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cAMP-Response Element-Binding 3-like protein 1 (CREB3L1) is required for decidualization and its expression is decreased in women with endometriosis

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Abstract

Endometriosis is a major cause of infertility and pelvic pain, affecting more than 10% of reproductive-aged women. Progesterone resistance has been observed in the endometrium of women with this disease, as evidenced by alterations in progesterone-responsive gene and protein expression. *cAMP-Response Element-Binding 3-like protein 1* (*Creb3l1*) has previously been identified as a progesterone receptor (PR) target gene in mouse uterus via high density DNA microarray analysis. However, CREB3L1 function has not been studied in the context of endometriosis and uterine biology. In this study, we validated progesterone (P4) regulation of *Creb3l1* in the uteri of wild-type and progesterone receptor knockout (PRKO) mice. Furthermore, we observed that CREB3L1 expression was significantly higher in secretory phase human endometrium compared to proliferative phase and that CREB3L1 expression was significantly decreased in the endometrium of women with endometriosis. Lastly, by transfecting *CREB3L1* siRNA into cultured human endometrial stromal cells (hESCs) prior to hormonal induction of in

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

vitro decidualization, we showed that *CREB3L1* is required for the decidualization process. Interestingly, phosphorylation of ERK1/2, critical factor for decidualization, was also significantly reduced in *CREB3L1*-silenced hESCs. It is known that hESCs from patients with endometriosis show impaired decidualization and that dysregulation of the P4-PR signaling axis is linked to a variety of endometrial diseases including infertility and endometriosis. Therefore, these results suggest that CREB3L1 is required for decidualization in mice and humans and may be linked to the pathogenesis of endometriosis in a P4-dependent manner.

Keywords

CREB3L1; Decidualization; Endometrium; Endometriosis; Progesterone; Uterus

Introduction

The uterine endometrium is comprised of epithelial and stromal cell compartments that undergo dynamic molecular and morphological changes to allow for embryo implantation and development. These components are tightly regulated by the ovarian steroid hormones, estrogen (E2) and progesterone (P4) [1]. E2 is known to stimulate uterine epithelial cell proliferation whereas P4 inhibits this E2 effect, presumably through coordination of stromal-epithelial cell cross-talk. The progesterone receptor (PR) is required for successful implantation in both humans and rodents. PR signaling has been shown to regulate implantation-associated events including the development of uterine receptivity and endometrial stromal cell decidualization [2]. Studies utilizing a transgenic mouse model with a null mutation in the PR gene (PRKO) demonstrate the essential role for PR in P4-mediated uterine responses [3, 4], and have led to the identification of several P4-PR-signaling pathways within the uterus [5].

During early pregnancy, endometrial stromal cells are transformed into decidual cells in a P4-dependent manner. Decidualization is unique to species with hemichorial placentation, such as human, non-human primates and rodents and serves to protect the maternal uterus during trophoblast invasion and provide nourishment to the early embryo [6]. Endometrial stromal cells undergoing decidualization become plumper, acquire a secretory epithelioid-like morphology and secrete a variety of factors, including prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) [7]. Moreover, this transformation results in extensive changes in cellular gene expression, including alterations in steroid hormone receptor, extracellular matrix (ECM) and cytoskeletal gene profiles [8, 9]. Multiple transgenic mouse models have shown that the decidualization process is essential for the maintenance of pregnancy [3, 10, 11].

Imbalances in steroid signaling have been linked with multiple pathologies, including infertility, endometriosis, endometrial carcinoma, polycystic ovarian syndrome, and leiomyomas [12–15]. Each of these diseases displays a characteristic dysregulation of ER and PR expression profiles as well as their downstream target genes. All of these disorders are associated with increased E2 sensitivity, as characterized by decreased E2 metabolism and increased ER expression [12–15]. However, mounting evidence now links P4

dysregulation to these diseases as well. Loss of functional PR signaling has been shown to contribute to P4 resistance, at either the level of PR itself or its downstream target genes, which ultimately can disrupt critical epithelial-stromal cross talk within the uterus [16–18].

Endometriosis, a benign gynecological disease defined as the presence of endometrial cells outside the uterus, seriously impacts the quality of life and reproductive ability of 6–10% of reproductive-aged women and is strongly associated with chronic pelvic pain and infertility [19]. Disrupted steroidal control of uterine cell proliferation as well as abnormal decidualization have been shown in women with this disease [20]. Furthermore, P4 exposure is a negative risk factor for endometriosis [21], as pregnancy or progestin-based therapies are associated with disease regression in some women [22, 23]. However, a subset of women with this disease is non-responsive to frontline progestin treatments. Moreover, P4-induced molecular changes in the eutopic (intrauterine) endometrial tissue of this cohort are either blunted or undetectable. These in vivo observations suggest a resistance to P4 action in endometriosis [24]. Therefore, understanding the role of the progesterone receptor and its target genes will be key to the development of new therapeutic strategies [25, 26].

cAMP-Response Element-Binding 3-like protein 1 (CREB3L1), also known as Old Astrocyte Specifically Induced Substrate (OASIS), is an endoplasmic reticulum unfolded protein response (UPR) transducer [27]. In mammalian cells, the UPR family is comprised of three members: Inositol Requiring Element 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor (ATF-6) [27]. CREB3L1 is most structurally similar to ATF-6 [28, 29], a basic leucine zipper (bZIP) transcription factor, and located at the ER membrane under normal conditions. Upon UPR signaling, the N-terminal cytoplasmic domain of CREB3L1 is cleaved, translocates into the nucleus and alters target gene expression [29–31]. *CREB3L1* is expressed in a variety of tissues including pancreas, prostate, and intestine [32, 33]. Furthermore, previous studies suggest that CREB3L1 has a role in the differentiation and development of astrocytes, osteoblasts, odontoblasts, pancreatic beta-cells and large intestine goblet cells [28–30, 33–35].

Previous studies have identified *Creb3l1* as a PR target gene using high density DNA microarray analysis [36]. However, the function of CREB3L1 in the female reproductive tract is unclear. In this study, we examined the endometrial expression profile of CREB3L1 during the human menstrual cycle as well as its expression in women with and without endometriosis. To investigate the function of CREB3L1, we used the well-characterized in vitro primary human endometrial stromal cell decidualization model. Our results show that attenuation of CREB3L1 expression via small interfering RNA (siRNA) significantly reduced decidualization. We also observed reduced expression of CREB3L1 in the eutopic endometrium of women with endometriosis, as compared to healthy controls, suggesting that loss of CREB3L1 expression is an important factor in the pathogenesis of endometriosis.

Materials and Methods

Animal and tissue collection

Animals were maintained in a designated animal care facility according to the Michigan State University's Institutional Guidelines for the care and use of laboratory animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. To examine the P4 regulation of *Creb3l1* expression, wild type C57BL/6 mice and PRKO mice at six weeks age were ovariectomized. After two weeks, the mice were given a subcutaneous injection of either vehicle (sesame oil) or P4 (1 mg/mice) (n=3 per genotype). Six hours later, uterine tissues were dissected, flash-frozen and stored at −80°C for RNA /protein extraction and/or fixed in 4% (v/v) paraformaldehyde for immunohistochemistry.

Human endometrium samples

The human endometrial samples used to examine CREB3L1 expression patterns were obtained from Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository, the Greenville Hospital System, and the University of North Carolina in accordance with the guidelines set by the Institutional Review Boards of Michigan State University (Grand Rapids, MI), Greenville Health System (Greenville, SC) and University of North Carolina (Chapel Hill, NC), respectively. Written informed consent was obtained from all participants. For experiments examining CREB3L1 expression throughout the menstrual cycle, endometrial samples were analyzed from 25 (n=6 proliferative and n=19 secretory) cycling premenopausal women undergoing hysterectomy for benign conditions who were surgically negative for endometriosis and had not been on any hormonal therapies for at least three months prior to surgery. Endometrial menstrual staging was confirmed by an experienced pathologist familiar with female reproduction. To compare CREB3L1 expression patterns in the eutopic endometrium of women with and without endometriosis, 6 control samples were compared to 17 endometriotic samples obtained during the early secretory phase. Samples used for immunohistochemistry were fixed in 10% buffered formalin prior to embedding in paraffin wax.

Immunohistochemistry

Uterine cross sections from paraffin-embedded tissue were cut into 6 μm sections, mounted on silane-coated slides, deparaffinized and rehydrated in a graded alcohol series. Sections were pre-incubated with 10% normal goat serum in phosphate-buffered saline (PBS; pH 7.5) and then incubated with anti-CREB3L1 (Santa Cruz, Santa Cruz, CA) antibody in PBS supplemented with 10% normal goat serum overnight at 4°C. The next day, sections were washed with PBS and incubated with secondary antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Immunoreactivity was detected using diaminobenzidine (DAB-Vector Laboratories, Burlingame, CA) then counterstained with hematoxylin and coverslipped with permount. Imuunostaining was analyzed using microscopy software from NIS Elements, Inc. (Nikon, Melville, NY).

Human endometrial stromal cell culture and in vitro decidualization

Human endometrial stromal cells (hESCs), obtained from The Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository with MSU Biological Institutional Review Board approval, were isolated by digesting endometrial tissue samples with DNase I (Sigma-Aldrich, St. Louis, MO) and collagenase (Sigma-Aldrich, St. Louis, MO), followed by filtration. Isolated hESCs were then maintained in phenol red–free RPMI-1640 medium (Gibco, Grand Island, NY) containing 0.1 mM sodium pyruvate (Gibco, Grand Island, NY), 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) depleted of steroids by pre-treatment with dextran-coated charcoal (Sigma Aldrich, St. Louis, MO) (Charcoal-stripped FBS; CS-FBS), and 1% penicillin streptomycin (P/S; Gibco, Grand Island, NY). Cells were cultured in monolayer at 37°C in 5% CO_2. To induce in vitro decidualization, cells were washed with PBS and transferred to OPTI-MEM medium (Gibco, Grand Island, NY) containing 2% CS-FBS, 10 nM estradiol (E2; Sigma-Aldrich, St. Louis, MO), 1 mM medroxyprogesterone acetate (MPA; Sigma-Aldrich, St. Louis, MO), 50 μM cAMP (Sigma-Aldrich, St. Louis, MO), and 1% P/S. Differentiation medium was changed every 48 hours for a total of 6 days. For *CREB3L1* knockdown, small interfering RNA (siRNA) was obtained from Dharmacon (Lafayette, CO), RNAi Techologies. Human *CREB3L1* siRNA was transfected using Lipofectamine 2000 reagent (Invitrogen Crop., Carlsbad, CA) prior to in vitro decidualization.

Immunofluorescence

hESCs were grown on glass coverslips to 90% confluency and subjected to decidualization treatment as described above. Upon completion of treatment, coverslips were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% of Triton X-100 (Sigma-Aldrich, St. Louis, MO). After further washing, hESCs were exposed to anti-CREB3L1 (Santa Cruz, Santa Cruz, CA), anti-phospoho-ERK1/2 (Cell Signaling, Danvers, MA), or ERK1/2 (Cell Signaling, Danvers, MA) antibodies overnight at 4°C and secondary antibody for 2 hour at room temperature. Washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories, Burlingame, CA) to enable nuclear visualization and images captured via fluorescent microscopy (Nikon Instruments Inc., Melville, NY) using software from NIS Elements, Inc. (Nikon, Melville, NY).

Western blotting

Cellular proteins were extracted using lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, and 0.125% Nonidet P-40 (vol/vol)) supplemented with both a protease inhibitor cocktail (Roche, Indianapolis, IN) and a phosphatase inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Twenty μg of protein lysates were electrophoresed via SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membrane was blocked with Casein (0.5% v/v) prior to exposure to anti-phospho ERK1/2 (Cell Signaling, Danvers, MA), anti-ERK1/2 (Cell Signaling, Danvers, MA), or anti-Actin (Santa Cruz, Santa Cruz, CA) antibodies. Immunoreactivity was visualized by incubation with a horseradish peroxidase-linked secondary antibody followed

by exposure to Electrochemiluminescence reagents (ECL) according to manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ).

RNA isolation and quantitative real-time PCR

Total RNA was isolated using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's instruction. As a template for quantitative real-time PCR, cDNAs were synthesized using quantitative PCR random hexamers and MMLV Reverse Transcriptase (Invitrogen Crop., Carlsbad, CA). The expression of *CREB3L1, IGFBP1*, and *PRL* were quantified by real-time PCR using a CFX96 Real-time Detection System (Bio-Rad Laboratories, Hercules, CA) and iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). *RPL7* expression was included in each treatment group for normalization. Gene specific primers used are listed in Table 1.

Statistical Analysis

This semiquantitative approach was used to generate a H-Score for each immunohistochemistry sample: the percentage of positive tumor cells per slide (0%–100%) was multiplied by intensity strength of staining (0, negative or trace; 1, weak; 2, moderate; 3, intense). The overall score ranged from 0 to 300. Statistical analysis were performed using one-way ANOVA analysis Tukey's post hoc multiple range test or Student's t-tests using the Instat package from GraphPad (San Diego, CA). $p<0.05$ was considered statistically significant.

Results

Creb3l1 is identified as P4 and PR target gene in the murine uterus

P4-PR signaling is critical to implantation, decidualization, and glandular development [3, 37]. *Creb3l1* was previously identified as a PR target gene via high density DNA microarray analysis [36]. To validate whether *Creb3l1* is a P4-PR target gene, ovariectomized wild-type or PRKO mice were treated with vehicle (sesame oil) or P4 for 6 hours, then the levels of *Creb3l1* mRNA were examined using real-time PCR. As shown Figure 1A, the expression of *Creb3l1* mRNA was significantly increased in the uteri of wild-type mice treated with P4 compared to vehicle. However, this induction was significantly decreased in PRKO mice compared to wild-type mice. These results indicate that the expression of *Creb3l1* mRNA is mediated by PR. To analyze the uterine localization of CREB3L1 proteins following P4 treatment, we performed immunohistochemistry using tissues from vehicle or P4-treated wild-type and PRKO mice. In the control ovariectomized mouse uterus, CREB3L1 proteins were weakly detected in the stroma, and luminal and glandular epithelium. P4 treatment remarkably increased the localization of CREB3L1 proteins in the mouse uterus. However, CREB3L1 proteins were weakly detectable in PRKO mice treated with either vehicle and P4 (Figure 1B). These results suggest that *Creb3l1* is a novel target gene of the P4-PR signaling axis in the uterus.

To investigate the expression of CREB3L1 during early pregnancy, the level of CREB3L1 was examined in the uteri of female mice during early pregnancy (Figure 2). Immunohistochemical analysis of uterine sections revealed that the expression of CREB3L1

was weakly detected at 0.5 dpc (days post coitum). Interestingly, strong expression of CREB3L1 was present in luminal and glandular epithelium as well as stroma after 2.5 dpc. The expression of CREB3L1 was extended into the primary decidual zone at 5.5 dpc, and was next observed in the secondary decidual zone at 7.5 dpc. These results show that CREB3L1 may be involved in decidualization during early pregnancy.

CREB3L1 is decreased in human endometrium from women with endometriosis

We next used immunohistochemical analysis to examine the cell-specific expression of CREB3L1 in endometrium from proliferative phase (n=6), early (n=6), mid (n=8), and late (n=5) secretory phase in women (Figure 3). CREB3L1 expression was only weakly detectable in epithelial cells of the proliferative phase. CREB3L1 protein was significantly higher in endometrial stromal and epithelial cells of the secretory phase as compared to the proliferative phase (Figure 3B). Interestingly, we observed that decidual cells have strong CREB3L1 staining in the late secretory phase (Figure 3Ah). Because the secretory phase is a time of elevated circulating P4 levels, this result suggests P4 regulation of CREB3L1 in human endometrium.

We hypothesize that the molecular basis for progesterone resistance in endometriosis is related to an overall reduction in progesterone receptor levels (PRs), leading to dysregulation of downstream PR target genes [12, 17, 24]. Therefore, to confirm the association of CREB3L1 with PR, we examined CREB3L1 expression in secretory endometrium from women with (n=17) or without (n=19) endometriosis by immunohistochemistry. Interestingly, CREB3L1 protein is significantly lower in endometrium from women with endometriosis as compared to controls during the secretory phase (Figure 4).

CREB3L1 is activated in human endometrial stromal cells (hESCs) during in vitro decidualization

P4 signaling is crucial for the decidualization of the endometrial stromal cells during early pregnancy [2]. Previous studies have shown that eutopic human primary endometrial stromal cells (hESCs) from patients with endometriosis showed impaired decidualization [38, 39]. To examine the role of CREB3L1 in hESC decidualization, we induced decidualization in cultured hESCs by treating the cells with estrogen, MPA, and cAMP [54] and examined expression of the known decidualization markers, prolactin (*PRL*) and insulin-like growth factor-binding protein 1 (*IGFBP1*), as well as cellular morphology. *CREB3L1* mRNA expression levels were unaltered during in vitro decidualization in hESC (data not shown). Since CREB3L1 protein is activated by cleavage of its N-terminal domain which translocates from the ER membrane to the nucleus [28–31], we also examined CREB3L1 protein localization during decidualization by immunofluorescent staining. Our result shows that CREB3L1 was consistently expressed in cytoplasm of nondecidualized hESCs. However, upon induction of in vitro decidualization, CREB3L1 protein translocated to the nucleus on day 3 and day 6 (Figure 5) of treatment. This result suggests that CREB3L1 protein is activated during in vitro decidualization of hESCs.

CREB3L1 is required for decidualization in hESCs

To further analyze the role of CREB3L1 in hESC decidualization, we performed siRNA-mediated knockdown of *CREB3L1* expression (Figure 6A). To confirm *CREB3L1* attenuation, we performed real-time PCR and observed decreased *CREB3L1* expression in the *CREB3L1* siRNA group as compared to non-targeting pool siRNA (Figure 6A). Surprisingly, hESCs morphology changed markedly upon decidualization treatment, showing a reduction in size and shape on day 6 (Figure 6B). The expression of decidualization marker genes, *IGFBP1* and *PRL*, were also significantly reduced on day 6 of decidualization treatment (Figures 6C and D). These results suggest that *CREB3L1* is required for both marker gene expression and morphology changes accompanying decidualization in hESCs.

CREB3L1 regulates phosphorylation of ERK1/2 in decidualization process

ERK1/2 is a member of the MAPK pathway previously shown to be required for endometrial stromal cell decidualization [40]. To determine whether phosphorylation of ERK1/2 is regulated by CREB3L1 during in vitro decidualization, we performed western blot analysis on protein from hESCs decidualized in the presence or absence of *CREB3L1* siRNA prior to in vitro decidualization. We observed decreased phosphorylation of ERK1/2 (phospho-ERK1/2) on day 3 and day 6 in hESC treated with *CREB3L1* siRNA as compared to the non-targeting pool siRNA (Figure 7A). Quantification of phospho-ERK1/2 revealed a statistically significantly decrease in phospho-ERK1/2 expression on day 6, as well as a marked decrease in phospho-ERK1/2 on day 3, but the latter did not reach statistical significance (Figure 7B). It is known that phospho-ERK1/2 translocates from the cytoplasm to the nucleus, where it binds to and transcriptionally activates downstream target genes [41, 42]. Thus, we investigated the localization of both total and phospho-ERK1/2 throughout in vitro decidualization of hESCs in the presence or absence of *CREB3L1* siRNA transduction. This immunofluorescence result showed that levels of nuclear phospho-ERK1/2 were markedly reduced in decidualized hESCs transfected with *CREB3L1* siRNA as compared to those transfected with non-targeting pool siRNA (Figure 7C). However, total ERK1/2 levels in hESCs were unchanged by *CREB3L1* siRNA transfection (Supplemental Figure 1). These results suggest that CREB3L1 regulates the activation of phospho-ERK1/2 during the decidualization process.

Discussion

In this study, we have characterized *Creb3l1* as a P4-PR regulated gene in mouse uterus. P4 is a known critical regulator of the reproductive events associated with embryo implantation, decidualization and the maintenance of pregnancy [43–45]. Previous studies have shown that P4 regulation is dependent upon progesterone receptor (PR) [3, 4]. The binding of P4 to PR results in PR nuclear translocation and subsequent alteration of P4 target gene transcription. Recently, Rubel *et al.* identified uterine PR-regulated mechanisms and downstream targets via chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) [36]. This study provided an important dataset for identification of target genes in the uterus regulated by the P4-PR axis. These ChIP-Seq results also show that PR directly binds to the proximal promoter region of *Creb3l1* gene in uteri of ovariectomized mice treated with P4. Our study

has shown that CREB3L1 proteins were strongly detected in luminal and glandular epithelium as well as stroma of mouse uteri after 2.5 dpc. CREB3L1 proteins were significantly increased in both epithelial and stromal compartments of the human endometrium at the secretory phase as compared to the proliferative phase. The secretory phase of humans and 2.5 dpc of mice are characterized by elevated P4 levels [46]. Furthermore, decidual cells also have strong CREB3L1 staining in mice and humans. Therefore, our results support that CREB3L1 may play an important role for P4-PR signaling in both mice and humans.

Progesterone resistance has been hypothesized as a crucial element in the development of endometriosis [12, 24]. The interruption of P4 signaling, occurring from either loss of the PR itself or its interacting partners/downstream effectors, leads to a physiological state of P4 resistance [36]. Aberrant expression of critical P4 target genes such as aromatase, leukaemia-inhibitory factor, matrix metalloproteinases and the progesterone receptor has previously been shown in the endometrium of women with endometriosis [47–50]. This study demonstrates that CREB3L1 expression is also altered in the endometrium from women with endometriosis. Similar identification of P4 target genes and signaling pathways will be crucial for understanding the uterine dysfunction caused by this disease and contribute to the development of new therapeutic interventions.

Our study shows that inhibition of *CREB3L1* by siRNA treatment impaired decidualization capacity in hESCs, as evidenced by suppression of *PRL* and *IGFBP1* expression, known decidualization marker genes. P4 is a steroid hormone closely associated with endometrial stromal cell decidualization [2]. hESC from patients with endometriosis [39] and PRKO mice demonstrate a decidualization defect, supporting a critical role for P4-PR signaling in decidualization in both humans and mice [3]. Previous studies have shown that P4 activates Ihh signaling to induce expression of chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) during decidualization in hESCs [51, 52]. COUP-TFII, has been shown to promote decidualization of hESCs via induction of bone morphogenetic protein 2 (BMP2) and inhibition of ERα activation [51]. It has also been shown that STAT3 and PR crosstalk is required for successful embryo implantation and stromal cell decidualization in mice [53, 54]. However, the expression profiles of IHH, COUP-TFII, BMP2, and STAT3 were not altered in our decidualized hESCs following treatment with *CREB3L1* siRNA (data not shown), suggesting that CREB3L1 is acting down-stream of these key molecules or has an independent function with respect to decidualization.

CREB3L1 functions within the endoplasmic reticulum as an unfolding protein response (UPR) sensor [55]. Activation of CREB3L1 is stimulated by the UPR, leading to cleavage of its N-terminal domain by the basic leucine zipper (b-ZIP), causing translocation from the cytoplasm into the nucleus [29–31]. The cleaved CREB3L1 N-terminal domain then transcriptionally activates downstream target genes, such as collagen type I genes, protein-folding chaperones, and the antiproliferative gene [29–31]. This study reveals that during endometrial stromal cell decidualization, CREB3L1 also translocates from the cytoplasm to the nucleus, suggesting a role for activated CREB3L1 in decidualization.

ERK1/2 is activated by phosphorylation, resulting in its translocation from the cytoplasm into the nucleus where it phosphorylates target transcription factors [56, 57]. Previous studies have shown that ERK1/2 activity is increased in hESCs from women with endometriosis [58] and have linked the enhanced proliferation and survival of hESCs derived from women with endometriosis with alterations in ERK1/2 signaling [59, 60]. Abnormal decidualization of endometrial stromal cells has been correlated with unexplained infertility, miscarriage and endometrial pathologies such as endometriosis [20, 61–63]. It has been reported that endometriosis patients have a reduced decidualization capacity. Our previous studies showed that activation of ERK1/2 signaling coincided with the onset of decidualization in mice and humans [40]. Inhibition of ERK1/2 phosphorylation significantly decreased this decidualization process. TGF-β superfamily members are key regulators of female reproduction including decidualization. TGF-β stimulates activation of CREB3L1 [64] and ERK1/2 [65]. Additionally, many studies have demonstrated a potential link between ERK1/2 signaling and endometriosis [58, 59, 66]. These results suggest that tight regulation of CREB3L1 and ERK1/2 activity is required during the process of decidualization.

In this study, total ERK1/2 levels and its localization within hESCs was unchanged by *CREB3L1* silencing during decidualization. However, phosphorylation of ERK1/2 was significantly reduced by *CREB3L1* silencing and CREB3L1 expression was required for nuclear translocation of phospho-ERK1/2 during hESC decidualization. These results suggest that *CREB3L1* regulates the accumulation and translocation of phospho-ERK1/2 during the decidualization process. Previous studies have also shown that in vitro decidualization of hESCs from women with endometriosis is characterized by aberrantly regulated ERK1/2 phosphorylation [58] and that pretreatment with the ERK1/2 inhibitor, U0126, can prevent decidualization [40]. In light of these previous results, we hypothesize that phospho-ERK1/2 expression is tightly regulated by CREB3L1 during decidualization.

In summary, *Creb3l1* is identified as a target gene of P4-PR in the uterus. Our study observed dysregulation of CREB3L1 in endometrium from women with endometriosis as compared with healthy controls. Inhibition of *CREB3L1* activity by siRNA-mediated knockdown suppresses decidualization of hESCs. We also show that phosphorylation of ERK1/2, a critical factor for decidualization, was regulated by *CREB3L1*. These results suggest that *CREB3L1* is required for successful decidualization in mice and humans and have implications for our understanding of endometriosis and other endometrial pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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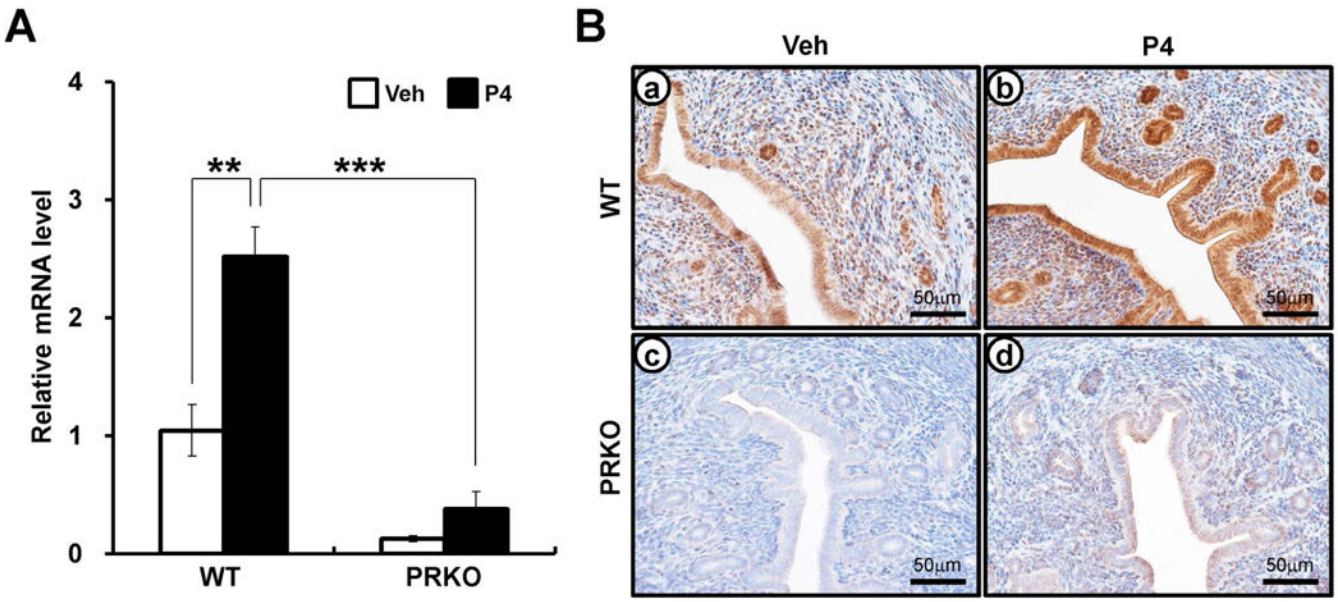

Figure 1.

The expression of CREB3L1 in wild-type (WT) or progesterone receptor knock-out (PRKO) mouse. (A) The expression level of *Creb3l1* from Progesterone (P4) injected WT or PRKO uteri by real-time RT-PCR assay. The samples were prepared from wild-type or PRKO uterus that was injected vehicle (Sesami oil) or P4 after 6 hours. The results represent the mean ± SEM. **p<0.01, ***p<0.001. (B) The localization pattern of CREB3L1 by immunohistochemistry in the vehicle or P4-treated uterus. Uterine sections were collected from P4 or vehicle treated WT and PRKO mice for 6 hours. Nuclei were counterstained with hematoxylin.

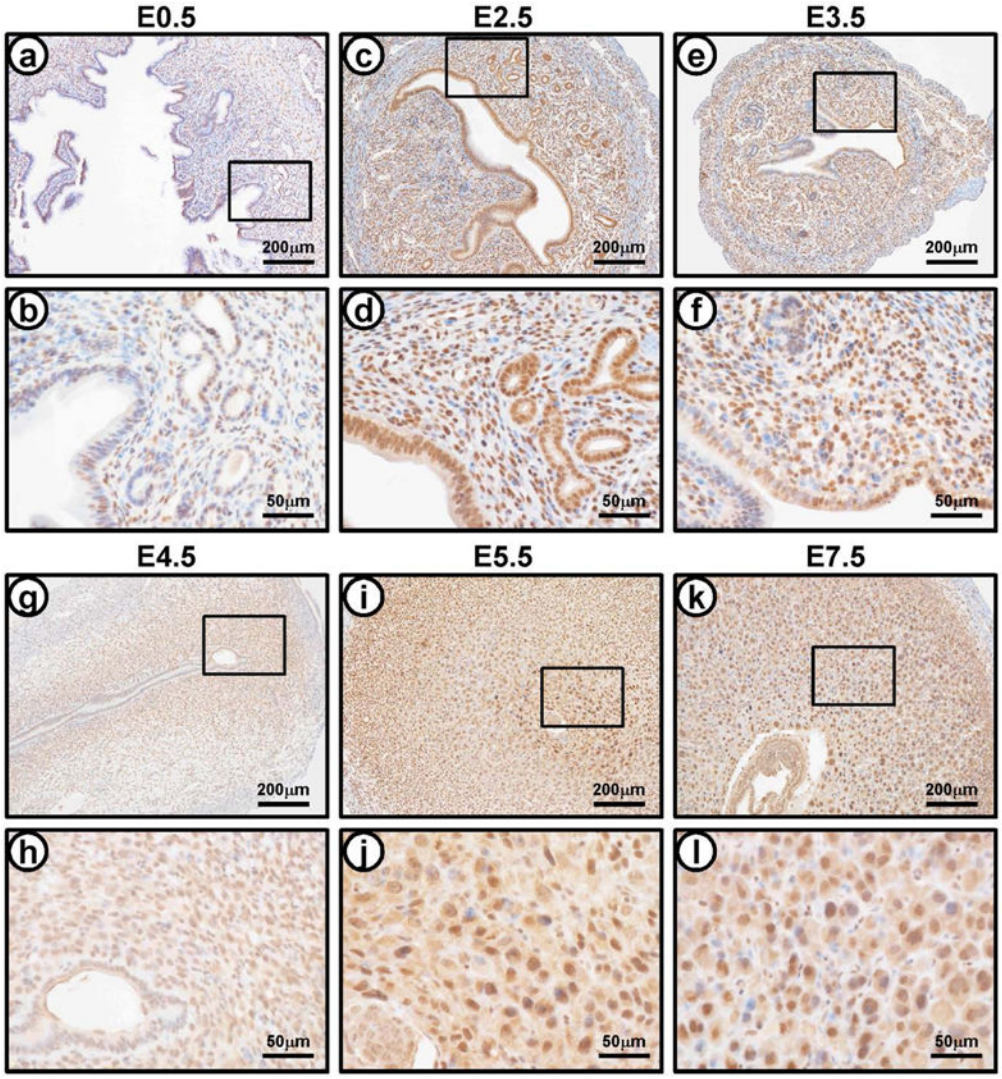

Figure 2.

The expression of CREB3L1 during early pregnancy. The localization pattern of CREB3L1 during natural pregnancy by immunohistochemistry were determined at 0.5 dpc (a and b), 2.5 dpc (c and d), 3.5 dpc (e and f), 4.5 dpc (g and h), 5.5 dpc (i and j), and 7.5 dpc (k and l).

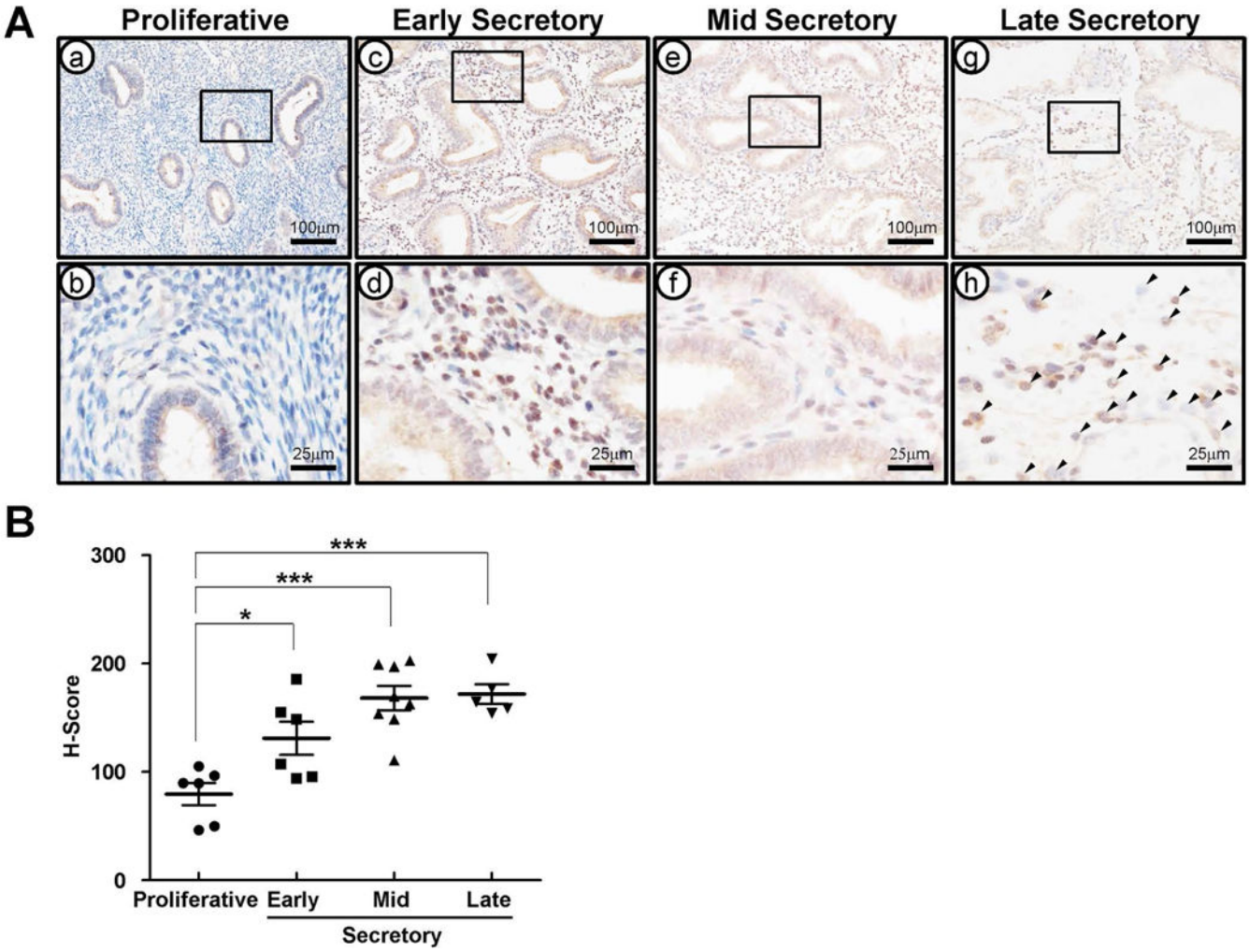

Figure 3.

The expression profile of CREB3L1 in human endometrium during estrus cycle. (A) The expression level and localization of CREB3L1 in proliferative (a and b) and early (c and d), mid (e and f), and late (g and h) secretory phase of human endometrium. CREB3L1 expression was high expression in secretary phase endometrial epithelium. Black arrow head indicates a decidualized cell. (B) was H-Score by measuring expression intensity of endometrial cells. CREB3L1 expression intensity was significantly high level in secretary phase endometrium. The results represent the mean ± SEM. *p<0.05, ***p<0.001.

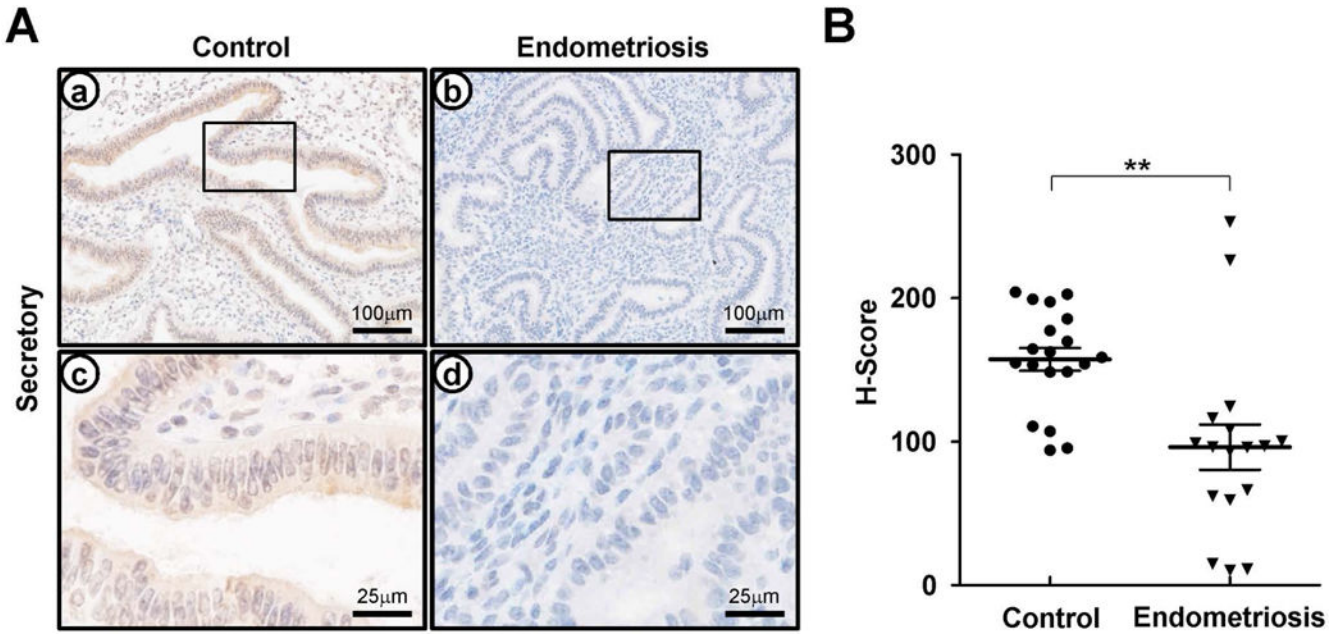

Figure 4.

The expression profile of CREB3L1 in secretory endometrium from women with or without endometriosis (A) The expression level and localization of CREB3L1 in human endometrium with or without endometriosis. CREB3L1 expression was low expression in human endometrium with endometriosis. Nuclei were counterstained with hematoxylin. (B) H-Score of CREB3L1 expression in human endometrium with or without endometriosis. CREB3L1 expression intensity was significantly low level in human endometrium with endometriosis. The results represent the mean ± SEM. **p<0.01.

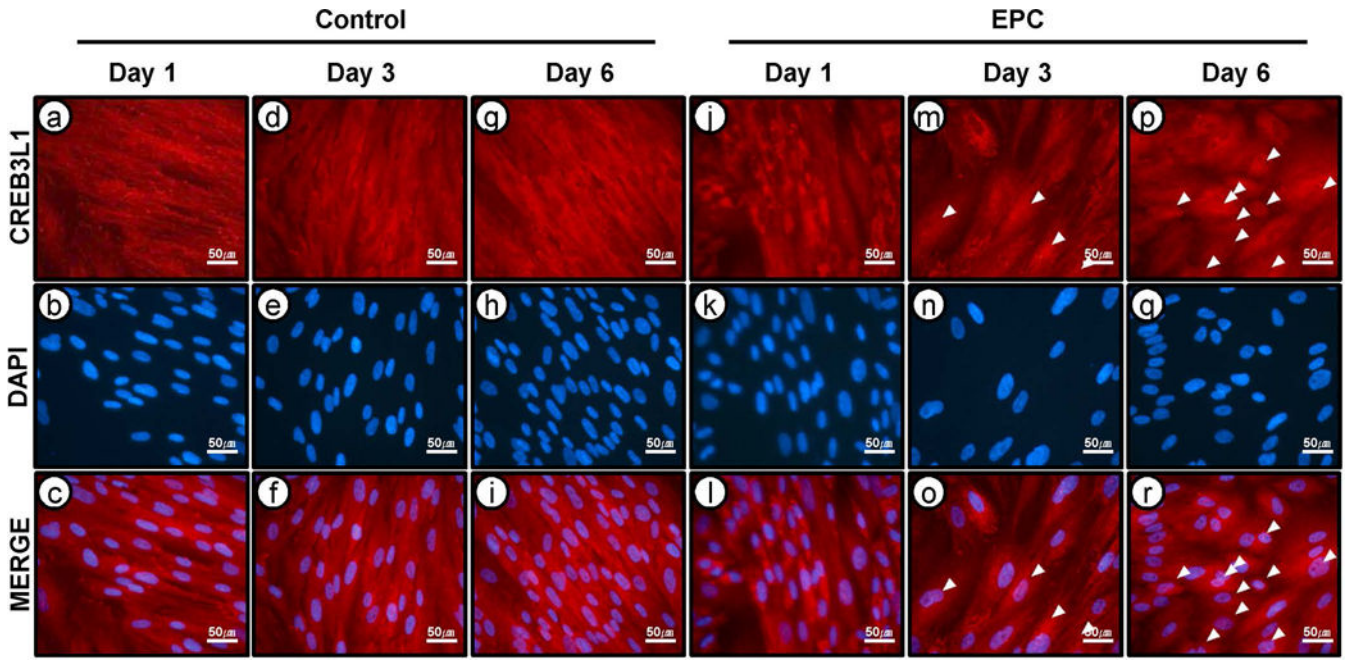

Figure 5.

The nuclear translocalization of *CREB3L1* during in vitro decidualization of human endometrial stromal cells (hESCs). Nuclear localization of CREB3L1 was detected at day 3 and day 6 in vitro decidualization hESCs with EPC. Neclei were conterstained with DAPI staining.

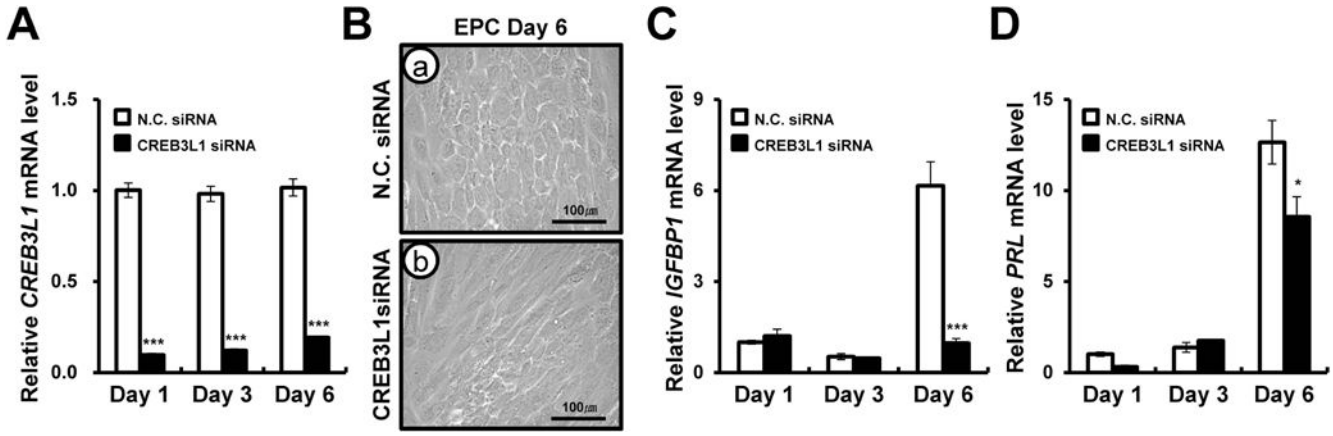

Figure 6.

The effect of inhibition of CREB3L1 in human endometrial stromal cells (hESCs) during in vitro decidualization. *CREB3L1* expression was inhibited by treatment with *CREB3L1* siRNA during in vitro decidualization. (A) The expression of *CREB3L1* gene was examined during in vitro decidualization. *CREB3L1* were significantly decreased in hESCs treated with *CREB3L1* siRNA during in vitro decidualization. The results represent the mean ± SEM. ***p<0.001. (B) Morphological change of hESCs was observed during in vitro decidualization on day 3 and day 6. The expression of decidualization marker gene *IGFBP1*(C) and *PRL* (D) were examined during in vitro decidualization. *PRL* and *IGFBP1* were significantly decreased in transfected *CREB3L1* siRNA hESC during in vitro decidualization. The results represent the mean ± SEM. *p<0.05, ***p<0.001.

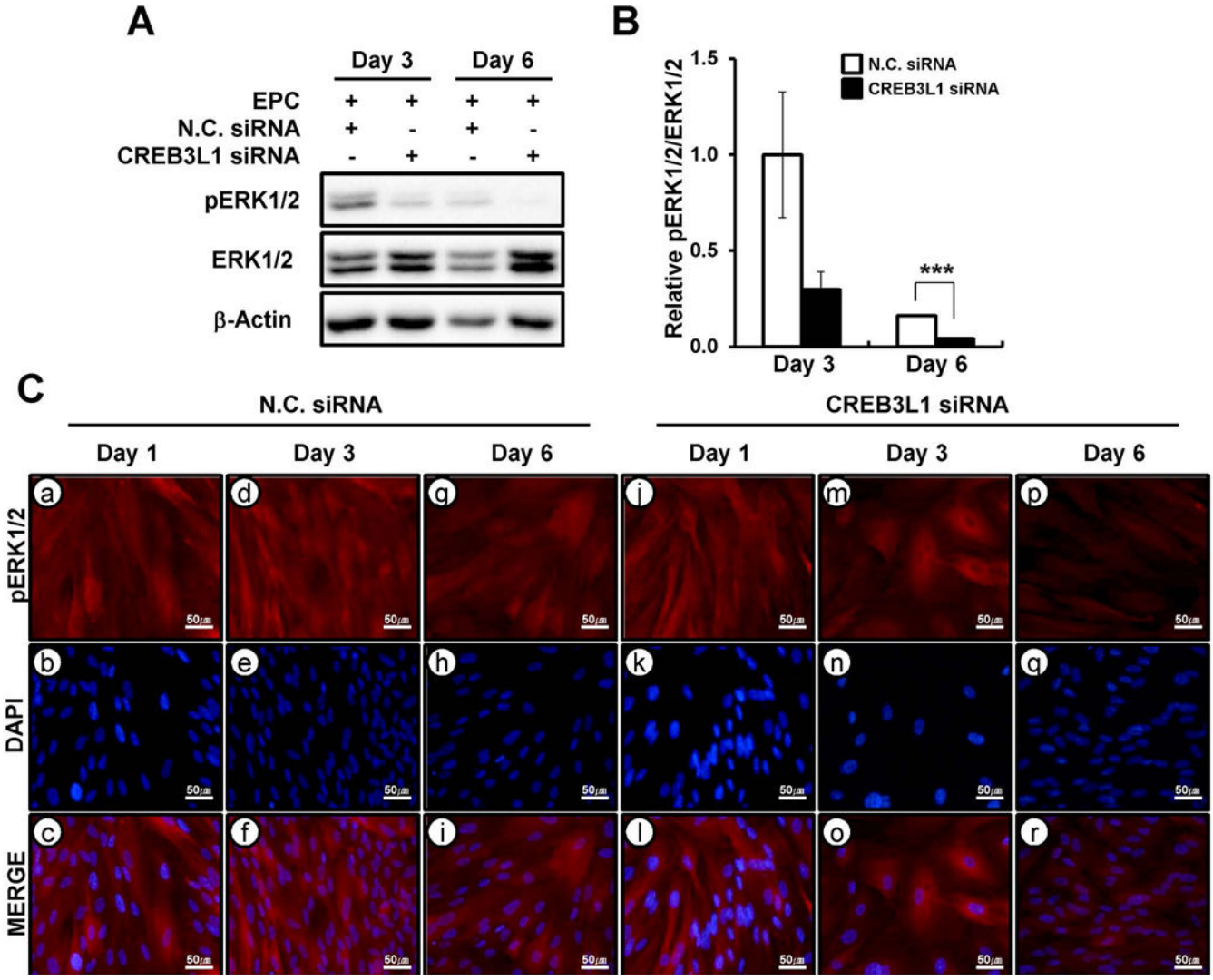

Figure 7.

The regulation of phospho-ERK1/2 by *CREB3L1* during hESCs decidualization. (A) The expression level of ERK1/2 phosphorylation during CREB3L1 inhibited hESCs decidualization. ERK1/2 phosphorylation was decreased at day 3 and day 6 in *CREB3L1* siRNA treatment. (B) Quantification of phospho-ERK1/2 protein level during in vitro decidualization with or without *CREB3L1* target siRNA treatment. The results represent the mean ± SEM. ***p<0.001. (C) Nuclear phospho-ERK1/2 was reduced at day 3 and day 6 in vitro decidualization hESCs with *CREB3L1* siRNA compared to transfected with non targeting pool siRNA. Neclei were conterstained with DAPI staining.

Table 1

List of gene specific primers used in real-time PCR analysis.

Species	Gene name	Primer sequence	
Mouse	*CREB3L1*	F	5′-GCCTTCCTGCATTCTCTTCC-3′
		R	5′-CCTCTGGCCAGGTCTCTCTC-3′
	RPL7	F	5′-TCAATGGAGTAAGCCCAAAG-3′
		R	5′-CAAGAGACCGAGCAATCAAG-3′
Human	*CREB3L1*	F	5′-ACAATGCGCACTTTCCTGAG-3′
		R	5′-GAGGGCTCTTCTCATCCAGC-3′
	IGFBP1	F	5′-CTATGATGGCTCGAAGGCTC-3′
		R	5′-TTCTTGTTGCAGTTTGGCAG -3′
	PRL	F	5′-CATCAACAGCTGCCACACTT-3′
		R	5′-CGTTTGGTTTGCTCCTCAAT-3′
	RPL7	F	5′-AAGAAGCGAATTGCTTTGAC-3′
		R	5′-CAAATCCTCCATGCAGATGA-3′