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ORIGINAL ARTICLE

TGF-β1 Neutralization Improves Pregnancy Outcomes by Restoring Endometrial Receptivity in Mice with Adenomyosis

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Abstract

The objective of this research is to study the effects of TGF-β1 inhibition on endometrial receptivity and pregnancy outcomes in mice with adenomyosis. Experiments were done using a mouse model of adenomyosis which took place in a hospital-affiliated laboratory. The mouse model used for this research is ICR mouse. Adenomyosis was induced by oral gavage of tamoxifen (TAM) from postnatal days (PNDs) 1 to 4 in ICR mice. Bilateral intrauterine injection of anti-TGF-β1-neutralizing antibody or isotype IgG or PBS was performed at PND42. The mice were then either sacrificed or mated at PND64 followed by sacrificing at gestational day (GD) 4 or proceeding to delivery. Implantation numbers, rate of dams with live birth, live birth numbers, survival at 1 week old, and pup mortality rate after weaning were recorded. Collagen was demonstrated by Masson's trichrome and Van Gieson's stains. Uterine expression of a receptivity marker, leukemia inhibitory factor (LIF), was examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blot, and immunohistochemistry (IHC). Anti-TGF-β1 treatment increased the mean implantation numbers, fecundity rate, the rate of dams with live birth, pup survival rate at 1 week old, and pup mortality rate after weaning. Collagen expression in uteri with adenomyosis was attenuated by anti-TGF-β1 treatment. Increased LIF expression by anti-TGF-β1 treatment was detected by qRT-PCR, Western blot, and IHC. The results suggest that inhibition of TGF-β1 improves pregnancy outcomes by restoring endometrial receptivity in mice with adenomyosis.

Keywords Adenomyosis · Tamoxifen · Leukemia inhibitory factor · Endometrial receptivity · Transforming growth factor-β1

Introduction

Adenomyosis is defined as the invasion of endometrial glands and stromal cells deeply into the myometrium and is estrogen-dependent [1]. This disease is manifested by pelvic pain, dysmenorrhea, and menorrhagia and usually results in subfertility. Genetic factors and inflammation play critical roles in the pathogenesis of adenomyosis [2]. Risk factors of adenomyosis include multiparity, early menarche (< 10 years old), short menstrual cycle (< 24 days), and oral contraceptive usage [3]. Although adenomyosis is a hormone-dependent disease; treatment by progestogens or GnRH agonist exhibits limited effect [4]. The ineffectiveness of medical treatments remains enigmatic.

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Adenomyosis is suggested to be associated with fibrosis [5–7]. Slowly progressive epithelial-mesenchymal transition (EMT), fibroblast-to-myofibroblast trans-differentiation (FMT), and smooth muscle metaplasia (SMM) play important roles in the development of fibrosis [5–7] and are observed in murine adenomyosis [5, 8, 9]. Specifically, EMT is characterized by loss of epithelial cell polarity and cell-cell adhesion [10] leading to the gain of migratory and invasive properties by stromal cells [10]. Moreover, EMT is associated with embryogenesis/organ development, tissue regeneration/organ fibrosis, and cancer progression and metastasis [11]. Transforming growth factor-β (TGF-β), Wnt/β-catenin, Notch1/Numb/Slug signaling pathway, and estrogen are all involved in the activation of EMT [11, 12].

Anti-inflammatory TGF-β1 is the most abundant isoform of the TGF-β family molecules secreted by various cells [13]. TGF-β1-Smad2/3-signaling pathway induces fibroblast proliferation, extracellular matrix synthesis, EMT, FMT, and resulting fibrosis [13–15]. High levels of TGF-β1 in uterine lavage from patients with adenomyosis were observed [16]. Hyperstimulating uterus by TGF-β1 results in decreased receptivity in rats [17]. A mouse model provides evidence that TGF-β1-induced EMT and FMT result in fibrosis, suggesting their potential roles in the pathogenesis of adenomyosis [5]. Reduced blood flow caused by the high content of fibrotic tissues in adenomyotic foci plays a potential role in impeding the hormonal therapy of adenomyosis that ultimately leads to hysterectomy [18]. Although decreased endometrial receptivity is observed in patients with intrauterine adhesion accompanied by fibrosis, direct evidence demonstrating the association of uterine fibrosis and endometrial receptivity is lacking [19]. Therefore, TGF-β1 represents a potential therapeutic target for adenomyosis to reduce fibrosis and improve endometrial receptivity.

Blastocyst implantation into a receptive endometrium during the window of implantation (WOI) is critical for the maintenance of gestation [20]. In women with regular 28-day cycles, the WOI occurs around days 21–24 of a menstrual cycle [21], whereas it occurs between days 3 and 4 of gestation in mice [22]. Leukemia inhibitory factor (LIF) is a glycoprotein secreted by diverse cell types. LIF displays pleiotropic functions, including regulating blastocyst growth [21], endometrial decidualization, trophoblast differentiation, and invasiveness as well as blastocyst apposition, adhesion, and attachment to the pinopodes during implantation [23–25]. Endometrial receptivity is pivotal for blastocyst acceptance and implantation when the endometrial epithelium acquires a functional, but transient, ovarian steroid-dependent phenotype. Human blastocyst and endometrium both express LIF receptor (LIFR) and gp130 [24]. Binding of LIF and LIFRβ/gp130 leads to activation of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3), mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 phosphate kinase (PI3K) pathways [26, 27] that induce cell differentiation, survival, and resulting self-renewal [27]. LIF expression is increased from days 18 to 28 during a menstrual cycle with a peak at day 20 in endometrial biopsies [28]. In mice, LIF is specifically expressed in endometrial glands at gestational day (GD) 4 [29]. In women with adenomyosis, LIF was found in both glandular and stromal cells in the basal layer of the endometrium [30]. Moreover, LIF expression is decreased in infertile women during the WOI, compared with fertile controls [31, 32]. Thus, LIF is used as a marker for endometrial receptivity [33, 34].

Successful implantation depends on optimal endometrial receptivity and trophoblast invasion. Defective implantation causes infertility, miscarriage, intrauterine fetal growth restriction, and preeclampsia. Therefore, improvement of endometrial receptivity has become critical in treating patients with adenomyosis who desire to reproduce. This is the first study that investigates whether TGF-β1 neutralization can improve uterine receptivity and pregnancy outcomes in uteri with adenomyosis using a mouse model.

Materials and Methods

Adenomyosis Mouse Model

The animal studies were conducted under E-Da Hospital Institutional Animal Care and Use Committee approval (permit number: IACUC-105024). Pregnant ICR mice (gestational age around 15–16 days) were purchased from BioLASCO Taiwan Co., Ltd. After delivery, each dam and its pups were housed in the same cage under controlled conditions (24 °C, 12:12 light-dark cycle with lights on at 6:00 AM). Female neonates were treated with 1 μg/g body weight of tamoxifen (TAM) suspended in peanut oil/lecithin/condensed milk mixture (2:0.2:3 v/v/v)/day or the same volume of solvent by oral gavage from postnatal days (PNDs) 1 to 4 (Fig. 1) [35]. At PND42, both uterine horns of TAM-treated mice were injected with 10 μg of anti-mouse TGF-β1 antibody (BioLegend, San Diego, CA, USA), isotype IgG (Novus, Centennial, CO, USA), or 1x PBS, whereas the uterine horns from solvent-treated mice were treated with isotype IgG or 1x PBS only. The ideal dose of anti-TGF-β1 was determined by a pilot dose-dependent study. All mice were mated at PND64 and the pregnancy outcomes were examined after delivery (Fig. 1). Masson's trichrome staining and Van Gieson's staining were used to confirm fibrosis formation at PND64. Mice receiving sham surgery were used as a surgical control. Furthermore, the pregnancy outcomes include (1) implantation number (live delivery number at birth + miscarriage counting after sacrificing dams following weaning); (2) fecundity rate; (3) rate of dams with live birth; (4) live delivery number; (5) pup survival number, survival rate at 1 week

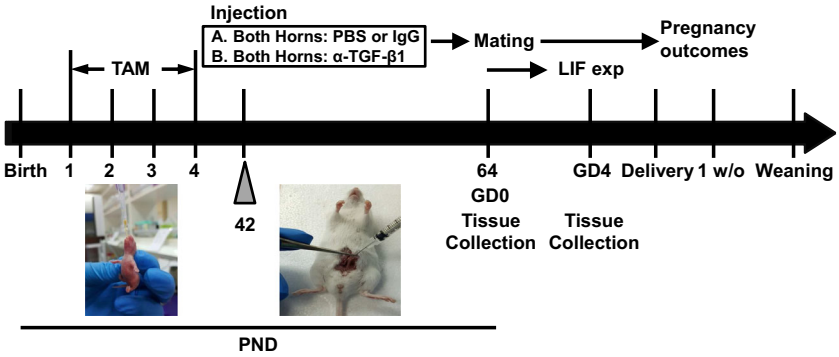

Fig. 1 Experimental design. Female neonates were treated with or without 1 μg/g body weight of TAM from PNDs 1 to 4. At PND42, the mice were injected with 1x PBS or isotype IgG or 10 μg of anti-mouse TGF-β1 antibody into both uterine horns. The mice were mated at PND64. Pregnancy outcomes at delivery, 1 week old (w/o), and after weaning were recorded. In additional groups, the uteri were collected at PND64 and GD4 to examine the formation of adenomyosis and implantation, respectively

old, and mortality rate after weaning. All of the uteri were harvested at GD4 for the evaluation of endometrial receptivity by examining LIF expression (Fig. 1).

H & E Staining

Paraffin-embedded tissue sections (4 μm) were prepared and placed on slides pre-coated with poly-L-lysine followed by deparaffinization and rehydration. Then, the sections were stained with hematoxylin and eosin. Finally, the slides were cleared with xylene for 5 min × 2 before mounting with DPX mounting medium (Electron Microscopy Science, Hatfield, PA, USA) and examined under a BX43 light microscope (Olympus, Tokyo, Japan).

Masson's Trichrome Staining

After deparaffinization and rehydration, tissue sections were incubated in Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) at 56 °C for 15 min. The slides were then stained by Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, and aniline blue solution following the manufacturer's instructions (Sigma-Aldrich). An Olympus BX43 light microscope was used to capture images.

Van Gieson Staining

Mice uterine sections were stained by following the standardized manufacturer's instructions (Abcam, Cambridge, UK). Slides were deparaffinized and rehydrated in distilled water. The slides were incubated with an elastic stain working solution for 15 min. After washing, the slides were treated with a differentiating solution for 1 min and washed for 3 min followed by treating with sodium thiosulfate for 1 min. After washing, the slides were stained with Van Gieson's solution for 3 min and rinsed in 95% ethanol × 2. Finally, the slides were dehydrated with 10% ethanol and mounted with Permount mounting medium (Leica Biosystems, Buffalo Grove, IL, USA). The images were obtained by a BX43 light microscope.

Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the uteri using a total RNA purification plus kit (Sigma-Aldrich). Reverse transcription used SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Specific primer sets for mouse LIF (forward sequence: TCAACTGGCACAGC TCAATGGC; reverse sequence: GGAAGTCTGTCATG TTAGGCGC; product length: 677 bp) and GAPDH (forward sequence: CATCACTGCCACCCAGAAGACTG; reverse sequence: ATGCCAGTGAGCTTCCCGTTCAG; product length: 153 bp) (ThermoFisher Scientific, Waltham, MA, USA) measured mRNA levels using PowerUp SYBR Green Master MIX based detection (Thermo Fisher Scientific) on a StepOneplus™ real-time PCR system (Thermo Fisher Scientific). Relative gene expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method. All samples were assayed in triplicate reactions. Melting curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation.

Western Blot

Mouse uteri were homogenized in 100 μL of RIPA lysis buffer (G-Bioscience, St. Louis, MO, USA) containing a protease inhibitor cocktail (Abcam) (v/v 1:200). The supernatants were collected after centrifugation (Hettich Co., Föhrenstr, Tuttlingen, Germany) at 5000 rpm for 10 min followed by 12,500 rpm for 20 min at 4 °C. Protein concentrations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of whole-tissue lysate was added to the sample-loading buffer (0.25 M Tris-HCl,

pH 6.8, 10% sodium dodecyl sulfate (SDS), 25% β-mercaptoethanol, 50% glycerol, and 0.25% bromophenol blue) and denatured at 100 °C for 10 min. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The membranes were blocked in 5% non-fat milk in PBST containing NaCl, 136.9 mM; KCl, 2.68 mM; $Na_2HPO_4{\cdot}2H_2O$, 6.39 mM; KH_2PO_4, 1.76 mM; and 0.5% Tween 20, pH 7.4 for 1 h at room temperature. Subsequently, the membranes were washed in PBST and incubated with anti-GAPDH (1:10,000 (v/v)) or -LIF (1:100 (v/v)) antibody overnight at 4 °C. The membranes were then washed in PBST for 15 min × 3 and incubated with a secondary antibody (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. After washing with PBST for 15 min × 3, chemiluminescent detection was performed using the chemiluminescent HRP substrate (Millipore-Sigma). Autoradiography was done by a BioSpectrum™ 500 Imaging System (UVP, Upland, CA, USA). Densitometry generated by ImageJ v1.46 DIA software (IHC Image Analysis Toolbox, National Institutes of Health, WA, USA; https://imagej.nih.gov/ij/index.html) was used to semi-quantify the protein expression. The protein abundance was normalized to each corresponding GAPDH expression.

Immunohistochemistry

The rehydrated paraffin-embedded sections were immersed in 3% H_2O_2 in 100% methanol for 10 min followed by a 3-min wash with 1x PBS × 3. Tissues were blocked with 0.5% bovine serum albumin (BSA) for 30 min and then incubated with rat monoclonal anti-mouse LIF antibody (1:100 (v/v), Abcam) for 2 h at room temperature. After washing, the slides were treated with an HRP-conjugated secondary antibody for 40 min at room temperature. Replacement of primary antibody by isotype IgG was used as a negative control. The immunoreactivity was displayed by 3,3′-diaminobenzidine (DAB) chromogen (Leica Biosystems). Sections were lightly counter-stained with hematoxylin for 20s, then, dehydrated in a gradient of alcohol and xylene, and finally mounted with Permount mounting medium (Leica Biosystems). The slides were examined using an Olympus BX43 light microscope.

Statistics

The quantification of Masson's trichrome staining, Van Gieson's staining, and IHC results was performed using ImageJ v1.46 DIA software.

The Kolmogorov-Smirnov one-sample test first examined the variance and normality of results. The statistical significance of results with equal variance was then examined by Student's *t* test. Results with an unequal variance that passed or failed normality testing were evaluated by Student's *t* test assuming unequal variance or the Mann-Whitney rank-sum test, respectively. $P < 0.05$ was considered statistically significant.

All statistical differences were analyzed using SigmaPlot software 11.0 (Systat Software, San Jose, CA, USA).

Results

Collagen Expression Was Reduced by Anti-TGF-β1 Treatment

The collagen fibers reflecting the levels of fibrosis were detected by Masson's trichrome stain (Fig. 2a) and Van Gieson's stain at PND64 (Fig. 3a). In Masson's trichrome staining, myometrial collagen expression was increased in the TAM-treated mice receiving sham surgery (7.78 ± 0.59), PBS (8.82 ± 0.71), or IgG (9.07 ± 0.86), compared with the control group (sham: 0.90 ± 0.08; PBS: 0.88 ± 0.14; IgG: 0.81 ± 0.18). Compared with PBS- (8.82 ± 0.71) or IgG-treated (9.07 ± 0.86) mice, anti-TGF-β1 reduced collagen expression (4.29 ± 0.56) in mice treated with TAM (Fig. 2b). Consistently, in Van Gieson's staining, TAM treatment enhanced myometrial collagen expression in mice receiving sham surgery (6.66 ± 0.52), PBS (6.20 ± 0.78), or IgG (7.25 ± 0.65), compared with the control groups (sham: 1.26 ± 0.06; PBS: 1.30 ± 0.21; IgG: 1.37 ± 0.34). In TAM-treated mice, anti-TGF-β1 suppressed collagen expression (2.60 ± 0.28), compared with the PBS-treated mice (6.20 ± 0.78) or IgG-treated mice (7.25 ± 0.65) (Fig. 3b).

Pregnancy Outcomes Were Improved by Anti-TGF-β1 Treatment in Adenomyotic Mice

Compared with control, TAM significantly decreased implantation numbers in mice receiving either sham surgery (12.9 ± 0.81 vs. 3.80 ± 0.57) or IgG (11.5 ± 0.43 vs. 4.00 ± 0.58) or PBS (11.7 ± 1.36 vs. 3.30 ± 0.88) treatment. Implantation numbers in TAM-treated mice receiving either IgG (4.00 ± 0.58) or PBS treatment (3.33 ± 0.88) were significantly fewer than those of the anti-TGF-β1-treated group (6.00 ± 0.44) (Fig. 4a). Mice fed with solvent showed a 100% fecundity rate in both sham surgery and PBS-treated groups. Fecundity rates in mice receiving either sham surgery or IgG or PBS treatment were significantly decreased by TAM treatment (sham surgery: 100 ± 0.00% vs. 38.43 ± 3.24%; IgG: 100 ± 0.00% vs. 50.0 ± 0.00%; PBS: 100 ± 0.00% vs. 50.0 ± 0.00%). In the TAM-treated mice, anti-TGF-β1 treatment (77.8 ± 11.11%) resulted in increased fecundity rate, compared with either the IgG- or PBS-treated group (both were 50.0 ± 0.00%) (Fig. 4b). Compared with control dams receiving sham surgery or IgG or PBS treatment (all were 100.0 ± 0.00%), TAM treatment led to a decrease in the rate of dams with live birth (sham: 22.69 ± 6.02%; IgG: 50.00 ± 0.00%;

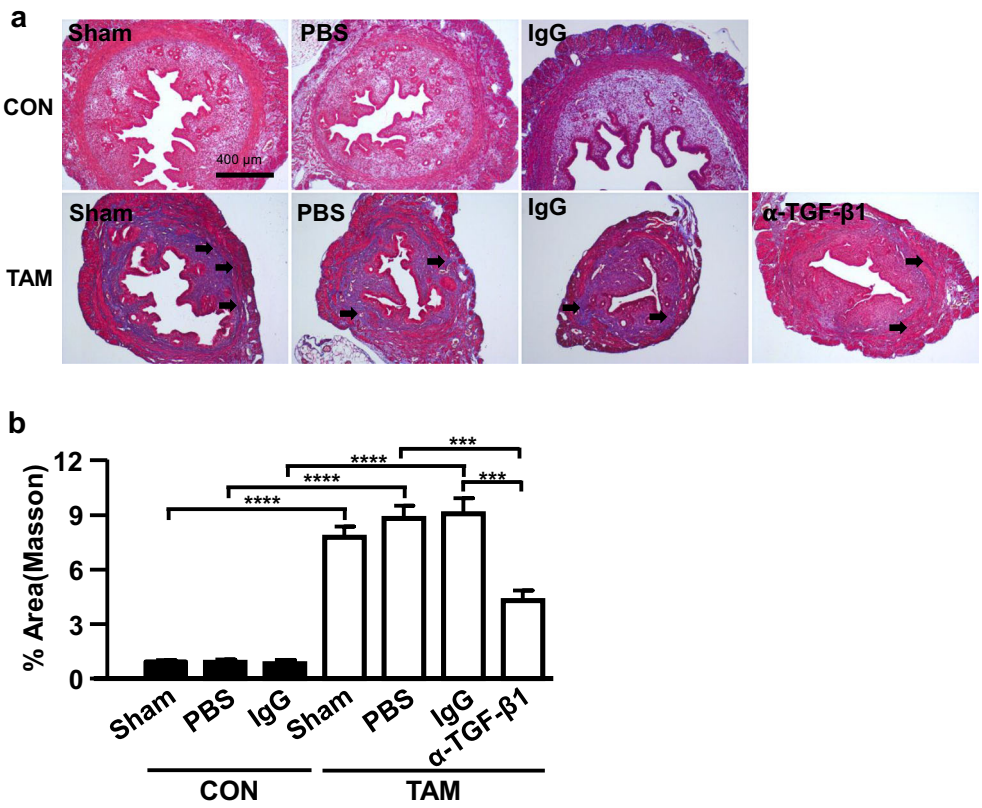

Fig. 2 Fibrosis formation in myometrium was reduced by anti-TGF-β1. Fibrosis formation in the uterus was detected by (**a**) Masson's trichrome stain: blue: collagen. (**b**) The expression of collagen fibers in the myometrium (arrows) was increased in TAM-treated mice and was decreased by anti-TGF-β1. The data were reported as mean ± SEM. $n = 4$; ***$p < 0.001$, ****$p < 0.0001$. Scale bar: 400 μm. Magnification: × 100

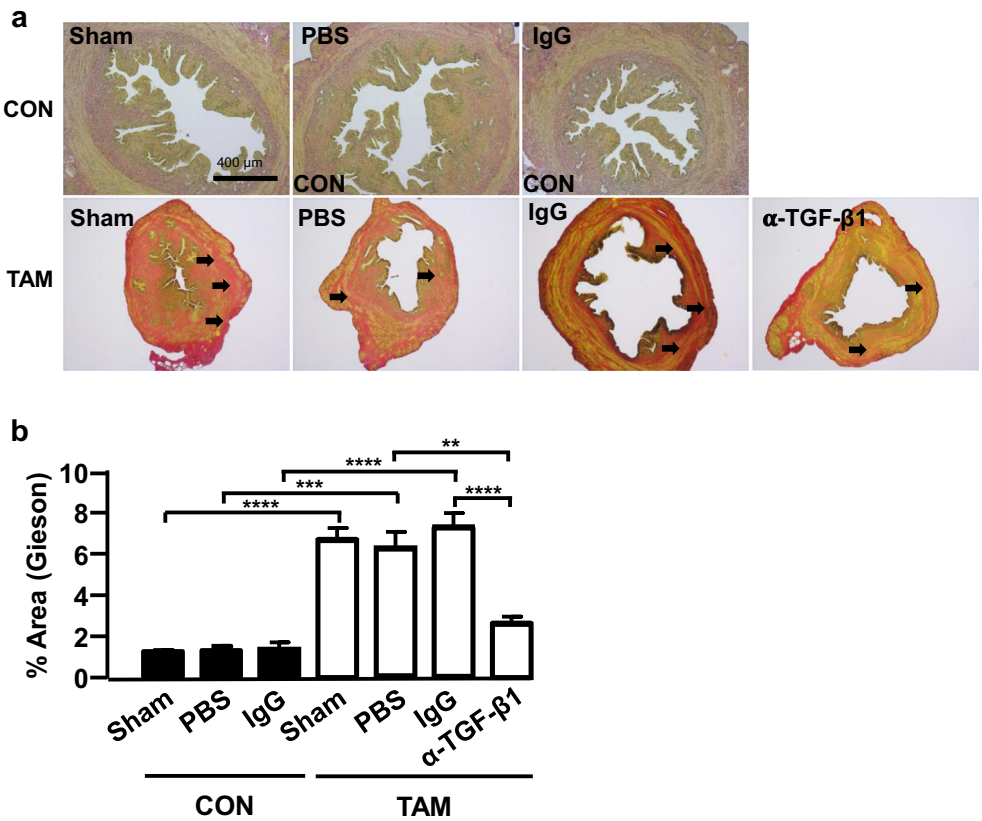

Fig. 3 Anti-TGF-β1 attenuated myometrial fibrosis. Fibrosis formation in the uterus was detected by (**a**) Van Gieson's stain: red: collagen; yellow: myometrium. (**b**) The expression of collagen fibers in the myometrium (arrows) was increased in TAM-treated mice and was decreased by anti-TGF-β1. The data were reported as mean ± SEM. $n = 4$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Scale bar: 400 μm. Magnification: × 100

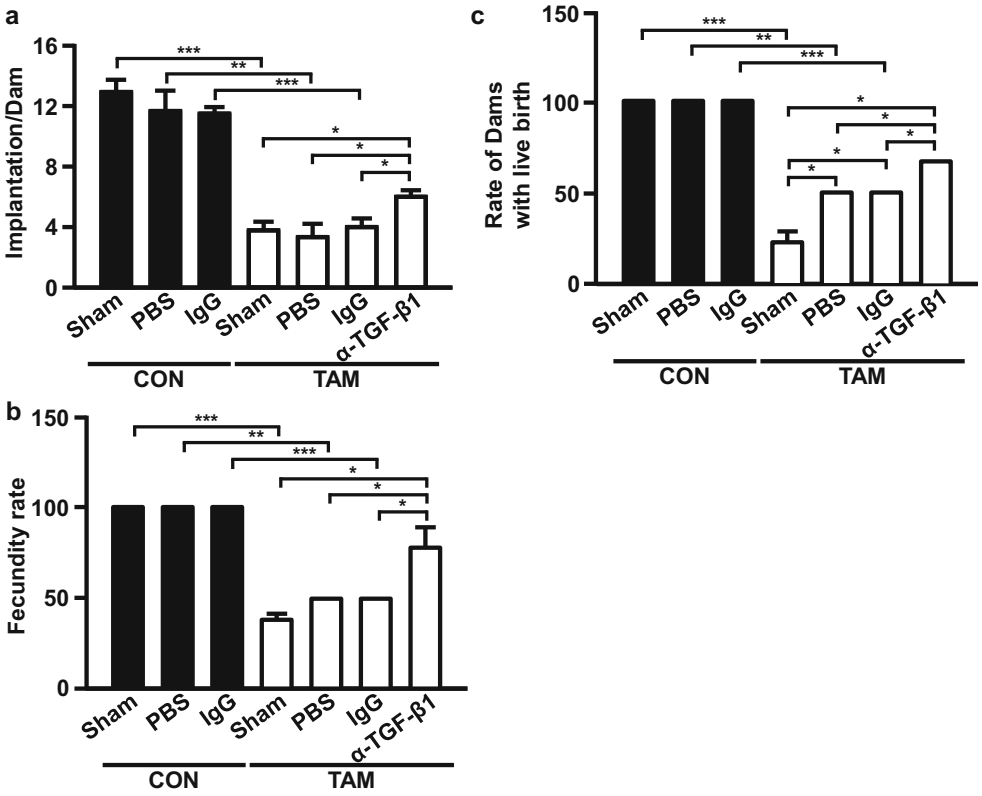

Fig. 4 Pregnancy outcomes were improved by anti-TGF-β1. Pregnancy outcomes were recorded in mice with TAM-induced adenomyosis in the presence or absence of anti-TGF-β1. Anti-TGF-β1 treatment improved (**a**) implantation number in each dam, (**b**) fecundity rate, and (**c**) rate of the dam with live birth. The data were reported as mean ± SEM. $n = 6–23$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

PBS: 50.00 ± 0.00%), which was elevated by anti-TGF-β1 treatment to 66.7 ± 0.00%. Moreover, PBS (50.00 ± 0.00%) or IgG (50.00 ± 0.00%) also improved the rate of dams with live birth in TAM-treated mice, compared with those receiving sham surgery (22.69 ± 6.02%) (Fig. 4c).

Live birth numbers per dam in TAM-treated mice with sham surgery (2.44 ± 0.82) or PBS (3.00 ± 0.58) or IgG (3.33 ± 0.67) treatment were significantly decreased, compared with controls receiving either sham surgery (12.93 ± 0.81) or PBS (11.67 ± 1.36) or IgG (11.50 ± 0.43) treatment. However, the live birth numbers were increased after anti-TGF-β1 treatment (5.67 ± 0.42) (Fig. 5a). Pup survival numbers and rates at 1 week old in TAM-treated mice receiving either PBS (number: 1.00 ± 0.58; rate: 33.33 ± 16.67%) or IgG (number: 1.00 ± 0.58; rate: 25.00 ± 14.33%) treatment were significantly lower than those of the control with either PBS (number: 11.17 ± 1.25; rate: 96.06 ± 2.50%) or IgG (number: 11.33 ± 0.56; rate: 98.33 ± 1.67%) treatment. After anti-TGF-β1 treatment, both pup survival numbers (4.50 ± 0.56) and rates (66.84 ± 13.43%) were significantly increased (Fig. 5b and c). Consistently, the pup mortality rate after weaning in TAM-treated mice receiving either PBS (66.67 ± 16.67%) or IgG (75.00 ± 14.43%) treatment was significantly higher than that of controls receiving either PBS (3.94 ± 2.50%) or IgG (1.67 ± 1.67%) treatment. Anti-TGF-β1 treatment significantly decreased the pup mortality rate to 21.55 ± 5.87% (Fig. 5d).

Endometrial Receptivity Marker Expression Was Improved by Anti-TGF-β1

Compared with controls (0.94 ± 0.02), TAM treatment led to lower uterine LIF mRNA expression (0.18 ± 0.03), whereas anti-TGF-β1-treated uteri exhibited higher levels of LIF expression (0.55 ± 0.03) (Fig. 6a). Consistently, the Western blotting analysis demonstrated that uterine LIF protein expression in TAM-treated mice was suppressed (0.50 ± 0.02), compared with controls (1.15 ± 0.12). After anti-TGF-β1 treatment, LIF levels were increased to 0.87 ± 0.09 (Fig. 6b and c). Similarly, immunostaining showed that LIF immunoreactivity in uteri from TAM-treated mice (Fig. 7a and b) was 0.63-fold of that in control mice (Fig. 7c). Compared with PBS and IgG treatment in TAM-treated mice, anti-TGF-β1 treatment correspondingly increased LIF immunoreactivity (Fig. 7a and b) by 1.36 ± 0.10-fold (Fig. 7d) and 1.56 ± 0.14-fold (Fig. 7e). LIF expression was primarily observed in the endometrium.

Discussion

Adenomyosis is a benign gynecologic disease with limited knowledge about its pathogenesis. An increasing body of evidence shows that adenomyosis causes subfertility by impeding implantation [36, 37]. Implantation failure is thought to be associated with altered uterine peristalsis [38], defective

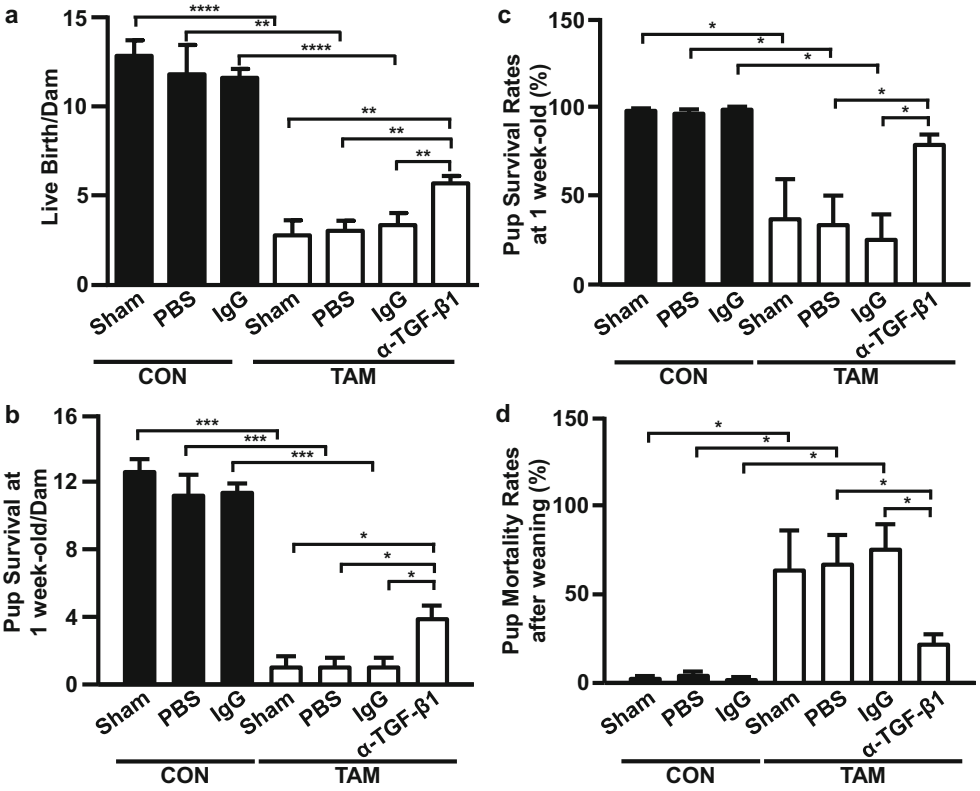

Fig. 5 Postnatal survival was enhanced by anti-TGF-β1. Postnatal survival was recorded in mice with TAM-induced adenomyosis in the presence or absence of anti-TGF-β1. Anti-TGF-β1 treatment increased (**a**) live birth number per dam, (**b**) pup survival number at 1 week old, (**c**) pup survival rate at 1 week old, and decreased (**d**) pup mortality rate after weaning. The data were reported as mean ± SEM. $n = 6–23$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

decidualization [39], and decreased endometrial receptivity [40]. Dysregulation of LIF, HOXA10, and integrins was shown to play important roles in impaired endometrial receptivity in adenomyosis [41–43]. Several studies demonstrated the negative effects of adenomyosis on reproduction, exemplified by reducing pregnancy and live birth rates as well as increasing miscarriage rates that perturb in vitro fertilization outcomes [44]. The obstetric complications associated with adenomyosis remain unclear. Several epidemiological studies showed an increased risk of preterm birth and preterm premature rupture of membranes in pregnancy complicated with adenomyosis [45, 46]. Adenomyosis was also implicated in impaired

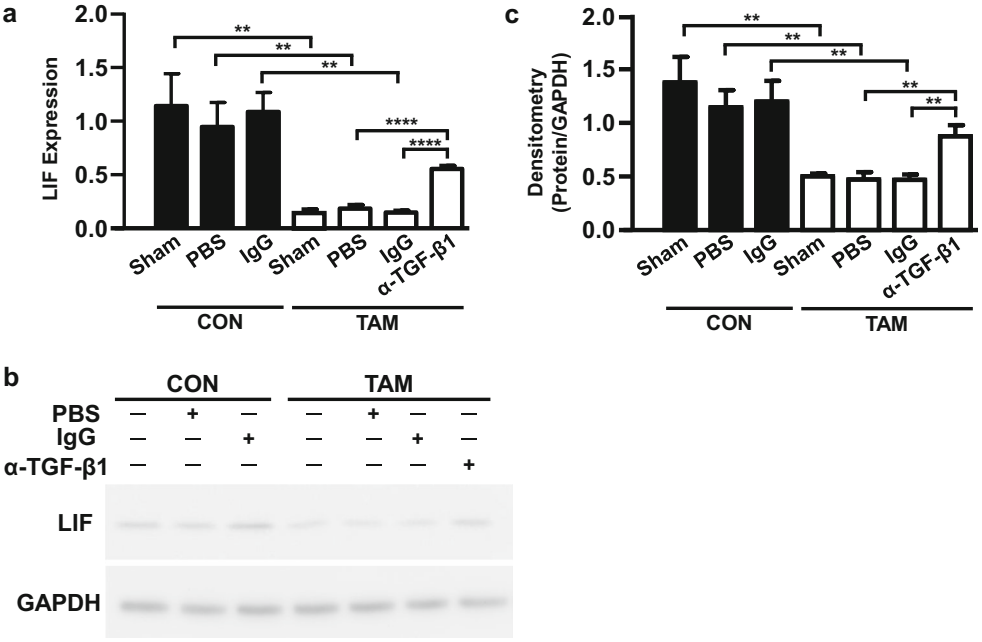

Fig. 6 Uterine LIF expression in mice with adenomyosis was augmented by anti-TGF-β1. Uterine expression of LIF at GD4 from pregnant mice with TAM-induced adenomyosis with or without anti-TGF-β1 treatment was examined. Anti-TGF-β1 treatment increased (**a**) LIF mRNA expression by qRT-PCR and (**b**) LIF protein expression by Western blotting analysis. (**c**) Densitometry was used for semi-quantification of the results from Western blots. The data were reported as mean ± SEM. $n = 4–6$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar: 100 μm. Magnification: × 400

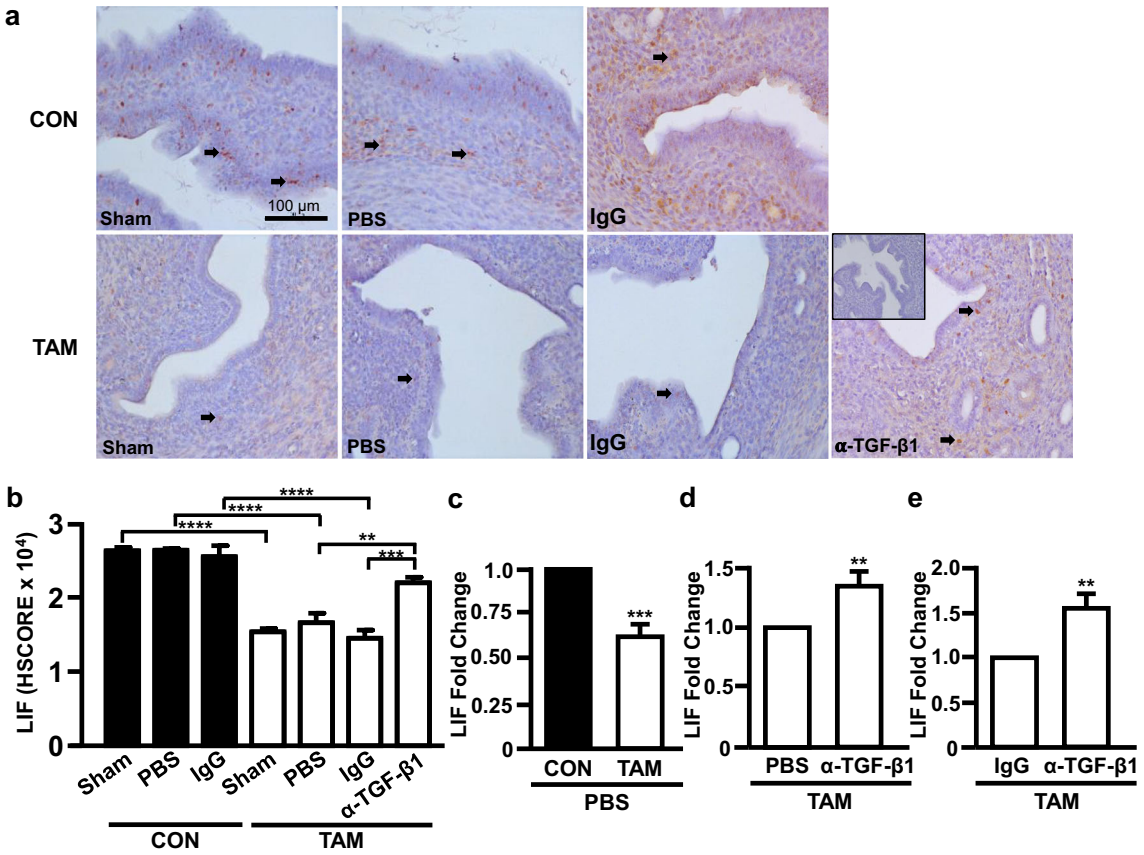

Fig. 7 The enhancement of uterine LIF expression by anti-TGF-β1 in mice with adenomyosis. (**a**) IHC showed LIF (arrows) was mainly expressed in the endometrium and its immunoreactivity was enhanced by anti-TGF-β1 treatment. Inset: isotype IgG staining. (**b**) HSCORE of LIF expression. The immunoreactivity of LIF was semi-quantitatively evaluated using the following intensity categories: 0, no staining; +, weak but detectable staining; ++, moderate or distinct staining; and +++, intense staining. A histological score (HSCORE) was calculated using the formula HSCORE = $\sum(P_i \times i)$, where i represents the intensity scores, and P_i is the corresponding percentage of the cells. Five fields/slide were evaluated by 2 investigators blinded to the tissue source. Fold changes of LIF expression (**c**) between mouse uteri with or without TAM-induced adenomyosis and (**d** & **e**) between mouse uteri with TAM-induced adenomyosis in the presence or absence of anti-TGF-β1 treatment were calculated. The data were reported as mean ± SEM. $n = 4–6$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar: 100 μm. Magnification: × 400

decidualization and placentation that result in placental insufficiency and compromise embryo development [47].

TGF-β1-induced EMT plays a critical role in the development of adenomyosis and other fibrotic diseases [48–50]. EMT, FMT, and SMM were shown to result in fibrosis [7, 51]. In other animal studies and human trials, anti-TGF-β1 was tested in treating various fibrotic diseases, such as renal, pulmonary, and cardiac fibrosis [52, 53]. Although the GnRH agonist is currently used as a major treatment to improve endometrial receptivity in patients with adenomyosis [41], such limitations as treatment duration, side effects, and its systemic administration, restrict its use. Furthermore, the effects of cytoreductive surgery remain controversial [36, 54]. Thus, an effective long-lasting therapy without systemic effects is required to improve endometrial receptivity in patients with adenomyosis. In the current study, anti-TGF-β1 reduced uterine collagen expression, suggesting its role in the inhibition of fibrosis in uteri with adenomyosis.

Implantation numbers, fecundity rate, rate of dams with live birth, live birth numbers, neonatal survival at 1 week old, and mortality rate after weaning were all significantly improved by intrauterine injection of anti-TGF-β1. Intrauterine development of fetuses and the well-being of neonates are highly dependent upon adequate implantation and resulting placentation. Impaired implantation has been documented to lead to abnormal prenatal and postnatal fetal development [55, 56]. In this study, the fecundity rate and implantation numbers were used to evaluate the sustainability of implantation. The rate of dams with live birth, live birth numbers, survival of 1-week-old pups, and mortality rate after weaning were used to evaluate the well-being of postnatal development, which had been programmed in utero. All of these endpoints were improved by anti-TGF-β1 in mice with adenomyosis. Although indirect, these observations indicate that anti-TGF-β1 improves implantation and thereafter the pregnancy outcomes in mice with adenomyosis. Unexpectedly, the rate of dams with live birth in TAM-treated mice was also improved

by PBS or IgG treatment. Since tubal flushing was shown to improve the rate of women with live birth, the injection of PBS or IgG may mimic the tubal flushing which resulted in an increased rate of the dam with live birth [57].

To identify the potential mechanism(s) responsible for the improvement of pregnancy outcomes, the uterine expression of LIF, a molecular marker of endometrial receptivity during the WOI [24], was assessed to determine the changes in endometrial receptivity. Endometrial LIF expression is increased in the WOI and however is reduced in patients with either unexplained infertility or adenomyosis [58]. The concentration of LIF in uterine lavage is significantly reduced in adenomyotic patients, compared with normal women [43]. Since the WOI in mice occurs around GD4 [59], this study collected uterine tissue at GD4 for the evaluation of endometrial receptivity. LIF mRNA and protein expression was significantly increased after anti-TGF-β1 treatment in mouse uteri with adenomyosis. IHC revealed endometrial LIF expression was elevated by anti-TGF-β1 treatment, indicating the therapeutic effect of anti-TGF-β1 on endometrial receptivity of uteri with adenomyosis. Although whole uterine tissue was used for mRNA and protein assays, based on the current IHC findings showing primary LIF expression in the endometrium, consistent results are expected if endometrium alone was used for these assays. Adenomyosis in mouse uteri was induced by TAM treatment in the current study. Clinically, adenomyosis is observed in patients taking TAM [60]. However, the mechanisms responsible for adenomyosis in the absence of predisposing factors remain unclear. Thus, further clinical studies are required to effectively translate the results of this study demonstrating the effects of anti-TGF-β1 on the compromised endometrial receptivity and pregnancy outcomes to clinical practice.

In a study by Guo et al. [41], administering the GnRH agonist by intraperitoneal injection at PND79 increased average litter size in TAM-treated mice. Nevertheless, no significant difference was found in the rate of dams with delivery. By comparison, the current study treated the mice with a single dose of anti-TGF-β1 by local injection into both uterine horns at PND42. The fecundity rate and the rate of dams with live delivery in TAM-treated were improved by anti-TGF-β1 treatment, suggesting the effectiveness of TGF-β1 neutralization in improving pregnancy outcomes in mice with adenomyosis.

In summary, the current study demonstrates that inhibition of TGF-β1 reduces fibrotic changes in the mouse uteri with adenomyosis. Anti-TGF-β1 treatment improves pregnancy outcomes potentially by restoring endometrial receptivity. Although the relationship between endometrial receptivity and uterine fibrosis remains unclear, our results reveal that the progression of fibrosis, pregnancy outcomes, and endometrial receptivity are all improved after anti-TGF-β1 treatment. Taken together, the current results provide a new insight in establishing a novel strategy for the treatment of adenomyosis.

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Compliance with Ethical Standards

The animal studies were conducted under the E-Da Hospital Institutional Animal Care and Use Committee approval (permit number: IACUC-105024).

Conflict of Interest The authors declare that they have no conflict of interest.

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