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The Mesenchymal–Epithelial Transition During In Vitro Decidualization

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Abstract

The epithelial–mesenchymal transition plays a critical role in embryonic development, cancer progression, and metastasis. Decidualization is the process by which the fibroblast-like endometrial stromal cells differentiate into polygonal epithelial-like cells. However, it is still unclear whether mesenchymal–epithelial transition (MET) occurs during decidualization. The aim of this study was to examine whether decidualization causes the downregulation of some mesenchymal markers and upregulation of some epithelial markers in cultured uterine stromal cells. We showed that decidualization causes the downregulation of snail and vimentin expression, and upregulation of E-cadherin and cytokeratin expression. During in vitro decidualization, cultured stromal cells lose elongated shape and show epithelium-like characteristics. Our data suggest that the process of MET may exist during decidualization.

Keywords

decidualization, MET, snail, E-cadherin

Introduction

Embryo implantation is a complex process that involves a precise interaction between receptive uterus and competent blastocyst. After blastocysts attach onto endometrium, the stromal cells at implantation sites begin to undergo decidualization.[1] Uterine decidualization is crucial for blastocyst implantation and maintenance of pregnancy. If maternal uterus does not undergo decidualization, the embryo will not develop normally. The differentiation of decidual cells is one of the earliest uterine adaptations to pregnancy,[2] including growth, change of shape, multinucleation, and establishment of intercellular junctions.[3] Many genes and signal pathways are involved in the process of decidualization. But the mechanism underlying decidualization remains largely unknown.

Decidualization could be artificially induced in either pregnant or pseudopregnant mice through uterine injection of oil or sepharose beads or mechanical stimulation. In addition, isolated endometrial stromal cells can also been induced to undergo decidualization in vitro.[4]

E-cadherin is expressed in luminal epithelial cells prior to implantation. With trophoblast invasion after the onset of implantation, both the trophoblast and the cells at the primary decidual zone (PDZ) express E-cadherin, suggesting that mesenchymal–epithelial transformation (MET) may occur during decidualization.[5] The MET is defined as the conversion of mesenchymal cells into epithelial derivatives, which is the reverse process of the epithelial–mesenchymal transition (EMT). A lot of genes are involved in the processes of EMT as well as MET. Among these genes, *snail* is the most widely studied during EMT or MET. *Snail* is capable of binding the promoter of E-cadherin and repressing the expression of E-cadherin, consequently inducing EMT.[6,7] Whether MET occurs during decidualization is still not clear. The aim of this study was to examine the expression level of EMT-related molecules (eg, snail, E-cadherin, N-cadherin, vimentin, and cytokeratin) and the morphological change during in vitro decidualization.

Materials and Methods

Animal Treatments

Adult CD-1 mice were maintained in a controlled environment with a 14-hour light/10-hour dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Xiamen University. From days 1 to 4, pregnancy was

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Table 1. Primer Sequences for Real-Time PCR.

Gene name	Primers	Accession no.	Length (bp)
Snail	5′-CGAGCCATAGAACTAAAGCC 5′-TGAGGGAGGTAGGGAAGTG	NM_011427	80
Slug	5′-CATTTCAACGCCTCCAAGA 5′-CTGCCGACGATGTCCATAC	NM_011415	163
Dtprp	5′-AGCCAGAAATCACTGCCACT 5′-TGATCCATGCACCCATAAAA	NM_010088	119
Rpl7	5′-GCAGATGTACCGCACTGAGATTC 5′-ACCTTTGGGCTTACTCCATTGATA	NM_29016	129
PRL	5′-GCTGTAGAGATTGAGGAGCA 5′-CATTTTCTTTGGTTTCAGGA	NM_000948	88
IGFBP1	5′-CCAAACTGCAACAAGAATG 5′-GTAGACGCACCAGCAGAG	NM_000596	87

Abbreviations: bp, base pair; PCR, polymerase chain reaction.

verified by recovering embryos from oviducts or uteri. The implantation sites on day 5 were visualized through intravenous injection of 0.1 mL of 1% Chicago blue dye (Sigma, St Louis, Missouri) in saline.

Isolation of Mouse Uterine Stromal Cells and In Vitro Decidualization

Endometrial stromal cells from 4-week-old mice were isolated and cultured as previously described.[8] Endometrial stromal cells were treated with 0.1 nmol/L of estradiol-17β (E2; Sigma) and 100 nmol/L of progesterone (P; Sigma) to induce in vitro decidualization.[4] Culture medium was changed every 2 days. Cells were harvested for further analysis after treating for 5 and 8 days, respectively.

Isolation of Human Uterine Stromal Cells and In Vitro Decidualization

Human uterine stromal cells were isolated as previously described.[9] Human endometrial samples were collected from normally cycling women undergoing hysterectomy or endometrial biopsy with written informed consent. All human procedures were approved by the Institutional Committee on the Use of Human Subjects in Medical Research of Bailu Hospital (Xiamen, China). Cells were cultured to 70% confluence and then induced for in vitro decidualization with 36 nmol/L E2, 1 μmol/L medroxyprogesterone acetate, and 0.1 mmol/L dibutyryl cyclic adenosine monophosphate (dbcAMP; Sigma) as previously described.[10] Cells were harvested for further analysis after 6 days of treatment.

Real-Time RT-PCR

Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously.[11] Briefly, total RNAs from cultured cells were isolated using TRIzol (Invitrogen, Carlsbad, California) according to the manufacturer's recommendations, digested with RQ1 deoxyribonuclease I (Promega, Madison, Wisconsin), and reverse transcribed into complementary DNA with PrimeScript reverse transcriptase reagent kit (Perfect real time; TaKaRa, Dalian, China). All the reactions were run in triplicate. Primers used for real-time RT-PCR were listed in Table 1.

Western Blot

Western blot was performed as described previously.[11] Cells were collected in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% triton X-100, and 0.25%

sodium deoxycholate). Protein concentration was measured by the bicinchoninic acid (BCA) Reagent kit (Applygen, Beijing, China). Samples were run on a 10% polyacrylamide gel electrophoresis gel and transferred onto nitrocellulose membranes. After being blocked in 5% nonfat milk powder in 0.1% Tween 20 in phosphate-buffered saline ([PBS] TPBS) for 1 hour, the membranes were incubated with primary antibody overnight at 4°C. After 3 washes in TPBS for 10 minutes each, the membranes were incubated with matched horseradish peroxide-conjugated secondary antibody for 1 hour followed by 2 washes in TPBS for 5 minutes each. The signals were developed in enhanced chemiluminescence (ECL). Antibodies for snail, E-cadherin, N-cadherin, lamin A/C, and tubulin were bought from Cell Signaling Technologies (Cell Signaling, Beverly, Massachusetts). Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was from Santa Cruz Biotechnology (Santa Cruz, California).

Living Cell Staining

Lyophilized Cell Tracker Green chloromethyl derivatives of fluorescein diacetate (CMFDA; Invitrogen) was dissolved in high-quality dimethyl sulfoxide to a concentration of 10 mmol/L as a stock solution. The stock solution was diluted to a final working concentration of 10 μmol/L in Dulbecco modified eagle medium -F12 containing 2% charcoal-treated FBS. In vitro decidualized cells were cultured in the medium containing Cell Tracker Green CMFDA dye for 30 minutes at 37°C. After the cells were washed with PBS thoroughly, the

morphology of the cells was examined under fluorescence microscopy.

Immunofluorescence

For immunofluorescence, the cultured cells were fixed in 4% paraformaldehyde solution for 30 minutes at 4°C, washed in PBS 3 times, and treated with 1% triton X-100 for 20 minutes at room temperature. After rinsed with PBS, the cell samples were blocked with 5% donkey serum (Jackson ImmunoResearch, Westgrove, Pennsylvania) for 1 hour at 37°C, followed by incubation with polyclonal goat anti-Vimentin antibody (1:50 dilution, Santa Cruz) or polyclonal rabbit anti-pan-Cytokeratin antibody (1:50 dilution, Santa Cruz) at 4°C overnight. Then the cells were incubated with Alex Fluor 488-conjugated AffiniPure Donkey Anti-Rabbit IgG or Alex Fluor 594-conjugated AffiniPure Donkey Anti-Goat IgG (1:200, Jackson ImmunoResearch) for 1 hour at 37°C. After washing in PBS for 3 times, the signal was detected by fluorescence microscope.

Preparation of Nuclear Extracts

Cellular extracts were prepared from induced and noninduced cells as described previously. After the culture medium was removed, the cells were washed with 10 mL of PBS, then collected using 1 mL buffer B (5 mmol/L EDTA in PBS, pH 7.4) in a 1.5 mL tube, and pelleted by centrifugation at 1000*g* for 2 minutes. The pellet was resuspended in 160 μL of buffer A (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride), then incubated on ice for 20 minutes. After 40 μL of buffer A containing 2.5% Nonidet P-40 was added, the tube was vigorously vortexed for 10 seconds. The homogenate was centrifuged for 5 minutes at 15000*g* at 4°C. The supernatant was designated as cytoplasmic proteins. The nuclear pellet was resuspended in 40 μL of ice-cold buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride) and the tube was vigorously rocked at 4°C for 25 minutes on a shaking platform. The nuclear extract was centrifuged for 5 minutes at 18000*g* at 4°C. Protein concentration was measured by the BCA Reagent kit.

Results

Expression of Snail *and* E-Cadherin *During In Vitro Decidualization in Mice*

Primary uterine stromal cells isolated on day 4 of pregnancy in mice were treated with the combination of E2 and P for 5 or 8 days to induce in vitro decidualization, respectively. Decidual prolactin-related protein (Dtprp) was examined as a reliable marker for decidualization in mice.[12,13] After uterine stromal cells were induced for decidualization for both 5 and 8 days, the expression of *Dtprp* was significantly upregulated, suggesting that the method inducing in vitro decidualization was successful. Under in vitro decidualization, the expression of *snail* messenger RNA was significantly downregulated, and Slug expression remained unchanged (Figure 1A). In order to observe the morphology of the uterine stromal cells isolated on day 4 of pregnancy under in vitro decidualization, we used Cell Tracker Green CMFDA to stain uterine stromal cells. The cells induced for in vitro decidualization lost elongated shape and showed epithelium-like characteristics (Figure 1B). After inducing for in vitro decidualization for 5 or 8 days, the expression of snail protein was significantly decreased and E-cadherin expression increased (Figure 1C). Cytokeratin, a marker of epithelial cells, was used to confirm the morphologic change. By immunofluorescence analysis, we detected a strong level cytokeratin expression in the cells induced for in vitro decidualization for 5 or 8 days, while the signals of vimentin were still present in the cells of control group (Figure 1D). Moreover, snail protein was significantly downregulated in nuclear extracts from the vitro induced-decidual cells compared to that of control group in cultured mouse stromal cells (Figure 1E).

Snail *Expression Under In Vitro Decidualization in Humans*

Human uterine stromal cells were treated with the combination of E2, medroxyprogesterone acetate, and dbcAMP for 6 days to induce in vitro decidualization. The expression of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1) was examined by real-time RT-PCR as the molecular markers of human decidualization.[14,15] Under in vitro decidualization, the expression of PRL and IGFBP1 was significantly upregulated compared to control (Figure 2A). The morphology of the human endometrial stromal cells was markedly different from the cells induced for in vitro decidualization. Cultured human stromal cells showed a change in shape from mesenchymal to epithelial appearance (Figure 2B), suggesting the induction of MET under in vitro decidualization. For cultured human stromal cells, snail and N-cadherin expression was also downregulated, while E-cadherin expression was upregulated under in vitro decidualization (Figure 2C). These data suggested that MET may occur during in vitro decidualization in humans.

Discussion

Based on our previous study, snail is mainly localized in the PDZ at implantation site on day 5 of pregnancy. On days 6 to 8 of pregnancy, *snail* is expressed at the second decidual zone.[16] Therefore, the expression of snail is complementary to that of E-cadherin, which is expressed at the PDZ after embryo implantation.[5] Our results show that cell morphology in treatment groups looks like epithelial cells under in vitro decidualization in mice and humans. In addition, snail and N-cadherin expression is significantly decreased, while E-cadherin expression is significantly increased in human decidual cells. For in vitro decidualization in mice, the change of the snail and E-cadherin expression is consistent with that in

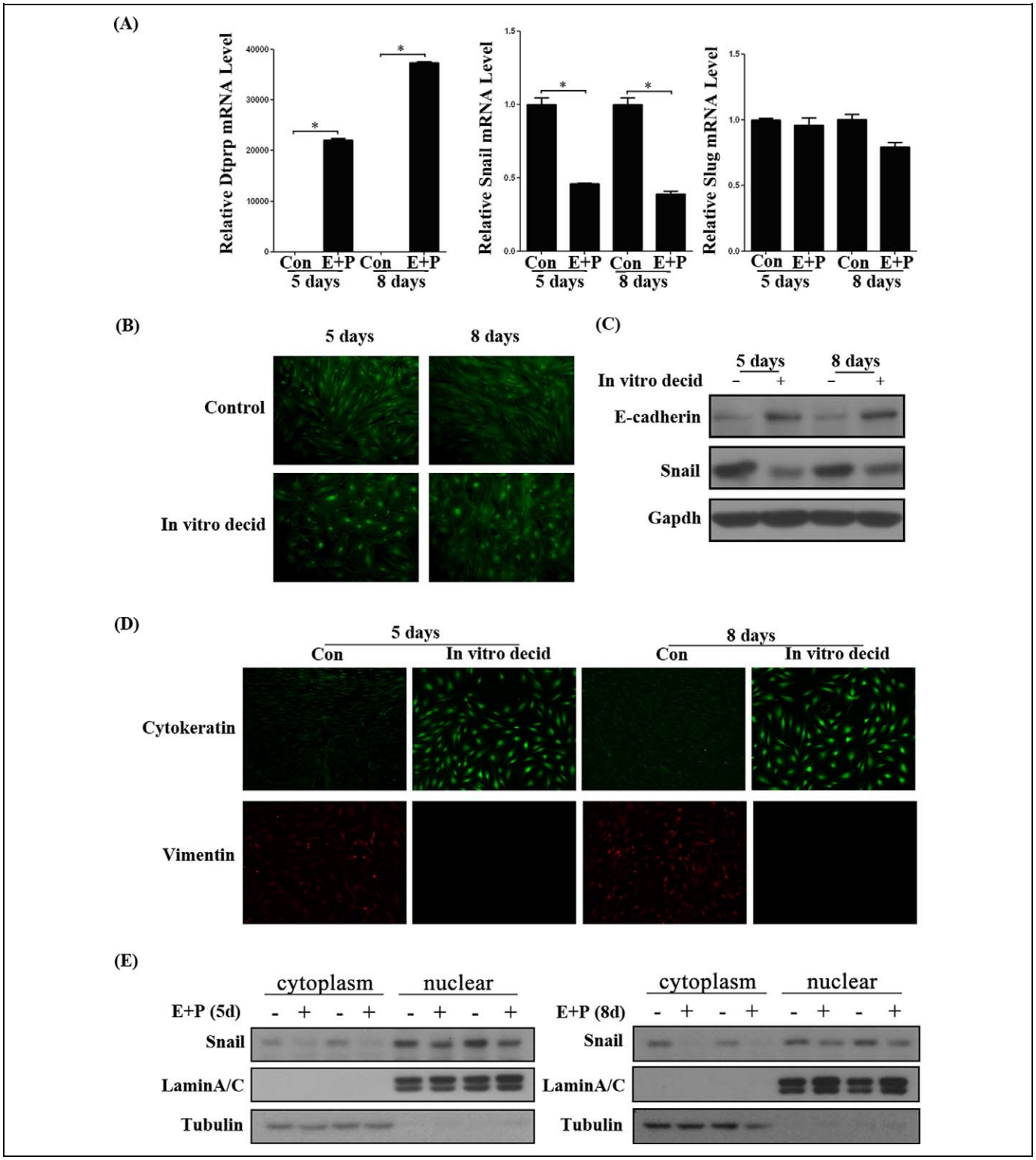

Figure 1. Snail expression during in vitro decidualization in mice. A, Cultured mouse stromal cells were induced for in vitro decidualization. Real-time PCR analysis was performed to examine the expression of Dtprp, snail, and slug. B, Cultured mouse stromal cells induced for in vitro decidualization were stained with Cell Tracker Green CMFDA (Invitrogen) to show morphologic changes. C, Western blot analysis of E-cadherin and snail expressions in cultured mouse stromal cells were induced for in vitro decidualization. D, The expression of cytokeratin was assessed by immunofluorescence with rabbit polyclonal pan-Cytokeratin antibody (green), and vimentin expression was detected with goat polyclonal vimentin antibody (red) in cultured mouse stromal cells induced for in vitro decidualization. E, Nuclear and cytoplasm extracts were used for immunoblot analysis. Snail expression was detected mainly in nuclear extracts. The expression of snail protein was downregulated in the decidual cells compared to the cells of control group. α-Tubulin and lamin A/C are used as loading controls for cytoplasm and nuclear extracts, respectively. CMFDA indicates chloromethyl derivatives of fluorescein diacetate; Dtprp, decidual prolactin-related protein; PCR, polymerase chain reaction.

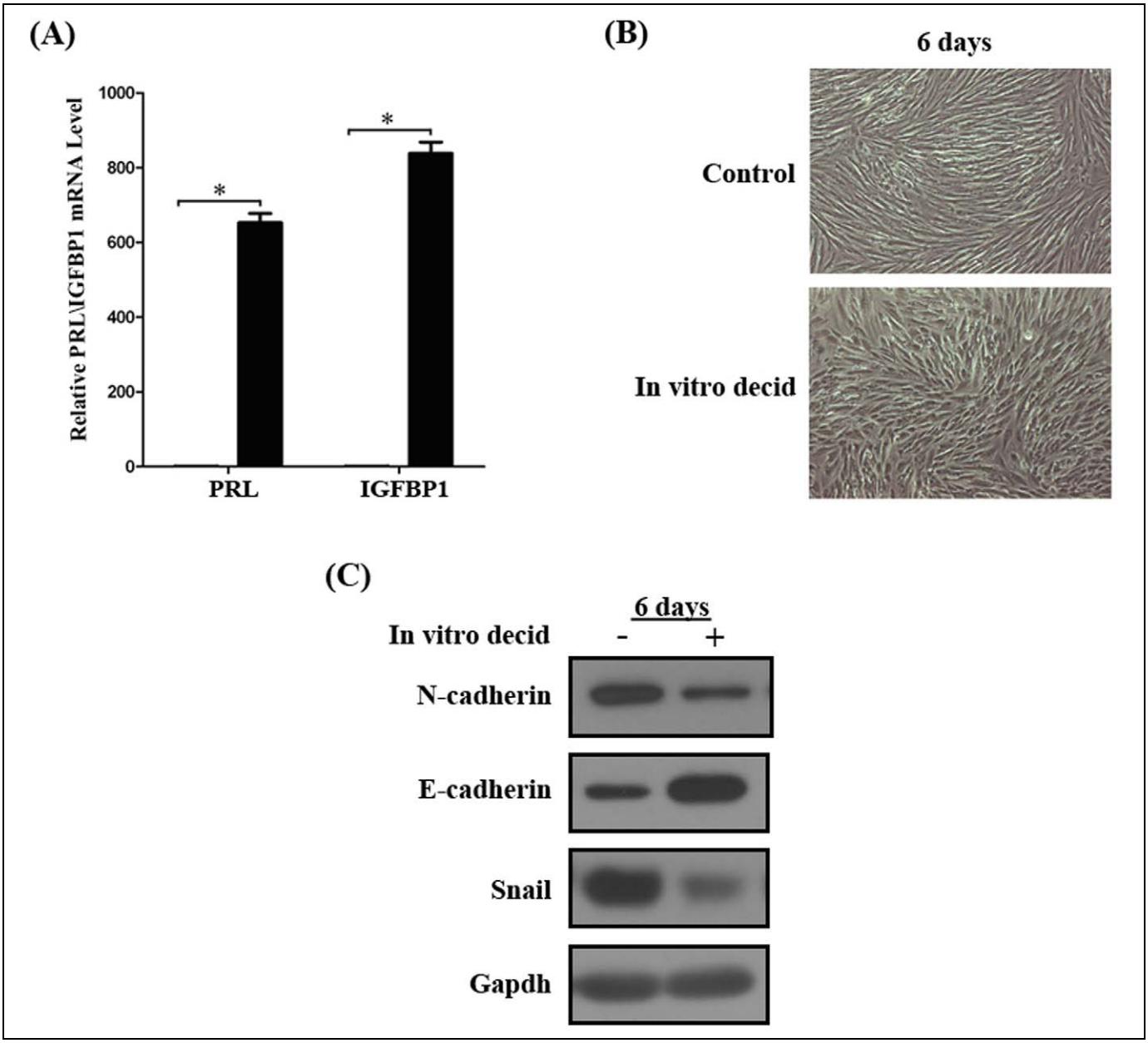

Figure 2. Snail expression during in vitro decidualization in humans. A, Cultured human endometrial stromal cells were induced for in vitro decidualization. Real-time RT-PCR analysis was performed to examine the expression of PRL and IGFBP1. B, Human endometrial stromal cells induced for in vitro decidualization were observed by phase contrast microscopy to show morphologic differences. C, Western blot analysis of N-cadherin, E-cadherin, and snail expressions in cultured human stromal cells. IGFBP1 indicates insulin-like growth factor-binding protein 1; RT-PCR, reverse-transcription polymerase chain reaction; PRL, prolactin.

human decidual cells, but the expression of N-cadherin has not been detected. Snail is a marker of mesenchymal cells, and E-cadherin is a marker of epithelial cells.[7] Overexpression of E-cadherin in cultured tumor cells which is deficient for this protein induces a more epithelial phenotype and decreases migration.[17,18] E-cadherin knockout mice were infertile because of the defects during implantation and decidualization.[19] N-cadherin was originally identified in developing neural tissue, but has now been shown to be expressed in many mesenchymally derived cell types. One frequent hallmark of EMT from the epithelial to migratory capacity is the switch from E-cadherin to N-cadherin, and N-cadherin may be altered during MET.[20,21] N-cadherin is frequently upregulated in invasive cells and is a marker for malignancy in many cancers. Additionally, N-cadherin is an indication of EMT in both normal and cancerous cells.[22] Recently, it is reported that EMT may play an important role in human embryo implantation by using an in vitro model. During the EMT, the phenomenon of ''cadherin switch'' with downregulation of E-cadherin and upregulation of N-cadherin was observed. The EMT of human

endometrial epithelial cells (EECs) can help embryo penetrate through the EECs into the endometrial stromal cell.[21] In addition, we detected other EMT-related markers (vimentin protein, a mesenchymal cell marker and cytokeratin protein, a epithelial cell marker).[23] Cytokeratin protein was strongly expressed in decidual cells, and the signal of vimentin protein was almost undetected in decidual cells. Consequently, our results suggest that MET may occur during the process of decidualization and be required for successful decidualization. Because epithelial cells are adherent cells and form coherent layers by intercellular adhesion complexes, the MET during decidualization may provide a stable developmental environment and an anchor point for embryos to invade into the uterus.

Snail, a transcriptional factor, can recognize and bind the E-box (5′-CACCTG-3′) and suppress E-cadherin expression. We detected snail expression mainly in nuclear compartments. Both snail and slug belong to the snail family of zinc-finger transcription factors and are similar in structure and function.[24] Slug also induces EMT-like events in adult epithelial cells.[25] Slug protein was mainly present in luminal epithelium from days 2 to 5 of pregnancy and in glandular epithelium from days 2 to 6 of pregnancy. The number of embryos implanted was greatly decreased after slug function in mouse endometrium was blocked by anti-slug polyclonal antibody before embryo implantation.[26] However, a few of implanted embryos were still detected when slug protein was blocked, suggesting that slug might not be the sole regulator during embryo implantation in mice. In our study, there was no detectable change in slug expression under in vitro decidualization. Therefore, how slug is involved in decidualization remains to be determined. In conclusion, our results suggest that the process of MET may occur during decidualization.

Declaration of Conflicting Interests

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