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Baicalin promotes embryo adhesion and implantation by upregulating fucosyltransferase IV (FUT4) via Wnt/beta-catenin signaling pathway

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ABSTRACT

Glycosylation plays a significant role in determining the receptivity of the uterine endometrium to embryo. Fucosyltransferase IV (FUT4) is expressed stage-specifically in the uterine endometrium of mammalians, and considered as a marker of the endometrial receptivity. Baicalin, a monomer of flavonoids, is known to have functions in improving reproduction. However, the mechanism by which baicalin regulates the expression of FUT4 in embryo-endometrium adhesion remains unclear. Our results showed that baicalin significantly increased FUT4 mRNA and protein expression levels both in human endometrial cells and mouse endometrial tissue, and consistently elevated embryo adhesion rate during implantation in vitro and embryonic implantation competence in pregnant mouse. This study suggests that baicalin facilitates endometrial reproduction via elevating FUT4 expression through Wnt/β-catenin signaling pathway.

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1. Introduction

Embryo implantation is crucial for a successful pregnancy. In this process, the mature blastocyst locates, adheres and embeds into the receptive uterine endometrium [1–3]. Embryo implantation occurs during a limited period named implantation window [4,5]. In this critical stage, numerous factors can regulate the molecular alterations in the endometrial cells, including hormones, growth factors, cytokines, et al. [6,7]. For example, progesterone plays an important role in the development of endometrial receptivity through the regulation of the uterus epithelial compartments [8]. Apart from the pathological alterations of the endometrial cavity, hydrosalpinx and embryonic abnormalities, female infertility mainly derived from the endometrial malfunction showing recently a high incidence [9–11]. Thus, exploring drugs which might improve endometrial condition could be a significant approach for the enhancement of embryo implantation rate.

The Chinese herbal medicine *Scutellaria baicalensis* (Huang-Qin in China) has multitudinous functions. The flavonoids extracted from *S. baicalensis* are exceptionally effective for anti-allergy, anti-inflammation and anti-tumor effects [12,13]. It has been used as an adjunctive reproduction agent for the improvement and regulation of menstrual cycle, infertility and abortion, as well as for the treatment of restless fetus in pregnant women [14–16]. However, the mechanism of *S. baicalensis* in the field of reproduction is largely unknown. In the female reproduction processes, Wnt, PI3K, MAPK signaling pathways have been found to be correlated with the functional states of uterine endometrium [17,18]. For instance, Wnt/β-catenin signaling pathway is involved in embryonic implantation and endometrial proliferation,

Abbreviations: FUTs, fucosyltransferases; Fuc, fucose; LeY, Lewis Y; DKK1, dickkopf 1; PMSG, pregnant mare serum gonadotropin; hCG, human chorionic gonadotropin; GD1, gestation day 1; NS, normal saline; SBD, *Scutellaria baicalensis* decoction; p-GSK3β, phosphorylated glycogen synthase kinase 3β; SP1 (5), specificity protein-1 (5); CMFDA, 5-chloromethylfluorescein diacetate; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; DAPI, 2-(4-amidinophenyl)-6-indolecarbamidine

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differentiation and decidualization [19,20]. Whether the role of *S. baicalensis* in the development of endometrial receptivity during implantation is mediated by Wnt/β-catenin signaling pathway, needs to be explored.

Glycosylation is a critical post-translational modification of the proteins, which participates in many physiological and pathological events, such as development, reproduction, cancer and immune diseases, et al. [21–23]. Fucosylation, an important type of glycosylation, is regulated by specific fucosyltransferases (FUTs) which catalyze the transfer of fucose (Fuc) residues from the donor substrate, GDP-Fuc, to the oligosaccharide acceptors in α 1–2, α 1–3/4 and α 1–6 linkage [24,25]. FUTs promote the synthesis of different fucosylated oligosaccharide chains of glycoconjugates that ultimately affect the adhesion and migration of the cells [26,27]. Evidences have shown that FUTs are stage-specifically expressed in mammalian reproduction processes [28,29]. In mice, the expression of FUT1, FUT4, FUT7 and FUT9 in the uterine endometrial cells reaches the peak on the day of implantation, and facilitates the establishment and maintenance of uterine receptivity [30,31]. FUT4, a member of α l-3 FUTs, is the key enzyme for the synthesis of Lewis Y (LeY) oligosaccharide antigen, and is significantly upregulated during the early and mid-secretory phase of human endometrium in menstrual cycle [32]. We have found that FUT4 could regulate embryo adhesion by regulating the synthesis of LeY on endometrial epithelial surface. Also, FUT4 over expression promoted the recognition and adhesion between uterine endometrial cells and embryonic cells [33]. The above data suggest that FUT4 expression level is closely related to the endometrial receptivity, and can be used as a vital marker for endometrial function evaluation.

To elucidate the reproductive functions of *S. baicalensis*, we explored the regulatory effects of baicalin which is a monomer of flavonoids, and *S. baicalensis* decoction (SBD) on FUT4 expression in human endometrial RL95-2 cells, as well as in murine endometrium during implantation window. We found that both baicalin and SBD could significantly upregulate the gene and protein expression of FUT4, and promote embryo implantation in vitro and in pregnant mouse.

2. Materials and methods

2.1. Cell culture

We purchased human endometrial RL95-2 and human embryonic JAR cells from the American Type Culture Collection (ATCC; Manassas, VA). RL95-2 cells were grown in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 medium (DMEM/F12; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 5 μg/ml insulin (Sigma, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime, China). JAR cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Both types of cells were maintained at 37 °C under 5% CO_2 in cell incubator. The growth medium was changed every 2–3 days.

2.2. Drug treatments

Baicalin (Keluoma, China) was dissolved in dimethylsulfoxide (DMSO; Sigma, USA) to a concentration of 6 mg/ml and stored at −20 °C. During the treatment, concentrations of 3 μg/ml and 6 μg/ml were used. Dickkopf 1 (DKK1; Peprotech, USA), an inhibitor of the Wnt signaling pathway, was dissolved in autoclaved distilled water to a final concentration of 0.05 mg/ml, and utilized at a dose of 100 ng/ml for 48 h. Cells were treated with DKK1 1 h before baicalin treatment.

2.3. Extraction of S. baicalensis decoction (SBD)

The herb *S. baicalensis* (Qiyunsheng, China) was authenticated by Professor Ming Gao from the department of traditional Chinese medicine in Dalian University. According to the extraction and preparation standard for Chinese traditional herb pharmacopoeia, the herb (10 g) was minced and filtered through No. 4 drug sieve (250 μm ± 9.9 μm). The big residue part was dried in the oven at 50–60 °C and minced again for sieving. This step was repeated, until the entire herb passed through the drug sieve. The herb powder was soaked in distilled water (100 ml) for 30 min, and then boiled with little bubbling at 100 °C for 1 h. After filtration of the supernatant by three-layer gauze, the herb residue was boiled again for 40 min in 100 ml of distilled water. After final filtration, the two parts of the supernatant were combined and simmered to the volume of 10 ml (1 g/ml).

2.4. Animal treatments and tissue collection

Mice of Kunming species (6–8 weeks) were from the Animal Center Laboratory of Dalian Medical University, China. All experimental procedures involved in the mouse studies were approved by the Institutional Review Board in Dalian Medical University. Mice were maintained under controlled environmental conditions (14L:10D, 22–25 °C, humidity 60%). Before mating, females were injected with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG; Chifengboen, China) to increase the number of ovulation. Pregnancy was obtained by housing two virgin females with one male. Gestation day 1 (GD 1) was defined as the day of finding a vaginal plug. Females were randomly divided into four groups: control, normal saline (NS), baicalin and *S. baicalensis* decoction (SBD); and each group had 12 mice. Mice in baicalin group were gavaged with baicalin, which was dissolved to a concentration of 7.53 mg/ml with sterile phosphate-buffered saline (PBS) containing 7.5% DMSO, at a dose of 100 mg/kg (0.4 ml per mice). Mice in SBD group were gavaged with 0.3 ml herbal decoction. Animals in NS group were given 0.4 ml PBS containing 7.5% DMSO. Mice were gavaged 7 days before GD 1, and until the day before specimen collection.

At GD 4 (8:30 AM), half of the pregnant mice in each group ($n = 5$) were sacrificed by cervical dislocation. One side of the uterine horns were fixed in 4% (v/v) paraformaldehyde, and processed for immunohistochemical analysis. The other sides were carefully cleaned fat, and removed the fetuses by washing with PBS. Then, the endometrial tissues were scraped off slightly on a pre-cooled ground glass with surgical blade at 4 °C, and kept in liquid nitrogen for RT-PCR and Western blot. At GD 8, the left half of mice was sacrificed and the number of implanted embryos was calculated.

2.5. Transient transfection

When RL95-2 cells reached 70% confluence in 6-well plates, sh-SP5 (Gene Pharma, China), SP1 cDNA (Trans Gen, China), the co-transfection, si-FUT4 (constructed by Xuesong Yang in our lab) and si-β-catenin (Gene Pharma, China) were transiently transfected into the cells using Lipofectamine 2000™ reagent (Life Technologies, USA) following the manufacturer's instructions. Sh-control, vector and mock were used as controls for sh-SP5, si-β-catenin and SP1 cDNA transfected cells, respectively. Cells were harvested 48 h or 72 h post-transfection.

2.6. RT-PCR

Total RNA was extracted from mouse endometrial tissues and RL95-2 cells using Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. The cDNA was synthesized

using an RNA PCR Kit (AMV), version 3.0 (Takara, Japan). The forward (F) and reverse (R) primer sequences used for the RT-PCR are shown below:

Primer	Size (bp)	Primer sequences (5′-3′)
Human FUT4	456	F:5′-cggacgtctttgtgccttat-3′ R:5′-cgaggaaaagcaggtacgag-3′
Human β-actin	838	F:5′-atctggcaccacaccttctacaatgagctgcg-3′ R:5′-cgtcatactcctgcttgctgatccacatctgc-3′
Mice FUT4	511	F:5′-ttgcagcctgcgcttcaacatcag-3′ R:5′-actcagctggtggtagtaacggac-3′
Mice SP5	295	F:5′-accgggacactttcgaggccactcc-3′ R:5′-cagcagcgactcccacaagcaaggc-3′
Mice β-actin	792	F:5′-gatatcgctgcgctggtcgtcgac-3′ R:5′-caagaaggaaggctggaaaagagc-3′

The PCR reactions were carried out as follows: initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C (mice FUT4) or 57 °C (human FUT4, mice SP5) for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min. The amplified products were analyzed by 1% agarose gel electrophoresis stained with ethidium bromide and the bands were visualized under U.V transilluminator.

2.7. Western blot

Total and nuclear proteins were extracted from mouse endometrial tissues and RL95-2 cells using KeyGEN mammalian cell lysis reagent and nuclear and cytoplasmic reagent, respectively. Blots obtained after transfer were incubated with specific antibodies for phosphorylated glycogen synthase kinase 3β (p-GSK3β), GSK3β, β-catenin (1:500, Affinity Biosciences, USA), human FUT4 (1:1000, Proteintech, USA), mouse FUT4 (1:1000, Abgent, USA), SP5 (1:500, Abcam, USA) and β-actin (1:5000, Proteintech, USA).

2.8. Immunofluorescence staining

Cells were grown on the cover-slips and fixed in 4% paraformaldehyde for 20 min. After blocking with 1% goat serum (Beyotime, China) for 2 h, cells were incubated with rabbit anti-FUT4 antibody (1:200) at 4 °C overnight. The following day, slides were washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200, Sigma, USA) for 30 min. Then slides were incubated with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI; Beyotime, China) for 10 min. After rinsing, slides were mounted in anti-fade solution (Beyotime, China) and subsequently monitored under an Olympus BX51 immunofluorescence microscope (Osaka, Japan).

2.9. Cell adhesion assay

RL95-2 cells of different treatments were grown on 96-well plates to form a confluent monolayer. All groups were replicated 3 times. Embryonic JAR cells were stained with Cell Tracker™ Green CMFDA (Life Technologies, USA) about 1 h before adhesion assay. After trypsinization, JAR cells (10^4) were gently seeded onto RL95-2 cell monolayer and cultured with JAR cells medium. After 1 h, unattached embryonic cells were removed by washing with PBS. An equal amount of pre-stained JAR cells (10^4) were added into 3 blank holes. After detected by multimode plate reader (PerkinElmer, USA), adhesion rate was calculated as percentage of attached JAR cells (e/b). e, average fluorescence intensity value in each experimental group; b, average fluorescence intensity value in the blank holes. Cell pictures were taken under a fluorescent phase microscope (Olympus, Japan).

2.10. Immunohistochemistry

Uterus serial histological sections (4 μm thick) were deparaffinized in xylene, and rehydrated in descending concentrations of ethanol, followed by antigen retrieval and cooling to room temperature. The following experiments were performed using Histostain-Plus Kits (ZSGB-BIO, China), and chromogenic reaction was performed with diaminobenzidine (ZSGB-BIO, China) according to the manufacturer's protocol. The primary antibodies used in this assay were rabbit monoclonal anti-FUT4 and anti-SP5 antibodies both at a 1:100 dilution. After counterstained and mounted, the images were captured by Olympus microscope (Osaka, Japan).

2.11. Statistical analysis

All data were expressed as means ± standard deviation (S.D.). Differences between groups were analyzed by one-way analysis of variance using the SPSS statistical software 13.0. In all tests, $^{*}P \leqslant 0.05$ was considered as statistical significant. Each experiment was repeated independently at least three times.

3. Results

3.1. Baicalin upregulates the expression of FUT4 in human endometrial cells

To assess the effect of baicalin on the expression of FUT4 in endometrial cells, RL95-2 cells were treated with different concentrations of baicalin (3 μg/ml and 6 μg/ml). RT-PCR and Western blot demonstrated that baicalin at a concentration about 3 μg/ml ($P < 0.05$) and 6 μg/ml ($P < 0.01$) could significantly enhance FUT4 mRNA and protein expression levels compared with the control and DMSO groups (Fig. 1B–E). Simultaneously, our immunofluorescent staining assay showed the same observations as above (Fig. 1F). These results indicate that baicalin promotes FUT4 expression in human endometrial cells.

3.2. Baicalin activates Wnt/β-catenin/SP5 signaling pathway

We have studied that short time fed of baicalin activates Wnt/β-catenin signaling pathway in vitro, which is crucial during the process of embryo development and implantation [19,34]. We have examined the levels of key molecules involved in Wnt signaling pathway, including p-GSK3β, GSK3β, β-catenin and SP5 in different treated groups by Western blot analysis. Baicalin (6 μg/ml) could obviously increase the expression of p-GSK3β, β-catenin (nuclear and cytoplast) and SP5 ($P < 0.001$) compared to the control (Fig. 2A, lane 3, B). However, DKK1 (100 ng/ml), an inhibitor of Wnt signaling pathway, dramatically decreased the expression of p-GSK3β, β-catenin (nuclear and cytoplast) and SP5 ($P < 0.001$) in comparison with the control (Fig. 2A, lane 4, B). In β-catenin siRNA group, the expression level of β-catenin (nuclear) was significantly reduced ($P < 0.001$), and the expression levels of β-catenin (cytoplast) and SP5 were lower than that in the control and vector groups, respectively (Fig. 2A, lane 7, B). We also found that baicalin combined with DKK1 could restore the expression of all the molecules mentioned above (Fig. 2A, lane 5), and in the same way, baicalin combined with β-catenin siRNA could also restore totally all the molecules except GSK3β (Fig. 2A, lane 8). Note that there was no significant difference in GSK3β expression among all the groups. These results suggest a role for baicalin in the activation of Wnt/β-catenin/SP5 signaling pathway in endometrial RL95-2 cells.

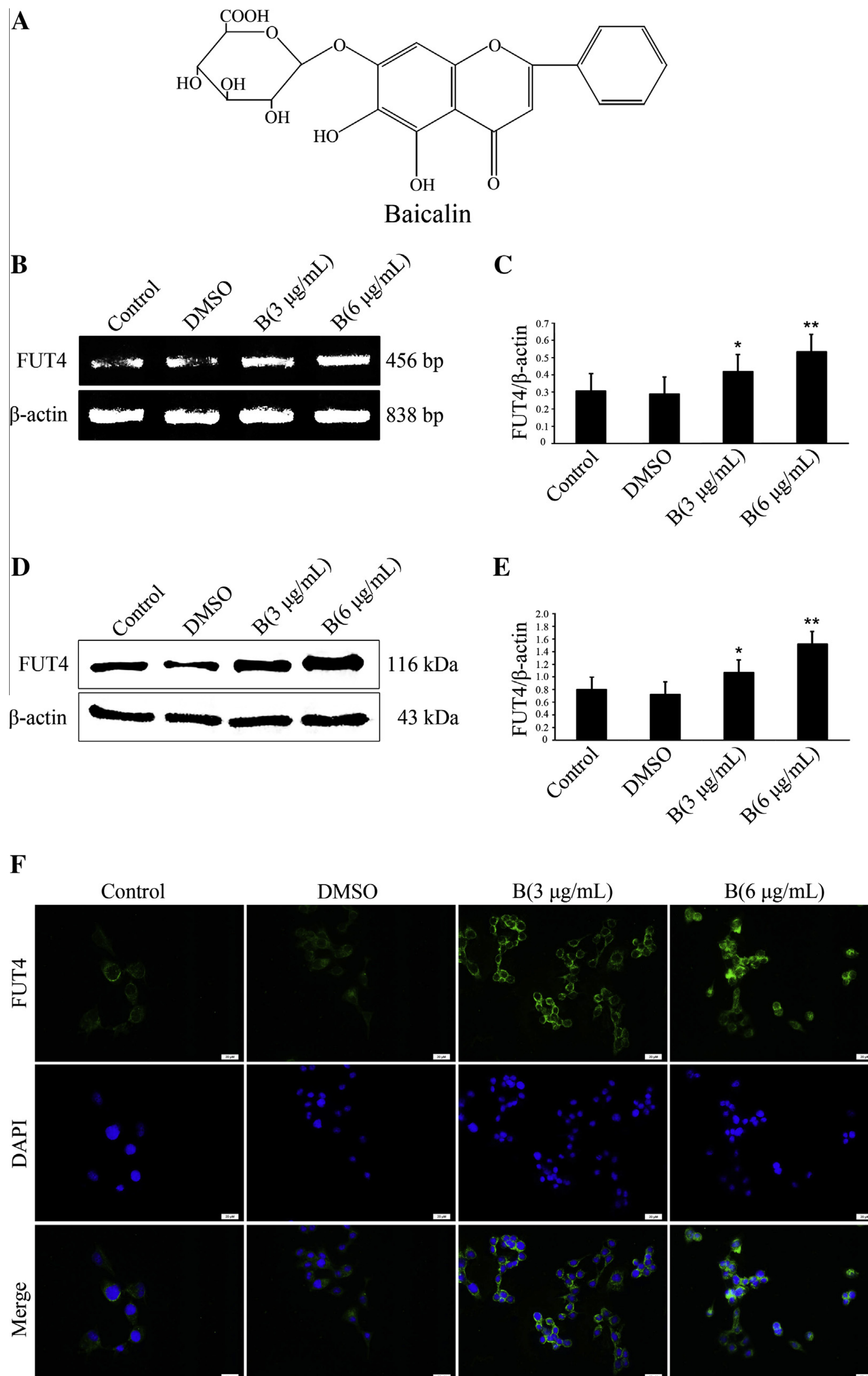

Fig. 1. Baicalin upregulates FUT4 expression in human uterine endometrial RL95-2 cells. (A) Molecular structure of baicalin. (B and D) FUT4 mRNA and protein expression in RL95-2 cells were analyzed using RT-PCR and Western blot. β-Actin was used as loading control. (C and E) Relative densitometric analysis of FUT4 vs β-actin both in gene and protein levels. $^{*}P < 0.05$; $^{**}P < 0.01$. (F) Immunofluorescence staining analysis of FUT4 expression and localization in RL95-2 cells (FITC, green; DAPI-counterstained nuclei, blue; magnification, ×400; bar = 20 μm).

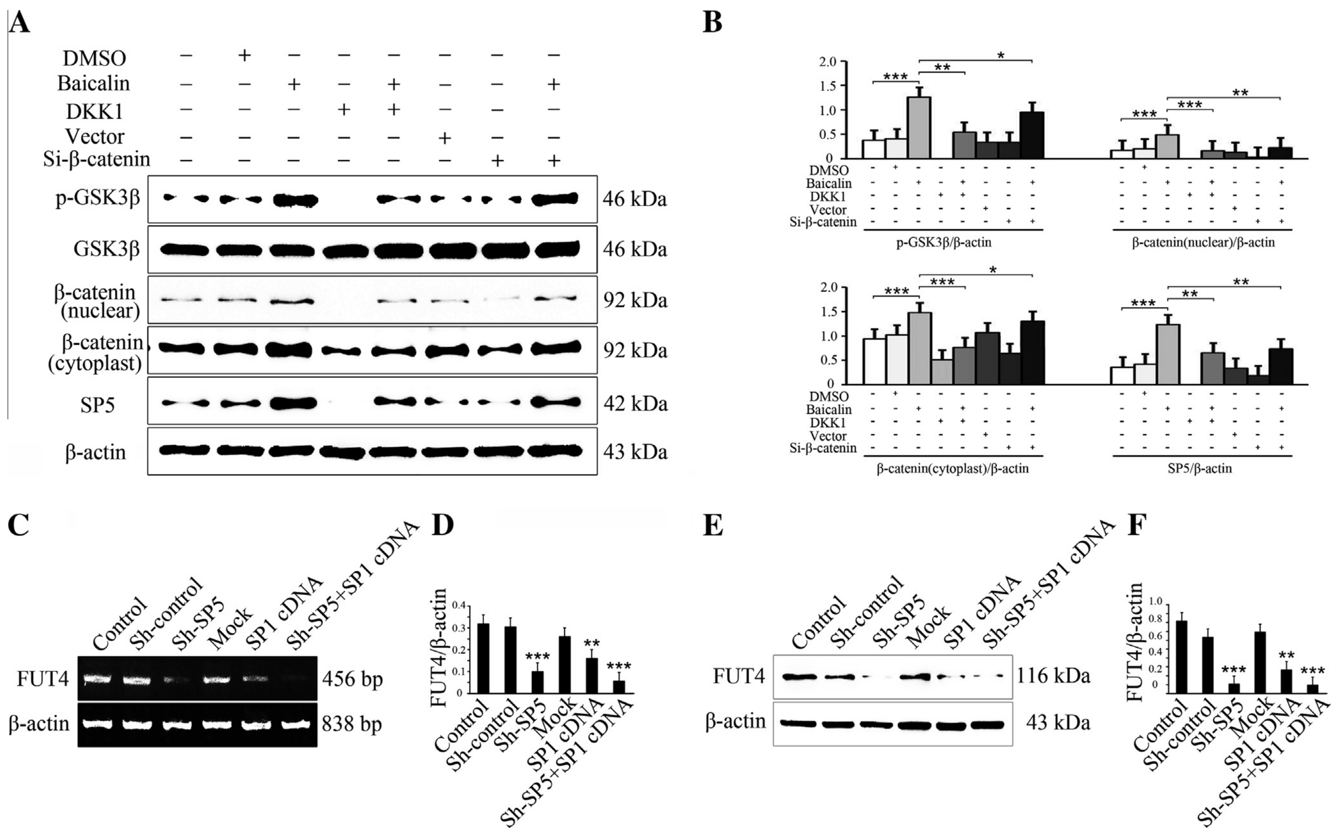

Fig. 2. Baicalin mediates FUT4 expression through Wnt/β-catenin/SP5 signaling pathway. (A) Wnt/β-catenin signaling pathway molecules, including p-GSK3β, GSK3β, β-catenin and SP5 were detected by Western blot. (B) Relative densitometric analysis of each protein vs β-actin. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (C and E) RT-PCR and Western blot analysis of FUT4 expression in different groups of transfected cells (Sh-SP5, SP1 cDNA or co-transfection). β-Actin was used as internal control. (D and F) Relative densitometric analysis of FUT4 vs β-actin. $^{**}P < 0.01$; $^{***}P < 0.001$.

3.3. SP5 promotes FUT4 expression via competing with SP1

To determine how SP5 induced the expression of FUT4, we analyzed FUT4 expression levels in different treated groups of RL95-2 cells. RT-PCR and Western blot assays showed a decreased expression of FUT4 both at mRNA and protein levels in sh-SP5, SP1 cDNA and the co-transfected groups compared to the control groups (Fig. 2C–F).

3.4. Baicalin enhances embryonic JAR cells adhesion to endometrial RL95-2 cells

The embryo adhesion rate among diverse treated groups was assayed in vitro with the co-culture of RL95-2 and JAR cells in implantation model. The results showed that the adhesion rate in the different groups treated with baicalin 3 μg/ml (Fig. 3A, c) and 6 μg/ml (Fig. 3A, d) was largely enhanced compared to the control and DMSO groups. Note that there was no statistical significance among the control (Fig. 3A, a), DMSO (Fig. 3A, b), sh-control (Fig. 3A, e) and mock groups (Fig. 3A, h). While the adhesion rate in sh-SP5 (Fig. 3A, f) or SP1 cDNA (Fig. 3A, i) groups was significantly decreased in comparison with the control groups; in baicalin combined with sh-SP5 (Fig. 3A, g) or SP1 cDNA (Fig. 3A, j) groups, the adhesion rate was largely restored, but still lower than that in baicalin groups. In addition, baicalin could considerably increase the adhesion rate in the co-transfected group (Fig. 3Ak, Al). Further, we demonstrated that baicalin significantly reversed the low embryo adhesion rate induced by FUT4 siRNA ($P < 0.001$) (Fig. 3C and D). The above results indicate that baicalin enhances the adhesion competence of endometrial cells to embryonic cells via Wnt/β-catenin/SP5 mediated FUT4 upregulation.

3.5. Baicalin upregulates SP5 and FUT4 expression in mouse uterine endometrium during implantation window

The expression of SP5 and FUT4 were further analyzed in mouse uterine endometrium at GD 4 in the different groups. By using RT-PCR and Western blot assays, we found that expression of SP5 and FUT4 increased relatively both at the mRNA and protein levels in baicalin ($P < 0.05$) and SBD groups compared to the control and NS groups. Corroboratively, our immunohistochemistry assay showed a strong staining of SP5 and FUT4 in the treated groups compared to the control group. It was noteworthy that we observed a higher staining intensity in baicalin group than in SBD group (Fig. 4E). These findings suggest that baicalin potentially upregulates SP5 and FUT4 expression in mouse uterine endometrium during the period of implantation window.

3.6. Baicalin facilitates embryo implantation in vivo

Using the mouse model, we calculated the embryo implantation number in different groups at GD 8. The results showed that implantated embryo in baicalin ($P < 0.01$) and SBD ($P < 0.05$) groups were highly increased compared to the control and NS groups (Fig. 5A and B).

4. Discussion

Adhesion is the foundation of successful embryo implantation. During mammalian reproductive processes, embryonic cells firstly adhere and followed by implant into the uterine endometrium for the consequent complex developmental events [35]. During embryo adhesion, adhesion-promoting factors play major roles in

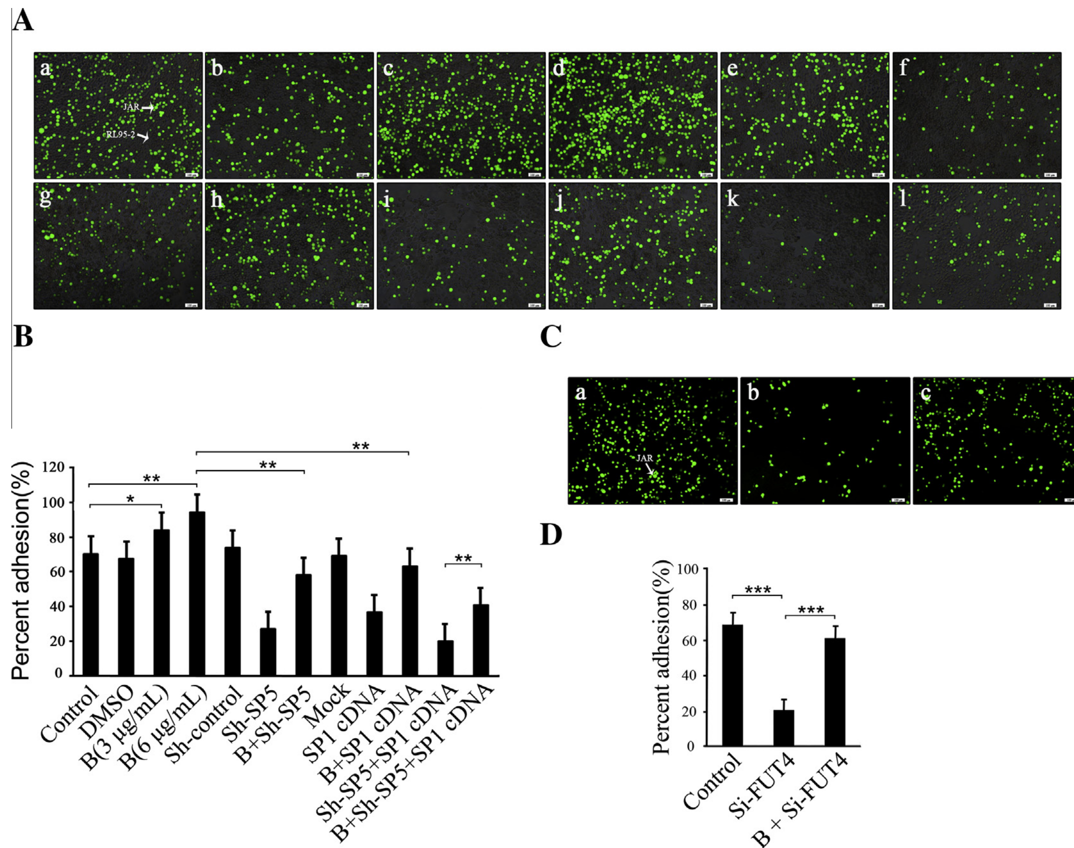

Fig. 3. Baicalin mediates JAR cells adhesion to RL95-2 cells in vitro. (A) RL95-2 cells were pre-incubated with a: control; b: 0.1% DMSO; c: baicalin (3 μg/ml); d: baicalin (6 μg/ml); e: sh-control; f: sh-SP5; g: baicalin (6 μg/ml) combined with sh-SP5; h: mock; i: SP1-cDNA; j: baicalin (6 μg/ml) combined with SP1-cDNA; k: sh-SP5 combined with SP1-cDNA; l: baicalin (6 μg/ml) combined with the co-transfection (CMFDA-counterstained JAR cells, green; magnification, ×100; bar = 100 μm). (B) Adhesion rate was calculated as percentage of attached JAR cells. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Adhesion of JAR cells to RL95-2 cells pre-incubated with a: control; b: si-FUT4; c: baicalin (6 μg/ml) combined with si-FUT4 (magnification, ×100; bar = 100 μm). (D) Adhesion rate of control, si-FUT4 and baicalin (6 μg/ml) combined with si-FUT4 groups. $^{***}P < 0.001$.

regulating adhesion molecules and cytokines [36–37] to the extent that barriers between embryo and endometrium will lead to an abortion or female infertility. Hence, increasing embryo adhesion rate is critical in mammalian reproduction improvement. Previous studies have shown that progesterone and leukocyte inhibiting factor (LIF) play an important role in the process of embryo and endometrium adhesion by regulating the expression level of adhesive molecule Jam2 [38]. In this study, in vitro implantation model emphasized that baicalin could significantly improve the adhesion rate between embryonic JAR cells and endometrial RL95-2 cells (Fig. 3Aa–d). Ma et al. demonstrated that baicalin could protect the mice from abortion through elevating the secretion of interleukin 10 (IL-10) in the uterine endometrium [39]. In our in vivo experiments, we demonstrated that baicalin significantly increased the number of implanted embryos (Fig. 5). Moreover, treatment with baicalin improves mammalian reproductive processes via promoting embryo adhesion. This is the first study reporting the facilitating role played by baicalin in the reproduction mechanism through its adhesive function. Based on its adhesion-promoting effect, baicalin might be used as a potential drug for infertility treatment. Baicalin improves the low rate of embryo implantation during in vitro fertilization and embryo transfer (IVF-ET), with a successful elevated pregnancy rate [40]. It can be used in combination with hormone therapies, such as progesterone and human chorionic gonadotropin (hCG), which promote embryo adhesion and decrease the side effects of hormone treatments in infertile patients [41–43] (see Fig. 6).

Glycosylation is intimately correlated with mammalian reproductive functions [21,44]. The specific changes of glycosylation in the surface of uterine endometrium are necessary for the initiation of embryo implantation [45,46]. It has been reported that baicalin plays a significant role in the process of aging and anti-fibrosis by regulating protein phosphorylation, acetylation and methylation [47,48]. However, the glycosylation regulation of baicalin in the process of female reproduction remains unknown. Our previous studies indicated that glycosylation modifications caused by the upregulation of the specific FUTs and oligosaccharides antigen, lead to the significant promotion of embryo adhesion and implantation [49,50]. FUT4, the key enzyme for the synthesis of implantation related LeY oligosaccharide, is significantly expressed in high-receptive endometrial cells (RL95-2) compared to low-receptive endometrial cells (HEC-1A). Overexpression of FUT4 in HEC-1A cells could prominently improve its adhesion capacity with embryonic cells [33]. Ponnampalam et al. revealed that progesterone promoted human endometrial FUT4 mRNA and protein expression [32]. Here we demonstrated that baicalin treatment notably elevated FUT4 mRNA and protein expression in human endometrial cells by RT-PCR, Western blot and immunofluorescence staining (Fig. 1). In accordance to our in vivo experiments, we also found that baicalin increased FUT4 mRNA and protein expression in mouse uterine endometrium during implantation window (Fig. 4). Currently, our researches reported for the first time that baicalin adjusts the gestation process of mammals' through specific regulation of glycosylation highlighting the roles of traditional Chinese medicine *S. baicalensis* in reproductive glycobiology.

Wnt/β-catenin pathway plays a key role in a wide variety of cellular differentiation and developmental processes, such as endometrial differentiation and embryo development [19].

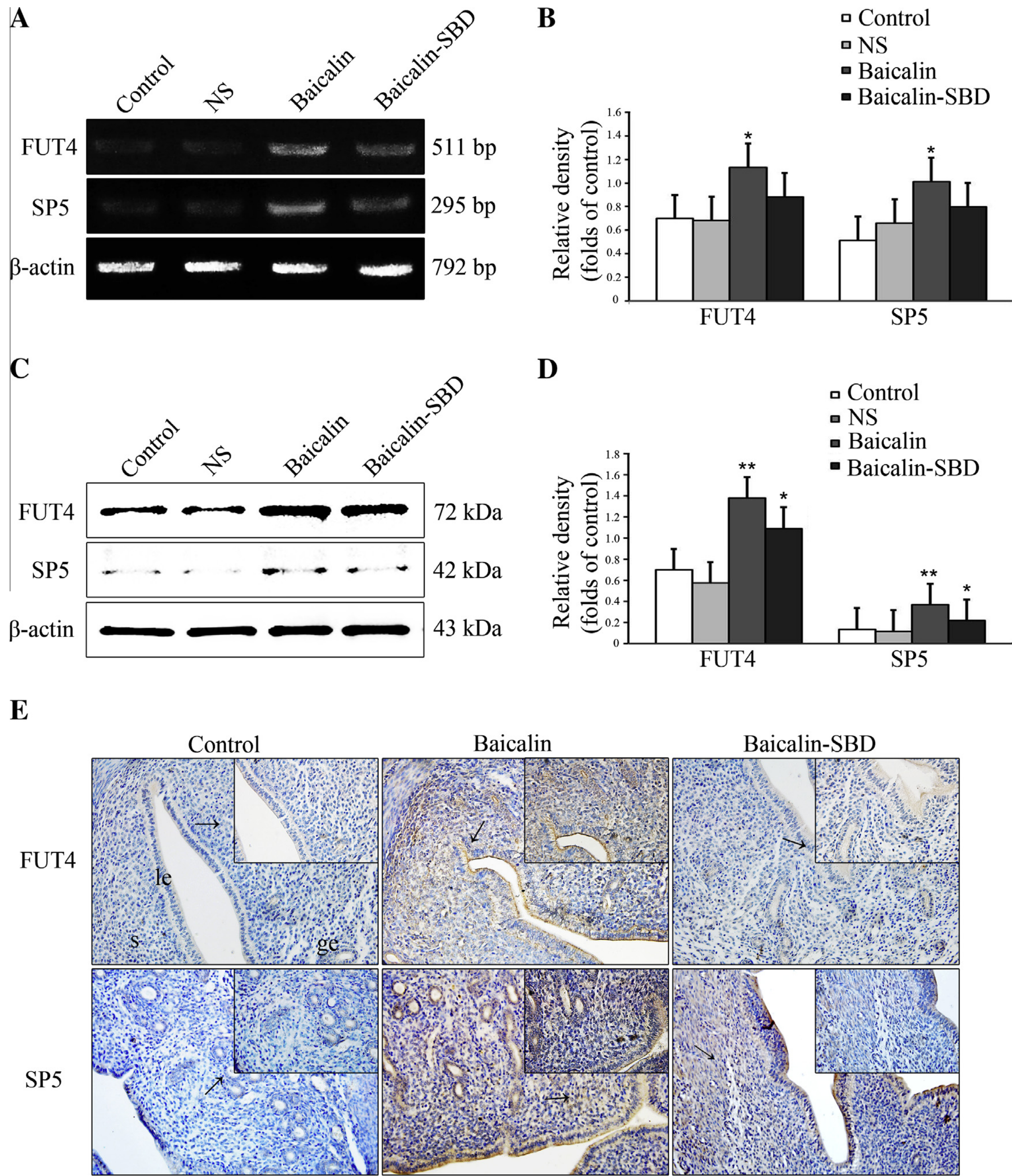

Fig. 4. Baicalin and SBD potentially regulate SP5 and FUT4 expression levels in mouse uterine endometrium at GD 4. (A and C) RT-PCR and Western blot analysis of SP5 and FUT4 expression in mouse endometrium. β-Actin was used as internal control. (B and D) Relative densitometric analysis of SP5 and FUT4 vs β-actin in mouse endometrium. $^{*}P < 0.05$; $^{**}P < 0.01$. (E) Immunohistochemistry staining analysis of SP5 and FUT4 expression in mouse endometrial tissue. le indicates luminal epithelium; ge, glandular epithelium; s, stroma. (magnification, ×200; bar = 50 μm).

Endometrial decidualization is developed during the process of embryo adherence onto the uterine endometrium, which also represents the differentiation of endometrium. Wnt/β-catenin pathway can be activated by different ways. Wnt ligands are capable to transduce extracellular signals into the cells by binding to their receptors frizzled (Fzd) protein and co-receptors lipoprotein receptor-related protein 5/6 (Lrp5/6) on cell membrane [51]. DKK1, an inhibitor of Wnt receptor, binds directly to Lrp5/6 and inhibits downstream Wnt pathway activation [52]. Guo et al. found that baicalin promoted differentiation of mouse osteoblasts cells similarly to Wnt3a [34]. We confirmed that baicalin significantly reversed the expression of p-GSK3β, β-catenin and SP5 in endometrial RL95-2 cells pretreated with DKK1 for 1 h, which indicated a recovery of the Wnt/β-catenin signaling activity (Fig. 2A and B). The findings of our study emphasize a role for baicalin acting as a ligand to activate Wnt/β-catenin signaling pathway in endometrial cells.

Previous researches have reported that SP5 transcription factor, a downstream target gene of Wnt/β-catenin signaling pathway, exerts momentous role in vertebrate embryonic development [53,54]. SP5 inactivation might result in developmental defects in mice mesoderm [55]. Our research reported that baicalin significantly increased the expression level of SP5 with activation of Wnt/β-catenin signaling pathway in vitro (Fig. 2A). From our previous bioinformatics predictions, we found that SP5 could bind to FUT4 DNA promoter region. Moreover, we have discovered that

Fig. 5. Baicalin facilitates embryo implantation in vivo. (A) Implanted embryos in different treated groups. (B) Analysis of implanted embryo is represented in the histogram. $^{*}P < 0.05$; $^{**}P < 0.01$.

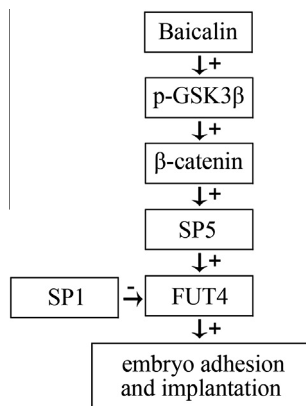

Fig. 6. Schematic diagram illustrates the mechanism underlying baicalin mediated FUT4 upregulation by activation of Wnt/β-catenin signaling pathway via transcription factors (SP5 and SP1).

baicalin stimulated FUT4 mRNA and protein expression both in vitro and in vivo. Other studies reported that SP1, a transcription factor of SP1 family as well as SP5, downregulated the expression of FUT4 via binding to FUT4 promoter region [56,57]. Hence, in order to determine the mechanism through which SP5 regulates the expression of FUT4, we transfect RL95-2 cells with sh-SP5, SP1 cDNA and their combination. The results demonstrated that all three groups could significantly downregulate FUT4 mRNA and protein expression (Fig. 2C–F). According to our above findings, we conclude that baicalin increases SP5 expression level via activating Wnt/β-catenin signaling pathway, and further promotes embryo adhesion and implantation derived from the boosted FUT4.

In conclusion, this study have identified a novel role of baicalin which upregulates FUT4 both in human endometrial cells and mouse endometrium during the period of implantation via Wnt/β-catenin pathway. We believe that the findings of this research could help the development of diagnosis and peculiar therapeutics for female infertility. Nevertheless, since there are complex regulatory network during pregnancy, further researches need to be carried out in order to reveal the glycobiological mechanisms of baicalin in reproduction.

Conflict of interest

The authors declare no competing financial interest.

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