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FULL LENGTH ARTICLE

Unique and independent role of the GABA$_{B1}$ subunit in embryo implantation and uterine decidualization in mice

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Abstract Embryo implantation and decidualization are crucial for successful pregnancy, which include multiple genes and signaling pathways, while the precise mechanism regarding embryo implantation and decidualization has yet to be explored. The GABA which activates GABA$_A$ or GABA$_B$ receptors has been found playing an important role in early pregnancy. Here we seek to investigate whether GABA$_B$ receptors participate in embryo implantation in mice. This study first characterized the spatiotemporal expression pattern of GABA$_B$ receptors in the uterus during the peri-implantation period and found that GABA$_{B1}$ expression was drastically upregulated in stromal cells on days 4–6, a period of embryo implantation and early stages of decidualization. Embryo delayed implantation and oil-induced decidualization models were further used to confirm that the GABA$_{B1}$ was associated with embryo implantation and decidualization. We also found estrogen or progesterone had no directly effect on expression of GABA$_{B1}$ in ovariectomized model. Because we were unable to detect significant GABA$_{B2}$ which couples with GABA$_{B1}$ to form whole GABA$_B$ receptors, and the agonist and antagonist of whole GABA$_B$ receptors had weak effect on the proliferation and differentiation of stromal cells as well, we excluded the possibility whole GABA$_B$ receptors function, and concluded it should be non-classical signals of GABA$_{B1}$ involving in embryo implantation and decidualization. Future studies should focus on investigating the roles and mechanisms of GABA$_{B1}$ during embryo implantation and decidualization.

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Introduction

During a normal pregnancy, the embryo attaches with the luminal epithelium later in day 4, while day 1 is generally defined as the first day with a vaginal plug.[1] The attachment leads to decidualization of endometrial stromal cells surrounding the blastocyst, which involves in stromal proliferation and differentiation, and is mainly regulated by progesterone.[2,3] Therefore, the process of embryo implantation is commonly regarded as being complicated, considering the potential participation of numerous genes as well as signaling pathways.[4,5]

Normally synthesized by glutamic acid decarboxylase, γ-aminobutyric acid (GABA) often functions as an inhibitory neurotransmitter.[6,7] There are reports about the involvement of GABA in mediating progression and migration of tumor cells.[8] Furthermore, GABA signaling is generally mediated by the ionic receptors ($GABA_A$ and $GABA_C$ receptors) and metabolic receptors ($GABA_B$ receptors).[9] For instance, the $GABA_A$ receptors have multiple roles in both non-neuronal and neuronal tissue.[10] In previous study, we found detectable levels of GABA in a pregnant uterus, and then, uncovered GABA/$GABA_A$ receptor-mediated suppression of decidualization and proliferation in endometrial stromal cells.[11] However, the distinctive roles of $GABA_B$ receptor-mediated signaling in early pregnancy have yet to be explored.

Traditionally, the $GABA_B$ receptors are mainly valued for its critical roles in GABA signaling during inhibitory synaptic transmission, neurodevelopmental processes, and pathogenesis and progression of neuropsychiatric diseases. These diseases include epilepsy, spasticity, anxiety, and neuropathic pain.[9] In recent years, however, there have been novel roles discovered for the $GABA_B$ subunit as an independent component in vital processes of autonomic ganglia and some visceral tissues, such as the stomach, intestine, heart, and spleen.[12,13] This contrasts with the traditional point of view that the $GABA_B$ receptors are obligatory heterodimeric complexes comprised of the $GABA_{B1}$ and $GABA_{B2}$ subunits.

In the present study, we aimed to detect the spatiotemporal expression pattern of the $GABA_{B1}$ subunit in mouse uterus during the peri-implantation period, and further explore its role in embryo implantation and decidualization *in vivo* and *in vitro*.

Methods

Animal models

Adult CD1 female mice were purchased from Chongqing Medical University. Guidelines for animal care and use were followed based on the animal ethic committee of Chongqing Medical University. Following female mice mating with healthy male ones in the wild type strain, the first day (D 1) in which the vaginal plug appeared was faithfully recorded for each female mouse. Among them, pregnant ones (anesthetized) received intravenous injection of 0.1 mL of 1% dye Chicago sky blue (C8679; Sigma—Aldrich) in order for confirmation of the implantation sites on day 5 and day 6. Oil-induced decidualization model were generated by transcervically infusing 20 μL sesame seed oil (S3547; Sigma—Aldrich) into the uterine horn of anesthetized female mice, and the heterolateral horn as the control group with no treatment.[14] The mice were sacrificed to collect the uteri 24—96 h after oil infusion. To observe whether $GABA_{B1}$ expression was influenced by ovarian hormone *in vivo*, adult CD1 female mice were ovariectomized and rested for two weeks prior to being subcutaneously injected with ovarian hormone (E2 100 ng/mouse [E2758; Sigma—Aldrich], P4 2mg/mouse [P0130; Sigma—Aldrich], or E2 plus P4). The uteri were collected after injection at 6 h and 24 h.[15] The delayed implantation model was generated by CD1 female mice ovariectomized on day 4 and daily injected with P4 (2 mg/mouse) for two days, the mice were then injected with P4 (2 mg/mouse), and a combination of E2 (50 ng/mouse) and P4 (2 mg/mouse) respectively on the third day. The uteri were collected at 6 h and 24 h after injection.

Quantitative real-time PCR

Refer to a previous study,[16] the total RNA was extracted from pregnant uterine tissues or isolated stromal cells through TRIzol reagent (Invitrogen, USA) based on the manufacturer's protocol. The total RNA (2 μg) was reverse transcribed into cDNA and then the amount of distinct gene expression was evaluated by using the corresponding primer in the ABI 7500 sequence detector system (Applied Biosystems, USA). At least three repeated experiments were performed for each assessment of gene expression. Primers sequences: GABBR1 (F: ACGTCACCTCGGAAGGTTG; R: CACAGGCAGGAAATTGATGGC); Prl8a2(F: TTATGGGTG-CATGGATCACTCC; R: CCCACGTAAGGTCATCATGGAT); GAPDH(F: GGTGAAGGTCGGTGTGAACG; R: CTCGCTCCTGGAAGATGGTG).

Western blotting analysis

Refer to a previous study,[17] protein samples from uterine tissue (isolated from pregnant mice). The protein was then transferred onto a nitrocellulose membrane and incubated with the following primary antibodies: $GABA_{B1}$ (1: 1000, No. 3835, Cell signaling technology, USA) and β-actin (1:5000, A4700, Sigma—Aldrich, USA) at 4 °C overnight. The blots were rinsed and then incubated with the corresponding secondary antibodies (1:5000; Jackson Immuno Research,

USA). West Pico PLUS chemiluminescent substrate (Pierce-34077; Thermo Scientific, USA) was added for band visualization by ChemiDoc™ XRS + System (Bio-Rad, USA).

In situ hybridization

Refer to a previous study,[18] frozen sections (10 μm) were gently transferred onto slides precoated with poly-L-lysine, followed by incubation with 4% paraformaldehyde (158127; Sigma–Aldrich, USA) for 15 min at 4 °C. Then the sections were incubated with 50% formamide solution containing sense or antisense cRNA probes labeled by digoxygenin for 4 h at 45 °C. Prior to assessment with liquid emulsion autoradiography, the sections were subsequently incubated with solution containing RNase A (20 μg/mL; No. 2158, Takara, Japan) for another 20 min at 37 °C. Additionally, the negative control group received sense probes on the slides.

Immunocytochemistry and immunofluorescence

Paraffin section (5 μm) of mouse uterus tissues obtained on days 1–8 were deparaffinized, rehydrated, and then incubated in 3% peroxide in methanol for 15 min at room temperature in order to block endogenous peroxidase activity. After washing three times with PBS, the sections were blocked by 5% albumin solution in PBS at room temperature for 1 h and incubated with GABA_B receptor 1 antibody (1:300, ab55051, Abcam, UK) at 4 °C overnight. Primary antibody was replaced by 0.5% albumin solution in PBS in control group. After rinsing with PBS three times again, the sections were incubated with the corresponding secondary antibody for another 30 min at room temperature. For Immunofluorescence microscopy, sections were mounted after nuclear counterstain using 4′, 6-diamidino-2-phenylindole (DAPI) (1:1000, no. P36931, Thermo Fisher Scientific), and fluorescence can detect by laser line. The peroxidase on each section were visualized through administration of 3,30-diaminobenzidine solution, and images were obtained under a Leica DM2500 light microscope.

Cell purification and culture

As previously described,[19] uterine stromal cells were isolated and cultured with a modified method. The uterine tissue isolated from pseudo-pregnant mice (female mice mating with male mice with vasectomy) on D4 were dissected into small pieces (2–3 mm). The tissues were first rinsed in HBSS containing penicillin and streptomycin (Gibco, USA), and then digested in HBSS supplemented with 6 mg/mL dispase II (Gibco, USA) and 25 mg/mL trypsin (Sigma–Aldrich, USA) for 1 h at 4 °C, for 1 h at room temperature, and for 10 min at 37 °C. The tissues were then incubated in 4 mL of fresh medium supplemented with 0.5 mg/mL collagenase (Sigma–Aldrich, USA) for 30 min at 37 °C. The stromal cells were subsequently harvested after the digested tissue passed through a 70-μm filter. Isolated stromal cells were plated in either 60-mm dishes or 96-well plates, which contained DMEM/F12 medium (Gibco, USA) supplemented with 10% FBS (Biological Industries, USA), penicillin, and streptomycin. The primary culture medium

was regularly refreshed with fresh medium. To evaluate the effect of GABA_B receptor on proliferation and differentiation, cells were cultured in modified DMEM/F12 medium (10% FBS, penicillin and streptomycin) with 100 μM baclofen (R-baclofen, abs47028346a, Absin, USA), or 100 μM saclofen (2-Hydroxysaclofen, 0245/10, TOCRIS, USA), or DMSO as a control for cell proliferation analysis and cultured in modified DMEM/F12 medium containing the necessary ingredients (1% FBS, 10 nM estrogen [E2; E2758, Sigma–Aldrich], 1 μM progesterone [P4; P0130, Sigma–Aldrich], penicillin and streptomycin) for cell differentiation analysis. Then cells were harvested after 0–96 h and proliferation and differentiation were evaluated by Cell Counting Kit-8 and Prl8a2 mRNA expression levels respectively.

Statistical analysis

The statistical analyses were carried out with SPSS 11.5 software (SPSS, USA). Data was shown as a mean plus or minus the standard error of the mean. Comparison of means was generally performed using a Student's t-test between two groups. For data comprising three or more groups, an unpaired Students' two-tailed t-test or a one-way ANOVA was performed, respectively. $P < 0.05$ was regarded as statistically significant, and $P < 0.001$ was regarded as statistically highly significant.

Results

Expression pattern of the GABA_{B1} on the uterus during decidualization

In order to evaluate the expression level of GABA_{B1}, Western blotting and quantitative RT-PCR were performed. The results showed a dynamically altered expression level of the GABA_{B1} subunit on days 4–8 in the mouse uterus (Fig. 1A–C). Besides, GABA_{B1} was weakly expressed in the uterus on days 1 and 4, while GABA_{B1} expression became obviously enhanced on days 5 and 6 (Fig. 1A–C). By using immunohistochemistry and in situ hybridization, it was further revealed that GABA_{B1} expression was drastically upregulated in almost all stromal cells, which normally surrounds the blastocysts implanted on day 5. Following blastocyst attachment, GABA_{B1} exhibited a similar pattern of expression on day 6 (Fig. 1D and E). Collectively, the spatiotemporal expression pattern of the GABA_{B1} subunit suggested that GABA_{B1} is potentially involved in an early decidualization during embryo implantation. This inspired us to further evaluate the association between the GABA_{B1} subunit and normal pregnancy in different mouse models.

Induction of GABA_{B1} expression in oil-induced decidualization model

As shown in Fig. 1, GABA_{B1} expression was significantly upregulated on days 5 and 6 during decidualization. It was reasonable to surmise that expression of GABA_{B1} was closely related to decidualization of stromal cells. Therefore, an oil-induced decidualization model was generated
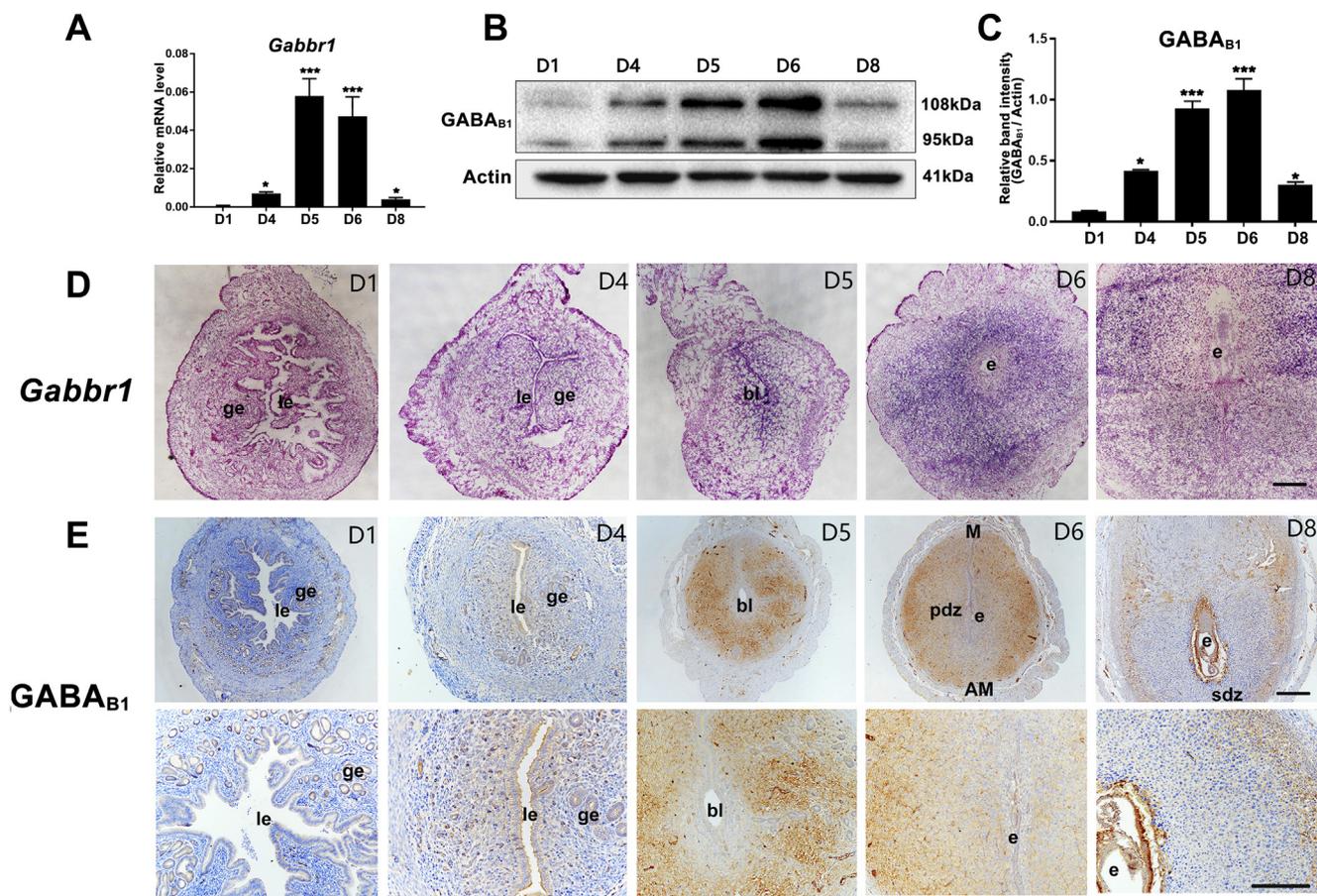
Figure 1 GABA expression in mouse uterus at each designated time point following pregnancy. **(A)** Quantitative RT-PCR analyses revealed expression level of the GABA$_{B1}$ subunit. Each mRNA expression level was normalized to GAPDH. The experiments were repeated three times and all the data were shown as a mean plus or minus the standard error of the mean. *$P < 0.05$,***$P < 0.001$ **(B)** Western blotting analyses of the GABA$_{B1}$ subunit in mouse uterus on days 1, 4, 5, 6 and 8. **(C)** The relative expression of GABA$_{B1}$ subunit by gray scanning analyses. *$P < 0.05$,***$P < 0.001$ **(D–E)** Analyses of uterine GABA$_{B1}$ expression via *in situ* hybridization and immunostaining on days 1, 4, 5, 6 and 8 following pregnancy. Scale bars, 200 μm e, embryo; bl, blastocyst; le, luminal epithelium; ge, glandular epithelium; m, mesometrial pole; am, antimesometrial pole; pdz, primary decidual zone; sdz, secondary decidual zone.

in order to further unscramble the underlying mechanism of the unique expression pattern of the GABA$_{B1}$ subunit (Fig. 2A). Here, the results showed that detected GABA$_{B1}$ was mainly displayed at 24 and 48 h compared to the corresponding control group (Fig. 2B). Moreover, most of the enhanced signal appeared around the uterine cavity (Fig. 2C). However, GABA$_{B1}$ expression was obviously reduced at 72 and 96 h (Fig. 2B and C). This change in continuous expression resembled the altered expression pattern of the GABA$_{B1}$ subunit on days 5–8 during normal pregnancy. Therefore, the oil-induced model indicated that GABA$_{B1}$ is most likely to be involved in an early decidualization.

Induction of GABA$_{B1}$ expression following embryo implantation in an embryo delayed implantation model

In order to further determine whether embryo attachment is a key element for GABA$_{B1}$ expression, we employed the

delayed implantation model. The pregnant mice were ovariectomized at day 4, and then were (1) subcutaneously injected with P4 for three days, (2) P4 for two days, and a combination of E2 and P4 on the third day, followed by sacrificing the mice after another 24 h. Accordingly, GABA$_{B1}$ expression increased following an injection of E2 plus P4 (Fig. 3A), which induces embryo implantation. However, GABA$_{B1}$ expression was relatively low in the P4 treatment group in which embryos generally failed to attach to the uterine epithelium (Fig. 3B). In addition, the results were also confirmed by quantitative real-time PCR (Fig. 3C). Combined with the GABA$_{B1}$ expression pattern in a normal pregnancy, the expression of GABA$_{B1}$ is very likely to be induced by embryo implantation during normal pregnancy.

E$_2$ and P$_4$ are not directly responsible for the induction of GABA$_{B1}$ in mouse uterus

Embryo implantation, decidualization, and other developmental events undergo a refined regulation of both E2 and
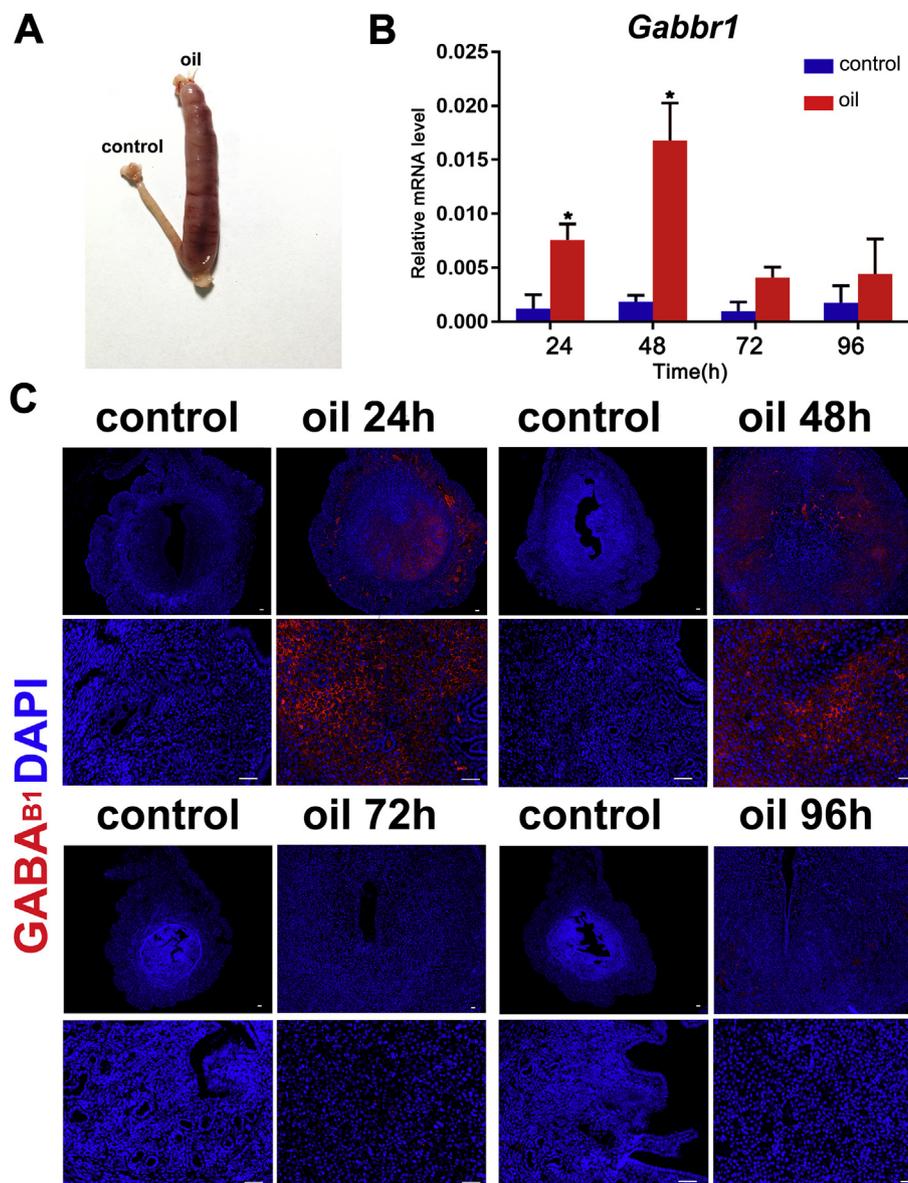
Figure 2 Expression of GABA$_{B1}$ in oil-induced decidualization models. **(A)** The oil-induced decidualization uterine model at 96 h after oil infusion. One uterus was infused with sesame oil to induce decidualization, and the other with no treatment as a control. **(B)** Quantitative RT-PCR analysis revealed a different expression level of GABA$_{B1}$ at 24–96 h in oil-induced models. The mRNA expression levels were normalized to GAPDH. Experiments were repeated three times and all the data was shown as a mean plus or minus the standard error of the mean. *$P < 0.05$. **(C)** Immunochemical analyses of GABA$_{B1}$ subunit expression at 24–96 h in oil-induced model. Scale Bars, 50 μm.

P4.[20,21] So ovariectomized mice with estrogen and progesterone injection were applied to assess the correlation between ovarian hormones and GABA$_{B1}$. There was no marked difference in GABA$_{B1}$ expression level and the related distribution at 24 h following injection of E2, P4, or combination of E2 and P4, respectively (Fig. 4A). Furthermore, GABA$_{B1}$ expression appeared at a minimal level according to immunohistochemical analyses at 6 h and 24 h after injection of ovarian hormones (Fig. 4B). Together, these results clearly clarify that E2 and P4 are not directly involved in the induction of GABA$_{B1}$ expression.

Minimal function of the GABA$_B$ receptor on the proliferation and differentiation of endometrial stromal cells

Both GABA$_{B1}$ and GABA$_{B2}$ subunits have been shown to be required for the formation of a functional GABA$_B$ receptor.[22] However, there was no detectable amount of GABA$_{B2}$ expression coded by the *Gabbr2* gene on uteri *in vivo* and *in vitro* (date not shown), the results suggested that there were weak signals of classical whole GABA$_B$ receptors. Nevertheless, further analyses displayed GABA$_{B1}$ expression
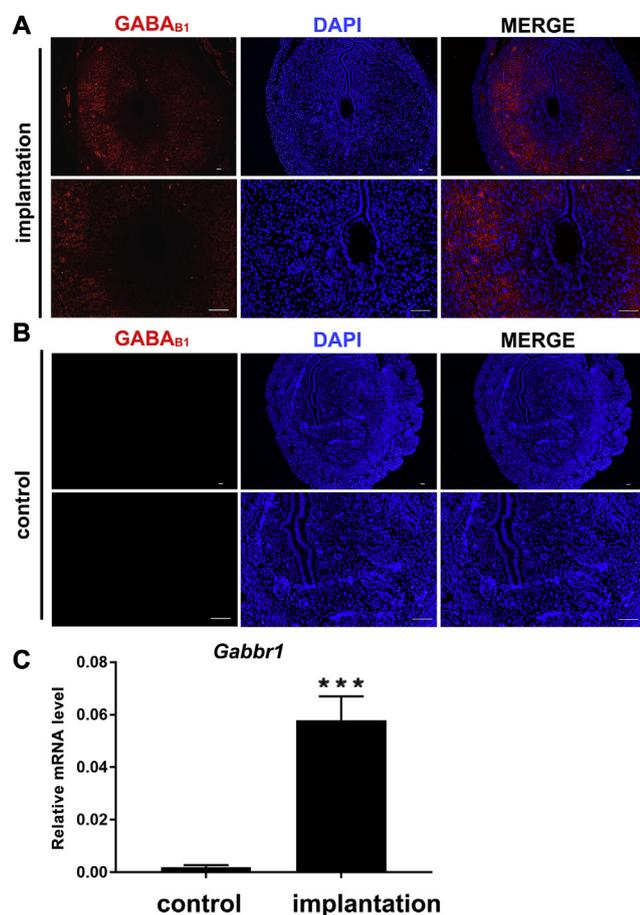
Figure 3 GABA$_{B1}$ upregulation following embryo implantation in embryo delayed implantation models. **(A)** Obvious expression of GABA$_{B1}$ subunit in stromal cells following embryo implantation; **(B)** Undetectable expression levels of GABA$_{B1}$ subunit in stromal cells in the non-implantation group. The right panel is a close-up of the left panel. Scale Bars, 50 μm. **(C)** Quantitative RT-PCR analyses revealed that GABBR1 mRNA was higher in the implantation group compared to that in the control group, both of which were normalized to GAPDH expression. Experiments were repeated three times and all the data was shown as a mean plus or mine the standard error of the mean. ***$P < 0.001$.

and distribution in primary stromal cells *in vitro* (Fig. 5A), which purity was confirmed by vimentin, a stromal cell marker, and cytokeratin, an epithelial marker.[23] Subsequently, roles of the GABA$_B$ receptors in primary stromal cells proliferation were evaluated with the specific inhibitor and activator of the GABA$_B$ subunit (i.e., saclofen and baclofen). Accordingly, no apparent promotion or inhibition of cell proliferation was detected based on proliferation or cytotoxicity assays by using a Cell Counting Kit-8 (CCK8) (Fig. 5B). On the other hand, cell differentiation was assessed through the expression level of Prl8a2 mRNA, which is known as a verified marker for decidual stromal cell[24,25] in stromal cells treated with estradiol and progesterone, in addition to baclofen or saclofen. As expected, the GABA$_B$ activator and inhibitor were unable to effect

differentiation of stromal cells compared to the control group (Fig. 5C). These results suggested that the GABA$_B$ receptors did not have any detectable influence on proliferation as well as differentiation regarding endometrial stromal cells in culture.

Discussion

The establishment of normal pregnancy requires proper decidualization[26]; therefore, it is critical to clarify the precise mechanism regulating decidualization in the early stages of pregnancy.[27] As the major type of inhibitory transmitter in the vertebrate central nervous system, GABA has recently been regarded as an option for alternative treatment or even dietary supplements.[28] In the present study, the expression level of a subtype of the GABA$_{B1}$ subunit was found to be significantly enhanced in uteri of pregnant mice, especially for the critical period of peri-implantation.

GABA$_B$ receptors belong to a super-family of G protein-coupled receptors, which are composed of two subunits, GABA$_{B1}$ and GABA$_{B2}$. GABA$_{B1}$ is often more active than GABA$_{B1}$ under many physiological and pathological conditions.[29,30] In addition to E2 and P4, GABA/GABA receptor signaling also regulates the secretion of luteinizing hormone releasing hormone, luteinizing hormone, and prolactin through the hypothalamic-pituitary-gonadal axis.[31] Previous reports have shown that the GABA$_A$ receptor π subunit is expressed in a variety of peripheral tissues, it has also been suggested that the π subunit is possibly the only subunit expressed in the human uterine epithelium and stromal cells, and increases in human secretory endometrium.[32] In previous study, we found GABA and GABA$_A$ receptors suppressed decidualization of mouse uterine stromal cells by down-regulating cyclin D3.[11] Hence, we further explored the expression and roles of GABA$_B$ receptor in peri-implantaion period.

According to the data collected in multiple mouse models of the present study, GABA$_{B1}$ spatiotemporal expression in early decidualization gave us cause to further explore the potential role of GABA$_{B1}$ in normal pregnancy. In the present study, although GABA$_{B2}$ has undetectable expression at least in stromal cells both *in vivo* and *in vitro* (date not shown), a number of studies had suggested that various cellular populations in the nervous system express GABA$_{B1}$ without GABA$_{B2}$, and the functions of GABA$_{B1}$ still are not clear.[33−36] We thought that there is a non-classical signal of GABA$_{B1}$ instead of the whole GABA$_B$ receptors.

Oil-induced decidualization, and embryo delayed implantation mice models were all generated to analyze potential roles of GABA$_{B1}$ in uterine decidualization and embryo implantation during the peri-implantation period. Spatiotemporal pattern of GABA$_{B1}$ expression during days 4−8 suggested that GABA$_{B1}$ was closely related to embryo implantation and uterine decidualization. Meanwhile, GABA$_{B1}$ also exhibited an almost identical pattern in oil-induced decidualization model and embryo delayed implantation model, thus further confirming the relevance of GABA$_{B1}$ in embryo implantation and early uterine
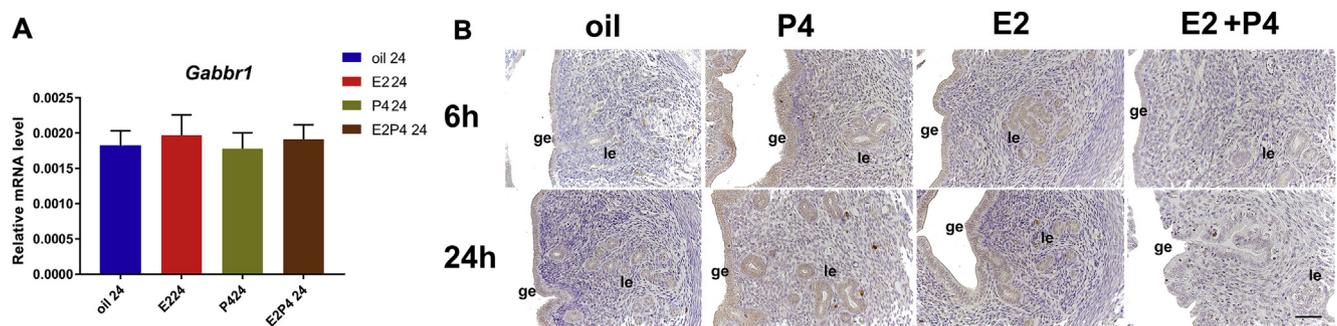
Figure 4 GABA$_{B1}$ is not respond to E$_2$ and P$_4$. **(A)** Quantitative RT-PCR analyses revealed a similar level of GABA$_{B1}$ expression in uteri of ovariectomized mice after injected with E2, P4, and a combination of E2 and P4 respectively for 24 h. The mRNA expression levels were normalized to GAPDH. Experiments were repeated three times and all the data was shown as a mean plus or mine the standard error of the mean. **(B)** Analyses of uterine GABA$_{B1}$ expression via immunostaining in ovariectomized adult mice injected with E2, P4, and a combination of E2 and P4 respectively for 6 h and 24 h. Scale bars, 50 μm le, luminal epithelium; ge, glandular epithelium.
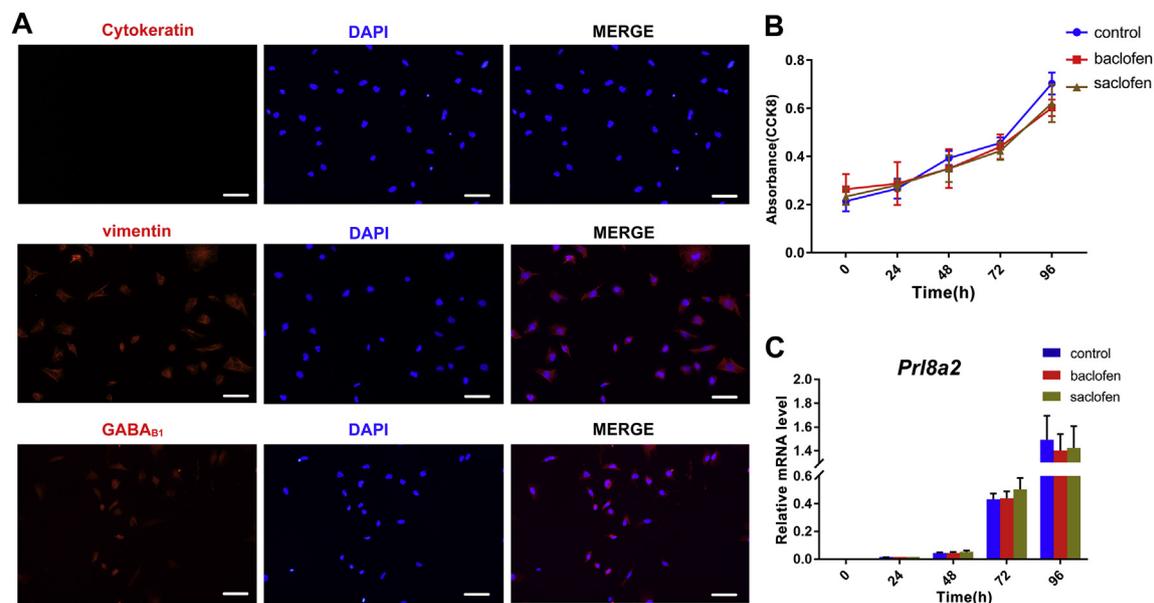

Figure 5 Minimal function of the GABA$_B$ receptor for proliferation and differentiation of primary stromal cells. **(A)** GABA$_{B1}$ expression in isolated endometrial stromal cells shown by a combination of vimentin-positive and cytokeratin-negative signals via immunofluorescence analyses. Scale bars, 50 μm. **(B)** 100 μM saclofen or 100 μM baclofen exhibited a weak influence on proliferation of stromal cells. **(C)** Quantitative RT-PCR analyses revealed that *Prl8a2* mRNA levels were not significantly changed in experimental groups compared to the control group at all time points, and all the mRNA expression were normalized to GAPDH. Experiments were repeated three times, and all the data were shown as a mean plus or minus the standard error of the mean.

decidualization. Most pregnancy events are under direction of E2 and P4, However, E2 and P4 failed to induce GABA$_{B1}$ significant expression in either a separate or a combined manner. From this fact, we infer that ovarian hormones may indirectly but not directly regulate GABA$_{B1}$. All those reliable evidences showed that GABA$_{B1}$ expression may be closely related to embryo implantation and decidualization.

upregulated in stromal cells on days 4—6. Furthermore, non-classical signals of GABA$_{B1}$ may involve in embryo implantation and early uterine decidualization.

Conflict of Interests

The authors declare that they have no conflict of interests.

Conclusion

The present study has evidentially shown that GABA$_{B1}$ is expressed epithelial cells and stromal cells of mice uteri during the peri-implantation period and is particularly

Acknowledgements

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