Int J Clin Exp Pathol 2017;10(2):1756-1764
www.ijcep.com /ISSN:1936-2625/IJCEP0039664

Original Article

Wnt/beta-catenin signaling pathway regulates beta1,4-galactosyltransferase I expression in the endometrium to affect the embryo implantation

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Received September 7, 2016; Accepted September 26, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Successful implantation depends on two aspects: blastocyst invasion ability and endometrial receptivity. β1,4-GalT-I plays an important role in fertilization, cell adhesion, neurite growth and tumor cell migration process. To explore the impact of β1,4-GalT-I in the process of embryo implantation and study on regulation of β1,4-GalT-I expression by Wnt/β-catenin signaling pathway. We established mouse model of early pregnancy, taking D4 pregnant mice uterine horn injected β1,4-GalT-I polyclonal antibody to detect the impact on blastocyst implantation and used reverse immunohistochemistry for detecting β1,4-GalT-I antibody effectiveness. Established in vitro implantation model, using immunofluorescence, Western-blot and cells adhesion experiments to detect whether Wnt/β-catenin signaling pathway regulated β1,4-GalT-I. We found that side of the uterine cavity of pregnant mice injected β1,4-GalT-I antibody, the number of embryo implantation (3.3±0.4) compared with the other side injected with saline embryo implantation count (10.1±1.7) was statistically significant ($P<0.05$). Immunohistochemically staining showed that brown particles precipitate on endometrial cells. Regulation of Wnt/β-catenin signaling pathway may regulate the expression of β1,4-GalT-I. Raised β-catenin expression in RL95-2 cells, the adhesion rate of JAR to RL95-2 cells (85.7±3.3%) was significantly higher compared with non-transfected group (53.6±2.1%). However, reduced β-catenin expression in RL95-2 cells, the adhesion rate of JAR to RL95-2 cells (35.3±1.6%) declined. β1,4-GalT-I participate in the process of embryo implantation. β1,4-GalT-I expression in endometrium during implantation and Wnt/β-catenin signaling pathway molecule expression has correlation and it may be located in Wnt/β-catenin signaling pathway downstream. In short, Wnt/β-catenin signaling pathway may regulate β1,4-GalT-I expression in the endometrium to involve the adhesion of JAR to RL95-2 cells.

Keywords: β1,4-GalT-I, Wnt/β-catenin signaling pathway, endometrial, embryo, adhesion

Introduction

Embryo implantation is a unique physiological phenomenon in mammalian reproductive process, which involves complex interactions and molecules signal transduction between the embryo and the endometrium [1]. Successful implantation depends on two aspects majorly: blastocyst invasion ability and endometrial receptivity. A series of related hormones (estrogen and progesterone), growth factors (EGF, TGFβ), cytokines (IFN, CSF), adhesion molecules (selectin family, the immunoglobulin superfamily, ICAM-1) and cell surface glycans (LeY, sLeX) and other substances play pivotal roles in embryo implantation process [2].

β1,4-galactosyltransferase I (β1,4-GalT-I) ubiquitously expresses in human tissues with the exception of the brain. It exists in two subcellular compartments where it performs two distinct functions [3]. Short β1,4-GalT-I distributes in the Golgi apparatus for forming Galβ1→4GlcNAc glycoside bond [4] and involves in the synthesis of glycoconjugates. Long β1,4-GalT-I locates in the cell membrane, owning more than 13 amino acids compare to the short in the cytoplasmic domain [5], which play

an important role in fertilization, cell adhesion, neurite growth and tumor cell migration process [6-8]. Studies have shown that the expression of β1,4-GalT-I is increased with the increase of tumor invasion capacity. The process of the embryos implantation is very similar to tumor invasion. So many biological factors participating in the tumor invasion also play a role in embryo implantation. We hypothesize that β1,4-GalT-I may take part in embryo implantation.

β-catenin plays an extremely important role in the canonical Wnt signaling pathway, which is a key molecule in the whole pathway. β-catenin not only is the core factor of the canonical Wnt signaling pathway but also plays an important role in mediating cell and cell adhesion [9, 10]. On the one hand, β-catenin and E-cadherin form β-catenin/E-cad complex, which plays an important role in maintaining cells-cells firmly connected [11], epithelial polarity and complete maintenance [12, 13]; On the other hand, β-catenin is located in the activation center of the canonical Wnt signaling pathway and impeded degradation causes cytoplasmic accumulation of free β-catenin into the nucleus and T cell factor/lymphocyte enhancer factor (TCF/LEF) binding, thereby activating transcription of downstream target genes. Axin involve in the formation of Axin-GSK-APC degradation complex, the concentration of Axin is a limiting factor of complex formation. After complex formation, β-catenin is identified and degraded by the proteasome through a series of phosphorylation, and ubiquitination. The cytoplasm Axin concentration decreased steadily can cause the disintegration of Axin-GSK-APC degradation complex. β-catenin cannot be phosphorylated and degraded, thus accumulate in the cytoplasm into the nucleus. After activating Wnt/β-catenin signaling pathway, β-catenin into the nucleus and transcription factor TCF/LEF binding to regulate a variety of downstream target genes transcription and thus regulate cell invasion and migration [14]. Wnt signaling pathway target gene c-myc has positive cell cycle regulators and transcription factors can combine with DNA to regulate endometrial cell division, proliferation and differentiation activities [15], and involve in blastocyst implantation. *CyclinD1* is also Wnt/β-catenin regulation of downstream target gene, which plays an important role in the cell cycle G1-S phase progression [16], β-catenin in the nucleus raises a lot of to activate the expression of cyclin genes and regulate cell proliferation, differentiation, adhesion and polarity. Wnt/β-catenin signaling pathway is a complex system of a variety of molecular interaction, mutual restraint and synergies, which is a key way of embryonic development, regulation of cell growth and proliferation [17].

To investigate whether β1,4-GalT-I could be regulated by Wnt, we used human uterine epithelial cell lines as in vitro models: RL95-2. RL95-2 was used as a model of receptive endometrium [18-21]. The cell lines were chosen based on earlier studies which have demonstrated that RL95-2 cells have stronger adhesiveness for human JAR choriocarcinoma multicellular spheroids compared to HEC-1A cells [22-24] and are considered as a model of the receptive endometrium.

In the process of embryo implantation, Wnt/β-catenin signaling pathway and β1,4-GalT-I correlation studies have not been reported in the literature. Investigate the effect of β1,4-GalT-I in the process of embryo implantation, and whether Wnt/β-catenin signaling pathway regulated β1,4-GalT-I expression. Provide certain basis for further research human reproduction and contraception and other aspects.

Material and methods

Animals

Mice of Kunming species were from Lab Animal Center in Dalian Medical University of China. All experimental procedures involves in the mouse studies were approved by the Institutional Animal Care and Use Committee in Dalian Medical University. Adult female mice and adult male mice were maintained under controlled environmental conditions. The mice were housed in a temperature 22-25°C, humidity 60%, and light-controlled (12 h light: 12 h darkness) with ad libitum access to water and food.

Antibody injection

Females were placed with males (one female with one male per cage). Females were checked for the presence of a vaginal plug in the next morning, which was defined as D1 if the vaginal plug came out. On day4 of pregnancy at 10

o'clock, after 10 mice were anesthetized with pentobarbital sodium (50 mg/kg), β1,4-GalT-I polyclonal antibody (10 μl, 200 μg/mL, Santa), was injected into the right uterus horn, and IgG injected into the left uterus horn. All mice were euthanized at 10 o'clock on day8 of pregnancy, and the number of embryo implanted was counted.

Reverse immunohistochemistry

Sliced fresh tissue immediately in a cryostat frozen microtome (can also be stored at -80°C), the thickness of the 5-6 μm, 4% paraformaldehyde 15 min, washed with PBS, 5 min × 3, 0.1% sodium citrate + 0.2% TritonX-100 perforated 15 min, washed with PBS, 5 min × 3, incubated with 3% H_2O_2 in the dark 20 min, to inactivate endogenous enzymes, washed with PBS, 5 min × 3. Wipe the water surrounding tissue and tissue was kept moist state, normal rabbit serum blocking solution was added dropwise, placed 15 min in 37°C, added anti-β1,4-GalT-I-Ab (1:100) from rabbit and PBS was used instead of primary antibody as a negative control, incubated overnight at 4°C. The next day dropping biotin labeled goat anti rabbit secondary antibody, incubated 40 min at 37°C, washed with PBS, 5 min × 3, dropping horseradish peroxidase labeled streptavidin, incubated 40 min at 37°C, washed with PBS, 5 min × 3. With DAB color, degree of staining was observed under a microscope, washed 10 min with tap water. And then stained 13 s with hematoxylin, tap water washed 10 min. The slides were soaked 3 min sequentially in 75% ethanol, 80% ethanol, 95% ethanol, anhydrous ethanol, and then placed in xylene 5 min. Finally mounted with neutral gum and observed by a microscope.

Cells culture and transfection

RL95-2 cells were cultured in DMEM/F12 (1:1) medium (containing 10% fetal bovine serum, 10 mmol/L HEPERS, 10 g/ml insulin, 100 IU/ml penicillin and 100 g/ml streptomycin). JAR cells were cultured in 1640 medium (containing 15% fetal calf serum, 100 IU/ml penicillin and 100 g/ml streptomycin).These cells were cultured in 37°C, 5% CO_2, 90% humidity incubator. To be cultured to the logarithmic growth phase, use lipofectamine 2000 to transfect. After trypsinization, cells were seeded onto six-well plates. When cells reached 80% confluence, siRNA and over-plasmid (purchased from Genepharma) was transiently transfected into the cells using 4 μL of LipofectamineTM reagent, following the manufacturer's instructions. Medium removed 6 h later and the cells were harvested after 72 h for protein. Three independent experiments were performed.

Immunofluorescence

After washing with PBS, cells grown on coverslips were fixed in 4% paraformaldehyde following 20 min and washed in PBS three times. Nonspecific binding sites were blocked by incubation with 1% goat serum in PBS at room temperature for 1 h. Slides were incubated primary antibody overnight at 4°C. After washing three times with PBS, slides were incubated with TRITC-conjugated goat anti-rabbit antibody (1:100) for 1 h at 37°C. After washed three times, slides were incubated with DAPI for 5 min at room temperature and washed three times again. Specimens were mounted in PBS containing 90% glycerol and 1.0% p-phenylenediamine and subsequently monitored under an Olympus BX51 immunofluorescence microscope.

Western blot

Proteins from uterine tissues or cells were extracted using Lysis Buffer (KeyGen Biotech Co., Ltd., Nanjing, China) and the determination of protein concentration was tested by the BCA assay (KeyGen Biotech Co., Ltd., Nanjing, China). Equal amounts of protein extracts (30 μg) were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filter (NC) membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 hours at room temperature and probed with primary antibodies β1,4-GalT-I, Axin2 , LEF/TCF, CCND1 (1:500, Santa Cruz), or β-actin (1:2000; Bioworld Technology Co., Ltd.,) overnight at 4°C. The membranes were washed with TBST three times. Then the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000; ZSGB-Bio Co., Ltd, Beijing, China) for 1 hour at room temperature. After washed with TBST four times, the membranes were detected using an enhanced chemiluminescence detection system (ECL, GE Healthcare) and visualized using Bio-Rad Laboratories. Mem-

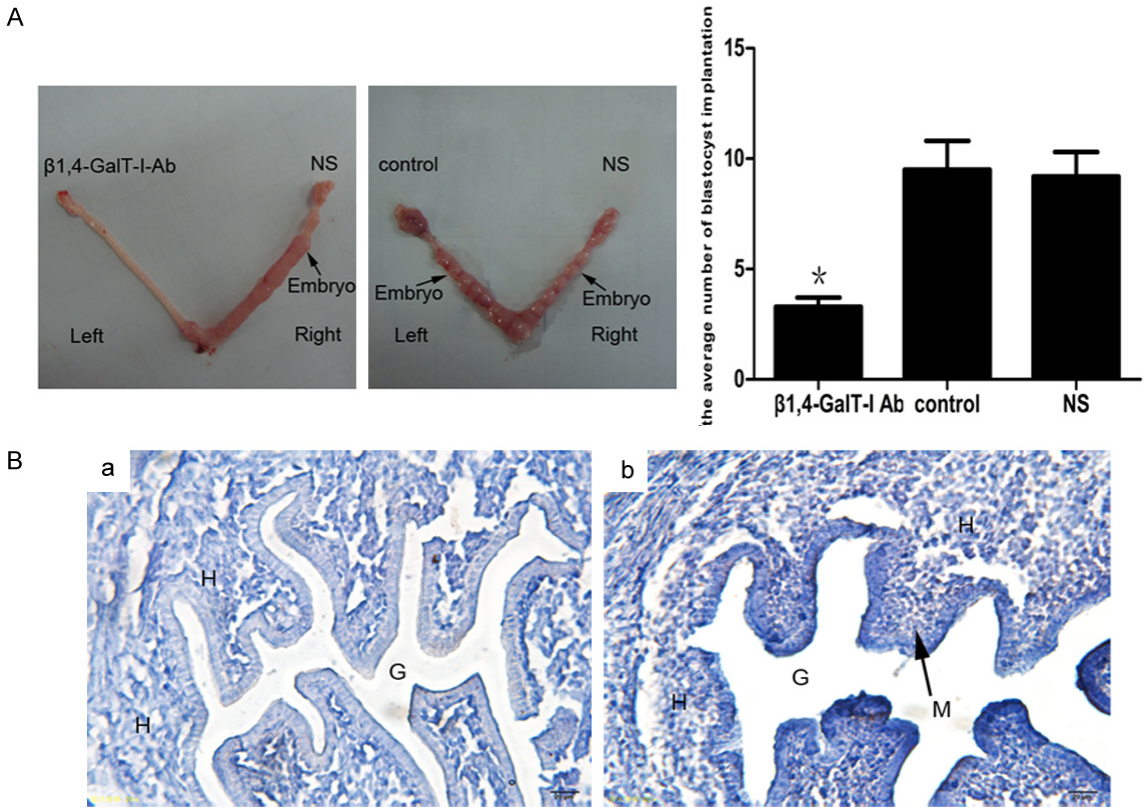

Figure 1. Intervention effect of β1,4-GalT-I polyclonal antibodies on mouse blastocyst implantation. A. The left and right side of the uterus of mice were treated differently in the experimental group and the control group. Quantitative data are presented as the mean (± S.D.) the average number of blastocyst implantation (n = 20). *Indicates a significant difference compared with the control (P<0.05). B. Reverse immunohistochemical detected β1,4-GalT-I antibody that binds with β1,4-GalT-I on endometrial cells. a. Immunohistochemical staining uterine tissue injected with saline. b. Immunohistochemical staining uterine tissue injected with β1,4-GalT-I polyclonal antibody. M: Brown particles precipitate represent that β1,4-GalT-I polyclonal antibody bind with β1,4-GalT-I on the endometrium. H: mouse endometrium, G: mouse uterus.

branes were tested with β-actin or GAPDH as a loading control.

Cells adhesion experiment

The RL95-2 monolayer cells were plated in 6-well culture plate and grew to 80% for cell transfection. Digested the JAR cells with 0.25% trypsin, and shifted into a centrifugal tube to centrifuge, suspend and count. The JAR cells were average added to RL95-2 cells in overgrow 6-well culture plate and cultured 1h at 37°C. After the 6-well culture plate was centrifuged for 5 min at 1,000 rpm/min, medium was collected and counted no adhesion the JAR cells. Calculation of embryonic cell adhesion rate = (the number of added JAR cells-no adhesion the JAR cell/the number of added JAR cells) × 100%. The experiment was repeated three to six times to calculate the adhesion rate.

Statistical analysis

The data were analyzed using SPSS13.0 software. The values were expressed as means ± SD. One-way ANOVA was used in comparison between groups. Difference between the means was considered statistically significant at P<0.05.

Results

Injected β1,4-GalT-I polyclonal antibodies into uterine horn to affect blastocyst implantation

Experimental results are shown in (**Figure 1A**): the experimental group of 10 mice had seven pregnant, the pregnancy rate was 70%, injected β1,4-GalT-I polyclonal antibody into mouse left uterine and blastocyst implantation number was 1-4, the average number of blastocyst implantation was 3.3±0.4, injected with saline

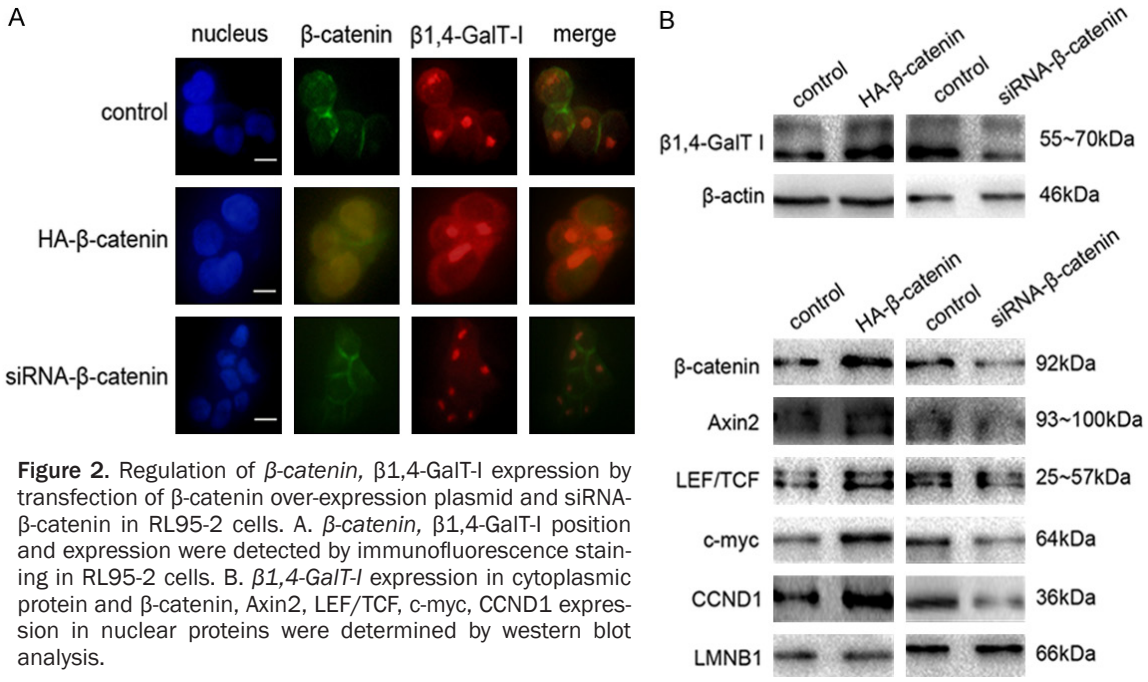

Figure 2. Regulation of *β-catenin,* β1,4-GalT-I expression by transfection of β-catenin over-expression plasmid and siRNA-β-catenin in RL95-2 cells. A. *β-catenin,* β1,4-GalT-I position and expression were detected by immunofluorescence staining in RL95-2 cells. B. *β1,4-GalT-I* expression in cytoplasmic protein and β-catenin, Axin2, LEF/TCF, c-myc, CCND1 expression in nuclear proteins were determined by western blot analysis.

on the right was 7-11, the average number of blastocyst implantation was 10.1±1.7; pregnancy rate was 90% in the control group, the average number of blastocyst implantation on the left and right side of the uterus were 10.3±1.6 and 9.8±1.7. Experimental group were injected with β1,4-GalT-I polyclonal antibody on the left, the number of blastocyst implantation significantly decreased compared to the right, $P<0.5$, the difference was statistically significant. However, the control group had no significant difference in the left and right side of the uterus about the number of blastocyst implantation.

Reverse immunohistochemical detected β1,4-GalT-I polyclonal antibody that binds with β1,4-GalT-I on endometrial cells

Injection of β1,4-GalT-I polyclonal antibody hindered the mouse embryos implantation, we speculate that β1,4-GalT-I polyclonal antibody combined with the corresponding antigen on the endometrium, hindered β1,4-GalT-I combined with the corresponding receptor or ligand, thereby blocking the adhesion of the blastocyst and the endometrium, resulting in anti-implantation effect. To confirm β1,4-GalT-I polyclonal antibody effectiveness, using anti-β1,4-GalT-I-Ab IgG to do reverse immunohistochemistry in the experiment. In **Figure 1B**, after injected β1,4-GalT-I polyclonal antibody into uterus of pregnant mice, using immunohistochemical staining tissue sections showed that many brown particles precipitate on endometrial cells, however, the saline control group did not find brown particles precipitate, indicating that the antibody had combined with β1,4-GalT-I on endometrial cells. It is prompted that the mouse blastocyst implantation was effectively interfered by injected β1,4-GalT-I polyclonal antibody into the mice uterine cavity.

Explore the situation of wnt/β-catenin signaling pathway regulating β1,4-GalT-I expression

Immunofluorescence staining showed that the expression of β1,4-GalT-I in cell membrane and Golgi apparatus and β-catenin in the nucleus were significantly increased as compared to the normal group and the interference group (**Figure 2A**). Western-blot results showed that β1,4-GalT-I, β-catenin and Axin2, LEF/TCF, c-myc, CCND1 in canonical Wnt pathway were increased at the protein level after RL95-2 cells were transfected with β-catenin over-expression plasmid. However, after RL95-2 cells were transfected with siRNA-β-catenin, the above expressions were reduced (**Figure 2B**).

Wnt/β-catenin signaling pathway regulation of β1,4-GalT-I can influence the adhesion rate of JAR cells and RL95-2 cells

In this study, we used two cell lines as *in vitro* implantation model which contains human

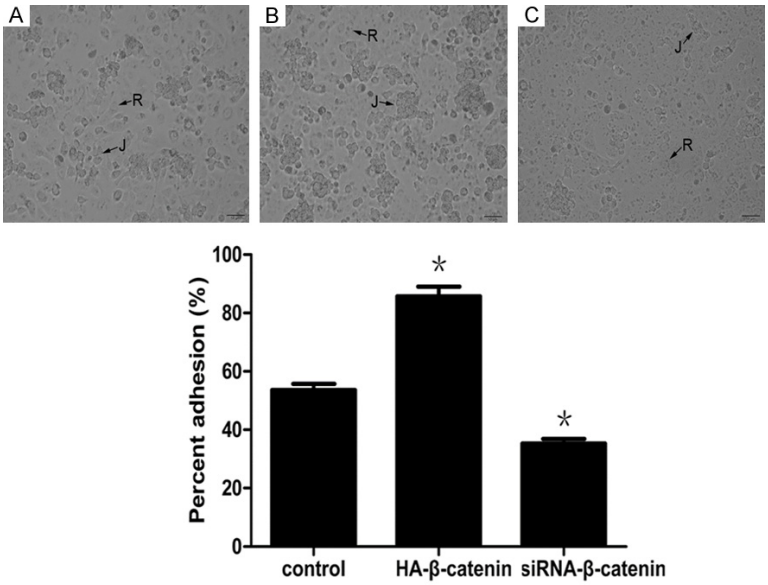

Figure 3. Adhesion analysis of JAR embryonic cells to RL95-2 uterine epithelial cells. A. A representative image of JAR cells attached to RL95-2 cells under normal circumstances. B. A representative image of JAR cells attached to RL95-2 cells after transfection of β-catenin over-expression plasmid. C. A representative image of JAR cells attached to RL95-2 cells after transfection of siRNA-β-catenin. R: RL95-2. J: JAR. Percentage adhesion was calculated as stated in the Materials and Methods. *Indicates a significant difference compared with the control (P<0.05). Bar = 50 μm.

endometrial cancer cells RL95-2 simulate pre-implantation endometrial and villi cancer cells JAR simulate pre-implantation embryo. After RL95-2 cells were treated with different treatments, calculate the adhesion rate of JAR to RL95-2 cells. Cell adhesion experiments showed that raised β-catenin expression in RL95-2 cells, the adhesion rate of JAR to RL95-2 cells (85.7±3.3%) was significantly higher as compared to non-transfected group (53.6±2.1%). However, reduced β-catenin expression in RL95-2 cells, the adhesion rate of JAR to RL95-2 cells (35.3±1.6%) decreased (**Figure 3**).

Discussion

Embryo implantation is closely intercellular communication links process which contains embryonic trophoblast cells having invasion ability and maternal endometrial cells at the receptivity. This period is known as “implantation window”. At this point, multi-factor such as adhesion molecules, cytokines, proto-oncogenes etc. carry out complex, fine molecular dialogue and signal transduction between the embryo and the uterine endometrial stromal, regulating the recognition and adhesion of the embryo and the endometrium to implant [25, 26]. Therefore, understanding the mechanism of embryo development and embryo implantation becomes a hot research in developmental biology.

β1,4-GalT-I locate on Golgi can catalyze Galβ1→4GlcNAc glycoside bond formation, involve in the synthesis of glycoconjugates; β1,4-GalT-I locate on the cell surface, as a cell adhesion molecule to involve in the interactions of cells and matrix and cells to cells, and has an important role in fertilization, cell adhesion, neurite growth, tumor cell migration and other processes. However, research is less about the adhesion of the blastocyst and the endometrium. The results of this preliminary study in our laboratory display: β1,4-GalT-I is highly expressed in the endometrium during implantation, and regulation of β1,4-GalT-I can affect the synthesis of matrix metalloproteinase 9 (MMP9), endometrial cell surface oligosaccharides LeY, cytokine LIF and synthetic growth factor (EGF, HB-EGF, TGF-α). It is prompted that β1,4-GalT-I involve in regulating the recognition and adhesion of the embryo and the endometrium.

To further improve the mechanism about β1,4-GalT-I regulation of embryo implantation, we established mouse model of early pregnancy in this study, using β1,4-GalT-I polyclonal antibody to directly detect intervention on embryo implantation, confirmed β1,4-GalT-I in the importance of the process of embryo implantation. The results showed that the 4th day of pregnancy (D4) injected β1,4-GalT-I polyclonal antibody in mice uterine horns had neutralized β1,4-GalT-I in mouse uterine cavity. The number of mouse blastocyst implantation and pregnancy rate was lower than the saline control group (P<0.5). On this basis, using IgG as the anti-antibody of anti-β1,4-GalT-I-Ab to do reverse immunohistochemistry. The results showed that β1,4-GalT-I polyclonal antibody

effectively combined the endometrium β1,4-GalT-I. It is prompted that interfered endometrial β1,4-GalT-I expression will effectively inhibit mouse blastocyst implantation. We speculate that β1,4-GalT-I polyclonal antibody combined with the corresponding antigen on the endometrium, hindered β1,4-GalT-I combined with the corresponding receptor or ligand. Thereby blocking the adhesion of the blastocyst and the endometrium, resulting in anti-implantation effect.

Wnt/β-catenin signaling pathway plays a pivotal position in the growth and development of animals. The related signal transduction changes will lead to abnormal activation of this pathway, thereby affecting the embryo development and embryo implantation and a series of physiological process. β-catenin is a critical component in Wnt/β-catenin signaling pathway. For detecting the activity of Wnt signaling pathway, Araki et al. constructed the TCF/Lef-Lac Z transgenic mice and confirmed regulation of Wnt/β-catenin signaling pathway may regulate endometrial decidualization and trophoblast cells' adhesion, invasion [27]. Studies have shown that endometrial β-catenin protein expression is a temporary down-regulation or deletion in implantation window. Speculate that this may be related to the synchronization of pre-implantation embryonic development and uterine differentiation [28], and promoting endometrial epithelial cells' separation and embryo implantation [29]. Therefore, Wnt/β-catenin signaling pathway is closely related to embryo implantation.

In order to explore the regulation mechanism of β1,4-GalT-I in embryo implantation process, and analyze the relevance about Wnt/β-catenin signaling pathway molecules and β1,4-GalT-I expression. In this study, human endometrial cancer RL95-2 cells were used as a model of receptive uterine epithelium. Transfected with β-catenin over-expression plasmid and siRNA-β-catenin to activate and blocking β-catenin expression in RL95-2 cells to detect regulation of β-catenin expression on β1,4-GalT-I influence. The results showed that activation of Wnt/β-catenin signaling pathway making the expressions of β1,4-GalT-I, β-catenin and Axin2, LEF/TCF, c-myc, CCND1 in canonical Wnt pathway upregulation in RL95-2 cells, and blocking Wnt/β-catenin signaling pathway making the above molecules downregulated. It is prompted that endometrium β1,4-GalT-I expression in the implantation period and Wnt/β-catenin signaling pathway molecules expression have correlation and β1,4-GalT-I may be located in Wnt/β-catenin signaling pathway downstream, but for the mechanism about Wnt/β-catenin signaling pathway regulation of β1,4-GalT-I need to be more in-depth study.

To explore Wnt/β-catenin signaling pathway regulation of β1,4-GalT-I on the adhesion conditions of embryo and endometrium. In this study, we used two cell lines as *in vitro* implantation model. Human endometrial cancer cells RL95-2 simulate pre-implantation endometrial and villi cancer cells JAR simulate pre-implantation embryo. Because RL95-2 as the ideal endometrial epithelial cell lines that is currently used *in vitro* implantation mechanism. It has no polarity, and has certain adhesion ability with embryonic cells. Cellular Dynamics studies showed that RL95-2 cells can accurately reflect high receptive endometrium changes [30]. The study found that, the adhesion rate of JAR cells and RL95-2 cells was 53.6±2.1% under normal circumstances. The rate was significantly increased to 85.7±3.3% when the RL95-2 cells were transfected with β-catenin overexpression plasmid. If it transfected with siRNA-β-catenin, the adhesion rate dropped to 35.3±1.6%. It shown that Wnt/β-catenin signaling pathway regulating β1,4-GalT-I can influence the adhesion rate of JAR cells and RL95-2 cells. It is prompted that Wnt/β-catenin signaling pathway in the complex implantation process may affect the endometrial β1,4-GalT-I expression to promote the adhesion of blastocyst and endometrium.

Implantation is a process which embryo attached to the endometrium. Placenta angiogenesis and maternal decidua trophoblast invasion are signs of successful implantation. Wnt/β-catenin signaling pathway in the regulation of embryo implantation process may include the following two aspects: [1] this pathway may activate circular smooth muscle of the uterus in prior to implantation, thus regulating the adhesion and invasion of the embryo and the endometrium; [2] activate implantation sites and regulate target genes expressions, vascular remodeling after implantation, and then the formation of the placenta.

Conclusions

Embryo implantation is a highly complex physiological and biochemical process, and involve in the adaptive response between the embryo and the matrix. A number of known and unknown substance information transfers between the blastocyst and the endometrium are essential to the implantation. Wnt/β-catenin signaling pathway regulate β1,4-GalT-I which plays a crucial role in embryo implantation process, but its regulation mechanisms are not yet fully elucidated. Further investigate the role of Wnt/β-catenin signaling pathway and β1,4-GalT-I in embryo implantation, providing new ideas for the developments of human reproduction, assist reproductive and anti-implantation technology.

Acknowledgements

This work was supported by National Natural Scientific Grants (No. 31570798, No. 3097-0464, No.81600197), China and Liaoning province natural science foundation of China (2014023055) and by the program for professor of special appointment in Liaoning province.

Disclosure of conflict of interest

None.

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