Original Research

TBC1D20 is Essential for Postnatal Uterine Development and Endometrial Decidualization in Mice

Wenpeng Min1,2, Xi Li3, Jing Zhou2, Wei Yuan1,2, Qing Yang1, Xinmei Ji4, Bing Liu4, Liqiong Luo2, Yuxia He5,*, Lina Cui6,*, Wen-Lin Chang2,4,0,*10

1 College of Veterinary Medicine, Hunan Agricultural University, 410128 Changsha, Hunan, China
2 Department of Obstetrics, The People’s Hospital of Longhua Shenzhen, 518109 Shenzhen, Guangdong, China
3 Department of Orthopaedics and Traumatology, The People’s Hospital of Longhua Shenzhen, 518109 Shenzhen, Guangdong, China
4 Department of Gynaecology, The People’s Hospital of Longhua Shenzhen, 518109 Shenzhen, Guangdong, China
5 Department of Obstetrics and Gynaecology, Center for Reproductive Medicine, Key Laboratory for Major Obstetric Diseases of Guangdong Province, The Third Affiliated Hospital of Guangzhou Medical University, 510150 Guangzhou, Guangdong, China
6 Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Peking University Shenzhen Hospital, Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center, 518036 Shenzhen, Guangdong, China

Correspondence: yuxia@664@163.com (Yuxia He); cuiлина52100@163.com (Lina Cui); wenlin.chang2006@163.com (Wen-Lin Chang)

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Abstract

Background: TBC1D20 is a conserved monofunctional protein that is highly expressed in the endometrial stromal cells and plays an important role in normal uterine development. However, the biological functions of TBC1D20 in postnatal uterine development are still unclear. Methods: As a prospective laboratory-based study, a mouse model with spontaneous functional mutations of Tbc1d20 (Tbc1d20−/−) was employed to investigate the impact of Tbc1d20 on postnatal uterine development and gonadal function. Results: Female Tbc1d20−/− mice were infertile. Functional mutations of Tbc1d20 exerted no obvious changes on the function of ovary, structure of fallopian tubes, and ability of early embryo implantation. However, Tbc1d20−/− mice presented marked reduction on the uterine size and weight at two-month-old, accompanied limitations on the myometrial thickness, the number of endometrial glands, and the density of blood vessels. Conclusions: The findings from this study indicated that TBC1D20 is necessary for normal postnatal uterine development and endometrial decidualization in mice.

Keywords: TBC1D20; uterine development; decidualization; endoplasmic reticulum stress

1. Introduction

The uterus is a part of the female reproductive tract and comprises the endometrial and myometrial layers. Uterine endometrium is organized and constructed by many different cell populations which cooperated playing important roles in embryonic development, implantation, and placentation [1–4]. The Uterine development including stratification of the uterine stroma, myometrial differentiation and growth, and the differentiation and development of endometrial glands, was accomplished in rodents, domestic animals and humans at the postnatal [5–7]. The processes of uterine development and uterine functions are exact regulated by intrinsic and/or extrinsic regulatory factors. Previous studies have demonstrated that loss of function mutations of certain genes cause defect related to female fertility [6,8,9]. The ovarian steroid hormones, estrogen and progesterone, play pivotal roles in regulating uterine functions for embryonic implantation, development, and pregnancy. Mice lacking functional estrogen receptors (ERs) are infertile and have hypoplastic uterus [10]. Various functional and morphological changes with disorder phenotype are observed in the endometrium of ERs mutation mice, relating leukocyte recruitment and the differentiation of endometrial stromal fibroblast cells (ESCs) into decidual stromal fibroblast cells (DFCs), to form the decidual lining during the implantation of blastocysts [11]. Proper decidualization controls conception and the exact time course of pregnancy, and is a critical determinant of the success of pregnancy in humans and mice. Progesterone receptor (PR) gene knockout resulted in uterine hyperplasia and loss of decidualization, which render the uterus non-receptive for blastocyst implantation in mice [12].
The blind sterile (bs) mouse model reported in 1983 exhibits the typical phenotypes of Warburg Micro syndrome (WARBM), including congenital cataract, male infertility and gonadal dysplasia [13]. A causative gene mutation of the TBC1D20 family member 20 (TBC1D20) is responsive for the abnormal of phenotype of bs mice, which is characterized by congenital cataracts and male infertility [14,15]. Our previous study also found that TBC1D20 is essential for maintaining the integrity of the blood-testis barrier postnatally and viability of Sertoli cells by modulating the structure and function of the endoplasmic reticulum and Golgi apparatus [16,17]. Furthermore, during the breeding of bs mice, we also realized the phenomena of reducing reproductive capacity of female mouse with TBC1D20 deficiency. However, the exact role and cellular pathway of Tbc1d20 in female fertility remains to be elucidated to date. In this study, we used the female bs mouse (defined as Tbc1d20−/−) with a spontaneous mutation in the Tbc1d20 gene as an animal model to investigate the role of TBC1D20 in postnatal uterine development and endometrial decidualization.

2. Materials and Methods

2.1 Animal Procedures and Sampling

This research is a prospective laboratory-based study. The bs mouse (Tbc1d20−/−) model was obtained from Jackson Laboratory (Bar Harbor, ME, USA) and raised at Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center. All the animal procedures were performed in accordance with the guidelines of the ethics committee of Peking University Shenzhen Hospital. The animals were maintained under specific pathogen free (SPF) grade animal room. Approval for the study was obtained from Animal Ethics Committee of Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center (Ethics approval number: 2019-205s and Mounting onto slides precoated with polylysine. The protocol was approved by the Animal Ethics Committee of Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center (Ethics approval number: 2019-205).

2.2 Isolation of Uterine Stromal Cells and in Vitro Decidualization

The ovariectomized mice were injected with E2 for three days (100 ng/day) and the uterine stromal cells were isolated at D4. Briefly, uterine horn from mice was cut into 3–5 mm pieces and dissected longitudinally to expose the uterine lumen. Tissue was washed with phosphate-buffered saline (PBS) and then immersed in 0.25% trypsin (Sigma, St. Louis, MO, USA) for 30 min at 37 °C. 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) was used to terminate digestion and mixed gently to remove epithelial clumps by pipetting. The remaining tissues were reserved, then was washed twice in PBS and finally placed in PBS containing 0.5% collagenase Type II (Sigma, St. Louis, MO, USA) for 30 min at 37 °C. 10% fetal bovine serum (FBS) was used to stop the digestion. After being washed twice in PBS, the tissues were mixed thoroughly by pipetting up and down to disperse stromal cells. The stromal cells were collected by centrifugation. The isolated primary endometrial stroma cells were first cultured in vitro for establishing a stromal cell proliferation model in 6-well cell culture plates seeded at a density of with 2.5 × 10^5 cells/well. The cells were cultured in a proliferation medium comprising DMEM (Dulbecco’s Modified Eagle Medium)/Ham’s F-12 nutrient mixture (1:1) supplemented with 10% charcoal-141 stripped FBS, (Invitrogen, Carlsbad, CA, USA) and 10 U/mL penicillin-streptomycin. The in vitro decidualization cell model was generated by culturing the cells in a culture medium containing 10 nM E2 and 1 μM P4.

2.3 Histomorphology and Immunohistochemistry Assays

Sections of uterine tissue were deparaffinized and rehydrated before assigning to hematoxylin and eosin (H&E)
staining or immunohistochemical assays. Immunohistochemical assays were performed using a Streptavidin Peroxidase Histostain-Plus Kit (SAP-9100, Beijing Zhong Shan Goldenbridge Biotech., Beijing, China), according to the manufacturer’s instructions. Briefly, sections were treated with 3% hydrogen peroxide for 15 min, washed thrice with PBS, permeabilized with 0.05% Triton-X 100 for 2 min, and subsequently rinsed with PBS. Then sections were blocked with a blocking buffer for 1 h and incubated overnight at 4 °C with primary antibodies (anti-TBC1D20, 1:100 diluted, NB1-92478, Novus Biologicals, Inc., Littleton, CO, USA; Ki67, 1:200 diluted, ab16667, Abcam, Cambridge, MA, USA; Cytokeratin, 1:200 diluted, Z0622, DAKO, Carpinteria, CA, USA; Vimentin, 1:100 diluted, ab92547, Abcam, Cambridge, MA, USA; and anti-estrogen receptor alpha antibody, 1:100 diluted, IR657, DAKO, Carpinteria, CA, USA). Section incubated with rabbit IgG was employed as negative control. Sections were subsequently incubated with the secondary antibody for 1 h at room temperature. The peroxidase reaction was performed with a SignalStain® DAB Substrate Kit (S058s, CST), and the signals were measured by light microscopy (Nikon ECLIPSE Ci-L, Nikon, Tokyo, Japan).

For immunofluorescence assay, the dewaxed uterine tissue sections were subjected to antigen retrieval in citrate antigen retrieval solution (pH 6.0), and subsequently incubated with 5% bovine serum albumin for 1 h followed by overnight incubation with the primary antibodies (anti-alpha smooth muscle actin, ab5694, Abcam, Cambridge, MA, USA; anti-CD34 antibody, PA5-89536, Thermo Fisher Scientific, Cleveland, OH, USA) at 4 °C. The tissue sections were washed with PBS and incubated with the fluorescent secondary antibody (Thermo Fisher Scientific, Cleveland, OH, USA) for 1 h at room temperature. The tissue sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min and mounted with SlowFade Gold Antifade reagent (S36936, Life Technologies, Carlsbad, CA, USA). The sections were examined with a Leica laser confocal microscope system (Leica TCS SP8, Leica, Wetzlar, Germany). For postnatal uterine morphological phenotype analysis, the results of each individual were averaged value based on three tissue sections from the proximal cervical segment, the middle segment of the uterine horn and the tail segment of the uterine horn of the same uterus, respectively.

For postnatal uterine morphological and histological measurement, the results from H&E assay, immunohistochemistry analysis and immunofluorescence assay of each individual, were average valued based on three tissue sections from the proximal cervical segment, the middle segment of the uterine horn and the tail segment of the uterine horn of the same uterus, respectively. To measure the thickness of the longitudinal and circular layers of the myometrium, starting from the side of the uterine mesentery, lines perpendicular to the circumference of the longitudinal and circular layers of the myometrium.
USA). Information regarding the primary antibodies used for western blotting are provided in Supplementary Table 2.

2.6 Radiometric assay (RIA)

Blood samples were collected from two-month-old Tbc1d20+/− and Tbc1d20+/+ female mice at dioestrus, and the serum samples were obtained, aliquoted, and stored at −80 °C until further analyses. The serum levels of progesterone (P4), estrogen (E2), and follicle-stimulating hormone (FSH) were measured by RIA method. The assay was performed by Beijing North Institute of Biological Technology (Beijing, China) by running the Xiayang RIA counter (Xi’an Nuclear Instrument Factory, Xian, Shanxi, China), and each sample comprised six different individuals per genotype. Estradiol radiometric assay kit (B05TB, Beijing North Institute of Biotechnology Co., Ltd., Beijing, China) for serum E2 measurement (detection range, 0.12–36 ng/mL; sensitivity, 0.05 ng/mL), and Follicle-stimulating hormone immunoradiometric assay kit (KIP1458, Beijing North Institute of Biotechnology Co., Ltd., Beijing, China) for FSH measurement (detection range, 1.5–100 mIU/mL; sensitivity, 0.2 mIU/mL) was employed in serum hormones test.

2.7 Cell Proliferation Assay

Cell proliferation was detected using a CellLight™ EdU (5-ethyl-2-deoxyuridine) Apollo567 In Vitro Kit (RiboBio, Guangzhou, Guangdong, China) according to the Kit Operation Manual. Briefly, cell was seeded in 96-well plate. Culture medium containing EdU was added to well and incubation with cells for 1 h. Then the cells were fixed with polyformaldehyde (PFA) for 10 min at room temperature. The cell nuclei were double dyed with EdU and DAPI for assessing cell proliferation. The images were captured with a laser confocal microscope (Leica TCS SP8), and the cells were counted from six different microscopic fields.

2.8 Statistical Analysis

The data were presented as the mean ± standard error of mean (SEM) and analyzed with GraphPad Prism 9.0 (FAQ 2176, GraphPad, San Diego, CA, USA). The Chi-square test was used for determining whether the number of newborn mice with different genotypes conformed to the Mendelian laws of inheritance. The statistical differences of observed value between the WT and mutation null mice were assessed using Student’s t-test, and p < 0.05 was considered statistically significant.

3. Results

3.1 Loss-Functional Mutations in Tbc1d20 Results in Female Mice Infertile

The mutation in Tbc1d20 results in a p.Phe231Met substitution followed by an in-frame p.Arg232_Val235 deletion in the Tbc1d20 protein (Supplementary Fig. 1A), which disrupts the highly conserved amino acids in the TBC domain of Tbc1d20. Result of statistical assay of reproductive capacity showed that Tbc1d20−/− female mice were infertile (Table 1). However, there were no significant differences in fertility between the heterozygous mutant and WT mice (Table 1).

3.2 Normal Ovary Function and Embryo Implantation are Presented in Tbc1d20+/− Female Mice

In order to explore the causes of infertility in Tbc1d20−/− female mice, we examined the function of the ovaries and fallopian tubes at estrus, which were the first decision factor in time course of female reproductive capacity. As depicted in Fig. 1A–D, there were no obvious histological changes of ovary and fallopian tube between the Tbc1d20−/− and WT mice. We also observed the occurrence of implantation in the uterus of Tbc1d20−/− mice at day 6 of gestation (Fig. 1E). However, the number of implantations was significantly reduced compared to Tbc1d20+/+ mice (Fig. 1F). The Chi-square test revealed that the different genotypes of newborn mice was significantly deviated from Mendel’s laws of inheritance with reduced number of Tbc1d20−/− genotype survival until birth (Supplementary Table 3). The size of the uterus of two-month-old mutant mice was remarkably smaller than that of the WT mice at diestrus (Supplementary Fig. 1B), which implicated the important role of TBC1D20 in regulating postnatal uterine development in mice.

3.3 Loss-Functional Mutations in Tbc1d20 Affects Postnatal Uterine Morphology in Mice

The uterine histomorphology of the mutant Tbc1d20−/− mice was measured. Ovariectomy was performed for eliminating the interference of ovarian hormones in mice uterine at the age of 2 month. Two weeks later, the uteri were collected from the cycle-synchronized mutants and WT mice, and the uterine weights were found to be significantly lower in the mutant mice (mutant vs. WT was 0.022 g vs. 0.035 g, p = 0.0006, n = 6; Fig. 2A,B). The results of histological assays also revealed that the cross-sectional areas of the uteri of Tbc1d20−/− bs mice were smaller than those of WT mice (Fig. 2C). Moreover, the number of endometrial glands in each observed field differed significantly between the mutants and WT mice (p = 0.0033, n = 6; Fig. 2D,E). Structure of the myometrium was determined by measuring the expression of alpha-smooth muscle actin (α-SMA), which is a biomarker of myofibroblasts. Results of the immunofluorescence assays demonstrated that the thickness of the longitudinal (p =
3.4 TBC1D20 Mutation Disrupted the Production of Maternal Reproductive Hormones in Mice

Concentration of sex hormones, the E2, P4 and FSH in maternal serum at the diestrous stage, were determined by RIA. Results from RIA indicated that the levels of E2 \( (p = 0.0095, n = 6; \text{Fig. 3A}) \) and P4 \( (p = 0.0133, n = 6; \text{Fig. 3B}) \) decreased significantly in female \( \text{Tbc1d20}^{-/-} \) mice compared to those of WT mice, but there were no significant differences about the serum levels of FSH between \( \text{Tbc1d20}^{-/-} \) and WT mice \( (p = 0.2273, n = 6; \text{Fig. 3C}) \).

3.5 Decidualization is Impaired in Female \( \text{Tbc1d20}^{-/-} \) Mice

Results from our research indicated that the affected ovary function and embryo implantation ability does not seem to be the main cause of infertility in \( \text{Tbc1d20}^{-/-} \) mice. Thus, it was valuable to check whether the infertility of female \( \text{Tbc1d20}^{-/-} \) mice could be mainly attributed to other reasons, such as defects in uterine decidualization. In this study, we assessed the differentiation ability of the endometrial stromal cells into decidual stromal cells using an animal model of \textit{in vivo} artificial decidualization (Fig. 4A). Decidual response was induced in the WT mice, but not the \( \text{Tbc1d20}^{-/-} \) mice through sesame oil injection (Fig. 4B). The ratio of the weights of the stimulated horns to the unstimulated horns of the same individual was significantly lower in \( \text{Tbc1d20}^{-/-} \) mice than WT mice (Fig. 4C). Immunohistochemistry (IHC) assay exhibited that the expression of TBC1D20 increased significantly in the uterus of \( \text{Tbc1d20}^{+/+} \) mice along with decidualization (Fig. 4D). Moreover, the increasing expression of \( \text{Tbc1d20} \) was also proved by the results of qRT-PCR assays (Fig. 4E). Ki67, an indicator of proliferation, was highly expressed in the tissues of \( \text{Tbc1d20}^{+/+} \) mice subjected to artificially induced decidualization; however, the Ki67 positive cells were very rare in the uterine tissues of \( \text{Tbc1d20}^{-/-} \) mice (Fig. 4G).

Analysis of the expression of cytochrome C (CK) and vimentin, revealed that the degree of decidualization in the endometrial tissues of \( \text{Tbc1d20}^{-/-} \) mice was much lower than that of \( \text{Tbc1d20}^{+/+} \) mice (Fig. 4G). Notably, the expression of estrogen receptor alpha (ER\( \alpha \)) was limited in the uterus of decidualized \( \text{Tbc1d20}^{+/+} \) mice (Fig. 4G). The results of qRT-PCR demonstrated that the mRNA levels of the key decidualization factors, such as Bmp2, Bmp4, Hoxa10, Pgr and Wnt4, were significantly elevated in the uterus of \( \text{Tbc1d20}^{+/+} \) mice compared to those of the \( \text{Tbc1d20}^{-/-} \) group following the induction of decidualization (Fig. 4F).

3.6 TBC1D20 Regulates the Proliferation and Decidualization of Murine Uterine Stromal Cells in Vitro

Stromal cells were isolated from the uterus of diestrous mice and cell proliferation was detected with 5-Ethynyl-2’-deoxyuridine (EdU) assays, for 1 h \textit{in vitro}. The findings revealed a significant decrease in the number of EdU-positive uterine stromal cells lacking functional TBC1D20 (Fig. 5A,B). The expression levels of cyclin B1 and cyclin D3 decreased significantly in the proliferative uterine stroma cells of WT mice while the expression levels of cyclin D1 increased significantly (Fig. 5C,D) compared to those of the mutant mice. These findings suggested the occurrence of impaired DNA synthesis in the uterine stromal cells of \( \text{Tbc1d20}^{-/-} \) mice, which presented as G1/S phase arrest during cell proliferation. \textit{An in vitro} decidualization model of murine uterine stromal cells was generated by the addition of E2 and P4 in the culture medium, and results demonstrated that the expression of vimentin was higher in the TBC1D20-depleted cells in the decidualization model. The findings indicated that TBC1D20 deficiency inhibited the induced decidualization of the uterine stromal cells of \( \text{Tbc1d20}^{-/-} \) mice, which presented as G1/S phase arrest during cell proliferation. \textit{An in vitro} decidualization induction uterine stroma cells when compared to \( \text{Tbc1d20}^{-/-} \) (Fig. 5F).

3.7 TBC1D20 Disruption Induced Endoplasmic Reticulum Stress in Uterine Stromal Cells

In our previous study, we demonstrated that the deletion of TBC1D20 resulted in endoplasmic reticulum stress in mouse Sertoli cells, and excessive endoplasmic reticulum stress induced DNA damage and promoted cell death. We therefore examined the expression of the key proteins.
that regulate endoplasmic reticulum stress. The results demonstrated that the expression levels of C/EBP homologous protein (CHOP), inositol-requiring 1 alpha (IRE1α), PRKR-like endoplasmic reticulum kinase (P-PERK) and phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (P-eIF2α) increased significantly.
Fig. 2. Loss-functional mutations in Tbc1d20 impair postnatal uterine development in mice.  
(A–C) H&E staining revealed a reduction in the size of the uterus in Tbc1d20−/− mice, and the cross-sectional area of the uterus and uterine weights were significantly lower than those of the WT mice (n = 6). (D,E) The number of uterine glands was reduced in female Tbc1d20−/− mice compared to that of the WT mice.  
(F–H) Representative images of immunofluorescence due to α-smooth muscle actin (α-SMA) (marker of myofibroblasts) depicting that the thickness of the longitudinal and circular myometrium layers, indicated by red and yellow lines, respectively, was significantly restricted in female Tbc1d20−/− mice.  
(I–K) Representative images of immunofluorescence due to CD34, a marker of vascular endothelial cells, depicting a decrease in the number of uterine vessels in female Tbc1d20−/− mice. The white dashed line marks the boundary between the uterine stromal cell layer and the uterine circular muscle layer; n = 6, ** p < 0.01. Bar, 100 µm.
in Tbc1d20−/− endometrial stromal cells in both the proliferative and differentiation phases. The expression levels of binding-immunoglobulin protein (BIP) and protein disulfide-isomerase (PDI) decreased significantly (Fig. 5G) in Tbc1d20−/− endometrial stromal cells in the proliferative and differentiation phases. The findings also indicated that the primary stromal cell from uterine tissues of mice with TBC1D20 deficiency exhibited severe endoplasmic reticulum stress. These findings indicated that the deletion of TBC1D20 induces excessive endoplasmic reticulum stress in endometrial stromal cells and inhibits cell proliferation and differentiation that leads to the failure of uterine decidualization.

4. Discussion

Normal anatomical and histological structure of postnatal uterus is a prerequisite for successful gestation in mice and humans. The development of the uterus in humans and mice is not fully accomplished until the embryo implantation stage [18,19]. Prenatal uterine development, which is referred to as organogenesis, begins at the embryonic stage and comprises the formation, patterning, and fusion of the Müllerian ducts. Postnatal uterine development requires further morphogenesis and differentiation into functional adult components. Serial genes have been found that are important in postnatal uterine development in mice and humans [5,8,19,20]. However, most key genes related to uterine growth and development and their exact function pattern that are still unknown [5]. In this study, we found Tbc1d20 is essential for normal postnatal uterine development and decidualization in mice. Causative mutation of the Tbc1d20 gene in hs mouse model markedly limited postnatal uterine morphogenesis and resulting in failed decidualization. Expression pattern of TBC1D20 in uterine indicated the involvement of this gene in regulating the cellular activity of the stromal cells, myometrium, blood vessel endothelium, and the glandular and luminal uterine epithelia of prepubertal mice. The thickness of the endometrial stromal layer, longitudinal and circular layers of the myometrium, and the number of uterine glands and vessels were significantly reduced in the postnatal uterus of Tbc1d20−/− mice. The uterus of female mice develops from Müllerian ducts and consists of the epithelium and mesenchyme at the perinatal stage. The mesenchyme differentiates into the stroma and smooth muscle layers, and two muscle layers form in the postnatal stage, including the outer longitudinal smooth muscle layer and an inner circular smooth muscle layer [21,22]. In particular, the formation of uterine glands is a critical event in mice [3,6]. In this study, the postnatal formation of uterine glands was also significantly retarded in Tbc1d20−/− mice, which may further affect the physiological functions of the uterus, including embryonic implantation and supporting the conceptus during pregnancy.

Postnatal uterine morphogenesis in mice and humans is governed by a variety of hormonal, cellular, and molecular mechanisms [18,19,23]. In this study, we observed that the concentration of E2 and P4 in the peripheral blood of female Tbc1d20−/− mice at diestrus was significantly reduced compared to that of WT mice. Estrogen regulates peripubertal uterine growth in mice but does not regulate postnatal uterine development and endometrial adenogenesis [24]. Therefore, a reduction in the uterine weights of mice with mutations in Tbc1d20 is considered to be partially attributed to the gonadal dysplasia. In addition, increasing evidence suggests that the inactivation of TBC1D20 can seriously affect the development of the endocrine nervous system [14]. We speculate that the dysfunction of TBC1D20 may lead to overall physiological abnormalities in the entire hypothalamic pituitary gonadal axis.
Fig. 4. Loss-functional mutations in Tbc1d20 results in failure of uterine decidualization. (A) Procedure of artificial decidualization in vivo. (B) Morphology of the uterine tissues in which the left horn was stimulated with sesame oil and the right horn was left untreated as the normal control. (C) Ratio of the average weights of the stimulated uterine horns to those of the unstimulated uterine horns from WT and Tbc1d20−/− mice (n = 6); **p < 0.01. (D) Immunohistochemistry analysis revealed that TBC1D20 was abundantly expressed in the uterine GE, LE, and My of female mice on the first day of artificial decidualization and in the decidua on day 12. Serial sections incubated with rabbit IgG instead of the primary antibody were used as the normal control (NC). LE, luminal epithelium; GE, glandular epithelium; My, myometrium. (E) The levels of Tbc1d20 mRNA increased significantly in the oil-stimulated left horn that underwent decidualization compared to that of the unstimulated horn of WT mice. (F) The expression of the marker genes of decidualization, Bmp2, Bmp4, Hoxa10, Pgr, and Wnt4, were reduced in the stimulated uterine horns of Tbc1d20−/− mice compared to that of the WT mice. (G) Histological analysis of representative uterine sections from WT and Tbc1d20−/− mutant mice. Representative images of Ki67, cytokeratin (CK), vimentin, and estrogen receptor alpha (ERα) immunohistochemistry in the oil-stimulated uterine tissues of WT and Tbc1d20−/− mice; n = 6. **p < 0.01. Bar, 100 µm.
Fig. 5. Mutations in *Tbc1d20* retarded the proliferation and decidualization of uterine stromal cells *in vitro* due to the disrupted function of endoplasmic reticulum. (A,B) The 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay revealed that *Tbc1d20* depletion significantly inhibited the proliferation of endometrial stromal cells. (C) Western blotting was used to detect the levels of cyclin B1, cyclin D1, and cyclin D3 proteins in murine endometrial stromal cells during the proliferative phase *in vitro*. (D) The results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed that the deletion of *Tbc1d20* inhibited the expression of cyclin B1 and cyclin D3 mRNA in murine endometrial stromal cells during the proliferative phase *in vitro*. (E) Western blotting was used to detect the levels of vimentin protein in murine endometrial stromal cells that underwent decidualization following artificial induction. (F) The expression level change of five marker genes associated with uterine stroma cell decidualization differentiation were detected by qRT-PCR. (G) Western blotting was used to detect the expression levels of endoplasmic reticulum stress-related proteins, namely, CHOP, BiP, IREα, PDI, and PERK, P-PERK, eIF2α and P-eIF2α in murine endometrial stromal cells during the proliferation and decidualization phases *in vitro*. **p < 0.01. Bar, 100 µm.

In this study, successful embryo implantation was observed in TBC1D20 deficiency female mice. But the number of implantation embryo was reduced when compared to WT (Fig. 1E,F), which may be attributed to the negative effects of *Tbc1d20* mutation on the viability of embryos. The speculation that the *Tbc1d20*−/− embryo exhibited reduced viability was partially supported by the finding that the number of mutant homozygous newborn mice from heterozygous parents was significantly lower than expected (Supplementary Table 3). In our previous research, the widely expression pattern of TBC1D20 in multiple tissues and organs of human beings have been revealed by tissue-array [16]. TBC1D20 is a Rab1 GTPase-activating protein [14]. The widespread expression of TBC1D20 suggests that it also plays an important regulatory role in embryonic development. Targeted disruption of Rab1a could cause early embryonic lethality. Endometrial decidualization plays an important role in embryonic implantation and the main-
tenance of pregnancy following embryonic implantation [11,25]. However, the decline in embryonic implantation and survival ability of Tbc1d20−/− embryo seems to be not the main cause of infertility in Tbc1d20−/− female mice. In this study, the failure of decidualization of endometrium is the main reason for infertility in Tbc1d20−/− female mice (Fig. 4). To ensure successful decidualization, endometrial stromal cells rapidly proliferate and differentiate into decidual cells under the control of hormones, and the structure and function of uterine luminal epithelial cells undergo dramatic changes at the time of receptivity. Aberrant decidualization can lead to numerous complications during pregnancy, including infertility, abortion, premature delivery, preclampsia, and retardation of intrauterine growth [4,26,27].

Endoplasmic reticulum stress seems to play a role in female reproductive processes [28]. The phenomena that moderate elevation of endoplasmic reticulum stress markers during the decidualization of human endometrial stromal cells was reported [29]. Thus, appropriate and finely regulated endoplasmic reticulum stress in time course may be critical for uterine decidualization or to meet the requirement of accommodate protein synthesis during decidualization [30], whereas excessive and disorder endoplasmic reticulum stress leads to DNA damage and inhibits the proliferation and decidualization of stromal cells [28]. We previously identified that TBC1D20 assembles in the Golgi apparatus and endoplasmic reticulum of Sertoli cells, which induces irreversible endoplasmic reticulum stress, leading to the disruption of organelles [16]. The present findings revealed that the loss of function in TBC1D20 disrupted the metabolism of steroid hormones and the corresponding receptors in female mice. This could also be attributed to the structural and functional interference resulting from the endoplasmic reticulum and Golgi apparatus. In this research, checkpoint markers of the three typical signaling pathway in endoplasmic reticulum stress were detected by western blotting. And results indicate the activation of endoplasmic reticulum stress in the proliferative uterine stromal cells and differentiated decidual cells caused by Tbc1d20 mutations, is mainly regulated on PERK-eIF2α-ATF4 signaling pathway. These results indicated that the uterine tissues of Tbc1d20−/− mice exhibit irreversible endoplasmic reticulum stress.

5. Conclusions

Our findings suggested that TBC1D20 is involved in the regulation of postnatal uterine development and decidualization, and possibly controls the proliferation of endometrial stromal cells by regulating endoplasmic reticulum stress. However, further studies are necessary for well understanding the cellular and molecular mechanisms of TBC1D20 in regulating of postnatal uterine growth and development.

Availability of Data and Materials

The data are available from the corresponding author on reasonable request.

Author Contributions

WM conducted the whole experiments and prepared the manuscript. XL, JZ and WY performed parts of the experiments and analyzed the data. QY, XJ and BL supervised the data and revised the manuscript. LL, YH, LC and WLC designed the study, reviewed the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was performed with the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources for the National Research Council. The protocol was approval by the Animal Ethics Committee of Shenzhen Peking University–Hong Kong University of Science and Technology Medical Center (Ethics approval number: 2019-205).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.ceog5106146.

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