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Clusterin from endometrial glands plays a critical role in decidualization via *Trem2*

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Abstract

Background Decidualization is a critical step in establishing pregnancy in mammals. Successful decidualization depends on intricate gland-stromal crosstalk. Clusterin (*Clu*) is a ubiquitously secreted protein in physiological fluids that is involved in numerous physiological functions. However, the role of *Clu* in decidualization is not fully understood.

Results In this study, we examined the expression pattern of *Clu* during early pregnancy in mice and explored its potential function in decidualization. Our results revealed that *Clu* was expressed in the uterine glands on Days 1–2 of early pregnancy and on Days 5–8 during decidualization after embryo implantation, as well as in glands at the interimplantation site. Additionally, ovariectomized mice exhibited significant upregulation of *Clu* expression in the uterine glands 3 h after in vivo estrogen injection. *Trem2*, a receptor for *Clu*, was detected in the decidual region of mice on Days 5–8 of early pregnancy, where it mediates *Clu* to regulate the decidual region. Furthermore, we observed that recombinant CLU protein increased the expression of the decidualization marker molecules insulin-like growth factor binding protein 1 (*IGFBP1*) and prolactin (*PRL*) in decidual cells. However, this upregulation was not observed when *Trem2* expression was inhibited with siRNA.

Conclusions Uterine gland-derived *Clu*, a new paracrine modulator, may participate in early pregnancy by influencing the decidualization process mediated by *Trem2* in mice.

Keywords *Clu*, *Trem2*, Uterine glands, Decidualization, Early pregnancy

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Background

Successful pregnancy comprises discrete events, including implantation, decidualization, placentation and parturition. If any step of the process is disrupted, adverse ripple effects of the disruption during pregnancy result in poor outcomes [1]. Decidualization is crucial for successful gestation, necessary for blastocyst implantation, and the formation of a functional placenta [2, 3]. Initially, attachment occurs between the blastocyst and crypt luminal epithelium, followed by proliferation and decidualization of stromal cells at the implantation site [4]. During decidualization, stromal fibroblasts are transformed into secretory decidual cells [5], which enable the endometrium to defend against excessive trophoblast

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invasion, act as an embryo quality sensor, and provide nutrition to the developing embryo before the placenta is fully functional [2, 3]. Dysregulation of decidualization can result in preeclampsia or miscarriage.

In rodents and primates, the uterine glands are essential for stromal decidualization. The notion that uterine glands secrete paracrine-acting factors into the stroma to induce decidualization is a novel concept derived from mouse models that are not equipped with uterine glands [3]. No evidence of implantation or stromal cell decidualization was found in progesterone-induced uterine gland knockout mice on Day 6 after mating [6]. The absence of endometrial glands resulted in a lack of several pregnancy-related factors, including leukemia inhibitory factor (*Lif*), steroid hormone receptors, cytokines, growth factors, and several developmental factors, which are critical for gestation. The infertility phenotype of *Lif* null mice and conditional forehead box A2 (*Foxa2*) deletion models, as well as mice lacking uterine glands, affects gland-derived products, underscores the primary role of gland-derived products in establishing and maintaining pregnancy [7].

Clusterin (*Clu*), also known as apolipoprotein J, is a highly conserved 80-kDa disulfide-linked heterodimer glycoprotein found in various body fluids [8–10]. This multifunctional protein exists in two distinct forms: one is processed and matured in the endoplasmic reticulum and Golgi apparatus before being secreted into the extracellularly, and the other is secreted directly into the cytoplasm during cell stress [11]. *Clu* functions as a chaperone molecule and is a functional homolog of small heat shock proteins [12], binding to the hydrophobic domains of misfolded proteins and promoting their receptor family-mediated endocytosis and lysosomal degradation [12, 13].

Since its discovery in 1983, *Clu* has been implicated in various physiological processes, such as cell proliferation, invasion, apoptosis, oxidative stress, stimulation of inflammatory factors, and regulation of complement activity [14]. Numerous studies conducted in the last few decades have explored the connection between *Clu* and Alzheimer's disease. *Clu* facilitates the removal of amyloid Aβ from the blood−brain barrier, and *Clu* deficiency in Alzheimer's mice is associated with increased amyloid angiopathy in brain tissue. Additionally, previous studies have shown that *Clu* regulates both normal and abnormal pregnancy events.

Clu has been observed to respond to hormone regulation in the uterine gland epithelium and lumen epithelium during both the human menstrual cycle and the mouse estrous cycle and is involved in physiological remodeling of the uterus. Additionally, *Clu* may have clearance functions mediated by receptors that promote the apoptosis of cells and the endocytosis and degradation of cell debris by lysosomes [15]. In 1996, Thomas L. Brown published a study on *Clu* in early pregnancy in mice; however, owing to technical limitations, the results of this study were not entirely convincing [16]. Consequently, the function and mechanism of *Clu* in early pregnancy remain largely unknown. Abnormal expression of *Clu* is associated with pregnancy-induced hypertension (PIH). Evidence suggests that the concentration of *Clu* in the serum increases in proportion to the severity of PIH. *Clu* may function as a trigger for the expression of inflammatory factors, and its dysregulation could be a crucial factor in the development of PIH [9]. Furthermore, research has indicated that irregular *Clu* expression is also linked to other pregnancy-related diseases, such as intrauterine growth restriction, recurrent abortion, premature delivery and preeclampsia, and may be able to predict adverse pregnancy outcomes. However, the role of *Clu* in regulating early pregnancy in mice under physiological conditions is not fully understood.

Research has demonstrated that several members of the low-density lipoprotein receptor family (LDL receptor family), including low-density lipoprotein receptor-related protein 1 (*Lrp1)*, low-density lipoprotein receptor-related protein 2 (*Lrp2*), apolipoprotein E receptor 2 (*ApoER2*), and very-low-density lipoprotein receptor (*Vldlr*), serve as receptors for *Clu*, allowing the secreted *Clu* to perform its physiological function [17]. For example, research as early as 1995 revealed that LRP2 binds to CLU in vitro, and cells expressing LRP2 facilitate CLU endocytosis leading to lysosomal degradation [18, 19]. *Clu* amplifies central leptin signals through *Lrp2*-mediated endocytosis [20]. Furthermore, *ApoER2* /*Vldlr*-mediated *Clu* is implicated in inducing male germ cell meiosis [17]. Recent studies have also identified triggering receptor expressed on myeloid cells 2 (*Trem2*) as a receptor for *Clu*, promoting amyloid β uptake and potentially reducing Alzheimer's disease risk [21]. However, the receptors mediating *Clu* in the uterus in normal early pregnant mice remain unidentified, necessitating further research to investigate the expression, function, and regulatory mechanisms of *Clu* in this context.

Methods

Animals and treatment

CD1 mice (purchased from Guangdong Sijia Jingda Biotechnology Co., Ltd.) were housed in the SPF animal care facility under a light/dark cycle (12:12 h) and controlled temperature (22–24 °C). All animal procedures were approved by the Animal Care and Use Committee of South China Agricultural University (No. 2021f108). To establish pregnancy, female mice aged 8–10 weeks were mated with fertile mice. The pseudopregnant mice were produced by mating between the female and vasectomized males, and the day when the vaginal plugs

were checked was recognized as Day 1 of pregnancy. To induce artificial decidualization, sesame oil (10 μL, Sigma−Aldrich, St. Louis, MO, USA) was injected into one uterine horn on Day 4 of pseudopregnancy, and the noninfused contralateral horn served as the control.

On Day 4, the mice were anesthetized via intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg), followed by exposure to CO_2. After confirming that the animals were fully anesthetized, they were euthanized by cervical dislocation, and the uteri were collected for further analysis. Ovariectomies were conducted on adult mice to examine the impacts of steroid hormones, followed by a two-week period of rest to eradicate any circulating ovarian steroids. The mice were treated with a single injection of 17β-estradiol (100 ng per mouse, Sigma−Aldrich, St. Louis, MO, USA). The control mice were injected with vehicle. The uterus was harvested 3 h after injection. All the experiments were repeated at least three times.

Immunofluorescence

Frozen Sect. (10 μm) were fixed with 4% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS) for 10 min, followed by incubation with 0.1% Triton X-100 in PBS for 10 min. After the sections were blocked with FBS albumin in PBST for 30 min at 37 °C, they were incubated with primary antibody at 4 °C overnight. The primary antibodies used were as follows: rabbit anti-Clusterin antibody (1:200, Abcam, ab184100), rat anti-E-cadherin antibody (1:500, Abcam, ab11512), rabbit anti-FOXA2 antibody (1:400, Cell Signaling Technology, 8186); and rabbit anti-ERα antibody (1:200, Cell Signaling Technology, 13258). The next day, the cells were washed 3 times with PBST, incubated with anti-rabbit and anti-goat IgG (conjugated to Alexa Fluor 594 and Alexa Fluor 488 secondary antibodies, respectively (Jackson ImmunoResearch, William Seagrove, PA, USA)) for 30 min at room temperature, and then washed 3 times with PBST. Nuclei were counterstained with 6-diamino-2-phenylindole (DAPI, Sigma−Aldrich, St. Louis, MO, USA). The cells were observed under a fluorescence microscope (Leica).

In situ hybridization

In situ hybridization was performed as previously described [22]. Briefly, total RNA was isolated from mouse ovaries and amplified with *Clu* and *Trem2* primers after reverse transcription. The pGEMT plasmid (Promega) was used to clone the amplified fragments of *Clu* and *Trem2*, while primers for T7 and SP6 were used to amplify the *Clu* and *Trem2* fragments in the pGEM-*Clu*/*Trem2* plasmid, which were subsequently validated by sequencing. Digoxigenin-labeled antisense or sense complementary RNA probes were transcribed in vitro via a digoxigenin RNA labeling kit according to the manufacturer's instructions. The sequences used for in situ hybridization are listed in Table 1. Frozen 4% paraformaldehyde-fixed sections from the control and experimental groups were processed onto the same slide for 1 h and hybridized at 55 °C overnight. The sections were washed and incubated iwith alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5,000). The positive signal was visualized using a buffer containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 0.4 mM) and nitro blue tetrazolium (NBT, 0.4 mM) as reaction substrates. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole, the samples were counterstained with 1% methyl green, and the signal was dark brown. The sequences of the RNA-targeting probes are shown in Table 1.

Cell culture and in vitro induced decidualization

The stromal cell line 4003 was maintained in phenol red-free DMEM/F12 medium (Sigma) supplemented with 10% charcoal-stripped fetal bovine serum (cFBS), 500 ng/mL puromycin, 100 U/mL penicillin, 100 U/mL streptomycin, and 1% ITS (Sigma) in a 37 °C incubator containing a 5% CO_2 humidified atmosphere. To induce decidualization, the cells were treated with 1 μM medroxyprogesterone (MPA, Sigma) and 0.5 mM db-cAMP (Sigma). Recombinant CLU protein (RD, 2937) treatment was applied to decidual stromal cells. Stromal cells were seeded into 6-well plates. At 60-70% confluence, in each experimental group, the corresponding medium was replaced with fresh media containing 2% cFBS, and decidualization was induced in vitro by adding MPA (1 μm) or db-cAMP (0.5 mM), followed by treatment with or without recombinant CLU protein (1 μg per sample). The control group cells were also cultured in fresh media supplemented with 2% cFBS without MPA or db-cAMP. All of these cells were harvested after 2 days.

siRNA transfection

An siRNA kit for *TREM2* and nonspecific siRNA was synthesized by RiboBio Co., Ltd. (Guangzhou, China). Using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol, a random RNA sequence not specific for any specific gene (scramble) was used as a negative control (NC). The siRNAs with the most remarkable interference efficiency (50 nM) were used for transfection into stromal cells for 24 h, after which the cells were induced to undergo in vitro decidualization. The relative mRNA levels of *TREM2* were detected. The sequences of the targeted siRNAs are shown in Table 1.

Table 1 Sequences of the primers used in this study

Gene Name	Primer Sequences	Application	Accession Number
APOER2	GGACCTACTGACCAAGAAC GGATGAGGCGTGAATAGTT	RT–qPCR	NM_001018054.3
VLDLR	GACCACAGCAGTATCAGAG ATTCCGCCACATCAAGTAG	RT–qPCR	NM_001018056.3
LDLR	CAGCGAAGATGCGAAGAT AGAAGAGGTAGGCGATGG	RT–qPCR	NM_000527.5
Clu	CGAAGATGCTCAACACCTCA TCCTGCGGTATTCCTGTAGC	RT–qPCR	NM_013492.3
CLU	TCTTGTCTGTGGACTGTTC AGGAGGTGTTGAGCATCT	RT–qPCR	NM_001831.4
TREM2	CGGCTGCTCATCTTACTCTT CAAGTTGTGCGTGCTGAC	RT–qPCR	NM_001272078
RPL7	CTGCTGTGCCAGAAACCCTT TCTTGCCATCCTCGCCAT	RT–qPCR	NM_011291.5
Rpl7	GCAGATGTACCGCACTGAGATTC ACCTTTGGGCTTACTCCATTGATA	RT–qPCR	NM_011291.5
PRL	AAGCTGTAGAGATTGAGGAGCAAA TCAGGATGAACCTGGCTGACTA	RT–qPCR	NM_000948
IGFBP1	CCAAACTGCAACAAGAATG GTAGACGCACCAGCAGAG	RT–qPCR	NM_001013029
Clu	CGAAGATGCTCAACACCTCA TCCTGCGGTATTCCTGTAGC	ISH	NM_013492.3
Trem2	TGACGCCTTGAAGCACTG CCTCGGAGACTCTGACACT	ISH	NM_001272078
TREM2	CCCACAACACCACAGTGTT	siRNA	NM_001272078

Real-time quantitative polymerase chain reaction (RT–qPCR)

RT‑qPCR was performed as previously described [23]. Mouse uterine endometrium or cultured stromal cells were harvested with TRIzol reagent (Accurate Biotechnology, Hunan, China), and RNA was extracted according to the manufacturer's instructions. Reverse transcription into cDNA was performedwith the HiScript II Reverse Transcriptase kit (Vazyme). ChamQTM Universal SYBR® qPCR Master Mix (Vazyme) was used to perform RT‑qPCR. The sequences of the primers used for RT‑qPCR in this study were shown in Table 1.

Statistical analysis

All experiments were repeated at least three times independently. All the data are presented as the means ± standard deviations (SDs). Statistical analyses were performed with Student's t tests. Analysis of variance (ANOVA) was performed for multiple comparisons. $p < 0.05$ was considered to indicate statistical significance. All analyses were performed with GraphPad Prism® software (GraphPad Software Inc., San Diego, CA, USA).

Results

Localization of Clu expression in the mouse uteri during early pregnancy

The spatial expression profile of the CLU protein at implantation sites in the mouse uteri from Days 1 to 8 of pregnancy was determined by immunofluorescence. The presence of the endometrial epithelium at the implantation site was confirmed by measuring E-cadherin (E-cad) expression. CLU was strongly expressed in the endometrial glandular epithelium on Days 1 to 2 and Days 5 to 8 of pregnancy, but almost no signal was detected in the uteri on Days 3 and 4 (Fig. 1a). As previously reported, 3D visualization confirmed that glands connect directly to the chamber during implantation and that glands become more developed and continue to elongate and stretch following implantation on Day 4.5 [4]. CLU was detected in the expanding ductal regions of glands connecting the crypt encasing the embryo on Days 5 to 8 of pregnancy (Fig. 1a).

In situ hybridization revealed localized *Clu* mRNA expression in the endometrial glandular epithelium, similar to the results of the spatiotemporal expression pattern of immunofluorescence. In situ hybridization further confirmed the expression of *Clu* at implantation sites in the mouse uteri in early pregnancy (Fig. 1b).

Clu is expressed in the glandular epithelium of the whole Uterus

To further corroborate whether *Clu* was localized to the glandular epithelium, *Foxa2* was used to characterize the glands further, as it has been reported that *Foxa2* is expressed only in the glandular epithelium of the uterus [24, 25]. Immunofluorescence was used to analyze the

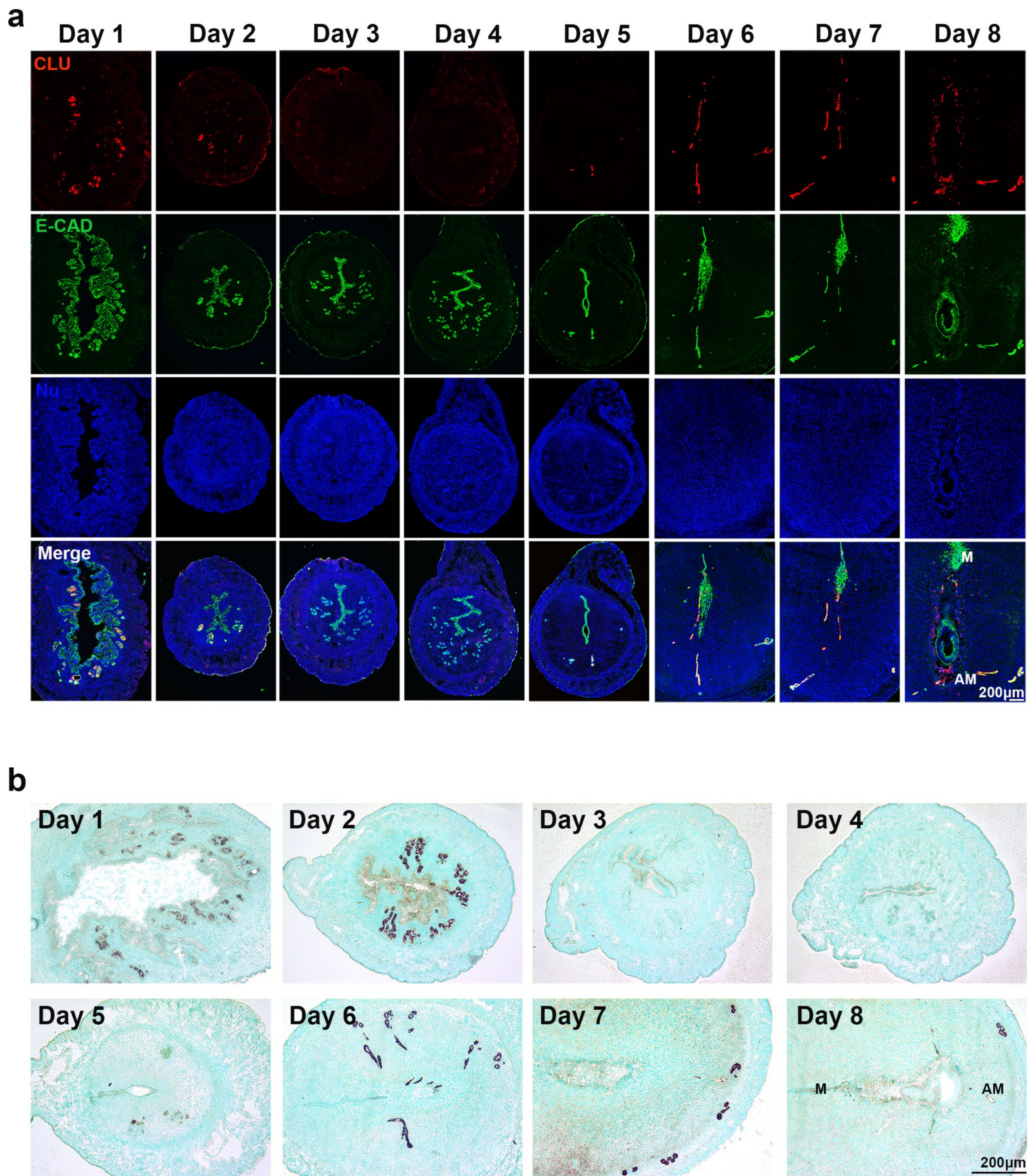

Fig. 1 Spatiotemporal expression of *Clu* protein and mRNA in the mouse uteri during early pregnancy. (**a**) Immunofluorescence image showing the expression of the CLU protein in mouse uteri from Days 1 to 8 of early pregnancy. (**b**) In situ hybridization showing the expression of *Clu* in mouse uteri from Days 1 to 8 of early pregnancy. Scale bar: 200 μm. M, mesometrial pole; AM, antimesometrial pole. All images are representative of three independent experiments

expression of the FOXA2 and CLU. Double immunofluorescence staining revealed complete colocalization of FOXA2 and CLU proteins in the glandular epithelium from Days 5 to 8 of pregnancy (Fig. 2a), which provided more concrete evidence of CLU expression in the glandular epithelium of the mouse uteri.

As a previous study showed, the glands at the interimplantation sites remain clustered together due to dilation of the implant chamber and pressure on the decidua from Days 5 to 8 of gestation [4]. Owing to the previous finding that CLU is located in the expanding glands, we hypothesized that CLU would also be detected in the glandular epithelium at interimplantation sites. To test this hypothesis, we used longitudinal sections of the implantation site and interimplantation site of the joint together and found that the glands clustered at the interimplantation site were positive for CLU expression (Fig. 2b). These results confirmed that *Clu* was expressed in the glands of the whole uterus from Days 5 to 8 and not only in the glands of the implantation sites.

Two secretory forms of Clu in Glands

Previous studies on *Clu* have demonstrated that it has two main forms, one extracellularly secreted from cells and another intracellularly retained, but the canonical pathway involves the synthesis of secretory CLU, which plays a major role in a broad range of physiological and pathophysiological functions [10]. Under enlarged visualization, we found that secretory CLU exists in two pathways and is secreted at implantation sites on Days 5 to 8 of gravidity. The results showed that in some glands, CLU was secreted into the ductal region, whereas in other glands, CLU was secreted into neighboring stromal cells through the paracrine pathway (Fig. 2c). These data indicate that *Clu* has two secretion patterns in the uterus during pregnancy, which may confer correspondingly diverse functions.

Estrogen regulation by Clu depends on estrogen receptor α (ERα)

In both mice and rats, the estrogen peak is present on Day 4 of gestation, which is essential for the progesterone (P_4)-primed uterus to turn into a receptive uterus [26]. If ovariectomized mice are treated with P_4 on the morning of Day 4 before the estrogen level increases, diapause of the embryo is induced, and implantation is delayed, while an injection of estrogen can induce implantation again [1]. As shown in Fig. 1a, CLU signals were almost undetectable in the uterus on Days 3 and 4; however, they reoccurred on Day 5, so we hypothesized that estrogen might regulate the expression of CLU. The above analysis demonstrated the function of estrogen through the ER and revealed that ERα plays a more essential role in female reproduction than does ERβ [2], emphasizing the gland's response to estrogen [27]. Thus, to test this hypothesis, we first investigated whether the glands expressed ERα, which responds to estrogen. Immunohistochemistry analysis revealed ERα in the endometrial glandular epithelium (Fig. 3a). It was suggested that estrogen could affect glands and colocalize with CLU.

We then used an ovariectomized mouse model to further verify whether estrogen might regulate the expression of CLU. Our results revealed that the protein levels of CLU were increased by estrogen in ovariectomized mouse uteri at 3 h after estrogen administration (Fig. 3b). Together, these studies revealed that estrogen could activate the expression of *Clu* in the mouse uteri in an ERα-dependent manner.

Trem2 is expressed in uterine stromal cells during decidualization

The above studies have shown that members of the LDL receptor gene family, such as *Lrp2*, *Lrp1*, *ApoER2*, and *Vldlr*, are responsible for the binding of *Clu* and that *Clu* acts via them [10, 28]. *Clu* was identified as a crucial ligand of *Trem2* from an unbiased protein microarray screen. Additionally, *Clu* can bind to *Trem2* to clear β-amyloid in the brain. *Trem2* deficiency in microglia reduces *Clu* uptake in mice [21]. Paracrine crosstalk between the glandular epithelium and stroma has been reported [3, 29]. Previously, we showed that *Clu* can be secreted by neighboring stromal cells through the paracrine pathway. Endometrial stromal cells initiate decidualization on Day 5, with maximal decidualization occurring on Day 8. Gland stretching occurs throughout the implantation site, and *Clu* is expressed throughout the gland; thus, the gland may secrete *Clu* to neighboring stromal cells through paracrine signaling to regulate decidualization. Overall, we predicted that *Clu* receptors might interact with the *Clu* secreted from the glands to influence decidualization in endometrial stromal cells. To explore this prediction, RT-qPCR was performed to quantify the mRNA expression levels of the LDL receptor gene family and *TREM2* in the cultured endometrial stromal cell line 4003. Under in vitro decidualization, the *VLDLR*, *LDLR*, and *APOER2* mRNA levels all decreased significantly, and only the *TREM2* mRNA level markedly increased (Fig. 4a). Therefore, we focused on *Trem2* in the following study. To investigate whether *Trem2* was expressed in vivo, we performed in situ hybridization to determine the localization of *Trem2* mRNA in mouse uteri from Days 5 to 8 of pregnancy. The *Trem2* mRNA signal was present in the stroma surrounding the implanting blastocyst on Day 5, at which point the zone began to form a decidua called the primary decidual zone (PDZ). The proliferating and differentiating stromal cell layer around the PDZ region subsequently expressed *Trem2* until it peaked on Day 8 (Fig. 4b). These results

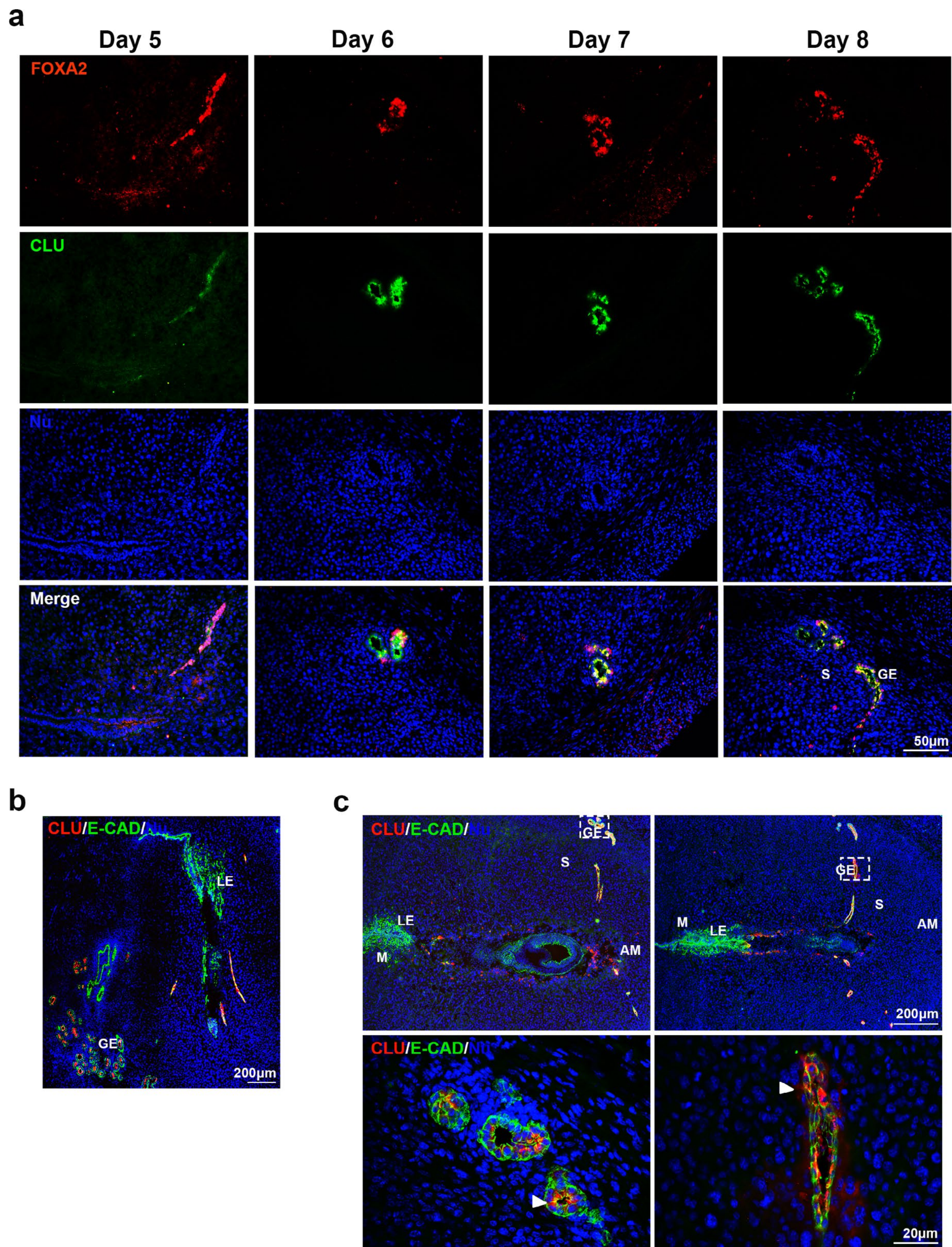

Fig. 2 *Clu* was expressed in the glandular epithelium of the whole uterus. (**a**) FOXA2 and CLU coimmunostaining during Days 5 to 8 of early pregnancy. (**b**) CLU is expressed in the glandular epithelium at the interimplantation site of the longitudinal section of early pregnancy. (**c**) Two CLU secretion pathways exist in the mouse uteri during pregnancy. Scale bar: 200 μm. The enlarged area depicts the two CLU secretion modes: secretion to the ductal regions and secretion outside the stromal cells. Scale bar: 20 μm. Arrowheads indicate the location of the CLU. M, mesometrial pole; AM, antimesometrial pole; S, stromal cells; GE, glandular epithelium; LE, luminal epithelium. All images are representative of three independent experiments

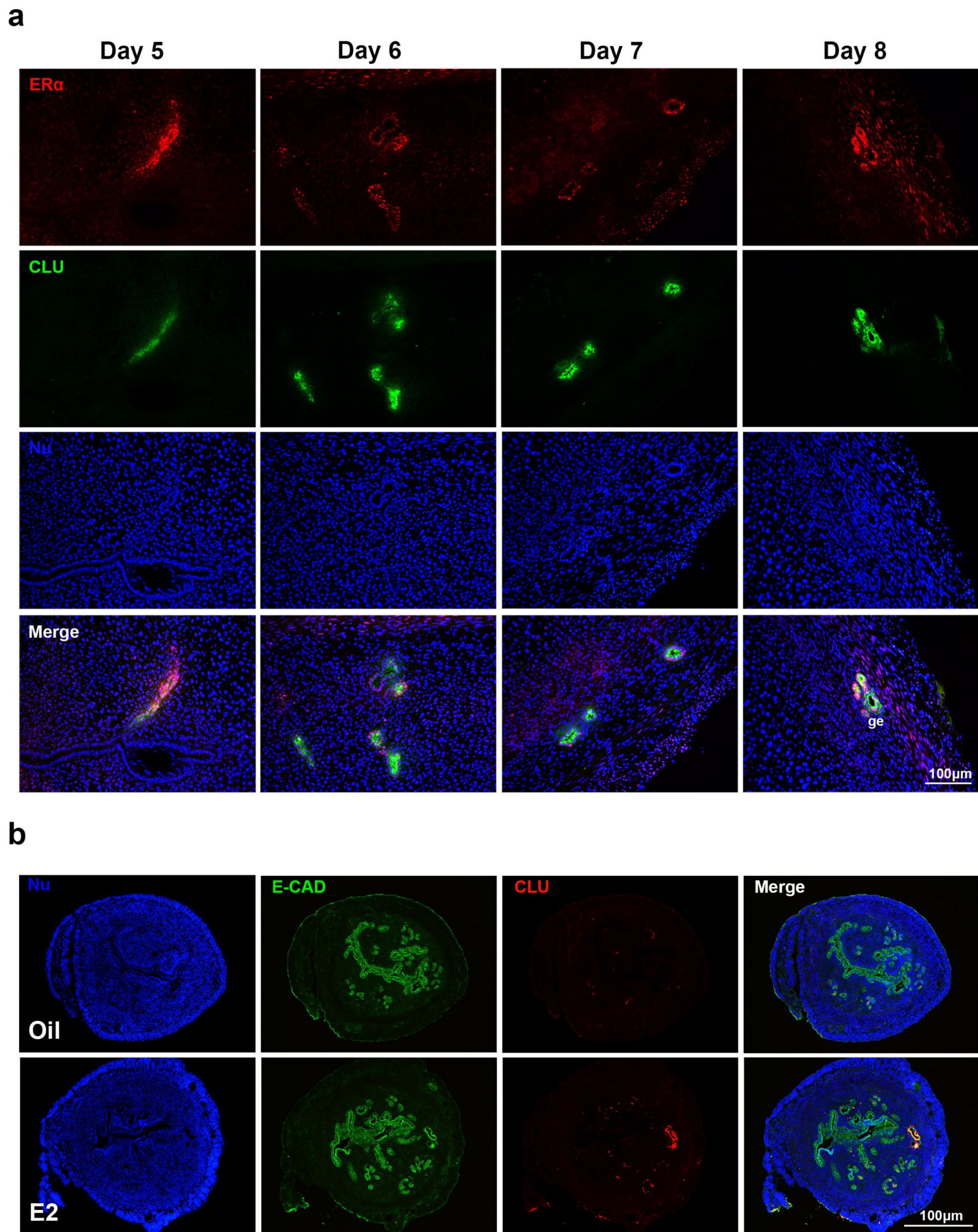

Fig. 3 *Clu* expression is regulated by estrogen through ERα. (**a**) The colocalization of ERα and CLU on the glandular epithelium in the mouse uteri during early pregnancy was detected by immunofluorescence. Scale bar: 100 μm. (**b**) Immunofluorescence showed that CLU was induced at 3 h after treatment with estrogen in ovariectomized mouse uteri. Scale bar: 100 μm. All images are representative of three independent experiments

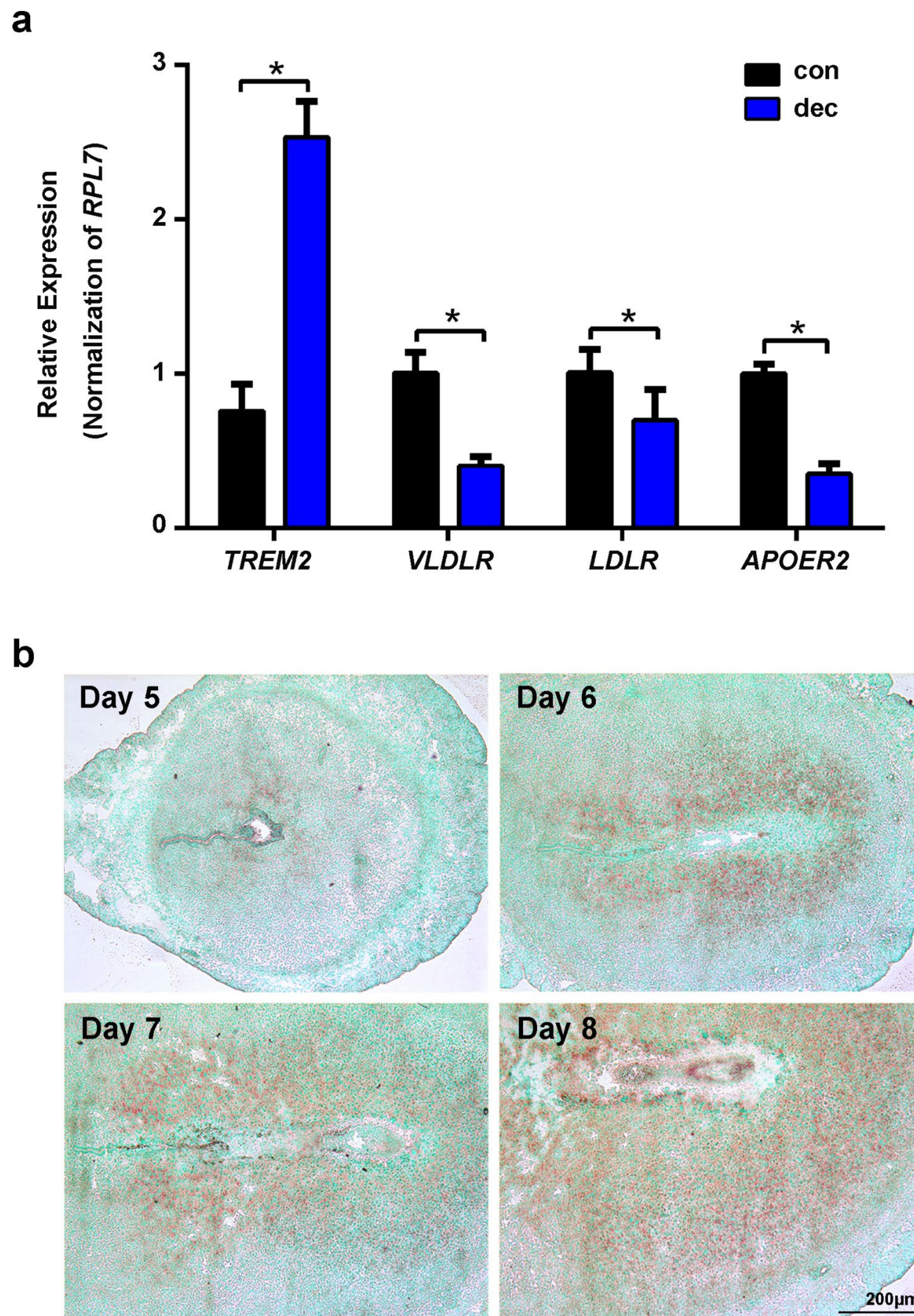

Fig. 4 *Trem2* is expressed on stromal cells during decidualization. (**a**) RT–qPCR analysis of *Clu* receptor genes in endometrial stromal cells under decidualization conditions for 2 days. The ΔΔCt method was used to calculate changes in gene expression relative to *RPL7*. (**b**) In situ hybridization showing the expression of *Trem2* in mouse uteri from Days 5 to 8 of pregnancy. Scale bar: 200 μm. The data are from three independent experiments. Con, control; dec, in vitro decidualization. *$p < 0.05$

confirmed that endometrial stromal cells expressed *Trem2* during decidualization.

Clu regulates decidualization via Trem2

To further investigate the prediction that the secretion of *Clu* from glands may affect decidualization through interactions with receptors, we carried out the following experiment. Intrauterine injection of sesame oil on Day 4 of pseudopregnancy in vivo can induce artificial decidualization [30, 31]. On Day 4, the pseudopregnant mice were injected with sesame oil into the uterine lumen to induce a decidual reaction. The uterus was harvested

4 days later. Compared with the Day 4 pseudopregnant uterus, in situ hybridization revealed that *Clu* and *Trem2* mRNA expression was distinctly increased in the glands and decidual cells, respectively, in artificial decidualization (Fig. 5b and c). Immunofluorescence also revealed that the CLU protein was highly expressed in the glands of the artificial decidua (Fig. 5a). Real-time RT-qPCR was then performed to quantify the mRNA expression levels of *Clu* and *Trem2*, which were markedly upregulated in the uterus after artificial induction for decidualization (Fig. 5d). To verify the role of secreted *Clu* during decidualization, stromal cells were treated with disulfide-linked heterodimers from the recombinant CLU protein. We found that the expression levels of the widely used decidualization markers *PRL* and *IGFBP1* in stromal cells under in vitro decidualization conditions were markedly increased by recombinant CLU (Fig. 6c and d). To assess the importance of *Trem2* during decidualization, we knocked down *TREM2* expression with siRNA in cultured endometrial stromal cells in vitro. Under in vitro decidualization, compared with that under normal conditions, the *TREM2* expression level was significantly lower and almost undetectable by *TREM2* siRNA, confirming that the knockdown efficiency was sufficient. Then, in our analysis of the expression levels of *PRL* and *IGFBP1*, we observed that *PRL* and *IGFBP1* expression was obviously attenuated by *TREM2* siRNA (Fig. 6a and b). These observations indicate that the suppression of *Trem2* perturbs the process of decidualization and potentially regulates *Clu* secretion during decidualization through *Trem2*.

To further examine the molecular mechanisms underlying whether the secretion of *Clu* impacts decidualization via *Trem2*, we treated endometrial stromal cells with recombinant CLU to knock down *TREM2* in vitro. Under in vitro decidualization, the expression of *PRL* and *IGFBP1* did not increase with the silencing of *TREM2* expression, which was contrary to the results obtained without *TREM2* siRNA treatment (Fig. 6c-g). Collectively, these results suggest that the secretion of *Clu* might mediate decidualization via *Trem2*.

Discussion

Decidualization is crucial for successful pregnancy, with defects potentially leading to preeclampsia or miscarriage [1]. *Foxa2*, a transcription factor uniquely expressed in uterine glands, regulates postnatal uterine gland differentiation in mice and is considered a distinct marker of these glands. Conditional knockout of *Foxa2* significantly reduces the number of uterine glands, causing severe defects in endometrial decidualization [3, 7, 32]. Our study revealed that *Clu* colocalized with *Foxa2*, specifically in uterine glands from days 5 to 8 of pregnancy in mice, coinciding with the timing of decidual formation. Silencing the *Clu* receptor*Trem2* expression downregulated the expression of the decidualization markers *IGFBP1* and *PRL* in the in vitro model of stromal cell decidualization. Treatment with recombinant CLU partially reversed this effect, suggesting that glandular-secreted *Clu* is involved in regulating the decidualization process.

The concept of uterine glands secreting paracrine agents to promote decidualization is relatively new. In this regard, the Kazal type 3 (*Spink3*), a gene specifically expressed in uterine gland, was revealed to be secreted into the lumen and decidual region during early pregnancy in mice, acting as a paracrine regulator to affect stromal decidualization [29]. Our experimental results showed that *Clu* is specifically expressed in glands and also observed in adjacent stromal cells, suggesting that *Clu* likely regulates decidualization through paracrine secretion.

Clu regulation of decidualization may depend on two key pathways: proteostasis (clearing misfolded proteins and maintaining protein stability) and anti-inflammatory action [33]. Decidualization is a dynamic process in which endometrial fibroblasts transform into decidual cells, leading to extensive remodeling of the endometrial extracellular matrix (ECM) occurs within the uterine stroma. This ECM remodeling is essential for decidualization. Gene expression analysis and immunohistochemistry reveal that decidualization defects are associated with abnormal expression and deposition of ECM molecules [34]. Matrix metalloproteinases (MMPs) are the primary enzymes responsible for degrading collagen and other proteins during ECM remodeling. Significant changes in the expression of MMPs family molecules are observed in uterine decidual tissues [35]. The overexpression of MMP9 is observed in the uteri of women with adenomyosis and preeclampsia, and CLU, which has a strong affinity for MMP9, inhibits its enzymatic activity [36]. In inflammatory diseases, uncontrolled MMP9 activity increases, whereas CLU prevents stress-induced MMP9 aggregation and inhibits its enzymatic activity. which is associated with the inhibition of ECM deposition. Additionally, CLU inhibits the enzymatic activities of MMP2, MMP3, and MMP7. *Clu* knockout mice exhibit elevated ECM protein levels, underscoring the critical role of *Clu* in maintaining ECM balance [37]. These findings suggest that uterine gland epithelial cell-expressed *Clu* may be secreted into the adjacent decidualization region, and regulates decidualization by maintaining protein homeostasis and balancing the hydrolysis of ECM-related proteins in the decidualization area.

Inflammatory responses are essential for decidualization in both rodents and humans [38]. This process commences with a robust proinflammatory stress response lasting several days, followed by the emergence

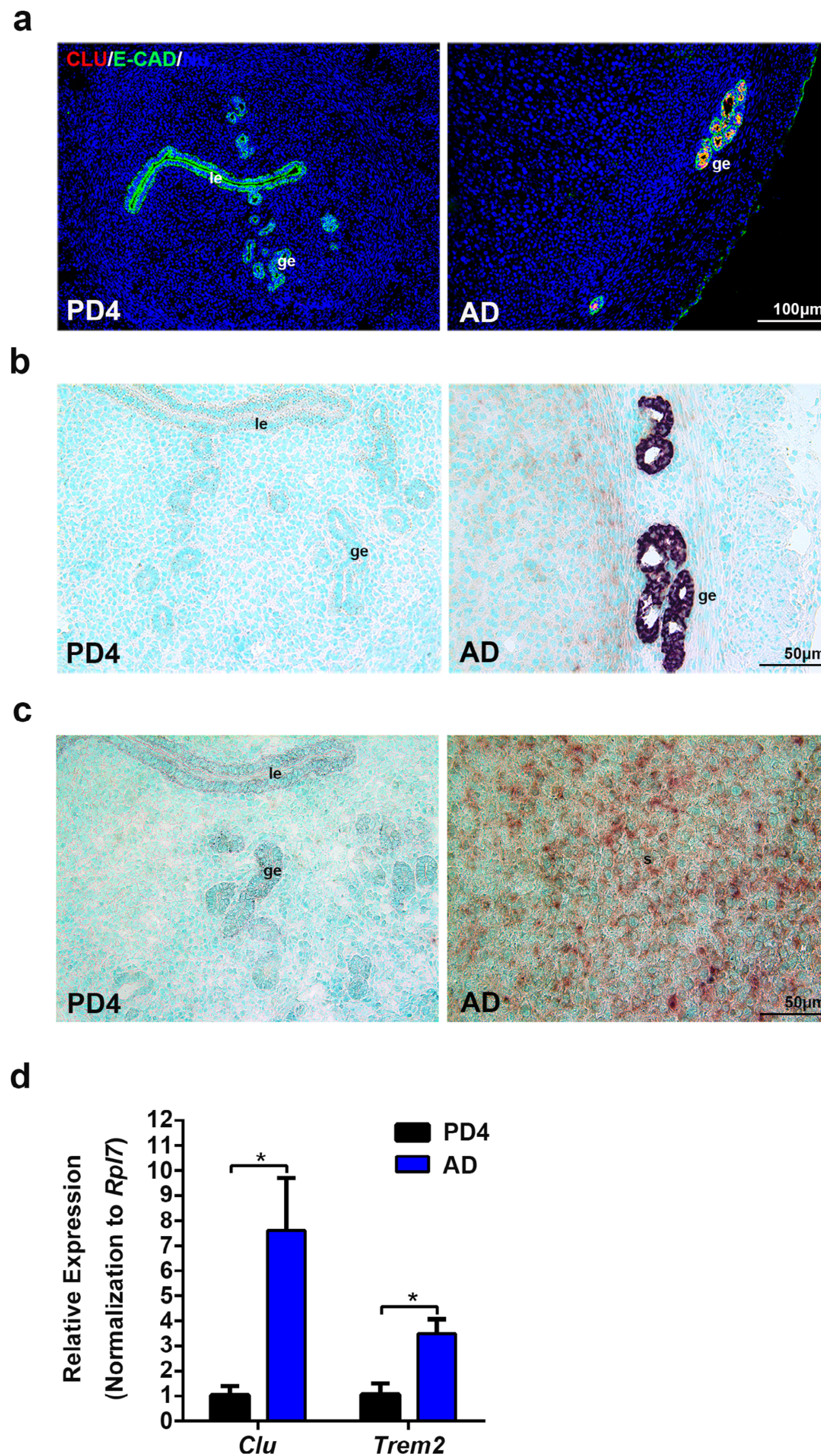

Fig. 5 *Clu* and *Trem2* are upregulated upon decidualization. (**a** and **b**) Expression of *Clu* in oil-induced decidualization in vivo. Immunofluorescence was used to measure the level of CLU protein in the uterus. Scale bar: 100 µm. The level of *Clu* mRNA was measured by in situ hybridization. Scale bar: 50 µm. (**c** and **d**) Expression of *Trem2* in artificial decidualization in vivo. In situ hybridization revealed that *Trem2* mRNA is expressed in decidual cells. Scale bar: 50 µm. RT–PCR was performed to quantify the mRNA expression level of *Trem2*. The ΔΔCt method was used to determine relative changes in gene expression with that of *Rpl7*. The data are from three independent experiments. PD4, Day 4 of pseudopregnancy; AD, artificial decidualization. *$p < 0.05$

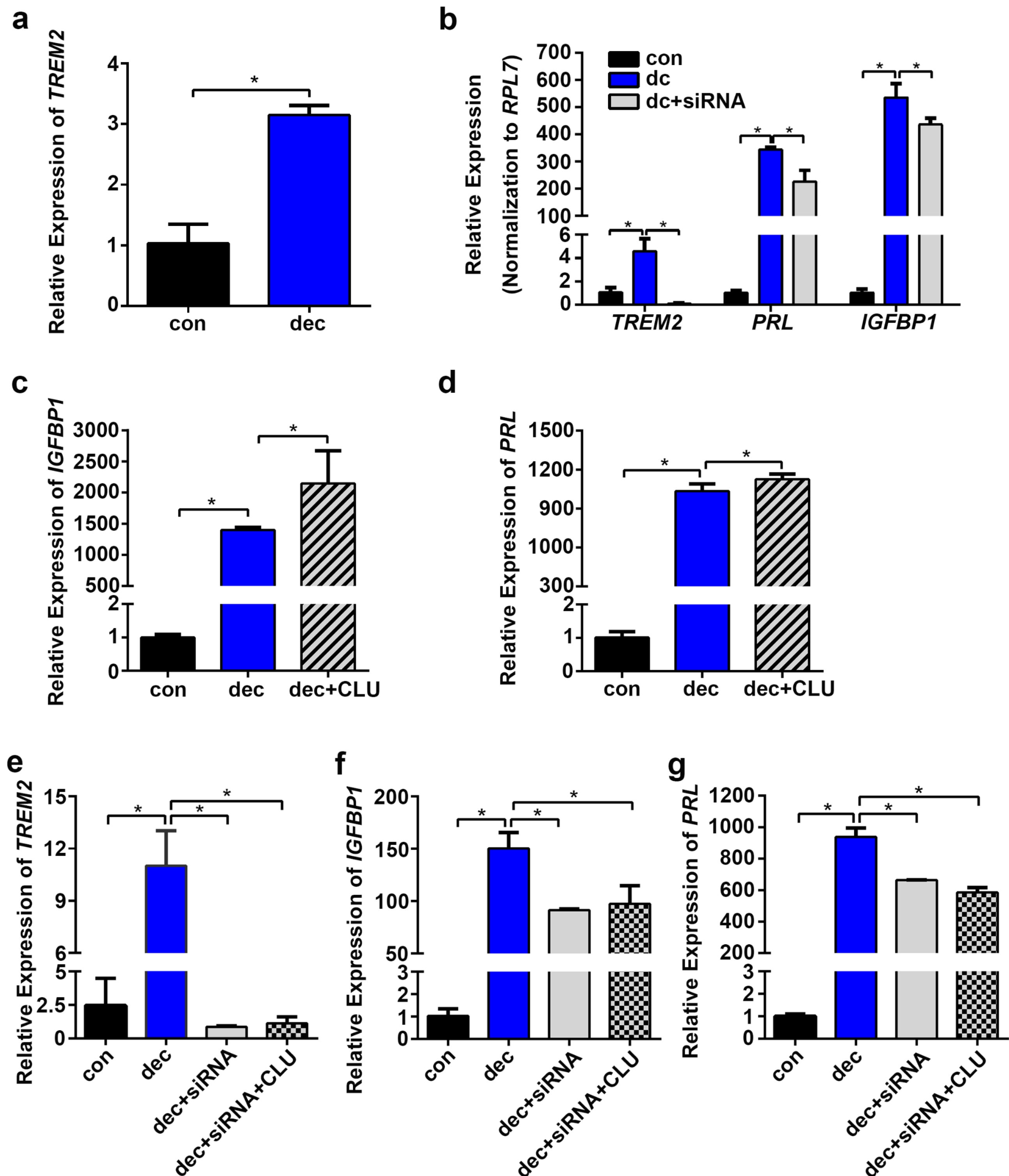

Fig. 6 *Clu* regulates decidualization via *Trem2*. (**a**) Real-time RT–PCR data showing *TREM2* expression in stromal cells after the induction of decidualization in vitro. (**b**) Knockdown of *TREM2* decreases *PRL* and *IGFBP1* expression in decidual stromal cells. (**c** and **d**) The expression of *PRL* and *IGFBP1* was upregulated upon recombinant CLU protein treatment in decidual stromal cells. (**e** and **f**) The effect of recombinant CLU protein in decidual stromal cells was blocked by *TRME2* siRNA. The data are from three independent experiments. The ΔΔCt method was used to calculate changes in gene expression relative to *RPL7*. Con, control; dec, in vitro decidualization. *$p < 0.05$

of anti-inflammatory and senescent decidual cells [39]. Compared to undifferentiated control cells, decidualization induction for 2 days resulted in upregulation of a large number of cytokines, interleukins, and their receptors, accounting for 70 out of 84 inflammatory mediators detected by PCR array. Persistent inflammation may underlie spontaneous decidualization dysfunction [40]. *Clu* is also an important anti-inflammatory factor. Studies have shown that *Clu* can bind to its receptor proteins, thereby mitigating severe endothelial inflammatory responses [33]. *Clu* knockout mice display increased severity of autoimmune myocarditis, accelerated progression from acute inflammation to myocardial scarring, and elevated levels of inflammatory factors in the lungs [41, 42]. *Trem2* activation antagonizes myeloid responsiveness to proinflammatory stimuli at the signaling level [43]. The interaction between *Trem2* and its ligands mitigates inflammatory responses [44, 45]. Knockdown of *Trem2* with short hairpin RNA suppresses inflammation in microglial cell cultures. These findings emphasize that *Trme2* plays a role in restricting inflammation. Our data indicated a specific expression of the *Clu* receptor protein *Trem2* in the decidualization regions of pregnant mice from days 5 to 8. The in vitro results demonstrated that *Clu* regulated the decidualization process through *Trem2*. The potential mechanism likely involves the role of *Clu* and *Trem2* in prevention of excessive inflammatory responses in the decidualization region. However, further investigation is required to elucidate the precise molecular mechanisms.

The evidence suggests that the *Clu* gene responds to steroid hormone regulation in various tissues. In castrated rats, metabolic ablation of hormones results in a significant upregulation of *Clu* mRNA [46]. Additionally, the endometrial cancer cell line ECC-1 exhibits increased *Clu* expression following estrogen treatment [47]. ERα is essential for the normal development of uterine glands postnatally and for increasing the number of glands after puberty. Protein-protein interaction between ERα and FOXA2 have been confirmed by immunoprecipitation experiments, indicating that glands and their secretions are likely regulated by estrogen through ERα [4]. While the localizations of CLU and ERα proteins were overlapped on Days 5–8 of early pregnancy, a 3-hour treatment of estrogen resulted in a significant upregulation of CLU expression in the uterus of ovariectomized mice. These data indicate that *Clu* is likely regulated by estrogen through ERα and is influenced in a short period. To gain a more comprehensive understanding of the role of *Clu* in early pregnancy, further experiments are necessary.

Conclusions

In our study, *Clu* expression was observed in the uterine glands during early pregnancy. Additionally, *Clu* has two secretory forms in glands. Notably, estrogen regulates *Clu* expression in the early stages of pregnancy through the ERα pathway. Our findings also suggest a potential role for *Trem2* as a mediator of *Clu* in the regulation of the decidual area. In conclusion, uterine gland-derived *Clu*, which serves as a novel paracrine modulator, may play a role in early pregnancy by influencing the decidualization process mediated by *Trem2* in mice. These new findings have significant implications for understanding the mechanisms underlying successful implantation and decidualization.

Abbreviations

Clu/CLU	Clusterin
LDL	Low-Density Lipoprotein
Lrp1/LRP1	Low-Density Lipoprotein Receptor-Related Protein 1
Lrp2/LRP2	Low-Density Lipoprotein Receptor-Related Protein 2
ApoER2/APOER2	Apolipoprotein E Receptor 2
Vldlr/VLDLR	Very-Low-Density Lipoprotein Receptor
Trem2/TREM2	Triggering receptor expressed on myeloid cells 2
M	Mesometrial
AM	Antimesometrial
ERα	Estrogen Receptor α
P_4	Progesterone
PDZ	Primary Decidual Zone
IGFBP1	Insulin-Like Growth Factor Binding Protein 1
PRL	Prolactin
PD4	Day 4 of Pseudopregnancy
AD	Artificial Decidualization
Foxa2/FOXA2	Forehead Box A2
PIH	Pregnancy-Induced Hypertension
E-cad	E-cadherin
Lif/LIF	Leukemia inhibitory factor
ECM	Extracellular Matrix
MMPs	Matrix metalloproteinases
scramble	Sequence not specific for any specific genes
NC	Negative Control
RT–qPCR	Real-Time Quantitative Polymerase Chain Reaction
cFBS	Charcoal-stripped Fetal Bovine Serum
MPA	Medroxyprogesterone
Spink3	Kazal type 3

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Author contributions

S.Y., X.L., Y.L. and W.L. designed the study. S.Y., R.C., and L.L. collected and treated the samples. S.Y. and J.Y. conducted the experiments and performed sequencing analyses. S.Y., Y.C., W.L. and X.L. drafted the manuscript. All the authors have read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate
All procedures involving animals were approved by the Ethical Committee of the Laboratory Animal Center of South China Agricultural University (No. 2021f108). All the authors were informed and provided consent for the use of the animals in this study. We confirmed that all methods were reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org) for the reporting of animal experiments, and all methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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