Title

Inhibition of TMEM16A impedes embryo implantation and decidualization in mice

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Abstract

Recent studies revealed that TMEM16A is involved in several reproductive processes, including ovarian estrogen secretion and ovulation, sperm motility and acrosome reaction, fertilization, and myometrium contraction. However, little is known about the expression and function of TMEM16A in embryo implantation and decidualization. In this study, we focused on the expression and regulation of TMEM16A in mouse uterus during early pregnancy. We found that TMEM16A is up-regulated in uterine endometrium in response to embryo implantation and decidualization. Progesterone treatment could induce TMEM16A expression in endometrial stromal cells through progesterone receptor/c-Myc pathway, which is blocked by progesterone receptor antagonist or the inhibitor of c-Myc signaling pathway. Inhibition of TMEM16A by small molecule inhibitor (T16Ainh-A01) resulted in impaired embryo implantation and decidualization in mice. Treatment with either specific siRNA of Tmem16a or T16Ainh-A01 inhibited the decidualization and proliferation of mouse endometrial stromal cells. In conclusion, our results revealed that TMEM16A is involved in embryo implantation and decidualization in mice, compromised function of TMEM16A may lead to impaired embryo implantation and decidualization.

KEYWORDS: TMEM16A; embryo implantation; decidualization; progesterone;
Introduction

In mammals, embryo implantation is an extremely complicated and elaborately organized process, which involves the intricate regulatory network of cytokines, chemokines and growth factors among embryonic cells, endometrial cells and immune cells (Zhang et al. 2013). Reproductive researchers have been engaged in clarifying this network in embryo implantation. However, there is limited information about the molecular changes that in response to embryo implantation in uterine endometrium. Therefore, it is necessary to identify the embryo implantation-related genes and their function, which may provide new therapeutic target for pregnancy failure and early pregnancy loss.

Calcium-activated chloride channels (CaCCs) are anionic channels that relate to Ca\(^{2+}\) and Cl\(^{-}\) transportation and involved in a variety of physiological activities. TMEM16A, which is also called ANO1 (anoctamin-1), is the major subunit of CaCCs (Paulino et al. 2017b). The gating of TMEM16A is a complex process, it results from the interaction between the binding of intracellular Ca\(^{2+}\), membrane depolarization, and permeant anions (Paulino et al. 2017a). TMEM16A is known to play a fundamental role in the epithelial fluid transport and responsible for fluid secretion of exocrine glands, participates in smooth muscle contraction, and regulates gastrointestinal contraction (Edlund et al. 2014, Kamikawa et al. 2016, Danielsson et al. 2018). TMEM16A is up-regulated in many types of cancers and thought to be involved in tumorigenesis, as well as cell migration and proliferation (Duvvuri et al. 2012, Wang et al. 2017). Knockout of TMEM16A in mice leads to embryonic lethality because of defects in the tracheal and gastrointestinal tract (Rock et al. 2008, Ousingsawat et al. 2009).

TMEM16A is expressed in mouse ovary granulosa cells (GCs), TMEM16A activation downregulates estrogen production in GCs through MEK-ERK signaling cascade. TMEM16A expression in GCs is stage-specific in the estrous cycle and is regulated by gonadotropin, and it may be involved in the regulation in ovulation induced by luteinizing hormone (LH) surge (Sun et al. 2014). Recently, TMEM16A has been found to promote uterine smooth muscle contraction, TMEM16A inhibition and genetic knockdown lead to an attenuation of contraction in pregnant human uterine smooth muscle, and TMEM16A is a potential target for tocolysis (Danielsson et al. 2018).
In addition, TMEM16A is expressed in telocytes and may contribute to preeclampsia (Nizyaeva et al. 2018). The presence of TMEM16A in human sperm is related to capacitation and progesterone-induced acrosome reaction, inhibition of TMEM16A may compromise the sperm capable of fertilizing oocytes (Cordero-Martinez et al. 2018). However, little is known about the role of TMEM16A in other reproductive processes such as embryo implantation and decidualization.

In this study, we demonstrated that TMEM16A is up-regulated in mouse endometrium during early pregnancy and decidualized mouse endometrial stromal cells (mESCs). The expression of TMEM16A is regulated by progesterone through progesterone receptor/c-Myc pathway. We further investigate the function of TMEM16A in embryo implantation and decidualization by using small molecule inhibitor of TMEM16A (T16Ainh-A01). TMEM16A inhibition could dramatically impede embryo implantation and decidualization. These findings support that TMEM16A is involved in reproductive physiology, especially in embryo implantation and decidualization.

**Materials and Methods**

**Animals and treatments**

All experiments were approved by the University Animal Ethics Committee and performed according to Wuhan University institutional and national guidelines. Sexual mature mice (CD-1 outbred strain, 25-30 g body weight) were housed in a controlled environment with a 14 h light: 10 h dark cycle. Female mice were mated with fertile or vasectomized males to induce pregnancy or pseudopregnancy (day 1 is the day of vaginal plug). From days 1 to 4, pregnancy was confirmed by flushing the embryos from the oviducts or uterus. The implantation sites on day 5 were visualized through intravenous injection of 0.1 ml of 1% Chicago blue dye (Sigma-Aldrich Inc., St. Louis, MO, USA) in saline.

Artificial decidualization experiments were performed as previous report (Qi et al. 2015). In brief, female mice were mated with vasectomized males of the same strain to induce pseudopregnancy. Artificial decidualization was induced by intraluminal injection of 10 µl sesame oil.
(Sigma-Aldrich) into one uterine horn on day 4 of pseudopregnancy, and contralateral uterine horn was used as control without any treatment. The uteri horn were collected on day 8 of pseudopregnancy.

Steroid hormonal treatments were initiated 2 weeks after ovariectomy. The ovariectomized mice were injected subcutaneously with estradiol-17β (E₂β, 0.1 µg/mouse, Sigma-Aldrich), progesterone (1 mg/mouse, Sigma-Aldrich), or a combination of E₂β and progesterone. The control mice only received vehicle (sesame oil, 0.1 ml/mouse, Sigma-Aldrich). At 24 h after injection, ovariectomized mice uteri were respectively collected for real-time PCR.

Freshly prepared TMEM16A inhibitor (T16Ainh-A01, 0.12 mg/0.1 ml/mouse, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) and injected subcutaneously once per day (22:00) from days 4 to 7 of pregnancy or artificial decidualization, respectively. DMSO was injected as a control. Uteri were collected on day 5 or day 8 to record: 1) the percentage of mice with implantation sites in each group; 2) the numbers of implantation sites for all mice with implantation sites; 3) the weights of implantation sites. The mouse serum and uteri tissues were respectively collected for further analysis.

**In situ hybridization**

Total RNAs from mouse uterus on day 8 of pregnancy were reverse-transcribed and each hybridization probe template was amplified with the corresponding primers (Table 1). pGEM-T vector plasmid (Promega, Madison, WI) was used for cloning the amplified fragment. Digoxigenin-labeled antisense or sense cRNA probes were transcribed *in vitro* by using digoxigenin RNA labeling kit (Roche Applied Science). In situ hybridization was performed as previously described (Qi *et al.* 2015). Briefly, frozen sections (10 µm) were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich)-treated slides and fixed in 4% paraformaldehyde solution in PBS. Hybridization was performed at 55°C for 16 h. Then, the sections were incubated with sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5000, Roche). The signal was visualized with the buffer containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitrobluetetrazolium. Endogenous alkaline phosphatase activity was inhibited with 2
mM levamisole (Sigma-Aldrich). The sections were counterstained with 1% methyl green. The positive signal of in situ hybridization was visualized as a dark brown color.

Cell culture and treatment

Primary mESCs were enzymatic isolated on day 4 of pregnancy and cultured with DMEM/F12 (Sigma-Aldrich) containing 10% charcoal-treated FBS (Biological Industries, Israel). As previously described (Qi et al. 2015), the mESCs were treated with 10 nM of E₂β (Sigma-Aldrich) plus 1 µM of progesterone (Sigma-Aldrich) to induce in vitro decidualization. Both E₂β and progesterone were dissolved in ethanol.

For steroid hormonal treatments in vitro, stromal cells were treated with 10 nM E₂β, 1 µM progesterone or a combination of E₂β and progesterone, respectively. For further studies, stromal cells were treated with RU486 (Sigma-Aldrich), c-Myc inhibitor (10058-F4, 20 µm, Sigma-Aldrich), TMEM16A inhibitor (T16Ainh-A01, 10 nM, Sigma-Aldrich), respectively.

siRNA targeted to Tmem16a was transferred into mESCs using Lipofectamine 2000 (Invitrogen, California, USA) according to the instructions. The efficiency of transfection was tested by real-time PCR after 24 h transfection.

For analyzing the effect of TMEM16A inhibition on mESC proliferation, mESC cultured in 96-well plate were treated with T16Ainh-A01 (10 nM) with or without in vitro decidualization. The medium was changed with serum-free medium containing Cell Counting Kit-8 (CCK-8, Dojindo) 24 h later cells were further cultured for 4 h. The absorbance at 450 nm was detected using a BioTekELx808 spectrophotometer.

RNA extraction and real-time PCR

Total RNAs from cultured cells and tissues were extracted by using TRIzol (TaKaRa Bio Inc., Tokyo, Japan) following the manufacturer’s recommendations. Small RNA was extracted using RNAiso for Small RNA (TaKaRa). RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser or Mir-X miRNA First-Strand Synthesis Kit.
cDNA for real-time PCR was amplified using a SYBR Premix Ex Taq™ II kit (TaKaRa), performing on the ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Rpl7 served as an internal control and all reactions were run in triplicate. The expression of Tmem16a, Dprp, and Alp mRNA is normalized by the level of Rpl7. The relative expression levels of all target genes were calculated using the \(2^{-\Delta\Delta CT} \). The sequences of real-time PCR primers are listed in Table 1.

**Western blot analysis**

Tissues and cultured cells proteins were extracted using lysis buffer supplemented and protein concentrations were measured using BCA Protein Assay Kit. Protein was loaded per well on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, UK). Membranes were blocked with 5% skim milk for 1 hour at room temperature. Membranes were incubated with primary antibodies in 5% BSA (1:1000) as follows: anti-TMEM16A (Santa Cruz Biotechnology, California, USA), anti-TUBULIN (Cell Signaling Technology, CST, Boston, MA), anti-PR (Dako), and anti-ERα (Santa Cruz Biotechnology, California, USA) overnight and washed. Then membranes were incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG secondary antibody in 5% skim milk (1:4000) at room temperature for 1 hour and visualized by enhanced chemiluminescence.

**Assay of serum hormones**

On day 8 of pregnancy, the orbital blood was collected from control and T16Ainh-A01 injection groups. The blood sample from each mouse was rested for 30 min at room temperature, then centrifuged at 1000 rpm for 15 min at 4°C. The supernatants were collected for serum estrogen (11-Esthu-E01, Alpco, MA USA) and progesterone (11-Prohu-E01, Alpco) concentration detection according to the manufacturer’s instruction.

**Statistical analysis**

For *in situ* hybridization, each experiment was repeated at least three times from four different mice. In cell culture and treatment experiments, there were three duplicates in each group, and the treatments were repeated three times. Statistical analyses were performed using SPSS statistical...
software (version 19.0). All results are presented as means ± standard deviation. ANOVA was used for comparison of multiple variables, and the Student’s t-test was used to analyze differences in the gene expression between groups. A value of $p < 0.05$ was considered statistically significant.

Results

Expression of TMEM16A in mouse uterus during early pregnancy

*In situ* hybridization was performed to detect the localization of *Tmem16a* mRNA in mouse uterus. *Tmem16a* mRNA signals were negative in mouse uterus from days 1 to 3 of pregnancy. However, the mRNA signal of *Tmem16a* was weakly localized in uterine luminal epithelium on day 4 of pregnancy. After implantation, *Tmem16a* was strongly expressed in the subluminal stromal cells surrounding the implanted blastocyst on day 5 of pregnancy, and spread to the decidual stroma from days 6 to 8 (Fig. 1A). Real-time PCR and western blot results verified that the mRNA and protein levels of TMEM16A are increased in implantation sites when compared with non-implantation sites from days 5 to 8 (Fig. 1B, C). In artificial decidualization model, *Tmem16a* mRNA was strongly localized in deciduoma induced by sesame oil injection, while the mRNA signal of *Tmem16a* was negative in control uterine endometrium (Fig. 1D). Compared to control uterine horn, artificial decidualization treatment stimulated a 19.4 ± 4.2 fold ($p < 0.001$) increase in *Tmem16a* mRNA expression in uterus (Fig. 1E).

Expression of TMEM16A in mESC during *in vitro* decidualization

Compared to vehicle-treated controls, *in vitro* decidualization treatment with estrogen (E$_2$β, 10 nM) and progesterone (1 mM) for 24 h and 48 h stimulated a 8.6 ± 2.5 fold ($p < 0.001$) and 14.8 ± 2.8 fold ($p < 0.001$) increase in *Tmem16a* mRNA expression in mESC, respectively (Fig. 2A). *In vitro* decidualization treatment also significantly stimulated TMEM16A protein in a time-dependent manner. Following treatment with estrogen (E$_2$β, 10 nM) and progesterone (1 mM), level of TMEM16A protein was remarkably increased at 24 h and 48 h (Fig. 2B).

Progesterone stimulates TMEM16A expression in mESC through progesterone receptor/c-Myc pathway

In ovariectomized mice, treatment with E$_2$β (0.1 µg/mouse) for 24 h had no significant effect on...
Tmem16a expression compared to vehicle controls (2.4 ± 1.0 fold, *P*=0.08). Tmem16a mRNA was significantly induced by the treatment with 1 mg progesterone (6.7 ± 1.5 fold vs. control, *P*<0.05) or progesterone plus with Eβ (7.8 ± 4.0 fold vs. control, *P*<0.05) for 24 h, respectively (Fig. 3A). Progesterone also significantly stimulated Tmem16a mRNA in primary mESC in vitro. At 24h post-treatment, 1 µM progesterone effectively stimulated Tmem16a mRNA expression (19.7 ± 3.7 fold vs. control, *P*<0.05). However, 10 nM Eβ had no effect on Tmem16a mRNA level in mESC (Fig. 3B).

Western blot results showed that ERα protein was down-regulated in implantation sites compared to non-implantation sites, but PR protein was significantly up-regulated in implantation sites compared to non-implantation sites on day 8 of pregnancy. In vitro decidualization treatment significantly inhibited ERα protein expression, but stimulated PR protein expression in mESC (Fig. 3C). The stimulation of progesterone on Tmem16a mRNA was blocked by pretreatment with PR antagonist-RU486. Moreover, treatment with the inhibitor of c-Myc pathway (10058-F4) was able to totally attenuated P4-induced Tmem16a expression (Fig. 3D).

TMEM16A inhibition impedes embryo implantation and decidualization in mice

To verify the biological function of TMEM16A in embryo implantation and decidualization, pregnant mice were treated once daily with small molecule inhibition of TMEM16A (T16Ainh-A01, 0.12 mg/0.1 ml/mouse) at 10:00 pm from days 4 to 7 of pregnancy. Compared to vehicle-treated controls (0.1 ml DMSO/mouse, *N*=10), one injection of T16Ainh-A01 (0.12 mg/mouse, *N*=10) at 10:00 pm on day 4 had no effect on embryo implantation rate (80% vs. 90%) and the number of implantation sites (9.4 ± 2.4 vs. 11.7 ± 1.2) on day 5 of pregnancy (Fig. 4A, C, D).

After four injections of T16Ainh-A01 (*N*=17), the embryo implantation rate was decreased to 47.1% compared to 87.5% in vehicle controls (*N*=16), the number of implantation sites was significantly reduced in T16Ainh-A01 group on day 8 of pregnancy (8.6 ± 1.4 vs. 11.4 ± 2.5, *P*<0.01), and the weight of implantation sites was significantly decreased in T16Ainh-A01 group on day 8 (16.7 ± 6.5 mg vs. 29.0 ± 2.5 mg, *P*<0.001), as shown in Fig. 4B, C, D and E. In addition, the implantation sites in T16Ainh-A01 treated mice showed a compromised decidua formation and significantly decreased levels of Alp and Dtprp mRNA, the reliable markers of decidualization.
Injection of T16Ainh-A01 had no effect on serum estrodial and progesterone concentrations compared to controls on day 8 of pregnancy (Fig. 4G, H).

In artificial decidualization model, mice with T16Ainh-A01 injection also showed a defective deciduoma formation compared to vehicle-treated mice (Fig. 4I). The percentage of mice with deciduoma formation in T16Ainh-A01 group was reduced to 50% (N=10) compared to control group (90%, N=10). Compared to control group, T16Ainh-A01 injection significantly decreased the weight of decidualized uterine horn (233.4 ± 204.8 mg vs. 573.4 ± 229.4 mg, P<0.05), as shown in Fig. 4K.

**TMEM16A is essential for mESC decidualization and proliferation**

In vitro decidualization treatment for 48 h significantly increased the mRNA level of Dtprp in mESC, the reliable marker of decidualization in mouse. Knockdown of Tmem16a or treatment with T16Ainh-A01 remarkably reduced the expression of Dtprp in mESC under in vitro decidualization (Fig. 5A, B). In vitro decidualization treatment showed a 1.7 ± 0.5 fold (P<0.005) increase in cell proliferation activity compared to controls. Knockdown of Tmem16a or treatment with T16Ainh-A01 significantly inhibited cell proliferation in mESC with or without in vitro decidualization treatment (Fig. 5C, D).

**Discussion**

The present study first reveal the expression pattern of TMEM16A in mouse uterine endometrium during early pregnancy. Our data suggests that the expression of TMEM16A is up-regulated in uterine endometrium in response to embryo implantation, TMEM16A is strongly induced in endometrial stromal cells under in vivo or in vitro decidualization. Progesterone is responsible for induce TMEM16A expression via progesterone receptor/c-Myc signal pathway. Inhibition of TMEM16A could lead to embryo implantation failure and compromised decidualization.

It has been confirmed that ion channels are closely related to the endometrial receptivity for embryo implantation. The activation of the epithelial Na⁺ channel induced the expression of COX2 and PGE2 through PKA signal pathway in mouse uterine epithelial cells (Ruan et al. 2012). Calcium channels and Ca²⁺ are playing a vital role in endometrial differentiation and implantation.
in both of humans and mice (Kusama et al. 2015). Calcium-activated potassium channels are expressed in human endometrium and affect embryo implantation by mediating endometrial receptivity and Ca\(^{2+}\) homeostasis (Zhang et al. 2012). Ca\(^{2+}\)-activated Cl\(^{-}\) currents are abundant and are present in nearly every cell type, TMEM16A is the major unit of Ca\(^{2+}\)-activated Cl\(^{-}\) channel and essential for Cl\(^{-}\) transportation. Our results found that TMEM16A is induced in mouse stromal cells during decidualization, suggesting that this Ca\(^{2+}\)-activated Cl\(^{-}\) channel may participate the process of embryo implantation and decidualization.

Previous results found that differential estrogen, progesterone and androgen signaling pathways may determine the specific expression and function of TMEM16A in different cells. TMEM16A stimulates cell proliferation in ER and PR positive breast cancer cells, but inhibited cell proliferation in ER and PR negative cells (Wu et al. 2017). In prostate cells, the promoter of Tmem16a gene contains an androgen response element, TMEM16A was induced in testosterone-treated prostate epithelia and contributed to testosterone-induced cell proliferation (Cha et al. 2015). Progesterone is essential to induce acrosome reaction in sperm, inhibition of TMEM16A blocked progesterone-induced acrosome reaction in previously capacitated sperm (Cordero-Martinez et al. 2018). In mESC, PR is up-regulated but ER \(\alpha\) is down-regulated during decidualization process. Our results showed that progesterone could significantly stimulate TMEM16A expression, but estrogen had no effect on TMEM16A expression in mESC. Progesterone receptor antagonist abrogated progesterone-induced TMEM16A expression in mESC. c-Myc signal pathway is involved in progesterone-mediated decidualization (Zuo et al. 2015), inhibitor of c-Myc signal pathway could totally eliminate progesterone-induced TMEM16A expression. Taken together, these results suggested the expression of TMEM16A is mediated by progesterone/c-Myc signal pathway in decidual cells.

T16Ainh-A01, a second-generation TMEM16A antagonist, was identified as the most potent blocker of Ca\(^{2+}\)-activated Cl\(^{-}\) currents produced by TMEM16A (Cordero-Martinez et al. 2018). T16Ainh-A01-treated mice displayed impaired embryo implantation and decidualization, indicating that Ca\(^{2+}\)-activated Cl\(^{-}\) currents is engaged in the process of embryo implantation. It remains unclear whether TMEM16A-involved embryo implantation and decidualization is due to...
the increased TMEM16A expression or TMEM16A-mediated ion channel activity. Knockdown of Tmem16a in mESC suppressed the expression of decidualization marker of Dprp during in vitro decidualization, which is similar to the effect of T16Ainh-A01 on mESC decidualization. This result implies that the expression level and channel activity of TMEM16A are both required for decidualization.

Chloride channels have been identified to be involved in cell proliferation and cell cycle regulation (Huang et al. 2014). TMEM16A is frequently overexpressed in tumor cells and contributes to tumor growth and proliferation. Knockdown of TMEM16A suppressed cell proliferation and cell cycle progress by ERK1/2 signal pathway and cyclin D1 in hepatoma carcinoma cell (Deng et al. 2016). Overexpression of TMEM16A demonstrated a reduction in apoptotic activity by inhibiting the pro-apoptotic protein Bim (Godse et al. 2017). Inhibition of TMEM16A by T16Ainh-A01 showed decreased proliferation activity in mESC during in vitro decidualization, suggesting that TMEM16A influences stromal cells proliferation during embryo implantation and decidualization.

In conclusion, we indentified the expression of TMEM16A in mouse uterus during embryo implantation and decidualization. TMEM16A expression in decidual cells is mediated by progesterone/c-Myc pathway. Inhibition of TMEM16A impedes the embryo implantation and decidualization possibly through affecting cell proliferation of decidual cells. We still need to further study on the molecular mechanism and function of TMEM16A during embryo implantation in mice and humans.

Declaration of conflicting interests
The authors report that no conflict of interest exists to prejudice the impartiality of the research reported.

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Acknowledgements

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References


**Figure 1. The expression of TMEM16A in mouse uterus during early embryo implantation and decidualization.** (A) The mRNA localization of *Tmem16a* in mouse uterus from days 1 to 8 of pregnancy (Black arrow: implanted embryo). (B) The mRNA level of *Tmem16a* between non-implantation sites and implantation sites in mouse endometrium from day 5 to day 8 of pregnancy. Data were expressed as mean ± SD from four different mice in each group, *p*<0.05. (C) The protein level of TMEM16A between non-implantation sites and implantation sites in mouse endometrium from day 5 to day 8 of pregnancy. NI: non-implantation sites; IS: implantation sites. (D) The mRNA localization of *Tmem16a* in mouse uterus under artificial decidualization, Con: control uterine horn. AD: artificial decidualization. (E) The mRNA level of *Tmem16a* between control uterine horn and artificial decidualization uterine horn. Data were expressed as mean ± SD from four different mice in each group, *p*<0.05.

**Figure 2. The expression of TMEM16A under in vitro decidualization.** (A) Real-time PCR was performed to quantify the mRNA level of *Tmem16a* in mESC during *in vitro* decidualization. Data were expressed as mean ± SD from three independent experiments using mESC from 7-9 different mice, *p*<0.05. (B) Western blot of TMEM16A protein in mESC during *in vitro* decidualization. Con: Control group; E+P: *in vitro* decidualization induced by estrogen and progesterone for 24h and 48 h. Data were derived from cell preparations from 3-5 different mice.

**Figure 3. The regulation of progesterone on TMEM16A in mESC.** (A) The mRNA level of *Tmem16a* in ovariectomized mouse uterus after steroids treatment. Data were expressed as mean ± SD from four different mice in each group, *p*<0.05. (B) The mRNA level of *Tmem16a* in mESC after steroids treatment. Data were expressed as mean ± SD from three independent experiments using mESC from 7-9 different mice, *p*<0.05. (C) The expression of estrogen receptor α (ERα) and progesterone receptor (PR) in mouse uterus and mESC. (D) The mRNA level of *Tmem16a* in mESC treated with progesterone, RU486 and 10058-F4. Data were derived from three independent experiments using mESC from 7-9 different mice, *p*<0.05.

**Figure 4. The effect of TMEM16A inhibition on embryo implantation and decidualization.** The representative photo showing Control (DMSO) and T16Ainh-A01-treated uterus on day 5 (A)
and day 8 (B) of pregnancy. Black arrow: embryo implantation site. (C) The implantation rate between Control and T16Ainh-A01 group on days 5 and 8 of pregnancy. Data were presented as the percentage of mice with implantation sites in each group. (D) The number of implantation sites between control and T16Ainh-A01-treated mice on days 5 and 8 of pregnancy. Data were presented as the number of implantation sites for all mice with implantation sites, * p<0.05. (E) The weight of implantation sites between control and T16Ainh-A01-treated mice on day 8 of pregnancy. Data were presented as mean ± SD from all mice with implantation sites (the average weight of implantation sites in each mouse as one data point), * p<0.05. (F) Levels of Alp and Dtgpr mRNA in control and T16Ainh-A01-treated mouse uteri on day 8 of pregnancy. Data were presented as mean ± SD from nine mice in each group, * p<0.05. The serum estradiol (G) and progesterone (H) concentrations in control and T16Ainh-A01-treated mouse on day 8 of pregnancy. Data were presented as mean ± SD from five mice in each group. (I) The representative photo showing control and T16Ainh-A01-treated uterus on day 8 of artificial decidualization. (J) The decidualization rate between control and T16Ainh-A01-treated group. Data were presented as the percentage of mice with deciduoma formation in each group. (K) The weight of decidual uterine horn between control and T16Ainh-A01-treated uterus on day 8 of artificial decidualization. Data were presented as mean ± SD from the mice with deciduoma formation in each group, * p<0.05.

Figure 5. The effect of TMEM16A knockdown or inhibition on decidualization and proliferation in mESC. (A) Real-time PCR was performed to quantify the knockdown effect of si-Tmem16a in mESC. (B) The effect of Tmem16a knockdown or T16Ainh-A01 treatment on Dtgpr expression during in vitro decidualization. Date were expressed as mean ± SD from three independent experiments using mESC from 7-9 different mice, * p<0.05. (C) The representative photo showing the effect of Tmem16a knockdown or T16Ainh-A01 treatment on cell proliferation during in vitro decidualization. (D) The effect of Tmem16a knockdown or T16Ainh-A01 treatment on mESC proliferation activity during in vitro decidualization. Date were expressed as mean ± SD from three independent experiments using mESC, * p<0.05.
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<th>Application</th>
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(A) Relative expression of Tmem16A

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(B) Western blot analysis

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* indicates statistical significance.