the proliferation of stromal cells begins. Decidual reaction starts in 5 days, and stromal cells surrounding the embryos are actively proliferate. Stromal cells adjacent to the embryo in 6 days stop the proliferation and form the primary decidual zone(PDZ). After the part is to stop the proliferation and apoptosis occurs, but the outer cells to proliferate continuously, to form a secondary decidual zone(SDZ). Eventually the decidual cells undergo apoptosis enlarging the implantation chamber to accommodate the growing embryo. This differentiation of the decidua is subject to the control of many factors. Among them, regulation of cell proliferation may be an important factor in differentiation, but the mechanism is not yet revealed well. Cdk2ap1 is known as growth suppressor identified and isolated from normal keratinocytes. Previous study, we observed that Cdk2ap1 mRNA is highly expressed during proestrus phage and is mainly localized to proliferating tissues. In this study, we profiled the Cdk2ap1 protein expression and localization and examined the functional roles of Cdk2ap1 during implantation. For that real-time PCR technology, Western blotting methodology, immunofluorescence method and in vitro decidualization-induction with transfect technology were employed. The levels of Cdk2ap1 proteins in the pregnant uteri were detected from day 1 of pregnancy but its level is low. At the time of implantation their level was increased until day 7 of pregnancy and then decreased. Cdk2ap1 localized in the stromal cell. Using in vitro primary endometrial stromal cell culture model, the functional role was examined. Cdk2ap1 knock-down suppressed decidual events but Cdk2ap1 overexpression accelerated decidualization. These results show that Cdk2ap1 regulate the stromal cells proliferation on the pregnant stage dependently and differentiation of endometrial cells during implantation.