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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.

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