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Protective role of GPR120 in the maintenance of pregnancy by promoting decidualization *via* regulation of glucose metabolism

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ABSTRACT

Background: Intake of ω-3 PUFAs have been demonstrated to have positive effects on pregnancy outcome, whose receptor, GPR120, regulates several cellular functions including differentiation, metabolism and immune reaction. However, whether GPR120 is involved in decidualization and pregnancy remains unknown.
Methods: Decidua tissue from women with normal pregnancy and spontaneous abortion were collected to determine the expression profile of GPR120. Abortion mouse models and artificially induced deciduoma in mice were established to evaluate the effect of GPR120 on pregnancy outcome and *in vivo* decidualization. HESCs and primary DSCs were used to explore the roles of GPR120 in decidualization and mechanisms involved.
Findings: We found that GPR120 functioned to promote decidualization by upregulating glucose uptake and pentose-phosphate pathway (PPP) of human endometrial stromal cells. Firstly, the expression of GPR120 in decidua of spontaneous abortion was downregulated compared to normal decidua. Lack of GPR120 predisposed mice to LPS or RU486 induced abortion. Decidualization was augmented by GPR120 *via* improving GLUT1-mediated glucose uptake and G6PD- mediated PPP. FOXO1 was upregulated by GPR120 *via* activation of ERK1/2 and AMPK signaling and increased the expression of GLUT1. Furthermore, the expression of chemokines and cytokines in decidual stromal cells was enhanced by GPR120. Lastly, GPR120 agonist ameliorated LPS-induced abortion in the mice.
Interpretation: GPR120 plays significant roles in decidualization and the maintenance of pregnancy, which might be a potential target for diagnosis and treatment of spontaneous abortion.
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1. Introduction

During early pregnancy, decidualization is essential for successful embryo implantation and the maintenance of pregnancy. Decidualization deficiency or abnormality is one of the major maternal causes of recurrent spontaneous abortions (RSA) [1,2]. Nevertheless, the critical molecular mechanisms governing decidualization and the pathology of decidualization deficiency-related pregnancy complications remain largely unknown.

Decidualization is characterized by the transformation of human endometrial stromal cells (HESCs) to decidua stromal cells (DSCs) that is initiated by dynamic change of progesterone (P_4) and estrodiol (E_2) level and continues to progress if conception occurs [3]. In response to P_4-mediated activation of cyclic adenosine monophosphate (cAMP) signaling, HESCs undergo vast expansion and differentiation. Morphologically, DSCs are charaterized by enlarged cellular size, rounded nucleus with increased number and complexity of the nucleoli, expanded secretory apparatus with dilatation of the rough endoplasmic accumulation of glycogen and lipid droplets, suggesting a need for accelerated biosynthesis and energy supply during decidualization [4]. Actually, it is known that enhanced glucose influx and metabolism are essential for

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Research in context

Evidence before this study

Recurrent spontaneous abortion (RSA) is a serious and potentially devastating health problem whose prevalence is as high as 5% of all pregnancies. Decidualization plays critical role in the maintenance of pregnancy, whose deficiency is closely correlated with RSA. Despite vast research efforts, the pathologensis of RSA related to defective decidualiztion is poorly understood and effective interventions need to be found. Numerous clinical studies and animal experiments verified that ω-3 PUFAs (DHA, EPA) addition during pregnancy was beneficial for pregnancy outcome. As the receptor of ω-3 PUFAs, GPR120 can regulate cell differentiation, proliferation, metabolism and immune reaction, which has been identified on the human placenta in a recent study. However, whether GPR120 functions during decidualization and the pathology of RSA remains unknown since no study was found on PubMed database with the searching term "GPR120" and "abortion" or "decidualization".

Added value of this study

In the current study, the expression level of GPR120 in the decidua from normal pregnant women and those with spontaneous abortion was determined and the mechanisms of the regulatory function of GPR120 on decidualization were clarified. It was found that GPR120 downregulation was correlated with decidualization deficiency and spontaneous abortion. Furthermore, GPR120 promoted decidualization by upregulating the glucose uptake and PPP of human endometrial stromal cells. Mechanistically, ERK1/2-AMPK-FOXO1 signaling was found to be involved. Animal experiments showed that GPR120 agonist was effective in ameliorating the symptoms of abortion.

Implications of all the available evidence

Our findings of GPR120 downregulation in abortion decidua and its functions in decidualization suggest that GPR120 may be implicated in the pathologenesis of RSA, indicating the clinical potential of GPR120 as the prognostic and therapeutic target for RSA. Moreover, the positive effect of GPR120 on the augmentation of decidualization and the maintenance of pregnancy further confirmed the benefits of ω-3 PUFAs addition during pregnancy.

decidualization and pregnancy [5]. DSCs produce insulin-like growth factor-binding protein-1 (IGFBP-1) and prolactin (PRL) which are commonly used as markers of decidualization [6]. DSCs function to regulate the proliferation and invasion of trophoblast and induce decidual differentiation of HESCs by autocrine secretion. Moreover, DSCs interact with decidual immune cells (DICs) to establish the Th2 type maternal-fetal interface, allowing normal decidualization and embryo implantation [7,8]. For example, the recruitment and education of decidual NK (dNK) cells, which account for the largest proportion of DICs, largely depend on chemokines and cytokines secreted by DSCs. Therefore, increased glucose metabolism and the immunoregulatory function of DSCs render sufficient energy, biosynthesis and proper immune microenvironment for decidualization.

ω-3 Polyunsaturated fatty acids (PUFAs) including Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) are commonly used nutritional supplements during pregnancy, which can maintain gestation length and fetal growth, prevent preterm birth and decrease the rate of gestational diabetes [9–11]. G-protein-coupled receptor 120 (GPR120), the receptor of ω-3 PUFAs, has been implicated in the pathogenesis of diabetes, cancers and inflammatory diseases [12,13]. GPR120 can regulate various celluar functions, including differentiation, proliferation, glucose metablism and immune response [14]. Although whether GPR120 plays a role in decidualization and pregnancy is still unknown, the expression of GPR120 in human placenta was determined in a recent paper [15]. Taken together, the previous studies indicate that GPR120 may function during decidualization and pregnancy.

In the present study, we found that the expression of GPR120 in decidua from women of spontaneous abortion was significantly downregulated compared to the decidua from that of normal pregnancy. Moreover, GPR120 deletion ($GPR120^{-/-}$) or inhibition predisposed mice to LPS or RU486 induced abortion, indicating that it may play a role in decidualization and the maintenance of pregnancy. Further experiments showed that GPR120 activation could promote decidualization by upregulating glucose transporter-1 (GLUT1) mediated glucose uptake and glucose-6-phophate dehydrogenase (G6PD) mediated pentose-phosphate pathway (PPP). FOXO1 was upregulated by GPR120 *via* ERK1/2 and AMPK signaling and was responsible for the upregulation of GLUT1. Besides, activation of GPR120 was found to upregulate CXCL12, TGFβ and IL-15 expression from DSCs, chemokine and cytokines dominant in dNK cell recruitment and education. Finally, we found that activation of GPR120 could ameliorate LPS induced abortion in the mice. Altogether, our results indicate that GPR120 plays protective role in maintaining pregnancy and reduces the incidence of spontaneous abortion by promoting decidualization during early pregnancy and, therefore, represents a potential target for diagnosis and treatment of RSA.

2. Materials and methods

2.1. Human sample collection

This study was approved by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China and the Human Research Ethics Committee of the First People's Hospital of Changzhou, Changzhou, China. Human decidua samples were collectd from Obstetrics and Gynecology Hospital, Fudan University and the First People's Hospital of Changzhou. All subjects gave informed written consent for the collection and study of tissue samples. First-trimester (gestational age 6–12 weeks) human decidual tissues were obtained from women with clinically normal pregnancies (terminated for non-medical reasons, $n = 20$) and women with unexplained spontaneous abortion ($n = 20$). Age of women with normal pregnancies: 30.9 ± 2.7 years; age of patients with abortion: 31.5 ± 1.2 years. Maternal body mass index (BMI) of women with normal pregnancies: 21.7 ± 1.8 kg/m^2; BMI of patients with abortion: 22.5 ± 2.9 kg/m^2. All data were presented as mean ± SEM. All tissues were collected under sterile conditions and transported to the laboratory on ice in DMEM (Dulbecco's modified Eagle's medium) /F-12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (All from Life Technologies).

2.2. Isolation of DSCs

Decidual tissues were washed with PBS plus P/S twice and DSCs were isolated according to the previous described method as soon as possible [16]. Briefly, decidual tissues were cut into pieces and digested with 0.1% collagenase type IV (Roche) and 0.002% DNase I (Sigma-Aldrich) in DMEM/F-12 medium for 30 to 60 min at 37 °C. After digestion, the tissue pieces were filtered through sterile gauzes pads (200 mesh) to remove cellular debris. Cell suspension was then centrifuged at 1500 rpm for 8 min and supernatant was discarded. Collected cells were resuspended in DMEM/F-12 medium and layered over different concentrations (60%, 40% and 20% from bottom top) of Percoll (GE Healthcare) and centrifuged at 2000 rpm for 20 min. The layer between 20% and 40% Percoll was collected and then washed with PBS. Finally,

isolated DSCs were cultured in dishes with DMEM/F-12 supplemented with 10% FBS and incubated in a humidified incubator with 5% CO_2 at 37 °C.

2.3. Animals

C57/BL6 wide type (WT) mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Constitutive GPR120 knock out (*GPR120*$^{-/-}$) mice were purchased from Shanghai Bioray Laboratory (Shanghai, China). Animals were housed in specific pathogen-free facilities at the Chinese Academy of Sciences. The knock out of GPR120 in decidua and other tissues were verified by protein expression detection. All animal experiments were performed according to the guide of the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and complied with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.4. Mouse models

Adult C57/BL6 WT or *GPR120*$^{-/-}$ female mice (8–10 weeks old) were mated with C57/BL6 male mice (8–10 weeks old) to establish pregnancy. Day 0.5 of gestation was determined when the vaginal plug was observed. Control and abortion group were injected i.p. with PBS, 0.1–0.5 mg/kg LPS (Sigma-Aldrich) or 2–10 mg/kg RU486 (Invitrogen) together with or without 0.5 mg/kg AH7614 (Tocris), respectively, on day 7.5 of pregnancy. In abortion-treated group, 10 mg/kg TUG891 (Tocris) or 10 mg/kg GW9508 (Sigma-Aldrich) was injected i.p. from gestational day 4.5 to 7.5 once a day, followed by 1 mg/kg LPS administration on gestational day 7.5. In the vehicle group, DMSO (Sigma-Aldrich) was administrated. Mice were sacrificed on gestational day (GD) 8.5 and decidual tissues were collected.

The artificially induced deciduoma was generated according to standard protocol [17]. Briefly,7- to 8-week old female mice were ovariectomized. 10 days after ovariectomy, female mice were treated with sc injection of 100 ng 17β-estradiol (Sigma-Aldrich), once a day for 3 days. After 2 days' rest, mice were then treated with daily injections of progesterone (1 mg, sc, Sellck Chemicals) and 17β-estradiol (6.7 ng, sc), once a day for 3 days. One uterine horn was traumatized by an intraluminal injection of 50 μL of sesame oil (Sigma-Aldrich) 6 h after the last injection while the contralateral uterine horn was served as a control. Mice were then given sc injections of progesterone (1 mg, sc) and 17β-estradiol (6.7 ng, sc), once a day. Mice were sacrificed 5 days after seasome oil injection and the wet weight of uterine horns was recorded. Uterine horns were dissected out and the tissues were collected.

2.5. In vitro decidualization

The immortal human ESC cell line T-HESCs (ATCC CAT# CRL-4003TM, RRID: CVCL_C464) was a gift from Professor Wang Haibin from Institute of Zoology, Chinese Academy of Sciences (Beijing, China) [18]. HESCs were cultured in DMEM/F-12 medium containing 10% FBS, 3.1 g/L glucose and 1 mM sodium pyruvate, supplemented with 1% P/S, 1% ITS and 500 ng/mL puromycin (All from Life Technologies). Cells were refreshed every 3 days. *In vitro* decidualization of HESCs was induced as previously described [19]. Briefly, HESCs were cultured in DMEM/F-12 medium supplemented with 2% FBS, 10 μM medroxy progesterone (MPA, Selleck Chemicals) and 0.5 mM 8-Br-cAMP (Selleck Chemicals) for 6 days and the medium was changed every 2 days. DMSO, TUG891(10 μM), AH7614 (10 μM), GW9508 (10 μM) or inhibitors of signaling pathways (Glucosamine hydrochloride, SCH772984, AS1842856, Compound C, All from Selleck Chemicals) were added during the induction of decidualization in certain experiments. Cells were harvested at indicated time points for subsequent experiments.

2.6. Cell transfection

To establish GPR120/GLUT1/G6PD knockdown HESCs, lentivirus construction and production were performed according to previously described protocols [20]. Briefly, specific shRNAs to GPR120, GLUT1 and G6PD or scramble shRNA were pre-designed and synthesized by Sangon Biotech (Shanghai, China). Lentivectors expressing specific or sramble shRNAs were constructed based on the pLKO.1 puro vector (Addgene). HEK 293 T cells were then transfected using Lipofectamine 2000 (Life Technologies) for lentivirus production. For lentiviral infection, HESCs were plated in a 6-well plate 24 h prior to transfection, and lentiviruses expressing GPR120/GLUT1/G6PD shRNAsor scramble shRNA were added to the culture medium supplemented with 8 μg/mL polybrene (Life Technologies). After 24 h of culture, the medium was replaced with fresh culture medium. Expand transfected cells for follow-up experiments.

2.7. Immunohistochemistry and immunofluorescence.

Human and mouse tissue samples fixed in 4% PFA were embedded into paraffin after dehydration and then sectioned at 5 μm thick. Paraffin sections were deparaffinized and rehydrated in graded alcohol series followed by H&E staining or immunohistochemistry (IHC). For IHC, the sections were boiled in 10 mM sodim citrate buffer, pH 6.0, by microwave for 10 min. After blocking, sections were incubated with mouse anti-human GPR120 (Santa Cruz, CAT# sc-390752) at 4 °C overnight, followed by HRP anti-rabbit IgG. The reaction was developed with DAB (GTVision III Detection System) and the sections were counterstained with hematoxylin.

Frozen samples of human decidual tissue embedded in the optimum cutting temperature (OCT, Sakura Finetek) compound were sectioned at 10 μm thick for immunofluorescence. After washed with PBS, the sections were fixed with MeOH/acetone (1:1) at −20 °C for 5 min and blocked with 1% BSA for 1 h at 37 °C. Mouse anti-human GPR120 (Santa Cruz CAT# sc-390752) and rabbit anti-human vimentin (Abcam CAT# ab92547, RRID:AB_10562134) was applied on cells overnight at 4 °C and then incubated with goat anti-mouse Alexa Fluor 488-IgG or goat anti-rabbit Alexa Fluor 555-IgG (eBioscience) for 1 h at 37 °C in the dark, followed by DAPI staining.

2.8. Real-time PCR

Total RNA were extracted from cells or tissue by using Trizol Reagent (Sigma-Aldrich) and then reverse-transcribed by a Reverse Tanscription System (Takara). Quantitative PCR was performed using FastStart Universal SYBR Green Master Kit (Roche) on an ViiA 7 Real-Time PCR System (Applied Biosystems). The reaction protocol used was 95 °C 5 min, 35 cycles with 95 °C 15 s, 60 °C 60 s, and 72 °C 5 min. The primer sequences used for PCR analysis were listed in the Supplementary Table.

2.9. Western blot

Cells or homogenized tissues were lysed with ice-cold RIPA buffer (Beyotime) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Merck Millipore). The lysates were fractionated by SDS-PAGE and analyzed by western blot with specific antibodies to IGFBP1 (Cell Signaling Technology, CST CAT#31025), GAPDH (CST CAT# 2118, RRID: AB_561053), β-ACTIN (CST CAT# 4970, RRID:AB_2223172), GLUT1 (CST CAT# 12939, RRID:AB_2687899), G6PD (CST CAT# 8866S, RRID:AB_10827744), HK2 (CST CAT# 2867, RRID:AB_2232946), PDH (CST CAT# 2784S, RRID:AB_2162928), LDHA (CST CAT# 2012S, RRID:AB_2137173), FOXO1 (CST CAT# 2880, RRID:AB_2106495), phosphorylated and total – AMPKα (CST CAT# 2535, RRID: AB_331250 and CST CAT# 5831, RRID:AB_10622186), ERK1/2 (CST CAT#4370, RRID:AB_2315112 and CST CAT# 4695, RRID:AB_390779), GPR120 (Abcam, CAT# ab97272, RRID:AB_10680852). Western blot

bands were quantified by densitometry using ImageJ software. Densities were normalized to control treatment and relative folds were normalized to GAPDH or β-ACTIN.

2.10. Detection of glucose uptake by 2-NBDG assay

HESCs were seeded in 24-well plate and incubated in culture medium or decidualization induction medium plus DMSO, TUG891, AH7614 or GW9508 for 72 h. For glucose uptake assessment, the media was replaced with DMEM/F-12 containing 30 μM fluorescent D-glucose derivative 2-NBDG (Invitrogen) and incubated for 2 h at 37 °C. Glucose uptake was stopped by washing three times with ice-cold PBS. Cells were then collected for flow cytometry as soon as possible. The uptake of glucose was determined by the fluorescence of 2-NBDG in cells, which typically displays excitation/emission maxima of ~465/540 nm. FACS analysis was performed on FACSCalibur Analyzer (BD Bioscience). Data were analyzed with FlowJo software.

2.11. Detection of apoptosis by Annexin V/PI staining

Cells cultured in 12-well plate of 80% confluent was refreshed with 0% FBS-DMEM/F-12 culture medium plus DMSO, TUG891, AH7614 or GW9508 for 72 h. After stimulation, cells were harvested and washed with ice-cold PBS. Cells were stained with Alexa Fluor 488 AnnexinV and Propidium Iodide (Invitrogen) at room temperature for 15 min and analyzed by flow cytometry as soon as possible.

2.12. Statistics

All the experiments were independently repeated at least 3 times. All data were expressed as mean ± SEM. Significant differences were evaluated using one-way ANOVA or the two-tailed Student's *t*-test with GraphPad Prism (version 6.0, GraphPad Software) and Statistical Package for Social Science software (version 22.0, SPSS). $P<0.05$ was considered statistically significant.

3. Results

3.1. Downregulation of GPR120 in decidua was correlated with human miscarriage

To define the expression profile of GPR120 in decidua, we first examined the expression of GPR120 and other PUFA receptors including GPR40, GPR41, GPR43 and GPR84 in normal human decidual tissue. Interestingly, GPR120 is distinctly highly expressed among the PUFA receptors (Supplementary Fig. 1a), indicating its possible role in pregnancy. Therefore, human decidua samples from women with normal pregnancy and women with spontaneous abortion were collected and the expression of GPR120 was determined. GPR120 was downregulated in abortion decidua at both protein and mRNA levels, accompanied by decreased expression of decidualization markers IGFBP1 and PRL (Fig. 1a,b). IHC staining of decidual tissue sections further verified the different expression of GPR120 in abortion-derived and normal decidua (Fig. 1c). Similarly, DSCs isolated from abortion-derived decidua also displayed decreased expression of GPR120 and reduced expression of

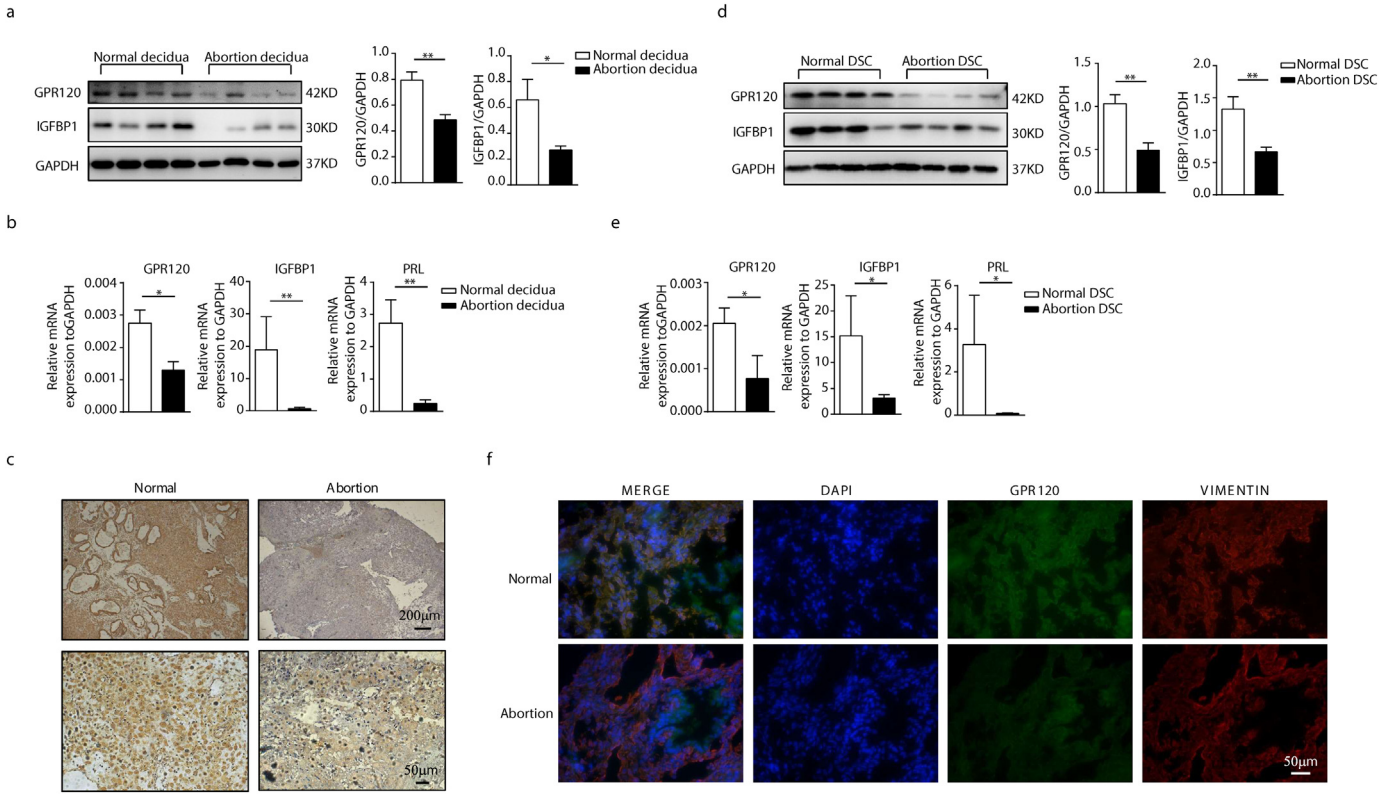

Fig. 1. GPR120 was downregulated in human spontaneous abortion decidua. Decidual tissues from women of normal pregnancies ($n = 20$) and women of spontaneous abortion ($n = 20$) were collected and the expression of GPR120 was determined. (a) Western blot was performed to detect the protein level of GPR120 and IGFBP1. (b) mRNA expression level of *GPR120*, *IGFBP1* and *PRL* were determined by real-time PCR. (c) Parraffin section of human decidual tissues were prepared and GPR120 was deteceted by IHC. (d, e) Normal and abortion DSCs were isolated from decidual tissues of normal pregnancy and abortion with collagnease and DNase. Protein level of GPR120 and IGFBP1 were measured by western blot (d) and real-time PCR was performed to detect mRNA expression level of *GPR120*, *IGFBP1* and *PRL* (e). (f) GPR120 (green) and the marker of DSCs vimentin (red) were detected in decidual tissues by IFH to confim the expression of GPR120 in vimentin$^+$ DSCs. Results are presented as mean ± SEM. $^*P < .05$, $^{**}P < .01$ (two-tailed Student's *t*-test).

decidualization markers IGFBP1 and PRL when compared to healthy controls (Fig. 1d,e). Vimentin marks DSCs in human decidua tissue. Immunofluorescence of GPR120 and vimentin further confirmed the downregulation of GPR120 in DSCs from abortion patients (Fig. 1f). Altogether, these data showed that GPR120 was significantly downregulated in decidua and DSCs from women with spontaneous abortion, indicating that GPR120 may contribute to the maintenance of normal pregnancy.

3.2. Lack of GPR120 predisposed mice to LPS or RU486 induced abortion

GPR120 expression was maintained at certain level during the early gestation in the mouse (Supplementary Fig. 1b). To evaluate the significance of GPR120 in pregnancy, we established normal pregnancy and abortion models in WT and *GPR120*$^{-/-}$ mice. Firstly, the fertility of *GPR120*$^{-/-}$ or WT female mice paired with *GPR120*$^{-/-}$ or WT male mice under natural laboratory condition was assessed. Compared to WT mice, the litter size decreased in *GPR120*$^{-/-}$ female mated with WT male mice and WT female mated with *GPR120*$^{-/-}$ male mice, which was even more significant when both parents were *GPR120*$^{-/-}$ (Fig. 2a,b), indicating that loss of GPR120 might have adverse effect on pregnancy. Next, abortion stimulators LPS or Mifepristone (RU486) was applied to *GPR120*$^{-/-}$ mice or to WT mice treated with GPR120 antagonist AH7614. WT mice presented healthy pregnancy under low dose of LPS administration and severe symptoms of pregnancy loss under high dose of LPS administration, manifested by hemorrhagic implantation sites, decreased litter size and restricted fetal growth. However, *GPR120*$^{-/-}$ mice exhibited severe abortion signs even under

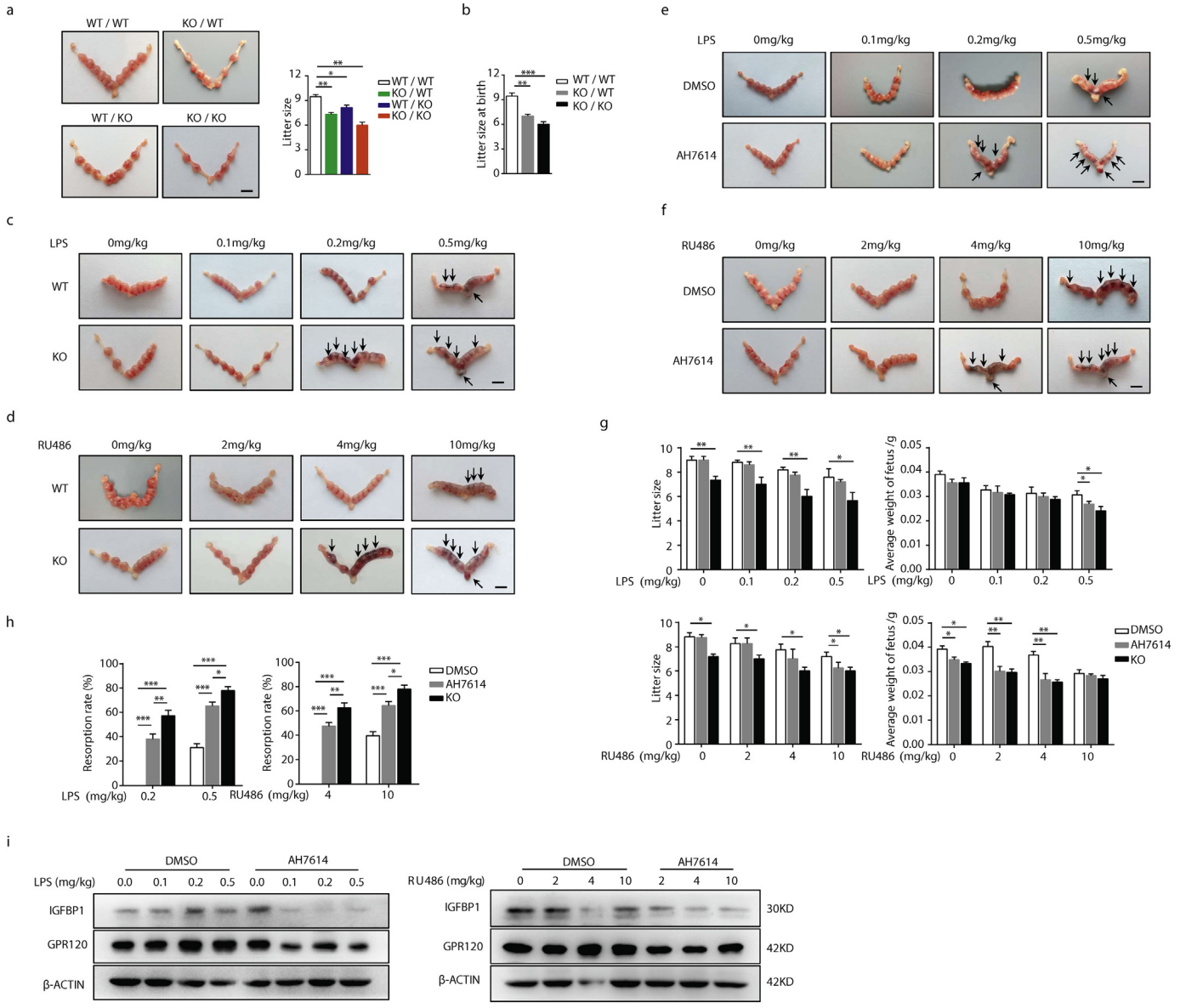

Fig. 2. Lack of GPR120 predisposed mice to LPS or RU486 induced abortion. (a) Representative images of embryos in the uterus of GD8.5 from WT female mice paired with WT male mice (WT/WT), *GPR120*$^{-/-}$ female mice paired with WT male mice (KO/WT), WT female mice mated with *GPR120*$^{-/-}$ male mice (WT/KO) and *GPR120*$^{-/-}$ female mice paired with *GPR120*$^{-/-}$ male mice (KO/KO) were shown. Litter size on GD8.5 was calculated and shown in the right panel while litter size at birth ($n = 6$) was shown in (b). (c, d) *GPR120*$^{-/-}$ and WT female mice were mated with WT male mice to induce pregnancy. Representative images of embryos in the uterus of GD8.5 from *GPR120*$^{-/-}$ (KO) and WT pregnant mice injected i.p. of different concentrations of LPS (0.1/0.2/0.5 mg/kg) or RU486 (2/4/10 mg/kg) on GD7.5 were shown. (e-i) On GD7.5, WT mice were injected i.p. of LPS (0.1/0.2/0.5 mg/kg) or RU486 (2/4/10 mg/kg) plus DMSO or GPR120 antagonist AH7614 (0.5 mg/kg). (e, f) Representative images of embryos in the uterus of GD8.5. (g) Mice were sacrificed on GD8.5 and fetus were dissected from uterus and weighed. Litter size and average weight of litter were recorded (n = 6). (h) Resorption rate (%) was calculated as the ratio of the number of resorption fetus/ (viable fetus + resorption fetus) (n = 6). (i) Uterine tissues were collected and western blot was performed to detect the expression of GPR120 and IGFBP1. Scale bar: 5 mm. Arrows: resorption implantation site. Results are presented as mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ (one-way ANOVA).

lower dose of LPS (Fig. 2c). Similarly, more severe abortion signs appeared in *GPR120*$^{-/-}$ mice than in WT mice when challenged with the same dose of RU486 (Fig. 2d). Application of GPR120 antagonist AH7614 also rendered the pregnancy in WT mice more susceptible to LPS or RU486, as in *GPR120*$^{-/-}$ mice (Fig. 2e,f). The litter size, average weight of litters in different groups were recorded on GD8.5 (Fig. 2g) and the resorption rate was shown in Fig. 2h. Besides, we determined the expression of GPR120 and IGFBP1 in murine uterus. As shown in Fig. 2i, IGFBP1 was downregulated in LPS or RU486 plus AH7614 induced abortion mice accompanied by decreased GPR120, showing the similar phenomenon in human abortion and indicating impaired decidualization. Together, these results showed that loss or inhibition of GPR120 sensitized mice to LPS or RU486 induced abortion and suggest that GPR120 is protective in maintaining normal pregnancy.

3.3. Decidualization was augmented by GPR120

To explore the function of GPR120 in decidualization, *in vitro* decidualization of HESCs was induced, as verified by increasing expression of IGFBP1 during induction (Supplementary Fig. 2a). GPR120 agonist TUG891 and antagonist AH7614 were then respectively applied to determine the effect of GPR120 on decidualization. We found that the expression level of GPR120 remained unchanged during the induction of decidualization or when stimulated with TUG891/AH7614 (Fig. 3a, Supplementary Fig. 2b,c). However, IGFBP1 was significantly upregulated by TUG891 and was downregulated by AH7614 (Fig. 3a,b). PRL showed a similar trend in response to the two treatments (Fig. 3b). Moreover, knock down of GPR120 with shRNA in HESCs produced the same result as GPR120 antagonist (Fig. 3c). Similar result was obtained when HESCs were treated with GW9508, another frequently used agonist of GPR120 (Supplementary Fig. 3). To exclude possible effect of GPR120 on HESC expansion, the proliferation and apoptosis of HESCs in induction or culture medium were determined. We found that neither TUG891 nor AH7614 had significant effects on proliferation of HESCs (Supplementary Fig. 4a) while apoptosis of HESCs was mildly inhibited by TUG891 (Supplementary Fig. 4b), which is beneficial to decidualization. Similar results were obtained in HESCs treated with GW9508 (Supplementary Fig. 4c,d). These results suggest a positive regulatory role of GPR120 in decidualization.

For *in vivo* evidence, we established artificially induced deciduoma in ovariectomized female mice according to a previously described protocol (Fig. 3d). GPR120 agonist or antagonist was injected i.p. to the mice 2 days after decidualization was induced by injection of seasome oil. As shown in Fig. 3e, oil-induced deciduoma in the uterus of

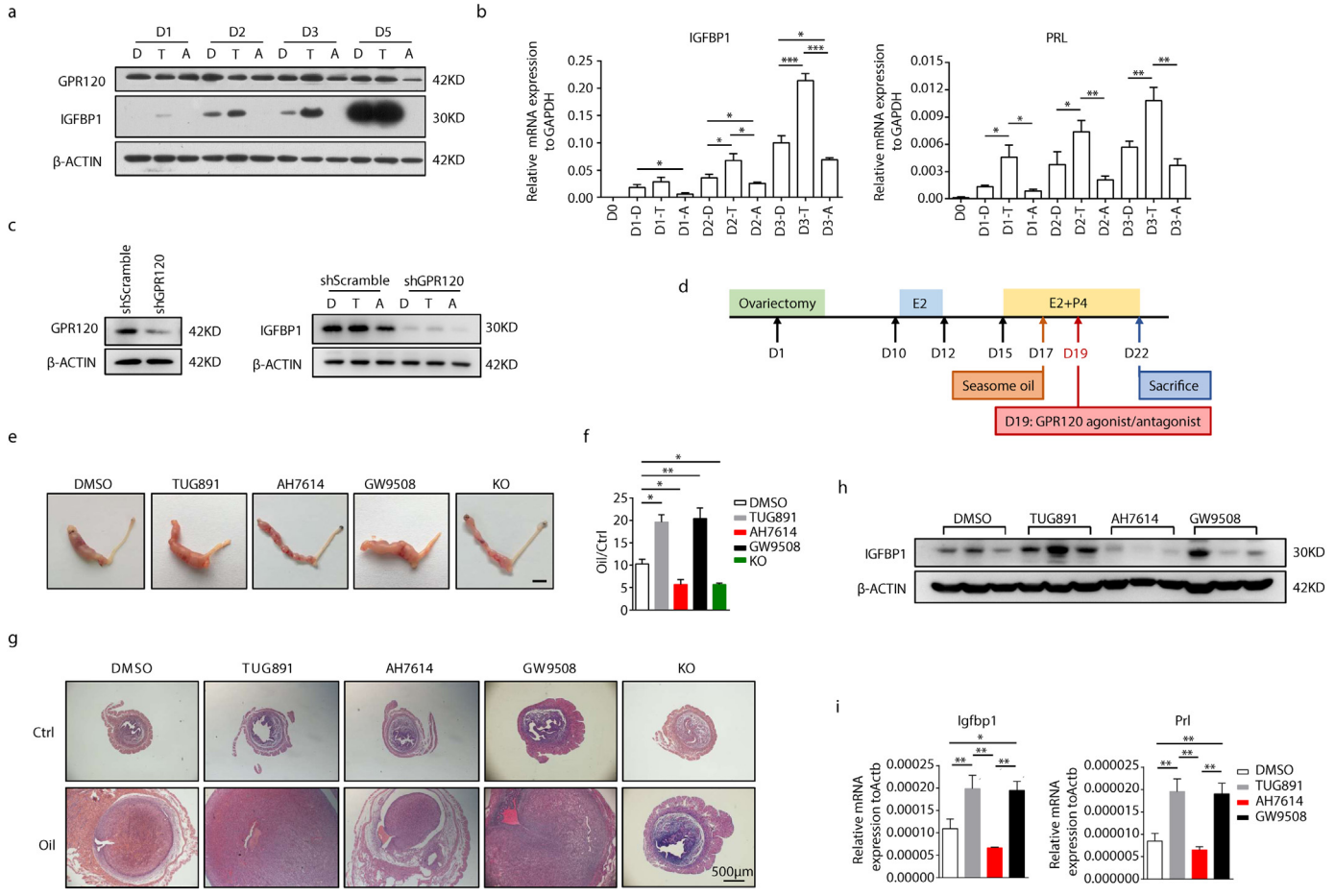

Fig. 3. Decidualization was augmented by GPR120. (a-c) To induce *in vitro* decidualization, HESCs were incubated in DMEM/F12 medium plus cAMP and MPA. DMSO (D), GPR120 agonist TUG891 (T, 10 μM) or GPR120 antagonist AH7614 (A, 10 μM) was added into the induction medium. (a) Cells were collected 1, 2, 3 or 5 days after induction and the protein level of GPR120 and IGFBP1 were examined by western blot. (b) Real-time PCR was used to determined the mRNA expression of IGFBP1 and PRL on day 3. (c) HESCs were transfected with sh*Scramble* or sh*GPR120* to knock down GPR120. GPR120 was detected by western blot to confirm the knock down of GPR120. Cells were incubated in induction medium for 3 days and IGFBP1 was determined. sh*Scramble* or sh*GPR120* HESCs were stimulated with DMSO (D), TUG891 (T) or AH7614 (A) during induction. Western blot was used to examine the expression of IGFBP1. (d) Schema of the induction of deciduoma and GPR120 agonist or antagonist treatment in mice. Deciduoma was induced by injection of seasome oil into the left side of uterine horn while the unstimulated right side was used as control. DMSO, GPR120 agonists (TUG891, GW9508, 5 mg/kg) or GPR120 antagonist (AH7614, 5 mg/kg) was injected i.p. on D19. (e) Representative images of uterine horns from mice sacrificed on D22. Scale bar: 5 mm. (f) The left side uterine horn injected with oil (Oil) and the right side uterine horn (Ctrl) were weighed. The ratio of deciduoma/ control was presented as oil/ctrl ($n = 6$). (g) Paraffin sections of deciduoma and the control uterine horn were used for H&E staining. Deciduoma of different groups were collected and the expression level of IGFBP1 and PRL were detected by western blot (h) and real-time PCR (i). Results are presented as mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$ (one-way ANOVA).

TUG891/GW9508 treated groups were much larger than those in DMSO treated group, while the deciduoma in AH7614 treated group and *GPR120*$^{-/-}$ mice were smaller. Measurement of the weight of uterine horn and HE staining of deciduoma confirmed the positive effect of GPR120 on decidualization (Fig. 3f,g). Moreover, IGFBP1 was increased in TUG891 or GW9508 treated groups but decreased in AH7614 treated group (Fig. 3h,i). Altogether, these results indicate that decidualization is augmented by GPR120.

3.4. GPR120 promoted decidualization by upregulating GLUT1 mediated glucose uptake and G6PD mediated PPP

Decidualization of HESCs requires increased supply of nutrients and biosynthesis, for which glucose metabolism plays important roles. Uptake of glucose, mediated by glucose transporters (GLUTs, SLC2 family), is the first step of glucose utilization. Therefore, the ability of HESCs to take up glucose was measured. We found that activation of GPR120 increased the glucose uptake of HESCs under normal culture condition or during decidualization (Fig. 4a, Supplementary Fig. 5), while inhibition or knock down of GPR120 inhibited the process (Fig. 4a,b). Examination of the expression of the various GLUTs in HESCs and DSCs revealed that *SLC2A1*, encoding GLUT1 highly expressed (Fig. 4c). In addition, it was increased during decidualization (Fig. 4d,e). When GLUT1 was knocked down in HESCs, the ability to take up glucose was inhibited, accompanied by impaired decidualization (Fig. 4f,g). More importantly, GLUT1 was further upregulated by TUG891 while inhibited by AH7614 during decidualization (Fig. 4h). Together these results indicat that GLUT1 mediates the increased uptake of glucose caused by GPR120.

Glucose taken up by cells may undergo glycolysis, PPP or glycogen synthesis. To elucidate the utilization pathway of glucose in HESCs, we analyzed the expression of key enzymes during decidualization and their responses to TUG891 or AH7614. G6PD, the key enzyme in PPP,

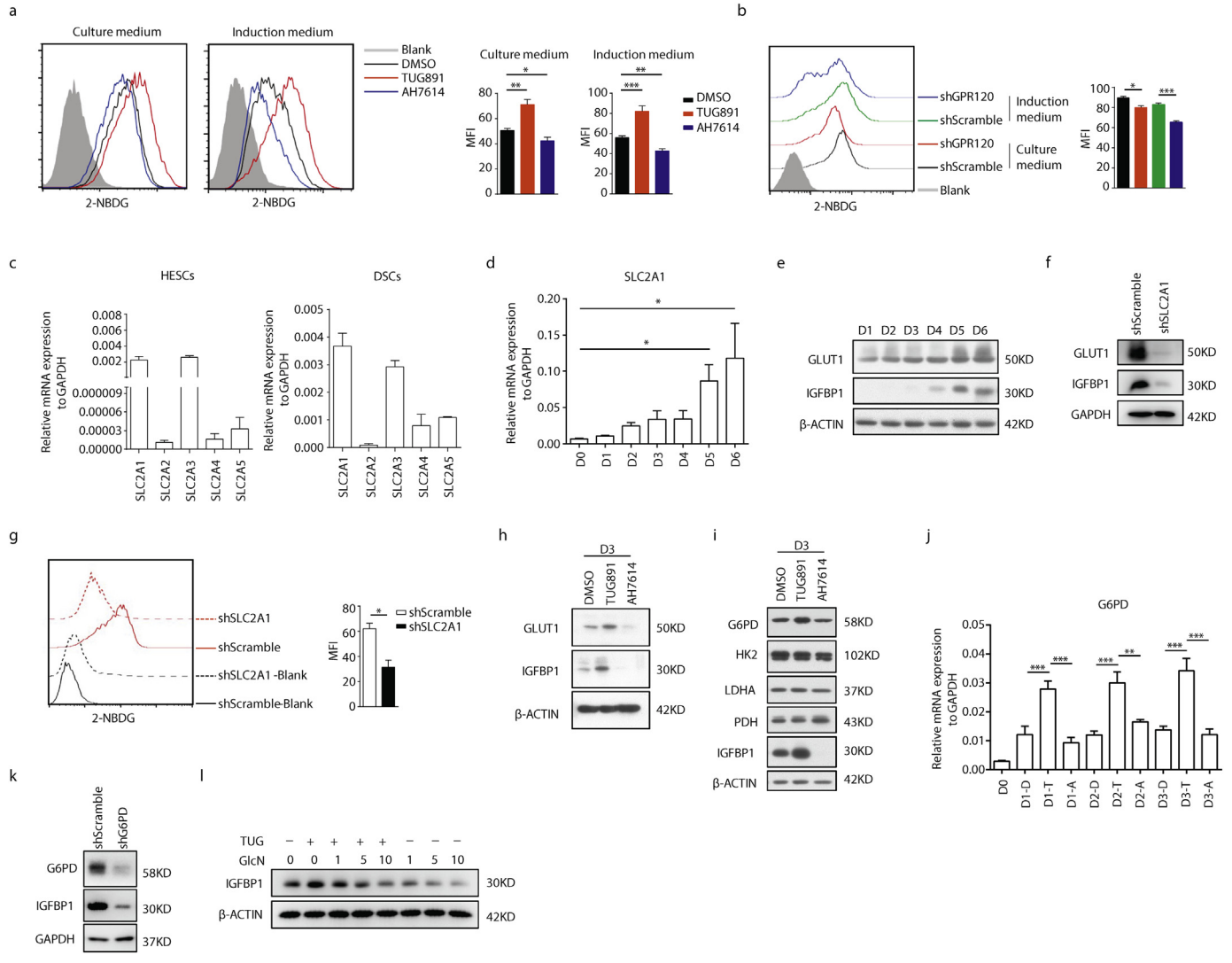

Fig. 4. GPR120 upregulated GLUT1 mediated glucose uptake and G6PD mediated PPP. (a) HESCs were incubated in culture or induction medium plus DMSO, TUG891 or AH7614 for 3 days. 2-NBDG was added and glucose uptake was determined by FACS analysis. MFI of 2-NBDG was shown (n = 6). (b) HESCs transfected with sh*Scramble* or sh*GPR120* were incubated in induction or culture medium for 3 days. Glucose uptake was detected by 2-NBDG assay and MFI was shown ($n = 5$). (c) mRNA expression of *SLC2As* were determined in HESCs and DSCs by real-time PCR. HESCs incubated in induction medium for D1-D6 were collected and mRNA expression level of *SLC2A1* was measured by real-time PCR (d) and protein level detected by western blot during decidualization (e). (f) HESCs were transfected with sh*Scramble* or sh*SLC2A1* to knock down GLUT1. Cells were cultured in induction medium for 3 days and western blot was performed to detect the expression of GLUT1 and IGFBP1. (g) Glucose uptake was detected by 2-NBDG assay in sh*Scramble* or sh*SLC2A1* HESCs. MFI was shown (n = 5). HESCs were incubated in induction medium plus DMSO, TUG891 or AH7614 for 3 days. (h) GLUT1, IGFBP1 and PRL were detected by western blot. (i) Expression level of key enzymes involved in glucose metabolism were determined by western blot. (j) mRNA expression of *G6PD* was examined by real-time PCR. (k) HESCs were transfected with sh*Scramble* or sh*G6PD* to knock down G6PD and incubated in induction medium for 3 days. G6PD and IGFBP1 were detected by western blot. (l) HESCs were incubated in induction medium with or without TUG891 (TUG, 10 μM) plus PPP inhibitor Glucosamine hydrochloride (GlcN, 1/5/10 mM). Decidualization was determined by protein level of IGFBP1. Results are presented as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ (one-way ANOVA in Fig. 4a-d and 4j, two-tailed Student's *t*-test in Fig. 4g).

was upregulated in TUG891 treated group (Fig. 4i,j). When G6PD was knocked down in HESCs, decidualization was inhibited (Fig. 4k). Furthermore, when PPP was inhibited by GlcN, the positive effect of TUG891 on decidualization was attenuated (Fig. 4l), indicating the functional significance of PPP in the augmentation of decidualization by GPR120. Thus, GPR120 augmented decidualization by upregulating glucose uptake mediated by GLUT1 and PPP mediated by G6PD.

3.5. Augmentation of decidualization by GPR120 was mediated by ERK1/2 signaling

To elucidate mechanisms underpinning GPR120-augmented decidualization, transcriptional factors involved in decidualization and the downstream pathways of GPR120 were examined. Firstly, as the critical transcriptional factor that regulates cell differentiation and cell cycle genes during decidualization, FOXO1 was found to be upregulated in HESCs undergoing *in vitro* decidualization (Fig. 5a). Moreover, it was upregulated by TUG891 while inhibited by AH7614 or GPR120 knockdown, a similar trend as IGFBP1 (Fig. 5b,c). We determined the role of FOXO1 in GPR120 regulated decidualization by applying FOXO1 inhibitor and found that the positive effect of GPR120 agonist on decidualization was abolished by FOXO1 inhibition (Fig. 5d), suggesting that the effect of GPR120 is mediated by FOXO1. More importantly, GLUT1 was significantly downregulated by FOXO1 inhibition (Fig. 5d), suggesting that the upregulation of GLUT1 by GPR120 depends on FOXO1. We next determined whether AMPK and ERK1/2 signaling pathway were involved in the regulation of FOXO1 by GPR120. Indeed, ERK1/2 and AMPK pathway were activated by TUG891 (Fig. 5e). Furthermore, inhibition of AMPK significantly diminished the positive effect of GPR120 on decidualization, as shown by the decreased

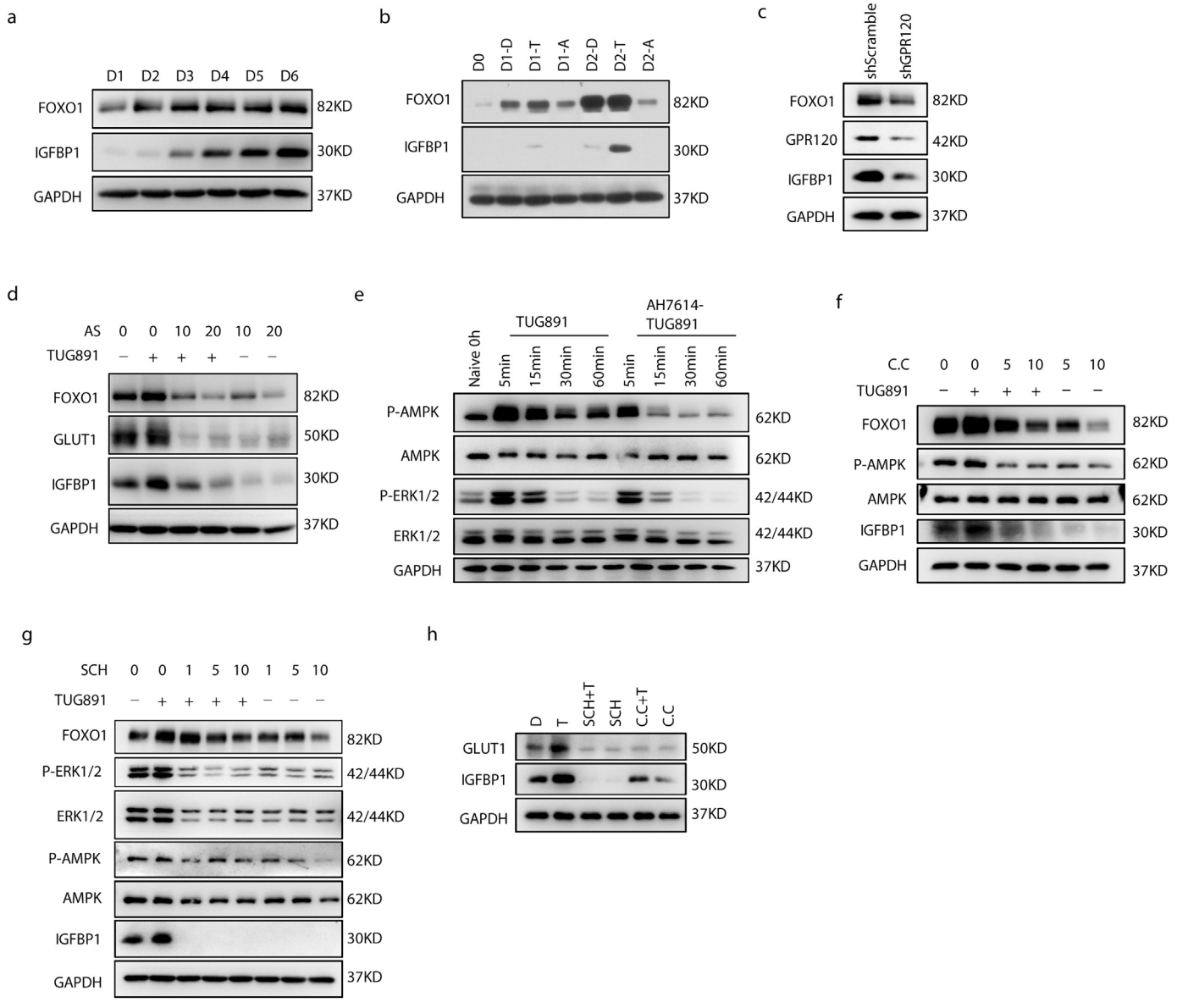

Fig. 5. Regulatory function of GPR120 in decidualization was mediated by ERK1/2 signaling. (a) Decidualization was induced in HESCs for 6 days. Expression of FOXO1 and IGFBP1 during decidualization were detected by western blot. (b) HESCs were treated with DMSO (D), TUG891 (T) or AH7614 (A) during decidualization. Expression of FOXO1 and IGFBP1 were detected by western blot. (c) sh*Scramble* or sh*GPR120* HESCs were incubated in induction medium for 3 days and western blot was performed. (d) Decidualization was induced in HESCs for 3 days and FOXO1 inhibitor AS1842856 (AS, 10/20 μM) was supplemented. Protein level of FOXO1, GLUT1 and IGFBP1 were examined by western blot. (e) HESCs were pretreated with 0% FBS medium for 24 h. Then cells were stimulated with TUG891 (10 μM) with or without 1 h of pretreatment of 10 μM AH7614 for 5, 15, 30 and 60 min. AMPK and ERK1/2 signaling pathways were determined by western blot. Decidualization was induced in HESCs for 3 days, supplemented with TUG891 (10 μM) and (f) AMPK inhibitor Compound C (C·C, 5/10 μM) or (g) ERK1/2 inhibitor SCH772984 (SCH, 1/5/10 μM). Western blot was performed. (h) HESCs were treated with DMSO (D), TUG891 (T, 10 μM) plus ERK1/2 inhibitor (SCH, 5 μM) or AMPK inhibitor (C·C, 5 μM) during decidualization for 3 days. Cells were collected and western blot was performed.

expression of FOXO1 and IGFBP1 (Fig. 5f). ERK1/2 inhibition similarly affected the levels of FOXO1 and IGFBP1, with or without TUG891 stimulation (Fig. 5g), implicating the involvement of ERK1/2 signaling in the augmentation of decidualization by GPR120. Finally, it was found that the GLUT1 upregulation by GPR120 was greatly attenuated by AMPK or ERK1/2 inhibition, substantiating the notion of GLUT1 as the downstream target of FOXO1 (Fig. 5h). Together, these results indicate that the augmentation of decidualization by GPR120 might be mediated by ERK1/2-AMPK-FOXO1 signaling pathway.

3.6. GPR120 affected the expression of chemokines and cytokines in DSCs

During the process of decidualization, HESCs differentiate into DSCs, which play a vital role in establishing Th2 type immune microenvironment in uterus to thereby support decidualization. Generally, DSCs recruit DICs by secreting chemokines and educate DICs toward Th2 type by producing cytokines and thus attenuate inflammation when infections occur. dNK cells account for the majority of DICs. We measured the expression of chemokines and found that CXCL12 was expressed at the highest level both in HESCs and DSCs (Fig. 6a). Interstingly, when challenged by LPS, CXCL12 was downregulated in DSCs (Fig. 6b), which might reduce the recruitment of dNK cells by DSCs. We explored the effect of GPR120 on the expression of CXCL12 in DSCs under LPS treatment. As shown in Fig. 6b, the reduction of CXCL12 by LPS was restored by TUG891. We further evaluated the expression of IL-15 and TGF-β in DSCs, cytokines pivotal for education of dNK cells [21], and found them to be upregulated during decidualization along with CXCL12 (Fig. 6c). Similarly, IL-15 and TGF-β were downregulated by LPS but were restored when co-treated with TUG891 (Fig. 6d), suggesting that education of dNK cells by DSCs might be impaired by LPS but normalized by GPR120 activation. Meanwhile, TUG891 inhibited the expression of proinflammatory cytokines IL-1β and TNF-α triggered by LPS (Fig. 6e). Taken together, these data suggest that GPR120 could maintain the expression profile of chemokines and cytokines in DSCs and propose the possibility that GPR120 may contribute to the immunosuppressive function of DSCs to establishes a Th2 type maternal-fetal immune microenviroment required for decidualization and therefore the maintenance of pregnancy.

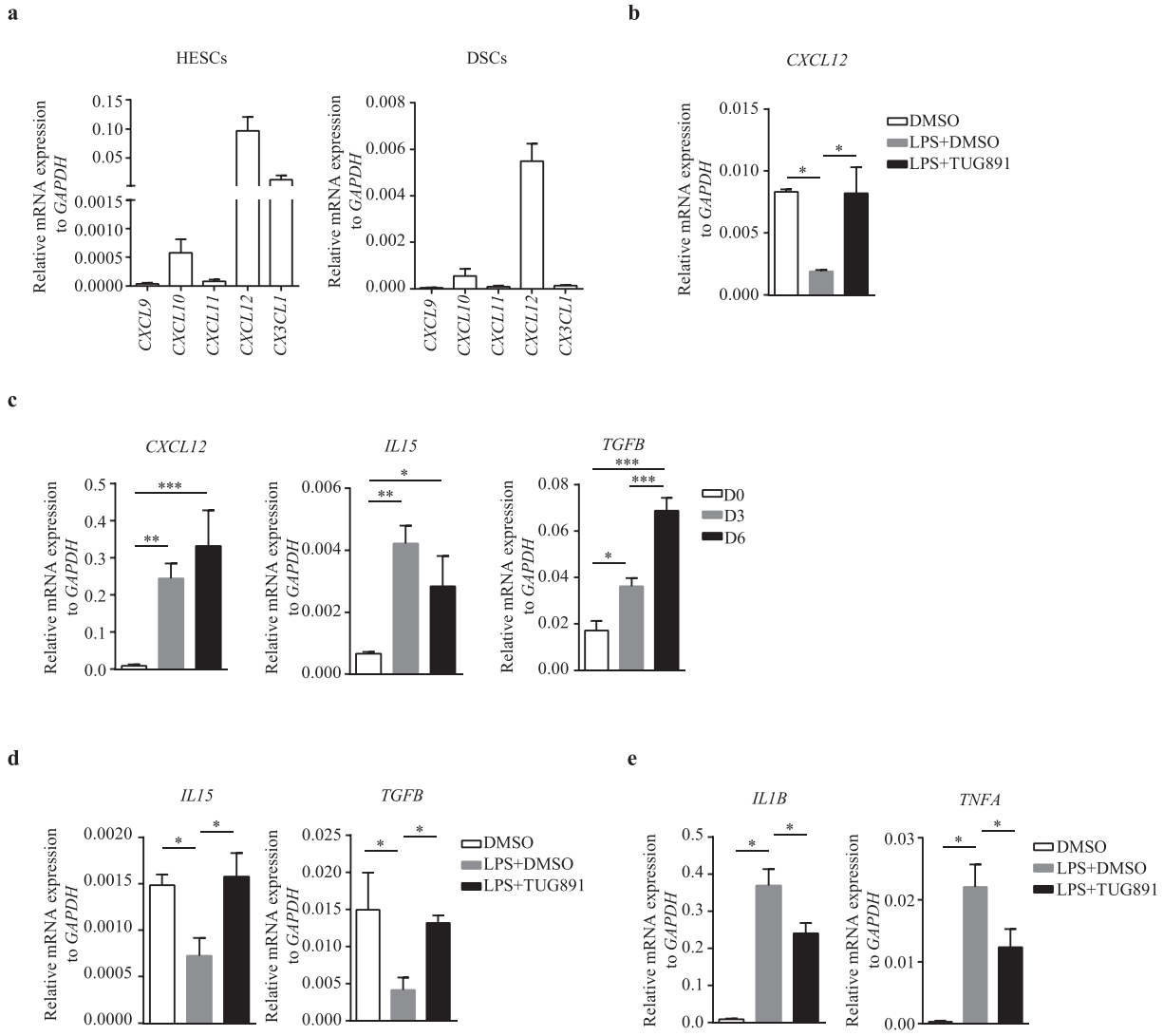

Fig. 6. GPR120 activation altered the expression profile of chemokines and cytokines in DSCs. (a) mRNA expression level of chemokines in HESCs and DSCs were examined by real-time PCR. DSCs were stimulated with LPS (1 μg/mL) plus DMSO or TUG891 (10 μM) for 6 h. Cells were collected and mRNA expression level of *CXCL12* (b) and cytokines (d) were detected. (c) HESCs were incubated in induction medium for 3 or 6 days to induce decidualization. Cells were collected to detect the mRNA expression level of *CXCL12*, *IL15* and *TGFB*. (e) DSCs were stimulated with LPS (1 μg/mL) plus DMSO or TUG891 (10 μM) for 6 h. Pro-inflammatory cytokines IL1-β and TNF-α were detected by real-time PCR. Results are presented as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ (one-way ANOVA).

3.7. GPR120 protected against abortion induced by LPS in the mice by promoting decidualization

Aforementioned results indiate that GPR120 may have protective effects on pregnancy challenged by abortion risk factors, we therefore investigated whether GPR120 could rescue or ameliorate LPS induced abortion in the mice. Mice treated with LPS produced hemorragic fetus, decreased litter size and lower fetal weight. In contrast, mice simultaneously treated with TUG891 or GW9508 produced healthier fetus, normal litter size and fetal weight (Fig. 7a, b), indicating that LPS-induced abortion was ameliorated by GPR120 activation. Finally, while decidualization marker IGFBP1 was downregulated by LPS, it was restored by TUG891 or GW9508 treatment (Fig. 7c). Thus, activation of GPR120 by agonists could amelioreate LPS-induced abortion by restoring decidualization.

As summarized in Fig. 7d, our studies reveal that GPR120 plays protective role in pregnancy by promoting decidualization. When activated by ω-3 PUFAs, GPR120 upregulated the expression of FOXO1 by activating ERK1/2 and AMPK signaling, which further increased the transcription of GLUT1. Glucose uptake was then increased and gluose underwent PPP mediated by GPR120-upregulated G6PD, to promote the process of biosynthesis and thereby augment decidualization, which is important for the maintenance of pregnancy.

4. Discussion

G-protein-coupled receptors (GPCRs) which are charaterized by a common motif with seven transmembrane domains constitute the largest membrane protein receptor family in mammals by far [14,22]. In recent years, some of the GPCRs are identified as fatty acid receptors, such as GPR40, GPR41, GPR84 and GPR120 [23–25]. GPR120, the receptor for ω-3 PUFAs, is reported to be involved in the regulation of various cellular and physiological functions. There are several lines of evidence suggest that GPR120 may play significant role in the maintenance of pregancy.

Firstly, ω-3 PUFAs such as DHA and EPA, the ligands of GPR120, are the acknowledged nutritional addition during pregnancy [26,27]. Human clinical trials and studies with animal models have suggested

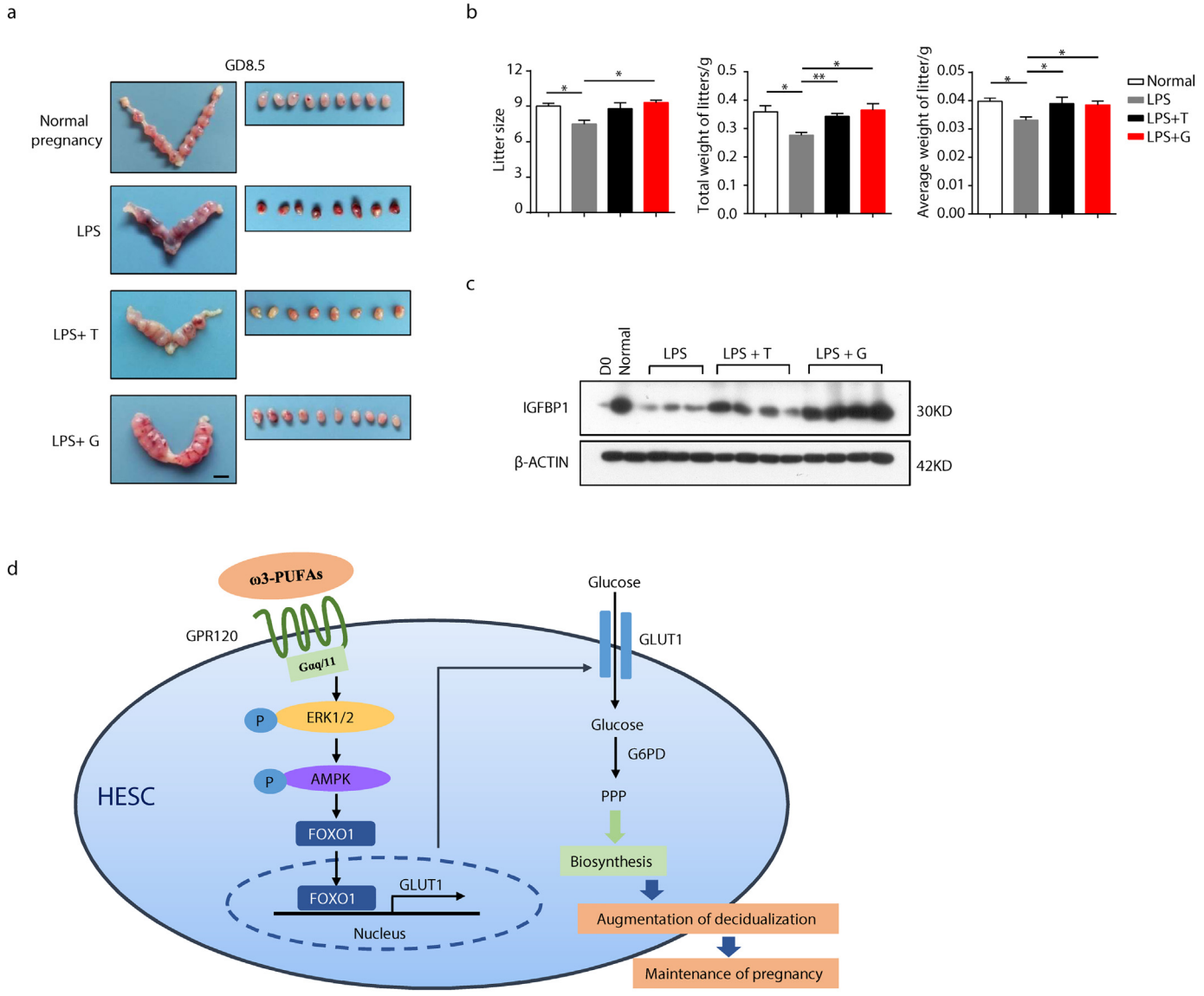

Fig. 7. GPR120 protected against mice from LPS induced abortion by promoting decidualization. WT female mice were mated with WT male mice to induce pregnancy. LPS (1 mg/kg) was injected i.p. on GD7.5. In treated groups, TUG891 (10 mg/kg) or GW9508 (10 mg/kg) was injected i.p. from GD4.5 to GD7.5. Mice were sacrificed on GD8.5. (a) Uterus and fetus were dissected. Scale bar: 5 mm. (b) Litter size was counted and litters were weighed (n = 6). (c) Uterine tissues were collected and protein level of IGFBP1 was detected by western blot. (d) Working model of the regulation of GPR120 on decidualization. Results are presented as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$ (one-way ANOVA).

that supplementation of ω-3 PUFAs during pregnancy can maintain gestation length and fetal growth, prevent preterm birth and decrease the rate of gestational diabetes [28]. ω-3 PUFAs are known to contribute to the maintenance of the immune defense system by promoting the differentiation of T cells to a Th2 phenotype in pregnancy and by shifting the Th1/Th2 ratio from a deleterious proinflammatory Th1 phenotype to a protective anti-inflammatory Th2 phenotype [9,29], indicative of their role in the regulation of maternal-fetal immune microenvironment. Secondly, GPR120 has been detected in human placenta [15]. As a multifunctional molecule, GPR120 is critical in the regulation of glucose metabolism [30]. Interestingly, the accummulated glycogen in DSCs demonstrated that upregulated glucose metabolism is indispensable for energy supply and biosynthesis during decidualization. Therefore, it is resonable to hypothesize that GPR120 activated by ω-3 PUFAs may contribute to maintain pregnancy by regulating decidualization. We tested this hypothesis by examining the expression level of GPR120 in human decidua tissues of spontaneous abortions and the effects of GPR120 agonist and antagonists in LPS or RU486 induced mouse abortions. We observed that GPR120 was downregulated in abortion decidua and DSCs, which might be relevant to defective decidualization. We found that mice with inhibition or deletion of GPR120 appeared to be more sensitive to LPS or RU486 induced abortion while activation of GPR120 could ameliorate the abortion symptoms caused by LPS. The augmentation of decidualization by GPR120 was also verified by *in vitro* and *in vivo* experiments, indicating a positive role of GPR120 in maintaining decidualizaiton and pregnancy.

Recently, the significance of metabolism in decidualization has been emphasized. It was demonstrated that Warburg-like glycolysis and lactate shuttle play critical roles during decidualization in mice [5]. While another study described the importantce of fatty acid beta-oxidation in decidualization and embryo implantation of human and mice [31]. However, the regulatory mechanisms of metabolism during decidualization still need to be explored. In the present study, we found that glucose metabolism mediated the function of GPR120 in decidualization while fatty acid beta-oxidation was not affected by GPR120 during decidualizaton (data not shown). Consistent with a previous study [32], GLUT1 was found to be abundantly expressed in HESCs and its expression determined the efficiency of glucose uptake during decidualization. Importantly, GPR120 could upregulate GLUT1 and subsequently the glucose uptake. Moreover, G6PD, the key enzyme in PPP, was upregulated by GPR120, indicating the importance of PPP in mediating the role of GPR120 in augmenting decidualization. PPP is the significant glucose metabolism pathway to supply ribose 5-phophate and NADPH for biosynthesis of nucleic acid, fatty caids and amino acids, *etc.*, in order to guarantee cell proliferation, differentiation, activation and other functions [33,34]. It has been described that maternal G6PD deficiency is embryonic letal and would cause severe abnormalities in the placenta [35]. Moreover, blockade of the PPP impaired decidualization and implantation [36]. Therefore, it is vital to preserve proper PPP flux during pregnancy. Given the regulatory role of GPR120 on G6PD and PPP shown in the present study, it can be hypothesized that downregulation of GPR120 damages the process of PPP, which causes defective decidualization and poor pregnancy outcome. While the upregulation of PPP by GPR120 could improve biosynthesis required for decidualization.

Molecular mechanisms of decidualization have been studied for years, revealing numerous signaling pathways and transcriptional factors participated in the regulation [37,38]. As a central controller of deicdualization, FOXO1 regulates the transcription of a large number of target genes, which are involved in differentiation, proliferation and cell cycle [39,40]. Although the mutual regulation between FOXO1 and metabolism has been mentioned [41,42], the concrete roles and mechanisms remain to be further explored. In the current study, we found that ERK1/2-AMPK pathway was activated by GPR120 signaling and upregulated the expression of FOXO1. Moreover, GLUT1 was positively regulated by FOXO1, indicating that the effect of GPR120 on glucose uptake and thereby decidualization was mediated by ERK1/2-AMPK-FOXO1 signaling pathway. However, how GPR120 activation drives G6PD and PPP remains to be elucidated in future study.

Immunomodulation is one of the important functions of decidua [21]. Considering its anti-inflammatory effect, GPR120 can be expected to augment decidualization *via* its immunomodulatory function. DSCs express high level of chemokines to recruit dNK cells. Moreover, peripheral NK (pNK) cells could be educated by TGF-β and IL-15 of DSC-origin into dNK cell phenotype, which is the dominant DICs in decidua [7]. CXCL12 is the main chemokine that recruits immuno-resistant dNK cells [43]. In the current study, while CXCL12, together with IL-15 and TGF-β, was decreased by LPS, GPR120 activation could restore the levels of CXCL12, IL-15 and TGF-β and simultaneously inhibited inflammatory cytokines TNF-α and IL-1β, thereby reduce pro-inflammaroty reactions induced by LPS in DSCs. Altogether, we demonstrate a positive role of GPR120 in decidualization possibly *via* regulating the immunomodulatory function of DSCs, which needed to be elucidated in future study.

In conclusion, our results suggest that GPR120 plays protective role in the maintenance of pregnancy by promoting decidualization during early pregnancy. Moreover, the augmentation of decidualization by GPR120 was mediated by improved glucose influx and metabolism of HESCs. Thus, these findings may provide new insights into the pathoghesesis and therapy of RSA.

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Declaration of interests

The authors declare that there is no conflict of interests.

Author contributions

Jiefang Huang and Yanyun Zhang designed the research. Jiefang Huang performed the experiments and wrote the paper. Jie Zhang, Meirong Du and Wenfeng Ye assisted with the collection of human samples. Mingxing Xue, Hongshuang Yu and Yuting Gu assisted with experiments. Min Jin and Bing Wan assisted with the data analysis. All authors discussed the results and commented on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.12.019.

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